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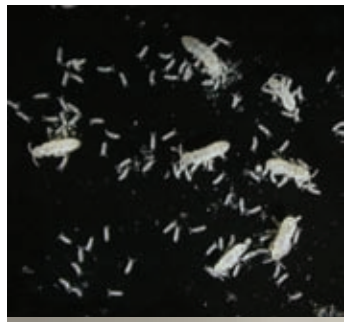

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Biological Test Method: Test for Measuring Survival and Reproduction of Springtails Exposed to Contaminants in Soil

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Environmental Protection Series

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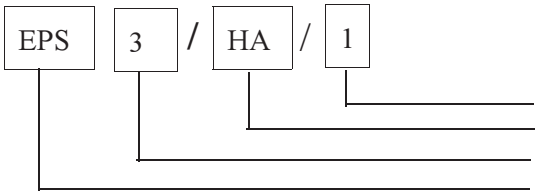

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PG Power Generation
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RA Refrigeration and Air Conditioning
RM Reference Methods
SF Surface Finishing
SP Oil and Chemical Spills
SRM Standard Reference Methods
TS Transportation Systems
TX Textiles
UP Urban Pollution
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Biological Test Method:
Test for Measuring Survival and Reproduction of Springtails Exposed to Contaminants in Soil

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

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Abstract

This document provides detailed procedures, conditions, and guidance for preparing for and conducting a biological test method for measuring soil toxicity using springtails (Collembola) (Orthonychiurus folsomi, Folsomia candida*, or* Folsomia fimetaria). *The test duration is species-specific at 21 days for* F. fimetaria*, and 28 days for* F. candida *and* O. folsomi*, at the end of which effects on the survival and reproduction of springtails exposed to one or more samples or concentrations of contaminated or potentially contaminated soil is determined. Each test method is conducted as a static (i.e., no renewal) test, using one or more samples of contaminated or potentially contaminated soil or one or more concentrations of chemical(s) or chemical product(s) spiked in negative control (or other) soil. Collembola are fed (activated dry yeast) during the test.*

The test is conducted at a mean temperature of 20 ± 2 °C in 100- to 125-mL glass jars containing a measured wet weight of approximately 30 g of soil. This test is initiated by placing test organisms [for O. folsomi*, 15 individuals (5 males and 10 females), 28 to 31 days old are used; for* F. candida*, 10 juveniles, 10 to 12 days old are used; and for* F. fimetaria *20 individuals (10 females and 10 males), 23 to 26 days old are used] into each replicate vessel containing test or clean (negative control or reference) soil. A minimum of three replicates for test soils and five replicates for clean (negative control or reference) soils are prepared for each treatment. At the end of the test, the survival rate for the replicate groups of adult springtails in each treatment is determined as well as the number of live juvenile springtails produced in each replicate and treatment. The treatment means are then compared.*

General or universal conditions and procedures are outlined for test preparation and performance. Additional conditions and procedures are stipulated that are specific to the intended use of each test. The biological test method described herein is suitable for measuring and assessing the toxicity of samples of field-collected soil, biosolids, sludge, or similar particulate material; or of natural or artificial soil spiked (mixed) in the laboratory with test chemical(s) or chemical product(s). Instructions and requirements are included on test facilities, sample collection, handling and storing samples, culturing test organisms, preparing soil or spiked-soil mixtures and initiating tests, specific test conditions, appropriate observations and measurements, endpoints and methods of calculation, and the use of a reference toxicant.

Résumé

*Le présent document renferme des indications précises et décrit en détail les procédures et conditions applicables à la préparation et à la conduite d'un essai biologique visant à mesurer la toxicité d'un sol à l'aide de collemboles (*Orthonychiurus folsomi, Folsomia candida *ou* Folsomia fimetaria*). La durée de l'essai est propre à l'espèce, soit 21 jours pour* F. fimetaria *et 28 jours pour* F. candida *et* O. folsomi*. Au terme de l'essai, on détermine les effets de l'exposition à un ou plusieurs échantillons ou concentrations de sol contaminé ou susceptible d'être contaminé sur la survie et la reproduction des collemboles. Il s'agit d'un essai sans renouvellement faisant appel à un échantillon ou plus de sol contaminé ou susceptible d'être contaminé, ou encore à une concentration ou plus d'au moins une substance ou un produit chimique que l'on mélange avec un sol témoin négatif (ou autre). Les collemboles sont nourris (avec une levure sèche active) pendant la durée de l'essai.*

*L'essai est mené à une température moyenne de 20 ºC ± 2 ºC; les bocaux de verre, d'une capacité de 100 à 125 mL, contiennent environ 30 g de sol (masse humide mesurée). Au début de l'essai, on transfère les organismes d'essai [*O. folsomi *: 15 individus (5 mâles et 10 femelles), âgés de 28 à 31 jours;* F. candida *: 10 juvéniles, âgés de 10 à 12 jours;* F. fimetaria *: 20 individus (10 femelles et 10 mâles), âgés de 23 à 26 jours)] dans chaque récipient de répétition contenant un échantillon de sol d'essai ou de sol propre (sol témoin négatif ou sol de référence). Il faut préparer au moins trois répétitions de sol d'essai et cinq répétitions de sol propre (sol témoin négatif ou sol de référence) pour chaque traitement. À la fin de l'essai, on établit le taux de survie des groupes de collemboles adultes ainsi que le nombre de collemboles juvéniles vivants produits, et ce, pour chaque répétition et chaque traitement. On compare ensuite les moyennes obtenues par traitement.*

Le présent document décrit les procédures et conditions générales ou universelles applicables à la préparation et à la conduite de l'essai. Il renferme aussi une description des autres procédures et conditions propres à l'usage prévu des résultats de chaque essai. La méthode d'essai biologique présentée ici convient à la mesure et à l'évaluation de la toxicité d'échantillons de sol, de biosolides, de boues ou de matériau particulaire semblable recueillis sur le terrain, ou encore de sol naturel ou artificiel enrichi, c'est-à-dire mélangé en laboratoire avec une substance ou un produit chimique d'essai ou plus. Des instructions et des exigences sont incluses sur les éléments suivants : installations d'essais; prélèvement, manipulation et entreposage des échantillons; élevage des organismes d'essai; préparation du sol ou des mélanges de sol enrichi; mise en route de l'essai; conditions propres à l'essai; observations et mesures pertinentes; paramètres et méthodes de calcul; utilisation d'un toxique de référence.

Foreword

This is one of a series of ***recommended methods*** *for measuring and assessing the toxic effect(s) on single species of terrestrial or aquatic 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 which 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 terrestrial or aquatic life 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, soil or similar particulate material, sediment or similar particulate material, effluent, elutriate, leachate, or receiving water. Appendix A lists the biological test methods and supporting guidance documents published to date by Environment Canada's Method Development and Applications Section in Ottawa, ON.

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

AES	atomic emission spectrophotometry
ANOVA	analysis of variance
$CaCl_2$	calcium chloride
$CaCO_3$	calcium carbonate
$Ca(OH)_2$	calcium hydroxide
CCME	Canadian Council of Ministers of the Environment
cm	centimetre(s)
CV	coefficient of variation
DDT	dichloro-diphenyl-trichloroethane
diam	diameter
ERA	ecological risk assessment
ES	effect size
g	gram(s)
h	hour(s)
HCl	hydrochloric acid
HNO_3	nitric acid
HPLC	high pressure liquid chromatography
H_2O	water
H_o	null hypothesis
ICAP	inductively coupled argon plasma
ICp	inhibiting concentration for a (specified) percent effect
KCl	potassium chloride
kg	kilogram(s)
L	litre(s)
LAS	linear alkyl benzene sulphonates
LC50	median lethal concentration
LOEC	lowest-observed-effect concentration
LSD	least significant difference
m	metre(s)
M	mole(s) (concentration)
mg	milligram(s)
mL	millilitre(s)
mm	millimetre(s)
mS	millisiemens
MW	molecular weight
n	sample size
NH_4	ammonium
nm	nanometre(s)
NOEC	no-observed-effect concentration
OM	organic matter
P	probability
PACs	polycyclic aromatic compounds
PAHs	polycyclic aromatic hydrocarbons
PAO	post antennal organ
SD	standard deviation
s	second
sp.	species (singular)
spp.	species (plural)
t	time
TOC	total organic carbon
TM (^{TM})	Trade Mark
v:m	volume-to-mass
v:v	volume-to-volume
WHC	water-holding capacity
wt	weight
wt:wt	weight-to-weight
°C	degree(s) Celsius
α	level of statistical significance (alpha)
µg	microgram(s)
µm	micrometre(s)
µmhos	micromhos
µmol	micromole(s)
>	greater than
<	less than
≥	greater than or equal to
≤	less than or equal to
%	percentage or percent
=	equals
+	plus
−	minus
±	plus or minus
×	times
÷	divided by
/	per; alternatively, “or” (e.g., survival/reproduction)
≅	approximately equal to
~	approximately

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.

Adult (springtail) is a Collembola that is sexually mature. (See also *juvenile*.)

Anal spines are chitinous structures extending from the anal segment of the Collembola.

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

Collembola refers to springtails which are members of the family Collembola.

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 micromhos per centimetre (µmhos/cm) or as millisiemens per metre (mS/m); 1 mS/m = 10 µmhos/cm.

Culture, as a noun, means 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.

Dentes (Dens, singular) is the pair of structures protruding from the manubrium (i.e., two arms forming the distal part of the furca).

Eclosion refers to hatching or escape of an insect larva (i.e., springtail) from its egg.

Ecological risk assessment (ERA) is the process of identifying and quantifying *risks* to nonhuman organisms and determining the acceptability of those risks.

Empodium is a small appendage on the pretarsus opposite the claw.

Euedaphic means permanent soil dwellers and refers to those species that live within the interstitial spaces of the soil. (See also *hemiedaphic*.)

Fungal hyphae is the long, slender, usually branched filaments of fungal mycelium.

Furca or jumping organ, evolved through the basal fusion of a pair of appendages on the fourth abdominal segment, and is capable of propelling some springtails many times their own body length in a fraction of a second. It evolved as an escape mechanism to avoid predators. Soil-dwelling species have reduced furca or have lost the structure entirely.

Hemiedaphic means living in the superficial soil layers and leaf litter. (See also *euedaphic*.)

Hormesis is an observed stimulation of performance (e.g., reproduction) among organisms, compared to the control organisms, at low concentrations in a toxicity test.

Instar refers to a stage of an insect or other arthropod between molts.

Juvenile (springtail) is a collembola that is sexually immature. (See also *adult*.)

Lamella refers to a thin sheet or plate of tissue.

Lux is a unit of illumination based on units per square metre. One lux = 0.0929 foot-candles and one foot-candle = 10.76 lux. For conversion of lux to quantal flux [µmol/(m^2 · s)], the spectral quality of 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 400–700 nm. The relationship between quantal flux and lux or foot-candles is highly variable and depends on the light source, the light meter used, the geometrical arrangement, and the possibilities of reflections (see ASTM, 1999a). Approximate conversions between quantal flux and lux, however, are:

- for cool-white fluorescent light: 1 lux ≅ 0.014 µmol/(m^2 · s);
- for full-spectrum fluorescent light (e.g., Vita-Lite® by Duro-Test®): 1 lux ≅ 0.016 µmol/(m^2 · s); and
- for incandescent light: 1 lux ≅ 0.019 µmol/(m^2 · s) (Deitzer, 1994; Sager and McFarlane, 1997).

Manubrium refers to the basal part of the furca.

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 soil quality variables, or the collection and testing of soil samples for toxicity.

Mucro is the hook-like (i.e., modified claw) structure on the ends of each of the dens. Springtails use these structures to push or hook against the ground, providing the leverage to enable them to jump.

Mycorrhizae is symbiotic association of the mycelium of a fungus with the roots of certain plants.

Ocelli is one of the types of photoreceptor organs in animals. Also called "simple eyes", Ocelli are miniature eyes, capable of just sensing light but not of distinguishing its direction.

Papillae are protuberances on the cuticle of Collembola that serve a sensory function.

Parthenogenetic means asexual, and refers to organisms in which females lay unfertilized eggs that develop into viable offspring and males are completely absent from the population.

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 0–14, 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 is the duration of illumination and darkness within a 24-h period.

Pollution is the addition of a substance or material, or a form of energy such as heat, to some component of the environment, in such an amount as to cause a discernible change that is deleterious to some organism(s) or to some human use of the environment. Some national and international agencies have formal definitions of pollution, which should be honoured in the appropriate contexts.

Post antennal organ (PAO) is dorsally located on the head at the base (posteriorally) of the antennae and it is believed to have an olfactory function.

Pretreatment means treatment of a sample of soil, or portion thereof, before exposure of the test organisms.

Progeny means the young or offspring (i.e., immediate descendants) of sexually mature (adult) springtails.

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

Pseudocelli are small areas of thin cuticle through which defensive fluid can be extruded. Pseudocelli can be distributed over the entire surface of the body including appendages. They are made from epicuticle and the glands located below each one are composed of secretory cells in direct contact with the haemolymph. The defensive fluid secreted by the pseudocelli will repel predators and other Collembola.

Quality assurance (QA) is a program within a laboratory, intended to provide precise and accurate results in scientific and technical work. It includes selection of proper procedures, sample collection, selection of limits, evaluation of data, *quality control*, and qualifications and training of personnel.

Quality control (QC) consists of specific actions within the program of *quality assurance*. It includes standardization, calibration, replication, control samples, and statistical estimates of limits for the data.

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.

Remediation is the management of a contaminated site to prevent, minimize, or mitigate damage to human health or the environment. *Remediation* can include both direct physical actions (e.g., removal, destruction, and containment of toxic substances) and institutional controls (e.g., zoning designations or orders).

Risk is the probability or likelihood that an adverse effect will occur.

Risk assessment – see *Ecological risk assessment*.

Setae are slender, usually rigid, bristles, hair, or spines distributed in characteristic patterns on the exoskeleton which function as sensory receptors or in locomotion.

Spermatophore is a capsule or compact mass of spermatozoa extruded by the males of certain invertebrates and directly transferred to the reproductive parts of the female.

Ventral tube is comprised of eversible sacs derived from a pair of appendages on the first abdominal segment. It is an organ that is important in fluid balance and as a means of adhering to surfaces.

Terms for Test Materials or Substances

Artificial soil is a laboratory-formulated soil, prepared to simulate a natural soil using a specific ratio of natural constituents of sand, clay, and peat. Artificial soil may be used as a *negative control soil*, and as a diluent to prepare multiple concentrations of *site soil(s)* or *chemical-spiked soil(s)*.

Batch means the total amount of a particular *test soil* (or specific concentration thereof) prepared for each treatment (concentration) in a test. A batch is any hydrated *test soil* ready for separation into replicates.

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 soil or water.

Chemical-spiked soil is natural or *artificial soil* (usually *negative control soil, reference soil*, or other *clean* soil) to which one or more chemicals or chemical products have been added, and mixed thoroughly to evenly distribute the substance(s) throughout the soil at a specific concentration to form a *batch* for use in a soil toxicity test. (See also *spiked soil*.)

Clean soil is soil that does not contain concentrations of any substance(s) or material(s) causing discernible toxic effects to the test organisms.

Concentration means, for this biological test method, the ratio of the weight of test substance of material to the weight of soil, and is frequently expressed as the weight of test substance or material per kg of dry soil (mg/kg). Concentration might also be expressed as a percentage of the test substance (e.g., *contaminated* site soil) or material per dry weight of soil.

Contaminant is a substance or material that is present in a natural system, or present at increased concentration, often because of some direct or indirect human activity. The term is frequently applied to substances or materials that are present at concentrations that have the potential to cause adverse biological effects.

Contaminated (soil) means (soil) containing chemical substances or materials at concentrations that pose a known or potential threat to environmental or human health.

Control is a treatment in an investigation or study that duplicates all the conditions and factors that might affect 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. The control is used as a check for the absence of toxicity due to basic test conditions such as temperature, health of test organisms, or effects due to their handling. Control is synonymous with *negative control*, unless indicated otherwise.

Control soil – see *negative control soil*.

Definitive (soil toxicity test) means decisive (as opposed to a preliminary, range-finding test). [See also *range-finding (test)*.]

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

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

Fertility (of soil) refers to the potential of a soil to supply nutrient elements in the amounts, forms, and proportions required for optimal plant growth. Soil fertility is measured directly in terms of the ions and compounds important for plant nutrition. The fundamental components of fertility are the essential nutrients (macronutrients including C, H, O, N, P, K, Ca, Mg, S and micronutrients including Fe, Mn, Mo, B, Cu, Zn, and Cl). Indirectly, soil fertility is measured by demonstrating its productivity (i.e., the capacity of the soil to produce plants that supply essential food and fibre; Hausenbuiller, 1985).

Hydration water means water used to hydrate test soils, to create a specific *moisture content* suitable for the test organisms. The water used for hydration is normally *test water*, and is frequently de-ionized or distilled water, reverse-osmosis water, or de-chlorinated tap water. Depending on study design and intent, a surface water or groundwater from the *site* might be used instead of de-ionized or distilled water for the hydration of each test soil (including negative control soil). (See also *test water, de-ionized water,* and *distilled water.*)

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

Moisture content is the percentage of water in a sample of test soil, based on its wet or dry mass. It is determined by measuring both the wet and dry weights of a subsample of the soil. The soil's moisture content is then calculated and expressed on a dry-weight basis, by dividing the mass of water in the subsample (wet mass - dry mass) by the mass of dry soil, and then multiplying by 100. Units for mass (i.e., g or mg) must be the same in each instance.

Negative control (see *control*).

Negative control soil is *clean* soil that does not contain concentrations of one or more contaminants that could affect the survival or reproduction of the test organisms. Negative control soil might be natural soil from an uncontaminated *site*, or artificial (formulated) soil. This soil must contain no added test material or substance, and must enable acceptable survival and performance of the test organisms during the test. The use of negative control soil provides a basis for interpreting data derived from toxicity tests using test soil(s) and gives information about the state of health (i.e., quality) of the test individuals coming from a culture.

Organic matter (OM) in soil consists primarily of plant and animal residues, at different stages of decomposition, including soil humus. The accumulation of OM within soil is a balance between the return or addition of plant and animal residues and their subsequent loss due to the decay of these residues by soil micro-organisms. For most types of soil, the following equation (from AESA, 2001) is suitable for estimating the total OM content of soil from *total organic carbon* (TOC) measurements: % OM = % TOC × 1.78. (See also *total organic carbon.*)

Positive control soil is *contaminated* soil that contains concentrations of one or more contaminants that adversely affect the survival and reproduction of the test organisms using the biological test method defined herein. Positive control soil might be used as a *reference toxicant* to assess the sensitivity of the test organisms at the time the test material or substance is evaluated, and to determine the precision of results obtained by the laboratory for that reference toxicant.

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

Range-finding (test) means a preliminary soil toxicity test, performed to provide an initial indication of the toxicity of the test material under defined conditions and to assist in choosing the range of concentrations to be used in a definitive multi-concentration test. [See also *definitive (soil toxicity test)*.]

Reference soil is typically *clean* field-collected soil or formulated (artificial) soil, that is selected for use in a particular toxicity test together with a *negative control soil* and one or more samples of *test soil*. The test soil might be either field-collected *site soil* that is *contaminated* or potentially so, or *chemical-spiked soil*. Reference soil used in a test frequently exhibits physicochemical properties (e.g., *texture*, compactness, *total organic carbon* content, pH) closely matching those of the test soil sample(s), except that it is free from the source of contamination being assessed. In tests involving samples of *site soil*, one or more samples of reference soil are often selected from the general location of test soil sampling, and thus might be subject to other sources of contamination aside from the one(s) being studied. Reference soil is used to describe matrix effects in the test, and may also be used as a diluent to prepare concentrations of the test soil. In tests involving chemical-spiked soil, one or more samples of artificial (formulated) soil with differing physicochemical characteristics might be chosen to investigate the influence of certain soil properties (e.g., soil texture, or percent organic matter) on the toxicity of a chemical mixed in each of these soil types. (See also *negative control soil*, *site soil*, *test soil*, *clean*, *artificial soil*, and *chemical-spiked soil.*)

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

Reference toxicity test is a test conducted using a *reference toxicant* in conjunction with a soil toxicity test, to appraise the sensitivity of the organisms and 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 and should be investigated as to the cause. A reference toxicity test with springtails is performed as a *spiked-soil* test, using a standard chemical.

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

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

Site soil is a field-collected sample of soil, taken from a location thought to be *contaminated* with one or more chemicals, and intended for use in the toxicity test with springtails. In some instances, the term includes *reference soil* or *negative control soil* from a site.

Soil is whole, intact material representative of the terrestrial environment, that has had minimal manipulation following collection or formulation. In the natural environment, it is formed by the physical, chemical, and biological weathering of rocks and the decomposition and recycling of nutrients from *organic matter* originating from plant and animal life. Its physicochemical characteristics are influenced by biological activities (e.g., microbial, invertebrate (including springtail), and plants) therein, and by anthropogenic activities.

Solvent control soil is a sample of (usually artificial) soil included in a test involving *chemical-spiked soil*, in which an organic solvent is required to solubilize the test chemical before mixing it in a measured quantity of *negative control soil*. The amount of solvent used when preparing the solvent control soil must contain the same concentration of solubilizing agent as that present in the highest concentration of the test chemical(s) in the sample of chemical-spiked soil to be tested. This concentration of solvent should not adversely affect the performance of springtails during the test. Any test that uses an organic solvent when preparing one or more concentrations of chemical-spiked soil must include a solvent control soil in the test. (See also *artificial soil*, *negative control soil*, and *chemical-spiked soil*.)

Spiked soil is natural or *artificial soil* (usually *negative control soil, reference soil*, or other *clean* soil) to which one or more chemicals, chemical products, or other test substances or materials (e.g., a sample of sludge or drilling mud) have been added in the laboratory, and mixed thoroughly to evenly distribute the substance(s) or material(s) throughout the soil at a specific concentration to form a *batch* for use in a soil toxicity test. (See also *chemical-spiked soil* and *spiking*.)

Spiking refers to the addition of a known amount of chemical(s), chemical product(s), or other test substance(s) or material(s) (e.g., a sample of sludge or drilling mud) to a natural or artificial soil. The substance(s) or material(s) is usually added to *negative control soil, reference soil*, or another *clean* soil, but sometimes to a *contaminated* or potentially contaminated soil. After the addition ("spiking"), the soil is mixed thoroughly. If the added test material is a *site soil*, Environment Canada documents typically do not call this spiking, but instead refer to the manipulation as "dilution", "amendment", or simply "addition". (See also *chemical-spiked soil* and *spiked soil*.)

Stock solution means a concentrated solution of the substance(s) to be tested, following the addition of a measured quantity of this solution to a sample of natural or *artificial soil* and thorough mixing to prepare a *batch* of *chemical-spiked soil*. To prepare the required strength of the stock solution, measured weights or volumes of test chemical(s) or chemical product(s) are added to test water (*de-ionized*, *distilled water*, or equivalent), with or without the inclusion of an organic solvent.

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 soil is a sample of field-collected soil or *chemical-spiked soil* to be evaluated for toxicity to springtails. In some instances, the term also applies to any solid-phase sample or mixture thereof (e.g., *negative control soil*, *positive control soil*, *reference soil*, sludge, drilling mud) used in a soil toxicity test.

Test water is water used to prepare *stock solutions*, rinse test organisms, or rinse glassware and other apparatus used for culturing springtails and for other purposes associated with the biological test method (e.g., to hydrate samples of test soil). *Test water* must be de-ionized or distilled water or better (e.g., reagent-grade water produced by a system of reverse osmosis, carbon, and ion-exchange cartridges). (See also *hydration water*.)

Texture is defined based on a measurement of the percentage by weight of sand, silt, and clay in the mineral fraction of soils. Classification as to texture confers information on the general character

and behaviour of substances in soils, especially when coupled with information on the structural state and *organic matter* content of the soil. Soil texture is determined in the laboratory by measuring the particle-size distribution using a two-step procedure whereby the sand particles (coarse fragments) are initially separated by sieving from the silt and clay particles; followed by separation of the silt and clay particles by their sedimentation in water. Textural classification systems typically refer to groupings of soil based on specific ranges in relative quantities of sand, silt, and clay.

There are three main textural classes:
(i) coarse texture (sands, loamy sands, sandy loams);
(ii) medium texture (loams, silt loams, silts, very fine sandy loams); and
(iii) fine texture (clays, silty clay loams, sandy clay loams, silty clays, and sandy clays).

Further distinction as to texture (e.g., "sandy clay", "silt loam", "loam") can be made based on different classification schemes using the relative amounts of percent sand, percent silt, and percent clay in the soil. The classification system used to define soil texture should be reported since these systems differ from country to country. (Hausenbuiller, 1985; SCWG, 1998; SSS, 1999).

Total organic carbon (TOC) refers to the organic carbon content of soil exclusive of carbon from undecayed plant and animal residues. TOC is determined by dry combustion analysis (ISO, 1995). (See also *organic matter*.)

Water-holding capacity (WHC) refers to the maximum quantity of water that a soil can retain, following complete saturation. It is usually determined gravimetrically, and is generally expressed as the percentage of water (by mass; wt water:wt dry soil) retained in a sample of soil that has been saturated with water.

Statistical and Toxicological Terms

Acute means within a short period (seconds, minutes, hours, or a few days) in relation to the life span of the test organism and is generally used to describe the length of a test or exposure duration.

Acute toxicity is a discernible adverse effect (lethal or sublethal) induced in the test organisms within a short period (usually a few days, and for purposes of this document within 7 or 14 days of exposure to *test soil(s)*.

Battery of toxicity tests is a combination of several toxicity tests, normally using different species of test organisms (e.g., a series of soil toxicity tests using springtails, plants, or earthworms), different biological endpoints (e.g., lethal and various sublethal), and different durations of exposure (e.g., acute and chronic).

Bioassay is a test (= assay) in which the strength or potency of a substance is measured by the response of living organisms. In standard pharmacological usage, a bioassay assesses the unknown potency of a given preparation of a drug, compared to the known potency of a standard preparation. *Toxicity test* is a more specific and preferred term for environmental studies.

Chronic means occurring during 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, and is generally used to describe the length of a test or exposure duration.

Chronic toxicity refers to discernable adverse effects observed during or after relatively long-term exposures to one or more contaminants, that are related to changes in reproduction, growth, metabolism, ability to survive, or other biological variables (e.g., behaviour) being observed.

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 ÷ mean).

Endpoint means the response(s) of the test organism that is measured (e.g., death or number of progeny), or the value(s) that characterize the results of a test (e.g., LC50, IC25).

Environmental toxicology is a branch of *toxicology* with the same general definition. However, the focus is on ecosystems, natural communities, and wild living species, without excluding humans as part of the ecosystems.

Geometric mean is the mean of repeated measurements, calculated logarithmically. 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.

Heteroscedasticity refers herein to data showing heterogeneity of the residuals within a scatter plot (see Figures I.2B and I.2C in Appendix I). This term applies when the variability of the residuals changes significantly with that of the independent variables (i.e., the test concentrations or treatment levels). When performing statistical analyses and assessing residuals (e.g., using Levine's test), for test data demonstrating heteroscedasticity (i.e., non-homogeneity of residuals), there is a significant difference in the variance of residuals across concentrations or treatment levels. (See also *homoscedasticity* and *residual*.)

Homoscedasticity refers herein to data showing homogeneity of the residuals within a scatter plot (see Figure I.2A in Appendix I). This term applies when the variability of the residuals does not change significantly with that of the independent variables (i.e., the test concentrations or treatment levels). When performing statistical analyses and assessing residuals (e.g., using Levine'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. (See also *heteroscedasticity* and *residual*.)

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 inhibition (*p*) compared to the control, in a *quantitative* (continuous) biological measurement such as number of progeny produced by individuals at the end of the test (e.g., IC25 or IC50).

LC50 is the *median lethal concentration*, i.e., the concentration (e.g., % or mg/kg) of substance(s) or material(s) in soil that is estimated to be lethal to 50% of the test organisms. The LC50 and its 95% confidence limits are usually derived by statistical analysis of percent mortalities in five or more test

concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 28-day LC50). Depending on the study objectives, an LCx other than LC50 (e.g., an LC25) might be calculated instead of or in addition to the LC50.

Lethal means causing death by direct action. Death of test organisms is defined as the cessation of all visible signs of movement or other activity indicating life.

LOEC is the *lowest-observed-effect concentration*. This is the lowest concentration of a test substance or material for which a statistically significant adverse effect on the test organisms was observed, relative to the control.

NOEC is the *no-observed-effect concentration*. This is the highest concentration of a test substance or material at which no statistically significant adverse effect on the test organisms was observed, relative to the control.

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

Precision refers to the closeness of repeated measurements of the same quantity to each other, i.e., the degree to which data generated from replicate measurements 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 effects in a toxicity test are those in which each test organism responds or does not respond. For example, an animal might respond by dying in or avoiding a contaminated test soil. Generally, quantal effects are expressed as numerical counts or percentages thereof. (See also *quantitative*.)

Quantitative effects in a toxicity test are those in which the measured effect is continuously variable on a numerical scale. An example would be number of progeny produced at test end. Generally, quantitative effects are determined and expressed as measurements. (See also *quantal*.)

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

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

Residual, in the context of Section 4.8.2.1 and Appendix I, refers to the difference between the predicted estimate (based on the model) and the actual value observed, as determined by subtracting the former from the latter. (See also *heteroscedasticity* and *homoscedasticity*.)

Static describes a toxicity test in which the *test soil* (nor any chemical or chemical product therein) is not renewed or replaced during the test.

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

Sublethal effect is an adverse effect on an organism, resulting from exposure to the concentration or level of contamination below that which directly causes death within the test period.

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

Toxicant is a toxic substance or material.

Toxicity is the inherent potential or capacity of a substance or material to cause adverse effect(s) on living organisms. These effect(s) could result from exposure to either lethal or sublethal concentrations of contaminants in soil.

Toxicity test is a determination of the adverse effect(s) of a substance or material that results from exposure of a group of selected organisms of a particular species (e.g., *Orthonychiurus folsomi* , *Folsomia candida*, or *Folsomia fimetaria*), under defined conditions. A toxicity test involving samples of *test soil* usually measures (a) the proportions of organisms affected (*quantal*), and/or (b) the degree of effect observed (*quantitative* or graded), after exposure of the test organisms to the whole sample (e.g., undiluted *site soil*) or specific concentrations thereof.

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. (See also *environmental toxicology*.)

Treatment refers to a specific *test soil* (e.g., a *site soil*, *reference soil*, or *negative control soil*) from a particular *sampling station*, or a concentration of *chemical-spiked soil* (or a mixture of test soil diluted with *clean* soil) prepared in the laboratory. Test soils representing a particular *treatment* are typically replicated in a toxicity test. (See also *replicate* and *replicate samples*.)

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

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

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Introduction

1.1 Background

The *Method Development and Applications Section* (*MDAS*) of Environment Canada is responsible for the development, standardization, and publication (see Appendix A) of a series of biological test methods for measuring and assessing the *toxic* effect(s) on single species of terrestrial or aquatic organisms, caused by their exposure to samples of test *materials* or *substances* under controlled and defined laboratory conditions. In 1994, MDAS, the Canadian Association of Petroleum Producers (CAPP), and the federal Program for Energy Research and Development (PERD) initiated a multi-year program to research, develop, validate, and publish a number of standardized biological test methods for measuring the *toxicity* of samples of *contaminated* or potentially-contaminated *soil*, using appropriate species of terrestrial test organisms. The goal was to develop biological test methods applicable to diverse types of Canadian soils using terrestrial species that were representative of Canadian soil ecosystems. The initial phase of this multi-year program involved a comprehensive review of existing biological test methods used internationally to evaluate the toxicity of contaminated soils to plants and soil invertebrates. The resulting report recommended that Environment Canada support the development, standardization, and publication of a number of single-species biological test methods for measuring soil toxicity, including those using springtails (Bonnell Environmental Consulting, 1994). This recommendation was endorsed by both the headquarters and regional offices of Environment Canada (Appendix B) and the Inter-Governmental Environmental Toxicity Group (IGETG) (Appendix C).

Since 1994, several years of research have been completed under the direction of the MDAS on the selection of suitable and sensitive test organisms for measuring soil toxicity to meet Canadian regulatory and *monitoring* requirements, and on the development of appropriate biological test methods. A technical report was produced describing the efforts and findings associated with the development of a *toxicity test* that uses a springtail *(Orthonychiurus folsomi)* for the assessment of *contaminated* soils (Aquaterra Environmental, 1998a). Two other technical reports written concurrently describe tests for assessing the toxicity of soils; specifically, a test for mortality, avoidance behaviour, and reproductive inhibition of earthworms (Aquaterra Environmental 1998b), and a test for adverse effects of contaminated soil on the survival and growth of terrestrial plants (Aquaterra Environmental, 1998c).

Numerous soil toxicity tests have been coordinated or supported by Environment Canada, using various species of springtails *(Orthonychiurus folsomi*, *Folsomia candida*, and *Folsomia fimetaria)* exposed to samples of *clean* soil and soils contaminated with pesticides, metals, petrochemical wastes, or prospective *reference toxicants*. These studies (Aquaterra Environmental, 1998a; Stephenson *et al.*, 1999a, b, 2000a; Aquaterra Environmental and ESG, 2000; ESG, 2000, 2001, 2002; ESG and Aquaterra Environmental, 2002, 2003; Becker-van Slooten *et al*., 2003, 2005; Stämpfli *et al*., 2005; EC, 2007a) focussed on the development and standardization of a biological test method for determining the *lethal* or *sublethal* toxicity of samples of contaminated soil to Collembola. Based on the results of these studies, together with the findings of a series on interlaboratory

method validation studies (EC, 2007b); Environment Canada proceeded with the preparation and finalization of a biological test method for conducting soil toxicity tests that measure the survival and reproduction of three species of springtails *(Orthonychiurus folsomi*, *Folsomia candida*, and *Folsomia fimetaria),* as described in this report.

A Scientific Advisory Group (see Appendix D) of international experts experienced with the design and implementation of soil toxicity tests using springtails provided key references which were reviewed and considered as part of this undertaking. These individuals also served actively in providing a critical peer review of two drafts of this methodology document. A larger group of knowledgeable persons (see Acknowledgements) provided further review comments in response to the final draft preceding this publication. The experience of the international scientific community when performing similar soil toxicity tests using springtails (see Appendices E and F) was relied on heavily when preparing this biological test method.

Detailed procedures and conditions for preparing and performing this biological test method are defined herein. Universal procedures for preparing and conducting soil toxicity tests using selected species of springtails (i.e., *Orthonychiurus folsomi*, *Folsomia candida*, or *Folsomia fimetaria*) are described. Guidance is also provided for specific sets of conditions and procedures that are required or recommended when using this biological test method for evaluating different types of *substances* or *materials* (e.g., samples of field-collected soil or similar particulate waste, or samples of one or more *chemicals* or chemical *products* experimentally mixed into or placed in contact with natural or formulated soil). The biological *endpoints* for this method are: (a) survival (mortality), and (b) reproductive success measured at the end of the test.

The flowchart in Figure 1 illustrates the universal topics covered herein, and lists topics specific to testing samples of field-collected soil, similar particulate waste (e.g., sludge, drilling mud, or dredged material), or soil spiked experimentally with chemical(s) or chemical product(s).

This biological test method is intended for use in evaluating the lethal and sublethal toxicity of samples of material such as the following:

(1) field-collected soil that is *contaminated* or potentially contaminated;

(2) soils under consideration for removal and disposal or *remediation* treatment;

(3) soils that have undergone remediation treatment;

(4) dredged material destined or under consideration for land disposal after dewatering;

(5) industrial or municipal sludge and similar particulate wastes that might be deposited on land; and

(6) *clean* or contaminated soil (natural or *artificial*), spiked with one or more chemicals or chemical products (e.g., for *risk assessment* of new or current-use chemicals).

In formulating this biological test method, an attempt has been made to balance scientific, practical, and cost considerations, and to ensure that the results will be sufficiently precise for the majority of situations in which they will be applied. It is assumed that the user has a certain degree of familiarity with soil toxicity tests. Explicit instructions that might be required in a regulatory *protocol* are not provided in this report, although it is intended as a guidance document useful for that and other applications.

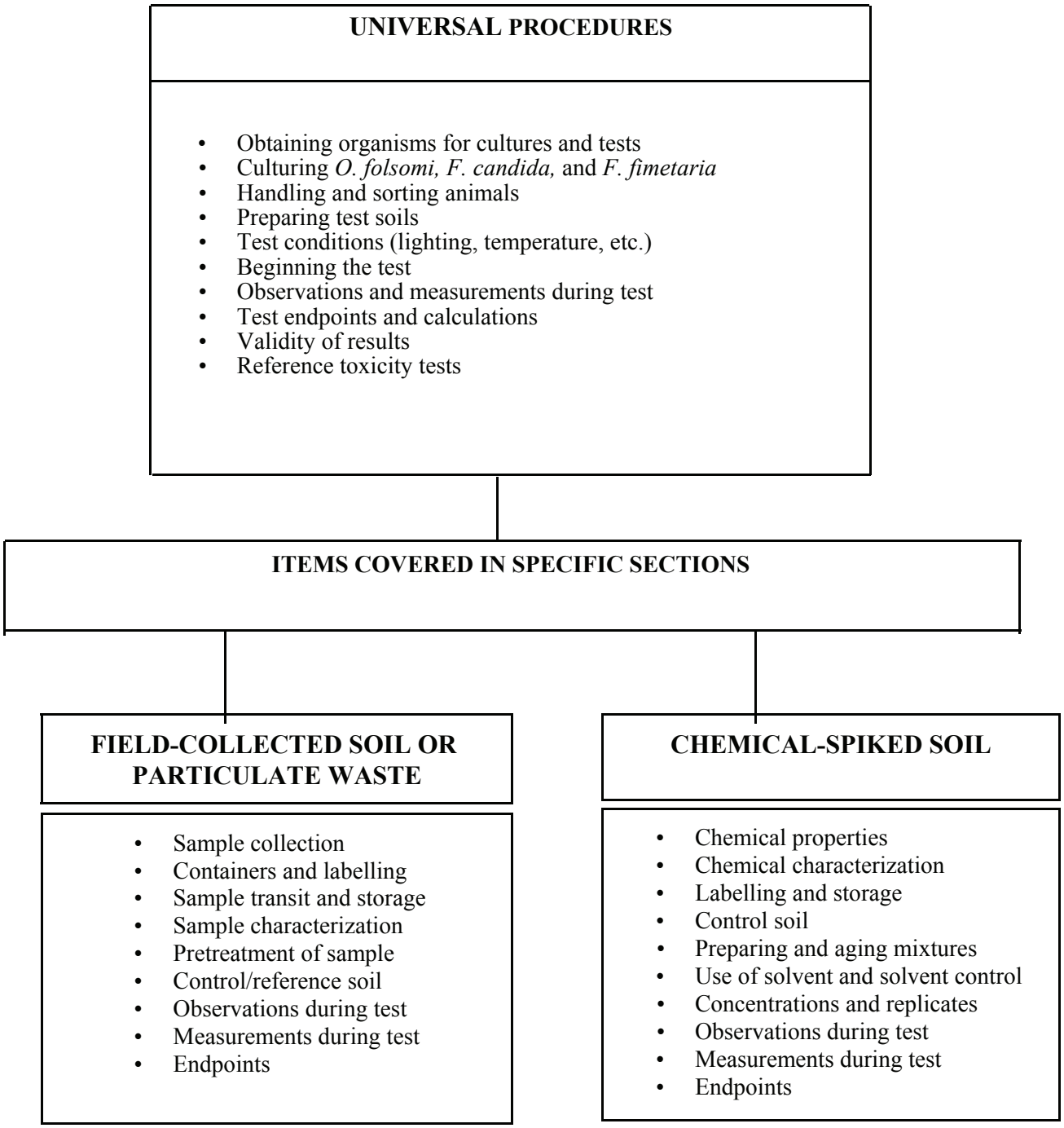

Figure 1 Considerations for Preparing and Performing Soil Toxicity Tests Using Springtails and Various Types of Test Materials or Substances

For guidance on the implementation of this and other biological test methods, and on the interpretation and application of endpoint data for soil toxicity, the reader should consult Sections 4.12, 5.5, and 5.6.4 in Environment Canada's *"Guidance Document on Application and Interpretation of Single-Species Tests in Environmental Toxicology"* (EC, 1999).

1.2 Identification, Distribution, and Life History of Orthonychiurus folsomi, Folsomia candida, *and* Folsomia fimetaria

The test species to be used for the biological test method described herein *(*i.e., *Orthonychiurus folsomi, Folsomia candida,* and *Folsomia fimetaria)* belong to the class Collembola (phylum, Arthropoda; subphylum, Pancrustacea; superclass, Hexapoda). The Collembola, commonly known as springtails, are currently considered to be a monophyletic (i.e., evolved from a single common ancestor) class of the phylum Arthropoda (Hopkin, 2002; Bellinger *et al.,* 2006). They are historically considered to be an order within the class Insecta; however, their position relative to other arthropods is subject to much debate and, based on modern theories of evolution and advancement in molecular phylogeny, their placement is yet to be settled (Hopkin, 1997, 2002). Collembola are the most abundant and widely occurring arthropods in terrestrial ecosystems and are ubiquitous to the wide variety of soil types occurring in Canada. Definitive information regarding the identification, systematics, distribution, biology, physiology, and life history of springtails, including *Orthonychiurus folsomi, Folsomia candida*, and *Folsomia fimetaria* can be found in several publications and Web sites, including: Hopkin, 1997; Bellinger *et al.,* 2006 (http://www.collembola.org); Fountain and Hopkin, 2005; and Hopkin, 2006 (http://www.stevehopkin.co.uk).
Collembola are apterygote (wingless) soil invertebrates. The basic body parts of the Collembola species to be used in this test method are illustrated in Figure 2. The Collembolan body can be divided into three main parts: (i) the head, which bears a pair of antennae, a pair of eyes (if present), and mouthparts, which are held inside the head capsule; (ii) the thorax, which consists of three segments, each bearing a pair of legs; and (iii) the abdomen, which is comprised of six segments. In several species, some of the abdominal segments are fused making it difficult to distinguish them (Hopkin, 1997; Bellinger *et al.*, 2006). The *furca* or the springing organ is what gives the Collembola their common name of springtails. If present (i.e., the furca is absent or has become a vestigial structure in some species confined to the soil), it is located on the ventral side of the fourth abdominal segment and is usually folded under the body. The furca originated from a pair of appendages, which fused basally to form the *manubrium*. The two distal parts remained separate and developed into a pair of structures called *dentes* (singlular, dens). On the end of each of these is a modified claw called a *mucro*. The springtails use their mucros to push or hook against the ground, providing the leverage to enable them to jump (Hopkin, 1997).
All Collembola have a *ventral tube* (a pair of thin-walled, closely apposed, eversible vessicles on the ventral side of the first abdominal segment) that plays an important role in fluid exchange with the external environment (i.e., the regulation of water and salt content) (Rundgren and van Gestel, 1998; Hopkin, 2000; Fountain and Hopkin, 2005), and which also plays an important role in the uptake of *toxicants* dissolved in porewater (Lock and Janssen, 2003). The ventral tube can also function as a sticky appendage to enable springtails to adhere to slippery surfaces (Hopkin, 2002).

Springtails occupy a key position in the soil food web being consumers of fungi, detritus, nematodes, and bacteria (Laskowski *et al.,* 1998; Lee and Widden, 1996). They are also one of the important prey groups for generalist invertebrate predators in agro-ecosystems such as mites,

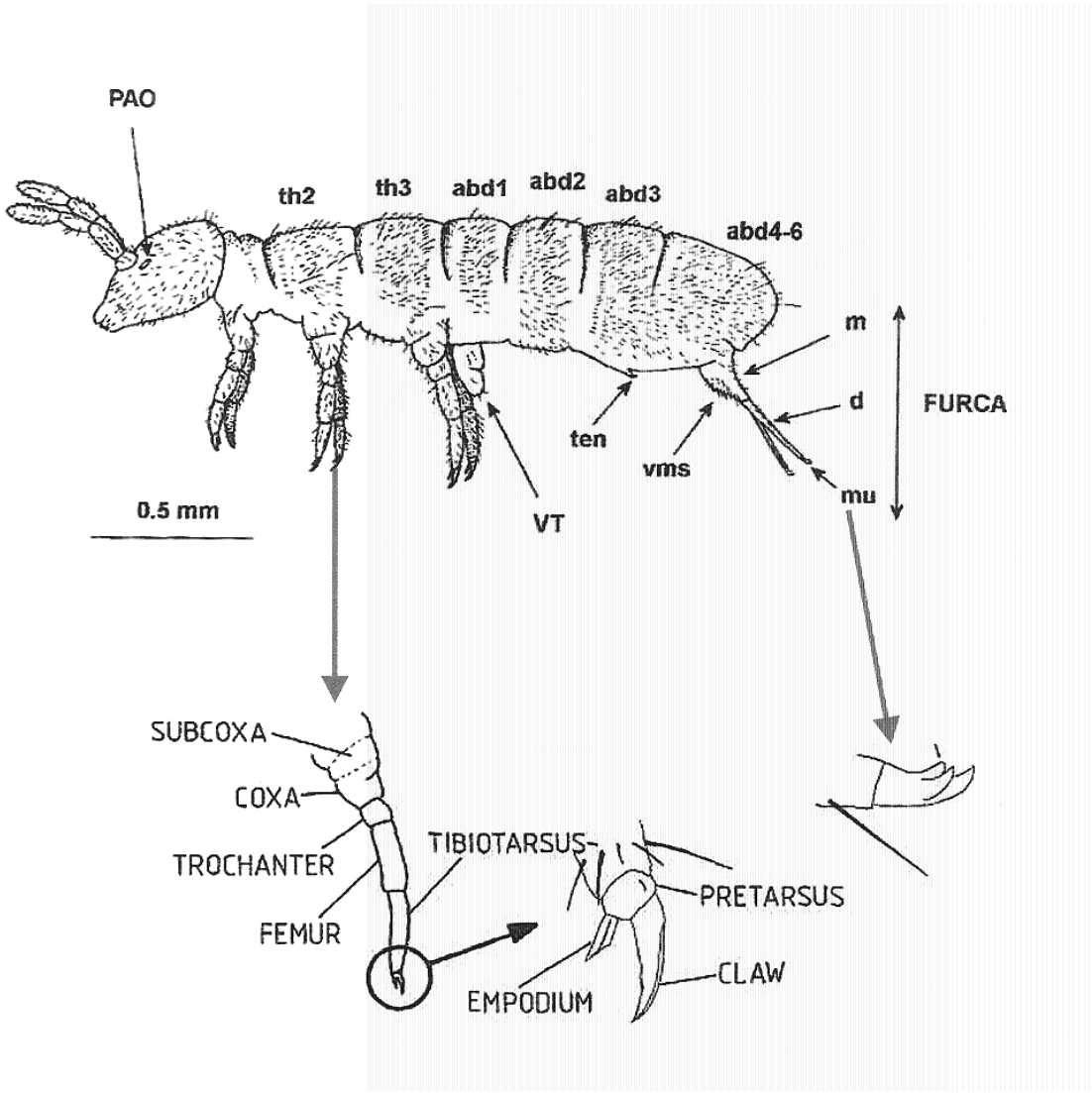

Figure 2 Adult Female *Folsomia candida*

This figure illustrates the basic body parts of all three species described in this test method document. The furca is normally held underneath the abdomen by the tenaculum (ten). The first thoracic segment is reduced dorsally compared with the second (th2) and third (th3) and the last three abdominal segments (abd 4–6) are fused together. Other key body parts include: d, dens; m, manubrium; mu, mucro; PAO, post-antennal organ; vms, ventral manubrial setae; VT ventral tube. (Reproduced from Fountain and Hopkin, 2005, Figure 1 and Hopkin, 1997, Figure 4.1, with permission from S. Hopkin).

centipedes, spiders, carabidae, and rove beetles (Bilde *et al.,* 2000; OECD 2005). Collembola contribute to decomposition and respiration processes in soil, mainly through feeding on *fungal hyphae* (Hopkin, 2000), although their role in humus formation is not well known. In soil, they have been shown to influence the growth of *mycorrhizae* and the control of fungal diseases of some plants (Laskowski *et al.,* 1998; Hopkin, 2000; Fountain and Hopkin, 2005). In acidic forest soils they may be the most important invertebrates as earthworms and diplopods are absent (OECD, 2005). Collembola population densities of $10^5/m^2$ are commonly observed in soil and leaf litter layers under favourable conditions (OECD, 2005). Springtails are important members of the soil fauna and are appropriate organisms for use in the assessment of potentially *toxic* soils, and compared to soft-bodied invertebrates (e.g., earthworms), the Collembola might represent organisms with a different route (or at least rate) of exposure (OECD, 2005).

1.2.1 Orthonychiurus folsomi

Orthonychiurus folsomi Schäffer 1900 *(*formerly identified as *Onychiurus folsomi)* belongs to the family Onychiuridae:

- class, Collembola;
- order, Poduromorpha;
- superfamily, Onychiuroidea;
- family, Onychiuridae;
- subfamily, Onychiurinae

(Bellinger *et al*., 2006). *O. folsomi* is a small, blind, poorly pigmented, *euedaphic* springtail that occupies the interstitial spaces between soil particles, or under stones and rotting wood on the soil surface. *O. folsomi* have several characteristics that are typical of those species that live permanently in the interstitial spaces in soil. These characteristics allow greater access to habitat space and enhance movement within soils (Kamplichler and Hauser, 1993) and include: lack of a *furca*, lack of eyes, pale white integument, elongate body (up to 1.9 mm in length) with rounded abdomen, downward-pointing mouthparts, and the absence of *anal spines.*

Diagnostic features of *O. folsomi* include:

- absence of *ocelli* (eye lenses);
- a complex elliptical *post antennal organ* (PAO) with 10–12 complex vesicles;
- the absence of *anal spines*;
- a dorsal sensory organ on the third antennal segment with four *papillae*;
- an inner unguis with a small tooth;
- an unguiculus slightly shorter than its unguis and without a *lamella*; and
- a *ventral tube* in the male consisting of four modified *setae* on the second abdominal segment.

The tibiotarsi of the legs bear nine distal setae and the *empodium* is long and filamentous reaching the same length as the claw (Figure 2). *Pseudocelli* are absent from the first thoracic segment, and form a dorsal pattern of 32/022/33342(or 3) and a ventral pattern of 2/010/0101 (Hopkin, 2006).[1] Pseudocelli are defensive pores (i.e., small areas of thin cuticle)

[1] Members of the family Onychiuridae possess numerous circular structures on the head, thorax, and abdomen called *pseudocelli*. The number and distribution of these pseudocelli have been used extensively by taxonomists for separating and identifying different species of onychiurids. The pseudocelli of *O. folsomi* form a dorsal pattern of 32/022/33342 (or 3). This formula describes the number and pattern of pseudocelli on each body segment (i.e., head/thoracic segments/ abdominal segments). *O. folsomi*, therefore, has a row of three, and a second row of two pseudocelli on the head, no pseudocelli on the first thoracic segment, and two pseudocelli on each of the second and third thoracic segments. They have three pseudocelli on each of the first three abdominal segments, followed by four and two (or three) pseudocelli on the 4th and 5th abdominal segments, respectively (Hopkin, 1997). Like the dorsal side, the ventral side of the organism has a distinct number and pattern of pseudocelli. In the case of *O. folsomi*, the ventral pattern is 2/010/0101 (Hopkin, 2006).

from which a fluid is extruded as a defense mechanism in response to perceived threats (e.g., predation).

Orthonychiurus folsomi is a species common in soil environments of North America. *O. folsomi* is a detritivore, playing an important functional role in nutrient cycling in soils. It is a sexually reproducing species with indirect sperm transfer. The sperm are produced from paired testes and ejaculated from a simple genital opening in a *spermatophore*, which is deposited on the substrate, or placed directly onto the female. Females have paired ovaries and the eggs are laid singly, but often in clumps or clutches. Conspicuous sexual dimorphism is rare and it is difficult to distinguish males from females. The females are generally slightly larger, especially if they are fecund. The organisms can be sexed by examining the genital plate but this requires high magnification. Subtle secondary sex characteristics can sometimes be used to distinguish males from females. For example, the setae on the males might be marginally shorter in comparison to the female and males occasionally have extra spines on their legs.

Snider (1983) conducted a study on the oviposition, egg development, and fecundity of *O. folsomi.* The author found that temperature affected the development time of eggs, in that the time to *eclosion* decreased with increased temperature. At 15 and 21 °C, eggs hatched in 21 and 14 days, respectively, and at 27 °C this time was reduced to 11 days. At 15 and 21 °C, time to eclosion was least variable and egg viability was highest. There was a shorter time to the onset of egg laying at 21 °C (four weeks), relative to 15 °C (five weeks). Egg mass size varied between 15 and 45 eggs at 15 °C, and between 12 and 36 eggs at 21 °C.

Snider (1983) also found that crowding negatively affected fecundity (i.e., there were four times more eggs in small *cultures* than in large ones) and that paired breeding was the most efficient technique for breeding. Aquaterra Environmental's results (1998a) differed from those of Snider in that cultures at greater population densities were more productive than cultures containing fewer individuals.

1.2.2 Folsomia candida

Folsomia candida Willem 1902, also known as the "compost" springtail (Römbke *et al.*, 2006) is among the most intensively studied of all species of Collembola (Hopkin, 1997). It belongs to the family Isotomidae:

- class, Collembola;
- order, Entomobryomorpha;
- superfamily, Isotomoidea;
- family, Isotomidae;
- subfamily, Proisotominae

(Bellinger *et al.,* 2006). *F. candida* resembles *O. folsomi* in that it is unpigmented, eyeless, and has no *anal spines*. Unlike *O. folsomi, F. candida* is *parthenogenetic* (i.e., asexual). Females lay unfertilized eggs that develop into viable offspring, and males are completely absent from the population. *F. candida* is *hemiedaphic* in nature (Schrader *et al.,* 1997) and possesses a well-developed *furca* (Hopkin, 1997). *Adults* are 1.5–3.0 mm in length at maturity (Fountain and Hopkin, 2005).

Diagnostic features of *F. candida* include:

- the absence of *ocelli*;
- the ratio of the length of the longest *setae* and the tip of the abdomen/length of *mucro* is between about two and four;
- the *manubrium* has numerous stout (16–32) ventral (anterior) setae;
- the dens has 20–40 ventral (anterior) setae and 7–10 dorsal (posterior) setae; and
- the POA is quite broad and is shorter than the width of the first antennal segment (Figure 2) (Fountain and Hopkin, 2005, Hopkin, 2006).

F. candida can be found in most regions of the world except for Africa and India (Hopkin,1997). Its original biogeographical locations are difficult to ascertain since it has been carried all over the world in small portions of soil (Fountain and Hopkin, 2005). In Canada, its distribution is limited mainly to southern areas (Christiansen and Bellinger, 1980). *F. candida* is an indigenous species to forest soils in Ontario and Quebec (Addison, 1996); however, it has low ecological relevance (i.e., it is not abundant) in soils of the Canadian boreal forests and northern lands (Römbke *et al.,* 2006). This species has also been recorded in British Columbia (Skidmore, 1995). *F. candida* is found in a variety of habitats including caves, mines, agricultural systems, soils high in *organic matter,* forests, stream banks, and greenhouses (Fountain and Hopkin, 2005; Hopkin, 2006). *F. candida* is well adapted to dry soil conditions. It has physiological adaptations to avoid dessication and the ability to absorb water vapor (Fountain and Hopkin, 2005). Oxygen uptake is via the cuticle (no tracheae) and they can survive for up to 18 h in completely anaerobic conditions, or under conditions of elevated carbon dioxide (Fountain and Hopkin, 2005).

Like other Collembola, *F. candida* feeds on *fungal hyphae*. In lab microcosm studies, *F. candida* showed a preference for fungi growing on the surfaces of leaf litter rather than on soil particles and there is good evidence that they are an important stimulant of decomposition (Fountain and Hopkin, 2005). The type of fungus on which *F. candida* feeds has been shown to influence their growth and fecundity (i.e., some taxa of fungi are more nutritious than others) (Fountain and Hopkin, 2005).

F. candida can reproduce 12–16 days after hatching (Spahr, 1981). Typically, however, the first egg laying occurs between 17 and 26 days, most often after 21–22 days (K. Becker-van Slooten, personal communication, Laboratory of Environmental Chemistry and Ecotoxicology, ENAC-ISTE, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland, 2006). It has a high reproductive rate, and populations consist exclusively of parthenogenetic females. Eggs are laid in small batches or on top of those already deposited by other females forming aggregates that can be easily seen with the naked eye in laboratory *cultures.* Crowding or high population densities reduce the number of eggs laid (Hopkin 1997; Fountain and Hopkin, 2005). During early *instars*, about 20 eggs are laid in each batch, but this increases to 100 around the 20th instar before declining back to 60 at the 30th instar (Snider, 1973; Hopkin, 1997). *F. candida* moults every 3 to 8 days, with a short reproductive instar (~1.5 days) alternating with longer nonreproductive instars (~8.5 days) (Fountain and Hopkin, 2005). Oviposition occurs every 5 to 10 days, depending on the age of the organism (Snider, 1973). Eggs, which are white, spherical and 80 to 110 µm in diameter, take 7 to 10 days to hatch. The optimal temperature for hatching success is 21 °C, and eggs maintained above 28 °C will fail to hatch (Fountain and Hopkin, 2005). *F. candida* lives for about 140 days (maximum 190 days) and goes through up to 45 moults under laboratory conditions at 21 °C. Longevity is almost doubled and egg production is ~30% greater at 15 °C compared with 21 °C (Hopkin, 1997). *F. candida* are used widely by ecotoxicologists in standard toxicity tests (Hopkin, 1997). These organisms are easily cultured in the laboratory and their biology and ecology is very well known.

1.2.3 Folsomia fimetaria

Like *Folsomia candida, Folsomia fimetaria* Linnaeus 1758 belongs to the family Isotomidae:

- class, Collembola;
- order, Entomobryomorpha;
- superfamily, Isotomoidea;
- family, Isotomidae;
- subfamily, Proisotominae

(Bellinger *et al.,* 2006). Also like *F. candida, F. fimetaria* is a *hemiedaphic* (Kanal, 1994; Folker-Hansen *et al.,* 1996; Bilde *et al.,* 2001), non-pigmented, eyeless species possessing a well developed *furca* (Jensen *et al.,* 2003). *F. fimetaria*, however, is a sexually reproducing species, unlike the *parthenogenetic F. candida* (see Section 1.2.2) and *adults* are smaller (0.8–1.4 mm long).

Diagnostic features of *F. fimetaria* (Figure 2) include:

- the absence of *ocelli*;
- the ratio of the length of the longest *setae* and the tip of the abdomen/length of *mucro* is between 3.2 and 4.0;
- the *manubrium* has 4 + 4 apical ventral (anterior) setae with 3 + 3 in a transverse row and 1 + 1 above them; and
- the dens has 18–24 ventral (anterior) setae and 5 dorsal (posterior) setae.

The PAO is narrow and is about the same length as the width of the first antennal segment (Hopkin, 2006). Discrimination from species of the same genus is not problematic today with the unique position of manubrial seta and other characteristics (Fjellberg, 1980); however, care should be taken to avoid confusion with other white and eyeless members of the same genus like *F. candida, F. lawrencei*, and *F. litsteri* (Krogh, 2004). *F. candida* (see Section 1.2.2) can be misidentified for *F. fimetaria* and vice versa; however, a good characteristic for separating the species is that *F. candida* has 2 + 2 or 3 + 3 setae on the ventral side of the third thoracic segment; these are absent in *F. fimetaria* (Hopkin, 2006).

Folsomia fimetaria is widely distributed and common in several soil types ranging from sandy to loamy soils and from mull to mor soils (OECD, 2005). It has been recorded in agricultural soils all over Europe (Römbke *et al.*, 2006); however, there is little evidence of this species inhabiting boreal forests or northern lands. In Canada, *F. fimetaria* has been found in the Northwest Territories, British Columbia, Alberta, Manitoba, Ontario, New Brunswick, and Newfoundland (Skidmore, 1995).

F. fimetaria has an omnivorous feeding habit, with a diet that includes *fungal hyphae*, bacteria, protozoa, and detritus (OECD, 2005). In farmland soils, it is considered to be an important prey for the beneficial arthropod predators which are recognized for their role of suppressing insect pests. Thus, the presence of *F. fimetaria* may stabilize these populations of beneficial insects at a level that is desirable in integrated and organic farming systems (Laskowski *et al.,* 1998). *F. fimetaria* has shown a high degree of food selectivity, preferring fungi that optimize their growth, survival, and fecundity. This species could even select the optimal food when a fungal species was grown in different soil substrates. The high degree of selectivity corresponding to food quality that was seen in this species might be due to a production of fungal odour that can be detected by the collembolans (Jørgensen *et al.,* 2003).

F. fimetaria is sexually mature after 18 days, when the sixth *instar* has been reached. Sexual differences between males and females are difficult to discern before 20 days after hatching. The males have a more slender body and they are only half the size of the females (Krogh, 2004). *F. fimetaria* has many characteristics desirable for a toxicity test species, including their ease of culturing in sufficient numbers, and they reproduce readily, continuously, and year-round, ensuring the routine availability of test organisms (Riepert and Kula, 1996).

1.3 Historical Use of Springtails in Toxicity Tests

The development of biological test methods for soil toxicity testing lags behind that for other media (e.g., water and sediment) (Bonnell Environmental Consulting, 1994). This delay is

partially due to the fact that research and regulators have focussed on the aquatic environment. Soil systems are more complex than aquatic systems with many problems inherent in its lack of homogeneity. The variety of exposure routes available to investigators (e.g., via pore water, soil vapours, or direct contact with soil particles), coupled with the high cost of running soil toxicity tests, in the past, have led investigators to rely on extrapolations from aquatic test methods to soil-based exposures (Bonnell Environmental Consulting, 1994).

Assessment of soil quality before the 1980s primarily involved the evaluation of the physicochemical properties of soil, and not until the 1980s did the initial use of standardized biological test methods for measuring soil toxicity emerge from agencies responsible for pesticide registration and application [e.g., the United States Environmental Protection Agency (USEPA), and the Office of Pesticides Programs (Holst and Ellanger, 1982)]. Historically, Collembola have been incorporated into a wide range of ecotoxicological assessments. One of the earliest laboratory studies involving Collembola was undertaken by Sheals (1956) who studied the effects of organochlorine compounds on microarthropod communities and screened various species for differences in susceptibility to DDT, using filter paper for the exposure (Wiles and Krogh, 1998). In a later study, Scopes and Lichtenstein (1967) used *F. fimetaria* in an *acute* test, also using the filter paper method of exposure. Thompson and Gore (1972) were among the first to promote the use of *F. candida* as a laboratory test species in their *bioassay* assessments of 29 insecticides. Many laboratory studies followed in the 1970s to 1990s using various species of Collembola, of which four species were used most commonly: *Folsomia candida, Folsomia fimetaria, Onychiurus armatus (Protaphorura armata),* and *Orchesella cincta* (Scott-Fordsmand and Krogh, 2005).

The toxicity of *site soils* became a "new" concern in the mid-1980s, and regulatory programs such as SUPERFUND in the United States, and the National Contaminated Sites Remediation Program (NCSRP) in Canada, were established to address the urgent need for guidance on the assessment and remediation of high-priority contaminated *sites*. Under the NCSRP, a review of existing whole-organism bioassays for soil, freshwater sediment, and fresh water (Keddy *et al.,* 1995) was conducted to lead to the establishment of a suite of tests that could be used immediately for contaminated-site assessment in Canada (Bonnell Environmental Consulting, 1994). Keddy *et al.* (1995) concluded that most of the existing methods or procedures for measuring the toxicity of samples of soil from contaminated sites were inadequate for proper ecotoxicological assessment, and recommended that attempts be made to develop a suite of standardized biological test methods for soil that used test species and conditions applicable to Canadian soil ecosystems. The Canadian Council of Ministers of the Environment (CCME) published a framework for *ecological risk assessment* (ERA) in 1994 (CCME, 1994) which had a subsequent impact on the management of contaminated sites (CCME, 1996, 1997). The ERA approach, which relied on the results of single-species toxicity tests, led to the need to develop reliable, reproducible, and realistic soil toxicity tests with ecologically relevant terrestrial test species for the assessment of *contaminated* site soils (Bonnell Environmental Consulting, 1994). In the late 1990s, biological assessments in the form of toxicity testing were becoming a useful complement to chemical analyses, especially when applied to site-specific *risk assessments.*

In 1998, Wiles and Krogh published a test procedure using three species of Collembola *(Isotoma viridis, Folsomia candida,* and *Folsomia fimetaria)*. The procedures were formatted like an ISO standard since it was the European Union's intention to standardize the method according to the ISO system of test

guidelines (Scott-Fordsmand and Krogh, 2005). The first standardized whole-soil toxicity test using springtails, applicable to both pesticide and non-pesticide exposures in *artificial soil*, was a reproduction test-method published by the International Organization for Standardization (ISO) in 1999. This method describes the use of *Folsomia candida* as the test species, and was developed to assess *chemical-spiked soils* only. In 2005, the National Environmental Research Institute in Denmark released a proposal to the Organization for Economic Cooperation and Development (OECD) for a new test guideline that assesses the effects of chemical-spiked soils on the reproduction of two species of Collembola *(Folsomia fimetaria* and *Folsomia candida)* (OECD, 2005).

Today, Collembola are widely used as test organisms in single-species toxicity tests intended to measure the toxicity of pure chemicals, chemical products, or samples of soil *contaminated* or potentially contaminated with chemicals in the field or (for experimental purposes) in the laboratory. Collembola play a key role in soil functioning and are vital indicators for soil ecotoxicology (Cortet *et al.,* 1999). They are frequently exposed to numerous toxic chemicals in soil such as fertilizers, insecticides, herbicides, and fungicides from agricultural and domestic applications, as well as heavy metals, petroleum hydrocarbons, or other chemicals such as wood preservatives (e.g., pentachlorophenol) or nitroaromatic explosive compounds in contaminated soils. Springtails possess many attributes that make them appropriate organisms for use in the assessment of potentially toxic soils. Their life history characteristics, distribution, and ecological function make them ecologically important (Riepert and Kula, 1996). They are ubiquitous in nature, widely distributed in diverse soil environments, often highly abundant, easily sampled in the field, can be cultured or maintained in the laboratory and have a relatively rapid life-cycle with a high reproductive rate (Scott-Fordsmand and Krogh, 2005). Besides the standard test using earthworms, tests involving Collembola are becoming more routine for testing the effects of chemicals on non-target organisms.

In Canada, the use of Collembola toxicity tests as "ecotoxicological assessment tools" for assessing the toxicity of contaminated or potentially contaminated site soil is also increasing (Aquaterra Environmental, 1998a; Stephenson *et al.,* 1999a, b, 2000a; Aquaterra Environmental and ESG, 2000; ESG 2000, 2001, 2002; ESG and Aquaterra Environmental, 2002, 2003) and results of soil toxicity tests are used to:

(i) derive national soil quality criteria,

(ii) establish site-specific, *risk*-based, cleanup objectives (e.g., remediation targets), and

(iii) assess the efficacy of remediation technologies (Stephenson *et al.,* 2002).

Extensive reviews on the use of springtail toxicity tests as "ecological assessment tools" for appraising the toxicity of *contaminated* or potentially contaminated soils in tiered testing or risk assessments have been carried out by various authors (NERI, 1993; Leon and van Gestel, 1994; Keddy *et al.,* 1995; Römbke *et al.,* 1996; Van Gestel *et al.,* 2001; Achazi, 2002; Lanno, 2003). Other ecotoxicological assessments involving the use of springtails include field monitoring of population trends (e.g., Neuhauser *et al.,* 1989), field bioassays (e.g., Wiles and Frampton, 1996), meso-and microcosm studies (e.g., Addison and Holmes 1995; Addison 1996; Cortet *et al.,* 2003), and a wide variety of laboratory tests (e.g., Crommentuijn *et al.,* 1993; Addison and Holmes, 1995, Martikainen and Krogh, 1999; Fountain and Hopkin, 2001).

A number of diverse laboratory methods have been investigated to measure the effects of specific chemicals or chemical products on springtails. Some of the less "standard"

endpoints reported include: growth (e.g., Folker-Hanset *et al.,* 1996), population growth (e.g., Crommentuijn *et al.,* 1993), bioaccumulation through ingestion of contaminated food, *toxicant* uptake, and body burden (e.g., Janssen *et al.,* 1991; Pedersen *et al.,* 2000; Fountain and Hopkin, 2001; Markweise *et al.,* 2001), and biomarkers (Stämpfli *et al.*, 2002). Test methodology improvements, as well as, the effects of variations on soil characteristics and/or laboratory test conditions have also been investigated and/or reviewed (Sandifer and Hopkin, 1996; 1997; Riepert and Kula, 1996; Smit and Van Gestel, 1996, 1997, 1998; Van Gestel and Van Diepen, 1997; Smit and Van Gestel, 1998; Crouau *et al.*, 1999; Martikainen and Krogh, 1999; Martikainen and Rantalainen, 1999; Lock and Janssen, 2001; Crouau and Cazes, 2003).

Toxic effects resulting from exposure of Collembola to a wide range of environmental *contaminants* have been documented in laboratory studies involving samples of soil spiked or contaminated with:

- **pesticides** (Thompson and Gore, 1972; Tomlin, 1975; Mola *et al.,* 1987; Addison and Holmes, 1995; Addison, 1996; Folker-Hanset *et al.,* 1996; Petersen and Gjelstrup, 1998; Martikainen and Krogh, 1999; ESG and Aquaterra Environmental, 2002; Indinger, 2002; Campiche *et al.,* 2006);

- **metals** (Crommentuijn *et al.,* 1993, 1997; Posthuma and Van Straalen, 1993; Pedersen *et al.*, 1997, 1999; Sandifer and Hopkin, 1996, 1997; Smit and Van Gestel, 1996, 1997, 1998; Van Gestel and Van Diepen, 1997; Scott-Fordsmand *et al.,* 1999; Aquaterra Environmental and ESG, 2000; Pedersen and Van Gestel 2001; Fountain and Hopkin, 2001);

- **petroleum hydrocarbons** (Neuhauser *et al.,* 1989; ESG 2000, 2001, 2003; Van Gestel *et al.,* 2001; Jensen and Sverdrup, 2002; Sverdrup *et al.,* 2002); and

- **other chemicals including reference toxicants** (Aquaterra Environmental, 1998a; Addison and Bright, 2002; Jensen *et al.,* 2003; Becker-van Slooten *et al.,* 2003, 2005; Stämpfli *et al.,* 2005; EC, 2007a).

In addition, database reviews have been summarized in reports discussing trends of Collembola toxicity to various contaminants (Leon and Van Gestel, 1994).

Historically, *Folsomia candida* has been the preferred species for studying the effects of prolonged exposure to contaminants on the survival and reproduction of springtails, due to widespread knowledge and experience in culturing this species, its rapid life cycle, its international distribution, and its frequent use in toxicity tests. The development, growth, and reproductive biology of *F. candida*, under laboratory conditions have been extensively studied and are well documented (Hopkin, 1997; Fountain and Hopkin, 2005). Following a review of the use of this species as a "standard" test organism, Fountain and Hopkin (2005) concluded that, although there has been some criticism toward the field relevance of the ISO test with *F. candida*, this species plays an important role in risk assessment and will continue to be included in the development of new environmental standards (Fountain and Hopkin, 2005).

Results from experiments on *F. candida* cannot, for the most part, be extrapolated to other species of Collembola because of the differences in sensitivity among species (Krogh, 1995; Hopkin 1997). For example, the NOEC for atrazine is 600 μg/g for *F. candida* but only 40 μg/g for *Orchessella cincta* (Badejo and Van Straalen, 1992). For dimethoate and copper, however, no differences in sensitivity between *F. candida*, and *F. fimetaria* were detected (Scott-Fordsmand

and Krogh, 2005) and for boric acid, the differences in sensitivity between these two species is small (K. Becker-van Slooten, personal communication, Laboratory of Environmental Chemistry and Ecotoxicology, ENAC-ISTE, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland, 2006).

Folsomia fimetaria is a relatively new test species for use in sublethal soil ecotoxicity tests. The development of a test using *F. fimetaria* was initiated in Denmark in 1990 while investigating the effects of pesticides (Wiles and Krogh, 1998). Since then, this species has been used for assessing the toxic effects of many different compounds such as copper, nickel, phthalates, linear alkyl benzene sulphonates (LAS), pyrene, dimethoate, polycyclic aromatic hydrocarbons (PAHs), polycyclic aromatic compounds (PACs), veterinary pharmaceutical products, and sewage sludge (Scott-Fordsmand *et al.,* 1997, 1999, 2000; Jensen *et al.*, 2001; Holmstrup and Krogh, 2001; Jensen and Sverdrup, 2002; Folker-Hansen *et al.*, 1996; Fabian and Petersen, 1994; Scott-Fordsmand and Krogh, 2004; Becker-van Slooten *et al.*, 2005; EC, 2007a).

Although the biology and ecological relevance of *Orthonychiurus folsomi* is well known (Snider, 1983 and Section 1.2.1), use of this species in laboratory toxicity testing is relatively unknown, and limited to several Canadian studies (Aquaterra Environmental, 1998a; Stephenson *et al.,* 1999a, b, 2000a; Aquaterra Environmental and ESG, 2000; ESG 2000, 2001, 2002; Addison and Bright, 2002; ESG and Aquaterra Environmental, 2002, 2003; EC, 2007a).

The methodology documents summarized in Appendices E and F have been used as guidance in developing Environment Canada's standardized biological test method for performing a test that measures the *toxic* effects of prolonged exposure to *chemical-spiked soil* or *site soil* on the survival and reproduction of Collembola. The resulting new biological test method is defined herein.

Test Organisms

2.1 Species and Life Stage

The biological method described herein must be performed using laboratory-cultured *Orthonychiurus folsomi* Schäffer 1900, *Folsomia candida* Willem 1902, or *Folsomia fimetaria* Linnaeus 1758. The identification, distribution, and life history of *O. folsomi, F. candida*, and *F. fimetaria* are summarized in Section 1.2. Species identification must be confirmed and documented by qualified personnel experienced with identifying the intended species (see Section 1.2) of Collembola to be used in the toxicity test. *Cultures* of *O. folsomi, F. candida,* and *F. fimetaria* held for a prolonged period at a testing laboratory should be identified to species at least once every two years. The soil toxicity test described herein must be started using either 28- to 31-day old *O. folsomi,* 10- to 12-day old *F. candida,* or 23- to 26-day old *F. fimetaria*.

2.2 Source

Laboratory-cultured springtails (see Section 2.3) must be used as the source of the test organisms. Sources of *Orthonychiurus folsomi, Folsomia candida,* and *Folsomia fimetaria* for establishing laboratory cultures may be government or private laboratories that are culturing these species of springtails for soil toxicity tests, or a commercial biological supplier.[2]

Breeding stock of *Orthonychiurus folsomi, Folsomia candida*, or *Folsomia fimetaria* can be obtained by contacting the following Canadian source:

Soil Toxicology Laboratory
Environmental Science and Technology Centre
Science and Technology Branch
Environment Canada
335 River Road
Ottawa, Ontario K1A 0H3
Phone: (613) 990-9544

All springtails used in a soil toxicity test must be derived from the same population. Springtails to be used as a source of breeding stock should be transported to the laboratory using a portion of the soil or other substrate to which they are adapted. Breeding stocks are best transported as a mixed-age culture in petri dishes containing the Plaster of Paris™ substrate described in Section 2.3.5[3] or in a small container of soil. Additional quantities of this substrate might be obtained for *acclimation* or culturing purposes, depending on culturing conditions and requirements (Section 2.3). Shipping and transport containers should be insulated to minimize changes in temperature

[2] Investigators might be concerned with the effects of excessive inbreeding of laboratory cultures for those species that reproduce sexually (i.e., *O. folsomi* and *F. fimetaria*), or might wish to use *progeny* produced from organisms that occupied a particular locale. Accordingly, cultures may be established using wild populations or may be genetically enhanced by introducing breeding stock from different sources. If animals are obtained from a wild population, their taxonomy should be confirmed and they or their progeny should be evaluated for sensitivity to *reference toxicant(s)* before being used in toxicity tests. Ideally, any site from which field-collected specimens are taken should be known to be free of any applications or sources of pesticides or fertilizers during the past five years or longer.

[3] The Plaster of Paris™ substrate may loosen from the bottom of the petri dish during transportation; therefore, steps should be taken to prevent the springtails from being crushed between the loosened substrate and the lid of the petri dish. Two pieces of folded paper inserted between the substrate and the lid of the petri dish during transportation should prevent the loosened substrate from damaging the Collembola on the substrate surface (K. Becker-van Slooten, personal communication, Laboratory of Environmental Chemistry and Ecotoxicology, ENAC-ISTE, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland, 2004).

during transit and the temperature should be maintained at ~20 °C. Live organisms should be transported quickly to ensure their prompt (i.e., within 24 h) delivery. Excessive crowding of animals during shipment or transport should be avoided to minimize stress in transit.

Upon arrival at the laboratory, organisms may be held in the substrate (i.e., soil or Plaster of Paris™) used in transit while temperature adjustments are made, or they may be transferred to new culturing substrate (Section 2.3.5). If the nature (including its *texture* and *moisture content*) of the substrate in which springtails were initially held (e.g., by a commercial supplier) or transported differs markedly from that in which they are to be cultured (Section 2.3.5), it is prudent to adapt the springtails to new substrate over several days.

Soil temperature should be adjusted gradually (e.g., ± 3 °C per day) to the exposure temperature to be used during culturing (Section 2.3.4). Guidance for handling Collembola given in Section 2.3.7 should be followed when transferring organisms from an outside source to culture vessels (Section 2.3.2). Other conditions during this interim holding period for *acclimation* of breeding stock or test organisms to laboratory conditions should be as similar as possible to those used for maintaining cultures (Section 2.3).

2.3 Culturing of Orthonychiurus folsomi, Folsomia candida*, and* Folsomia fimetaria

2.3.1 General

General guidance and recommendations for culturing *Orthonychiurus folsomi, Folsomia candida*, and *Folsomia fimetaria* in preparation for soil toxicity tests are provided here. In general, these three species are cultured under the same conditions and procedures. In keeping with the premise *"What might work well for one laboratory might not work as well for another laboratory"* (USEPA, 1994a; EC, 1997a, b, 2001), explicit directions regarding many aspects of culturing, including the choice of culture vessel, number of organisms per vessel, soil-renewal conditions, culturing substrate, and food type and ration, are left to the discretion and experience of laboratory personnel, although guidance and recommendations are provided herein. Performance-based indices[4] are used to evaluate the suitability of the cultured organisms for tests, and the acceptability of the test results. Cultures must have low mortalities to be suitable for use in tests, and the cultured organisms must appear healthy and behave and feed normally (see Section 2.3.9). Additionally, those used as controls in the test must have acceptably low mortality rates and meet all criteria for a valid toxicity test (see Section 4.4). The acceptability of the culture should also be demonstrated by concurrent or ongoing tests using a *reference toxicant* (see Section 4.9). If a culture of organisms fails to meet these criteria, it's cause should be investigated. Care must be taken to ensure that each culture is not cross-contaminated with another Collembola species, since the three species recommended herein are difficult to distinguish from each other. Periodic (e.g., annual) taxanomic checks of the laboratory's cultures are advisable to ensure that the springtail cultures maintained within the laboratory have not been cross-contaminated.

It is the responsibility of the laboratory to demonstrate its ability to obtain consistent, precise results using a *reference toxicant*, when initially setting up to perform soil toxicity tests with cultured *O. folsomi, F. candida*, and *F. fimetaria*. For this purpose, intralaboratory

[4] Performance-based indices include those related to the survival and condition of cultured organisms intended for use in the test (Section 2.3.9); as well as the criteria that must be met by control organisms for a test to be valid (Section 4.4), and those related to the performance of groups of animals in *reference toxicity tests* (Section 4.9).

precision, expressed as a *coefficient of variation* for the respective LC50 data, should be determined by performing five or more tests with different lots (groups) of test organisms from the same source, using the same reference toxicant and identical procedures and conditions for each test (see Section 4.9).

When routinely performing soil toxicity tests with *O. folsomi, F. candida*, or *F. fimetaria*, *reference toxicity tests* should be conducted every two months with the laboratory's cultures, using the conditions and procedures outlined in Section 4.9. If this bimonthly routine is not followed, the performance of individuals from the culture used to start a soil toxicity test should be evaluated in a reference toxicity test conducted concurrently. Additionally, the performance of any cultures that have been established recently using new breeding stock (Section 2.2) should be checked with a reference toxicity test, and the results determined to be acceptable (see Sections 2.3.9 and 4.9) before these cultures are used to provide test organisms.

Cultures of *O. folsomi, F. candida,* and *F. fimetaria* should be observed frequently (e.g., once or twice per week). Ideally, records should be maintained documenting:

- the date a culture is started with adults;
- dates of substrate renewal;
- feeding and watering regime (including type and quantity added on each occasion);
- facility and substrate quality (e.g., air temperature, *photoperiod* and light quality, *pH* of substrate); and
- observations of culture health (e.g., behaviour and appearance of springtails in culture, odour of substrate, location of springtails in the vessel, amount of uneaten food in vessel, presence of fungi).

A summary of the various conditions and procedures described in international methodology documents (Wiles and Krogh, 1998; ISO, 1999; OECD, 2005) for culturing various species of springtails is provided in Appendix E. A checklist of recommended conditions and procedures for culturing *O. folsomi, F. candida,* and *F. fimetaria* to generate organisms for use in soil toxicity tests is given in Table 1.

2.3.2 Facilities and Apparatus

Springtails should be cultured in a controlled-temperature laboratory facility. Equipment for temperature control (i.e., an incubator or a room with constant temperature) should be adequate to maintain temperature within the recommended limits (Section 2.3.4). The culturing area must be isolated from any testing, sample storage, or sample-preparation areas, to avoid contamination from these sources. It must be designed and constructed to prevent contamination of cultures (e.g., elimination of copper or galvanized piping or fixtures that could drip metal-contaminated condensation).

All equipment, vessels, and accessories that might contact the organisms or substrate within the culturing facility must be clean, rinsed as appropriate, and made of nontoxic materials (e.g., glass, Teflon™, type 316 stainless steel, nylon, Nalgene™, porcelain, polyethylene, polypropylene). Toxic materials including copper, zinc, brass, galvanized metal, lead, and natural rubber must not come in contact with this apparatus and equipment, or the culturing substrate or water.

A variety of culture vessels, such as plastic trays or breeding boxes of 1- to 6-litre capacity (e.g., white polystyrene rectangular boxes with dimensions of ~15 × 23 × 8 cm to ~20 × 33 × 11 cm), are suitable for culturing *O. folsomi* and *F. candida*. For *F. candida*, the sides and/or lid should be translucent or transparent, to enable light to contact the surface of the culturing substrate (see Section 2.3.3). Each vessel

Table 1 Checklist of Recommended Conditions and Procedures for Culturing *Orthonychiurus folsomi*, *Folsomia candida*, and *Folsomia fimetaria* to Provide Test Organisms for Use in Soil Toxicity Tests

Source of brood stock for culture	–	adults obtained from a government, private, or commercial culture; identification to species confirmed
Acclimation	–	gradually, for temperature (recommend ≤3 °C/day) and substrate differences upon arrival
Culture vessels	–	for ***O. folsomi*** and ***F. candida***: breeding boxes of ~1– 6 L capacity are suitable (e.g., plastic trays measuring ~15 × 23 × 8 cm to ~20 × 33 × 11 cm), covered with solid or perforated lids; sides and/or lid transparent or translucent to enable light to contact surface of culturing substrate for ***F. candida;*** for ***F. fimetaria***: Polystyrene Petri dishes (10 cm diam × 1.5 cm high), covered
Air temperature	–	daily average, 20 ± 2 °C; instantaneous, 20 ± 3 °C
Lighting	–	for ***O. folsomi*** and ***F. fimetaria***: continuous darkness; or incandescent or fluorescent; intensity, 400–800 lux at surface of culture vessel; fixed photoperiod (e.g., 16h L:8h D or 12h L:12h D); for ***F. candida***: incandescent or fluorescent; intensity, 400–800 lux at surface of culture vessel; fixed photoperiod (e.g., 16h L:8h D or 12h L:12h D)
Type of substrate	–	8:1 mixture of Plaster of Paris™ and activated charcoal; recommended minimum depth, 1 cm; for ***O. folsomi***, pie-shaped pieces filter paper coated with Plaster of Paris™ or a very thin layer of artificial soil placed on substrate surface
Hydration of substrate	–	hydrated with test water; moisture content sufficient to keep surface of substrate moist but with no standing water on the surface of the culture vessel
pH of substrate	–	6.0–7.0
Renewal of substrate	–	as required, and at least once every 1–2 months; transfer springtails to fresh breeding vessels manually; mix adults between culture vessels of same species for both ***O. folsomi*** and ***F. fimetaria***
Monitoring culture	–	air temperature of culture facility monitored weekly; pH measured on new batches of Plaster of Paris™
Maintaining culture	–	vessels aerated at least once/week by removing lids from culture vessels for several minutes; moisture level of substrate observed for each culture vessel at time of aeration; add several drops of test water to maintain humidity; record

		condition of culture; maintain loading density of springtails at ~2 to 3 organisms/ cm^2 for ***O. folsomi*** and ***F. candida***, and ~5 to 6 organisms/cm^2 for ***F. fimetaria***; recommend a minimum of 2 females/male for ***O. folsomi*** and ***F. fimetaria***
Feeding	–	granulated dry yeast (e.g., Fleischmann's ™); divided into 2 or 3 piles or sprinkled onto substrate surface; feed twice/week; for ***O. folsomi*** and ***F. candida***: ~100 mg (for 15 × 23 × 8 cm culture vessels); for ***F. fimetaria***: ~10 mg (for 10-cm Petri dishes)
Maintenance of age-synchronized cultures	–	for ***F. candida*** and ***F. fimetaria***: place 200–300 adults from existing culture onto new substrate to stimulate oviposition; feed; monitor daily for eggs; for ***O. folsomi*****, *F. candida*****,** and ***F. fimetaria***: monitor existing cultures for large egg clutches; 7 days after the first egg clutches appear in new culture vessels or large egg clutches appear in existing cultures, transfer egg clusters to hatching vessels (i.e., Petri dish ~10 cm diam. and ≥1 cm high) containing fresh substrate; feed; monitor daily for eclosion; remove unhatched eggs 48 h after appearance of juveniles for ***F. candida*** and 72 h after appearance of juveniles for ***O. folsomi*** and ***F. fimetaria***
Age/size for test	–	for ***O. folsomi***: 28- to 31-day-old adults; for ***F. candida***: 10- to 12-day-old juveniles; for ***F. fimetaria***: 23- to 26-day-old adults
Indices of culture health	–	considered healthy if (1) springtails move actively over the surface of the substrate, and (2) results for reference toxicity tests using springtails from the culture fall within historic warning limits

should have a lid, which can be solid, to minimize drying of the surface substrate and the *risk* of contamination, or perforated (e.g., holes covered with fibreglass mesh screening) to allow air exchange and prevent the springtails from escaping. Polystyrene Petri dishes (10 cm diam. × 1.5 cm high) are recommended for culturing *F. fimetaria*. Table 2 of Appendix E provides details of the type and size of various vessels recommended by international agencies for culturing several species of springtails in the laboratory, and to generate organisms for soil toxicity tests. The use of culture vessels constructed of wood is not recommended, due to the possible presence of toxic contaminants (e.g., plywood glues, antisapstain chemicals, or wood extractives such as resin acids, juvabiones, etc.). The choice of size and numbers of culture vessels required might be influenced by the number of adult springtails required by the testing facility for one or more series of soil toxicity tests. Each culture vessel should accommodate a minimum depth of 1 cm of culturing substrate.

2.3.3 Lighting

Cultures of *O. folsomi* and *F. fimetaria* can be cultured in complete darkness (e.g., inside a closed drawer or opaque breeding vessel), or with incandescent or fluorescent light and a regulated *photoperiod* (e.g., 16-h light:8-h dark or 12-h light:12-h dark). For *F. candida,* incandescent or fluorescent lights should illuminate the cultures, and the photoperiod should be regulated (e.g., 16-h light:8-h dark or

12-h light:12-h dark). Light intensity adjacent to the top of the culture vessels should range within 400–800 *lux*. This range is equivalent to a quantal flux of 5.6–11.2 μmol/(m^2 · s) for cool-white fluorescent, 6.4–12.8 μmol/(m^2 · s) for full-spectrum fluorescent, or 7.6–15.2 μmol/(m^2 · s) for incandescent. The lights should be positioned sufficiently far from the culture vessels to prevent evaporation caused by heat buildup.

2.3.4 Temperature

All three test species should be cultured in a facility with an air temperature of 20 ± 2 °C, as a daily average. Additionally, the instantaneous temperature of the facility should be 20 ± 3 °C.

2.3.5 Culturing Substrate

Various substrates have been used for culturing springtails in preparation for soil toxicity tests (see Table 4 of Appendix E). The choice of substrate for culturing this species is left to the discretion and experience of laboratory personnel; however, the following culture substrate is proven and recommended for all three test species.

A substrate comprising of 8 parts Plaster of Paris™ (Stucco)[5] and 1 part charcoal (e.g., analytical-grade activated charcoal 375 μm mesh; e.g., Fisher cat # 35-474) is recommended by EC (2007a), ISO (1999), OECD (2005), and Wiles and Krogh (1998) for culturing *F. candida, F. fimetaria,* and *O. folsomi,* and is also recommended here. Working in a chemical fumehood, the culture substrate is prepared in a 1-L glass or plastic bottle. First 120 g of Plaster of Paris™ and 15 g of charcoal are put into the 1-L bottle using a funnel and the bottle is shaken vigorously for approximately 30 seconds. Once the solids are homogenized, 130 mL of *test water* (ultra pure, e.g., MilliQ® water is recommended for use when preparing substrate for *F. fimetaria*) is added and the bottle is closed and shaken for another 30 seconds. The amount of water needed can vary depending the type of plaster used. Once prepared, the Plaster of Paris™ mixture is then poured into the culture vessel(s) to a depth of 1 cm (Becker-van Slooten *et al.,* 2003; Stämpfli *et al.,* 2005).[6, 7] This should be done fairly quickly to prevent the substrate from hardening before being poured into the breeding vessels. The vessels are gently tapped on the sides and on the laboratory bench top to release any air bubbles that may have formed during mixing, as well as to evenly distribute the culture substrate and to create a flat substrate surface.[8] The culture vessels should be placed on a level surface and allowed to air-dry flat for at least three hours. Once hardened, test water is added to the culture vessels to almost saturation (i.e., there should be no standing water on the substrate surface). If the prepared culture vessels are not being used immediately, they may be stored at room temperature for up to three weeks. Before storage, the substrate should be saturated with test water (i.e., slowly add ~1 cm of test water on top of the set surface) to prevent it from drying out during storage. Over-drying will

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[5] The quality of the Plaster of Paris™ may vary. If the Plaster of Paris™ has a strong odour and reproduction is low, a new batch of Plaster of Paris™ should be used.

[6] 120 g of Plaster of Paris™, 15 g of charcoal, and 130 mL of water make enough substrate for a 16 × 11 × 5.5 cm culture vessel (Becker-van Slooten *et al*., 2003).

[7] It is important not to pour the mixture onto the sides of the box, since the plaster will dry on the sides and then fall onto the substrate surface. Springtails will lay their eggs underneath the pieces of plaster, making it difficult to recover them (Stämpfli, 2001).

[8] Air bubbles leave crevasses on the surface of the culture substrate within which the Collembola reside and/or lay egg clutches. This makes the handling of eggs for synchronization purposes more difficult (EC, 2006a); however, egg production appears to be enhanced by the presence of crevasses and pieces of plaster (K. Becker-van Slooten, personnel communication, Laboratory of Environmental Chemistry and Ecotoxicology, ENAC-ISTE, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland, 2004).

result in the substrate shrinking away from the edges of the vessel, thereby creating a gap. If a gap is created between the sides of the vessels and the substrate due to over-drying, the substrate should be discarded since the springtails will reside and lay eggs down the sides and at the bottom of the vessel (i.e., where they are inaccessible). The substrate should be rinsed with test water before springtails are added. Approximately 1 cm of test water is added to the substrate and the edges and surface gently rubbed with a gloved fingertip to remove any sharp or un-even edges. The substrate should be rinsed three times. Excess water may be poured off, the surface lightly blotted with paper towel, and the vessels sealed with lids, after which the vessels are ready to use.
The pH of each new batch of substrate is verified by placing pH paper on the wet substrate surface. Substrate pH should between 6.0 and 7.0. Culture vessels should be re-hydrated with test water once or twice/week to maintain the humidity (e.g., optimum humidity is provided by keeping the Plaster of Paris™ moist). This is accomplished by adding several drops of test water with a pipette or by gently spraying the sides of the vessel using a fine mist spray bottle or squeeze bottle until the water just begins to remain on the surface. Care should be taken to not damage the springtails or to blow organisms out of the culture vessel during the re-hydration process.

The vessels must be aerated a minimum of once/week; however, twice/week is recommended if there is a history of fungal problems in the cultures, or for cultures of *F. fimetaria*, which are more sensitive to fungal growth. Aeration can be achieved during the weekly re-hydration process by simply removing the lids for $\geq$1 minute.

A mixture of freezer-free potting soil (manure/peat/loam blend), *Sphagnum* sp. peat moss, and *artificial soil*[9] has also proven to be a suitable culturing substrate for *O. folsomi* (ESG and Aquaterra Environmental, 2003) and *F. fimetaria* (J.I. Princz, personal communication,

[9] A 10-L batch of fertilizer-free potting soil can be prepared as follows:

- Mix ~3 L of potting soil with ~ 4.5 L of peat moss (both in their "dry form").
- Add *test water* (~1 L) to the substrate and mix mechanically (handheld mixer) until the moisture content, colour, and *texture* of this mixture appear to be homogeneous.
- Add ~1.5 L of artificial soil (see Section 3.3.2).
- Add more test water (~1 L) to this mixture while stirring mechanically, until a moisture content equivalent to ~70% of the *water-holding capacity* of the mixture is achieved.
- Measure the soil pH and, depending on the value, sprinkle ~30 g of calcium carbonate ($CaCO_3$) onto the surface of the culturing substrate using a fine sieve, and mix into the soil using a mechanical mixer until no white powder is visible (alternatively, for a more even distribution, calcium carbonate can be mixed into the dry substrate before the water is added; J. McCann, personal communication, University of Waterloo, Waterloo, Ontario, 2004).

This mixture is stored in a covered container at ambient laboratory temperature for three days. The culturing substrate is then stirred, and its pH measured (using a $CaCO_3$ slurry method; see Section 4.6) to ensure that it is between 6.0 and 7.5. If the pH is <6.0, additional $CaCO_3$ is added. If the pH is >7.5, the bedding should continue to be stored until the pH lowers naturally or more non-pH adjusted substrate can be added until the pH is <7.5. Once the pH has been properly adjusted, the laboratory cultures can be added to the bedding. A minimum substrate depth of 5 cm is recommended for maintaining springtail cultures in soil.

Substrate pH and moisture content should be measured periodically (e.g., weekly), and adjusted as necessary. On these occasions, the culturing substrate is gently stirred, a subsample of substrate collected, and its pH measured to ensure that it is between 6.0 and 7.5. If the pH is <6.0, additional bedding should be added and mixed into the existing culture until the pH is >6.0. Any standing water in the bin is discarded, and if the bedding appears too dry it should be thoroughly sprayed with test water.

Biological Methods Division, Environment Canada, Ottawa, Ontario, 2006), and can be used for maintaining mass or back-up cultures in the laboratory.[10]

2.3.6 Food and Feeding

Various types of food and feeding regimes have been used for culturing springtails in preparation for soil toxicity tests (see Table 5; Appendix E). Success in culturing the three species described in this test method document has been achieved using activated dry yeast (Aquaterra Environmental, 1998a; Wiles and Krogh, 1998; ISO, 1999; Aquaterra Environmental and ESG, 2000; ESG 2000, 2001, 2002; ESG and Aquaterra Environmental, 2002, 2003; Becker-van Slooten *et al.,* 2003, 2005; Stämpfli *et al.,* 2005; EC, 2006a).

Activated dry yeast, to be used as food for cultures, can be purchased from the grocery store. Fleischmann's™ is recommended for this purpose, since this brand has been used successfully during the development of this method (Aquaterra Environmental, 1998a; EC, 2006a, 2007a,b). The quantity of food added to each culture vessel depends on springtail density and developmental stage and, therefore, should be based on observations and records of food consumed or not consumed, during preceding weekly feedings. The quantities are species-specific as follows:

- for *O. folsomi* and *F. candida*, ~100 mg, (i.e., for a culture vessel that is ~ 15 × 23 × 8 cm); and
- for *F. fimetaria*, ~10 mg (i.e., for a 10-cm diam. Petri dish).

The food can be divided into 2 or 3 piles, or sprinkled over the surface of the culture vessel. The yeast should be placed onto the surface of the substrate of each vessel twice/week at the time of aeration and re-hydration.[11] The old, unconsumed yeast is removed (if remaining) before the new yeast is added.[12] Care should be taken to avoid excessive fungal and bacteria growth in the culture vessels, for *F. fimetaria*, especially, since this species is more sensitive to fungi and bacteria.[13] In order to activate the yeast, it should be added after the substrate has been hydrated. Alternatively, the yeast may be activated by hydrating it with a few drops of test water.

2.3.7 Handling Organisms and Maintaining Cultures

Springtails should be handled as little as possible, to avoid damage and undue stress. When handling is necessary, it should be done gently, carefully, and quickly to minimize stress to the animals. The use of a moist, fine-tipped paintbrush is suitable for moving springtails to and from the culture or test vessels; however, care must be taken to avoid damaging the organisms or their eggs. A low suction exhaustor system, described by ISO (1999) may also be used for transferring springtails. A glass

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[10] The problem with maintaining springtail cultures in soil is that it takes several months for the organisms to acclimate (i.e., such that enough eggs are produced) to the Plaster of Paris™ substrate required for age-synchronization (Rick Scroggins, personal communication, Biological Methods Division, Environment Canada, Ottawa, Ontario, 2006).

[11] Alternatively, more food can be added only once/week, provided that most of the food has been consumed by the next feeding date, and no excessive bacteria or fungi is present.

[12] It is important to completely remove the old uneaten yeast in order to avoid excessive formation of bacteria or fungi, which might be harmful to the cultures, especially *F. fimetaria* (Stämpfli, 2001; Stämpfli *et al*., 2005).

[13] Excessive fungal and bacterial growth in the culture vessels might be avoided with the following procedures: use ultra pure (e.g., Milli-Q®) water for culture substrate preparation and hydration, aerate the culture vessels more frequently (e.g., a minimum of twice/week), and remove any unconsumed yeast every four days (Stämpfli *et al*., 2005). If fungal and/or bacterial growth is excessive in any culture vessel, that culture vessel should be discarded.

Pasteur pipette, fitted with a suction bulb also functions well in this capacity. The suction strength should be modified to a very gentle level to prevent damage to the springtails. Collembola can also be transferred by gently tapping one vessel over another. When handled, any animals that are injured or appear stressed should be discarded, and must not be used for testing.

Table 6 of Appendix E summarizes useful guidance for maintaining cultures of various species of springtails, found in international guides and methodology documents on soil toxicity tests using these Collembola.

It is recommended that the contents of each culture vessel be inspected just before each weekly feeding, to determine the apparent condition of the springtails and the culture substrate. Records should be kept of the apparent condition of the culture (organisms and substrate) noted during each observation period (Section 2.3.1).

The loading density of springtails in each culture vessel should be restricted to prevent overcrowding and the resulting adverse effects on springtail growth, reproduction, and culture health (ISO, 1999). A loading density of ~ 2 to 3 adult Collembola per cm^2 is suggested for *O. folsomi* and *F. candida* (G.L. Stephenson, personal communication, Aquaterra Environmental, Orton, Ontario, 2006; K. Becker-van Slooten, personal communication, Laboratory of Environmental Chemistry and Ecotoxicology, ENAC-ISTE, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland, 2004) and ~ 5 to 6 adult Collembola per cm^2 is suggested for *F. fimetaria* (J.I. Princz, personal communication, Biological Methods Division, Environment Canada, Ottawa, Ontario, 2007).

The substrate in each culture vessel should be renewed as required and every 1 to 2 months, regardless of springtail densities. This can be achieved by preparing new culture vessels, and by transferring the springtails into the new vessels by tapping the old vessel over the new one. To reduce the population of springtails in a crowded culture vessel, only a portion of the total culture (e.g., 75% of individuals) are transferred when culturing vessels are renewed. The change of substrate will stimulate oviposition (Wiles and Krogh, 1998). For *O. folsomi* and *F. fimetaria* it is important that new cultures contain a mixture of males and females (i.e., a minimum of two females to every male) and that the organisms are mixed between independent culturing vessels to avoid inbreeding (Stämpfli *et al.,* 2005).

The air temperature of the culture facility should be monitored weekly and the moisture level of the culture substrate should be observed at the time of weekly aeration. Adjustments should be made as, and if necessary (see Sections 2.3.4 and 2.3.5).

2.3.8 Age-Synchronized Cultures for Toxicity Tests

To be successful, the culturing procedures used must produce the required number of healthy test organisms of a known developmental stage, and of similar age and size. Additionally, the cultured organisms must meet specific health- and performance-related indices (Section 2.3.9). The following paragraphs describe procedures that should be followed to obtain age-synchronized test organisms (i.e., 28–31 days old for *O. folsomi,* 10–12 days old for *F. candida,* and 23–26 days old for *F. fimetaria)* for use in the toxicity tests described in this method document.

For *F. candida* and *F. fimetaria*, new cultures can be created to begin the process of age-synchronization.[14] At least two new large cultures (culture vessels described for *F. candida*

[14] *O. folsomi* will not produce a sufficient numbers of eggs in new cultures established for age-synchronization, as described for *F. candida* and *F. fimetaria*; therefore, eggs should be taken from existing cultures to set up age-synchronized test organisms (EC, 2006a).

in Section 2.3.2) or several small ones (Petri dishes described for *F. fimetaria* in Section 2.3.2) should be prepared in order to obtain enough *juvenile* springtails for a toxicity test. This can be accomplished by transferring 200 to 300 mature *F. candida* or *F. fimetaria* from an existing culture by gently tapping the vessel of the existing culture and allowing the springtails to gently drop onto the newly prepared culture substrate of a culture vessel (see Section 2.3.5). Avoid transferring more than 300 individuals, since this would result in over-crowding and the inhibition of reproduction. Cultures are fed by adding 100–200 mg of yeast to each new culture vessel. The number of organisms transferred and the amount of yeast added to the cultures depends on the size of the culture vessels being used. These new cultures should be monitored daily for eggs. The springtails should begin to lay the first egg clutches within 24–48 hours of being transferred to new culture substrate.

For *O. folsomi*, existing cultures should be used to produce sufficient numbers of eggs for age-synchronization. Existing cultures may also be used to produce eggs for *F. candida* or *F. fimetaria* age-synchronization, in addition to the procedure of setting up new cultures, described previously.

Seven days after the first egg clutches appear in new cultures, or a large number of egg clutches appear in existing cultures, several (or all, if possible) egg clutches should be transferred onto moistened coated filter paper[15] which is then placed into new culture vessels or smaller hatching vessels.[16, 17] The eggs can be transferred using a fine spatula or a slightly dampened paint brush. The egg clutch should be "swept" by gently rolling the paint brush under and through the clutch and then tapping the paintbrush gently to deposit the eggs onto the moistened coated filter paper or plaster substrate in the hatching vessels. The plaster substrate in the new hatching vessels and the coated filter paper should be sufficiently moist, or the eggs will dehydrate (i.e., the Plaster of Paris™ substrate is sufficiently moist when a droplet of water will stay on the surface and only very slowly absorb into the substrate). For *O. folsomi* and *F. fimetaria*, a few adult females (i.e., ~6 of the biggest organisms) can be introduced into each hatching vessel to improve the rate of hatching.

Three to five grains of activated dry yeast should be added to the moistened substrate of each hatching vessel. The hatching vessels should

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[15] Plaster-coated filter paper should be prepared for the purpose of transferring and hatching *F. candida/fimetaria* eggs. First a piece of filter paper (~10 cm in diam.) should be cut into several pie-shaped segments (approximately 5-cm long and 3-cm wide). A Plaster of Paris™/charcoal mixture is prepared as described in Section 2.3.5; however, using only 120 mL of *de-ionized* or *distilled* water instead of 130 mL of water (i.e., the consistency should be slightly thicker than that used to prepare the culture substrate), as specified for the preparation of the culture substrate. The pie-shaped pieces of filter paper are then dipped into the Plaster of Paris™/charcoal mixture so that both sides of the filter paper are coated. The filter paper may be swept across the surface of the mixture in order to coat each side. Approximately 1 cm of the filter paper, at the wide edge, should be left free of the plaster mixture to allow for handling. The coated filter paper pieces are then hung (i.e., using paper clips or clothes pins) to dry. Once dry, the prepared filter paper can be stored in a container for future use (EC, 2006a).

[16] Smaller vessels (e.g., 125-mL canning jar or 10 × 1 cm glass Petri dishes) can be used for hatching springtails. These are prepared with the Plaster of Paris™/charcoal breeding substrate, as described in Section 2.3.5 (EC 2006a).

[17] Alternatively, eggs may also be transferred by placing a coated filter paper directly into the large plastic culture vessels, and allowing the adults to deposit their eggs directly onto the filter paper. The filter paper should be moistened and stuck to the substrate in the culture vessel to prevent the springtails from laying thier eggs underneath. The coated filter paper can then be moved into the Petri dishes for hatching.

then be sealed[18] and monitored daily for the appearance of juveniles. The substrate and coated filter paper should be continuously kept moist with test water. *F. candida* eggs should hatch within 2 to 3 days following their transfer (i.e., ~10-days after oviposition), whereas *F. fimetaria* eggs hatch within 3 to 4 days following their transfer. The coated filter papers containing any unhatched eggs should be removed 48 hours after the appearance of the first juveniles for *F. candida*, and 72 hours after the appearance of the first juveniles for *O. folsomi* and *F. fimetaria*, and placed into fresh hatching vessels. The process is repeated for the production of more age-synchronized test organisms. Since juvenile springtails tend to stay under or crawl on top of the coated filter paper, the filter paper should be tapped or brushed with a dry paint brush (being careful not to remove eggs from the filter paper in the process) before removing the filter paper. If adult female *F. fimetaria* were placed into the hatching vessels with the eggs, they should be removed when the coated filter paper is removed. *F. candida* juveniles can be used for testing 10 days after removal of the remaining unhatched eggs (i.e., *F. candida* used to start toxicity tests must be 10–12 days old); *F. fimetaria* organisms can be used for testing 23 days after the removal of the remaining unhatched eggs (i.e., *F. fimetaria* used to start toxicity tests must be 23–26 days old); and, *O. folsomi* organisms can be used for testing 28 days after the removal of remaining unhatched eggs (i.e., *O. folsomi* used to start toxicity tests must be 28–31 days old).

An alternative method for obtaining age-synchronized springtails is to place a number of adult springtails into many small vessels containing the Plaster of Paris™/charcoal substrate (see Section 2.3.5). The adults should be allowed to lay eggs over a two-day period. Adults are then removed two days after the first egg clutch appears. The small vessels should be monitored for the appearance of juveniles and organisms should be fed with 2 to 3 grains of active dry yeast, as necessary. Unhatched eggs are removed 48 hours after the appearance of juveniles for *F. candida*, and 72 hours after the appearance of juveniles for *O. folsomi* and *F. fimetaria.* The organisms can then be used in a toxicity test 10, 23, and 28 days after the first juveniles have emerged from the eggs, for *F. candida, F. fimetaria,* and *O. folsomi,* respectively.

Any laboratory-cultured *O. folsomi, F. candida*, or *F. fimetaria* used to start a toxicity test (including that with a reference toxicant) for effects on survival and reproduction should be acclimated in the laboratory as much as possible to conditions representing those in this toxicity test (Section 4.3). During the age-synchronizing period, temperature conditions must be the same as those to be used in the toxicity test, and springtails must be fed dry yeast (see Sections 2.3.4, 2.3.6, and 4.3).

2.3.9 Health and Performance Indices

Each culture vessel should be checked at least once per week, during which time culture performance should be monitored and recorded (see Sections 2.3.1, 2.3.6, and 2.3.7). Procedures and conditions used to maintain each culture should be evaluated routinely, and adjusted as necessary to maintain or restore the health of the culture. If the culture appears unhealthy or atypical during any weekly (or more frequent) check, it should be checked daily to make sure that "cascade mortality" (i.e., rate of death increasing exponentially over time) is not occurring. Cultures are considered healthy if springtails of differing sizes are moving actively over the substrate surface.

One or more reference toxicity tests (14-day test for *F. candida*, and 7-day test for *O. folsomi* and *F. fimetaria*) must be conducted using a portion of the population of age-synchronized springtails

[18] Canning jars should be sealed with a metal cap and screw-top ring, and the Petri dishes should be sealed with a glass cover.

taken from a particular culture to start a *definitive* soil toxicity test (see Section 4.9). Ideally, a reference toxicity test should be performed together with each soil toxicity test. Laboratories routinely undertaking soil toxicity tests using cultured Collembola may choose instead to routinely conduct one or more reference toxicity tests (i.e., at least once every two months), using age-synchronized springtails derived from the same culture(s) from which the test organisms for the soil toxicity test(s) are obtained. All tests with the reference toxicant(s) should be performed using the conditions and procedures outlined in Section 4.9. Test-related criteria used to judge the validity of a particular soil toxicity test (and, indirectly, the health of the culture), based on the performance of test organisms in the *negative control soil*, are given in Section 4.4.

A laboratory that routinely (e.g., once per month or more) performs toxicity tests with springtails might find it useful to monitor the data on number of *progeny* produced in negative control soil, as a measure of culture health and performance. A plot of such data over time can show problems with respect to reproductive success that are attributable to diet or other conditions to which cultures are exposed (G. Stephenson, personal communication, Stantec Consulting Ltd., Guelph, Ontario, 2004).

Test System

3.1 Facilities and Apparatus

Tests may be performed in an environmental chamber or equivalent facility having acceptable temperature and lighting control (see Section 4.3). The test facility should be well ventilated to prevent exposure of personnel to harmful fumes, and it should be isolated from physical disturbances or any contaminants that might affect the test organisms. The area used to prepare *test soils* should contain a fumehood and be properly ventilated.

The test facility should be isolated from the area where the springtails are cultured (Section 2.3) to avoid potential contamination. Additionally, the test facility should be removed from places where samples are stored or prepared, to prevent the possibility of contamination of test vessels and their contents from these sources. The ventilation system should be designed, inspected, and operated to prevent air within the testing facility from contaminating the culturing facilities. Return air from sample handling and storage facilities or those where chemicals are processed or tested should not be circulated to the area of the laboratory where tests are conducted.

Any construction materials that might contact the organisms, water, or test vessels within this facility must be nontoxic (see Section 2.3.2) and should minimize sorption of chemicals. Borosilicate glass, nylon, high-density polyethylene, high-density polystyrene, polycarbonate, fluorocarbon plastics, and type 316 stainless steel should be used whenever possible to minimize chemical sorption and leaching.

The test facility must have the basic instruments required to monitor the quality (e.g., temperature, pH) of the *test soil* and associated *test (hydration) water*. Additionally, the laboratory should be equipped to facilitate prompt and accurate analysis of the moisture content of test soils. Equipment requirements include a drying oven which can be set at 105 °C for drying soils, a weighing balance accurate to the nearest 0.1 mg, and a pH meter. Safety apparatus including a respirator with dust protection, gloves, laboratory clothing, and glasses for eye protection are required when preparing mixtures and aliquots of test soil.

All test vessels, equipment, and supplies that might contact *site soils*, test soils, test (hydration) water, *stock solutions*, or test solutions must be clean and rinsed with *de-ionized* or *distilled* water (i.e., test water), before use. All nondisposable materials should be washed after use. The following cleaning procedure is recommended (EC, 1997a, b, 2001, 2004a, 2005a):[19]

1. soak in tap water (with or without detergent added) for 15 minutes, then scrub with detergent or clean in an automatic dishwater;

2. rinse twice with tap water;

3. rinse carefully with fresh, dilute (10%, v:v[20]) nitric (HNO_3) or hydrochloric acid (HCl) (metal-free grade) to remove scale, metals, and bases;

[19] Steps 1–4 of the cleaning procedure should be used if metal contamination is of concern, steps 1, 2, 5, 6, and 7 should be used if contamination with organics is of concern, and all steps should be followed if both metal and organics contamination is suspected.

[20] To prepare a 10% solution of acid, carefully add 10 mL of concentrated acid to 90 mL of de-ionized water.

4. rinse twice with de-ionized water (or other test water);

5. rinse once with full-strength, pesticide-grade acetone to remove organic compounds and with reagent-grade (e.g., HPLC grade, ≥98.5% purity) hexane for oily residues (use a fume hood);[21]

6. allow organic solvent to volatilize from dishware in fume hood and rewash with detergent (scrub if necessary); and

7. rinse three times with de-ionized water (or other test water).

Test vessels and apparatus that might contact soil or test (hydration) water should be thoroughly rinsed with test water, before being used in the test.

3.2 Initial and Definitive Tests

3.2.1 Initial Tests

Before *definitive* soil toxicity tests, using the test method defined in Section 4, are performed for the first time by a testing laboratory, it is recommended that a minimum of five control performance tests with one or more samples of uncontaminated natural or *artificial soil* intended (or under consideration) for use in one or more definitive soil toxicity tests as *negative control soil* (see Section 3.3) be undertaken by laboratory personnel. Additionally, a minimum of five reference toxicity tests should be performed using one or more samples of a candidate artificial or natural negative control soil intended for routine use in conjunction with definitive soil toxicity tests (see Section 4.9). These initial tests are recommended to confirm that acceptable performance of the test species (*O. folsomi*, *F. candida*, or *F. fimetaria*) can be achieved in a candidate natural or artificial negative control soil using that laboratory and the culturing conditions and procedures specified in this report (see Section 2.3).

The conditions and procedures used to perform these initial tests with *negative control soil* should be identical and according to Section 4, whereas the conditions and procedures used to perform the initial reference toxicity tests should be identical and according to Section 4.9. Each test with *negative control soil* or *reference toxicant(s)* should be performed using a different lot of test organisms of the same species from the same source.

Data from the control performance tests (≥5) must show that the criteria for test validity (see Section 4.4) can be met for the intended test species using a natural or artificial soil intended for use as negative control soil in a definitive soil toxicity test. Data from the initial reference toxicity tests (≥5) should be compared by calculating and appraising the magnitude of the *coefficient of variation* (CV) for the respective series of tests and endpoint values (see Section 4.9).

3.2.2 Definitive Tests

Test vessels to be used in *definitive* tests must be inert to test and reference substances or *contaminant* mixtures (i.e., the test or reference substances, or mixtures should not adhere to, or react in any way with the test vessel). The volume of the vessel should be sufficiently large to accommodate springtail survival and reproduction for the duration of the test. Wide-mouthed glass jars (e.g., mason canning jars), with a capacity of 100–125 mL (~5 to 8 cm in diameter), are to be used as test vessels. Each glass jar must be cleaned thoroughly before and after use, and rinsed well with de-ionized or other test water before use. Each test vessel should be covered with a plastic or metal lid (i.e.,

[21] Rinsing Plexiglas™ or any plastic equipment or vessels with acetone or hexane is not recommended, since plastic can become pitted and etched by these solvents and can turn from transparent to opaque.

metal lid with rubber seal secured with metal screw-top ring).

3.3 Negative Control Soil

Each soil toxicity test must include *negative control soil* as one of the experimental *treatments*. Negative control soil is a soil that is essentially free of any contaminants that could adversely affect the performance of springtails during the test. The use of negative control soil provides a measure of test acceptability, evidence of the health and performance of the test organisms, assurance as to the suitability of the test conditions and procedures, and a basis for interpreting data derived from the *test soils*.

A soil toxicity test may use *clean* (uncontaminated) natural soil and/or *artificial soil* as the negative control soil. The selection of an appropriate negative control soil depends on considerations such as the study design, physicochemical characteristics of the test soil(s), and the availability of suitable clean natural soil with acceptable properties.[22] There should also be prior experimental evidence that the soil chosen for use as negative control soil will consistently and reliably meet the criteria for test validity defined herein for each test species (Section 4.4).

The biological test method described herein has been developed and tested using five negative control soils with diverse physicochemical characteristics (Aquaterra Environmental, 1998a; Stephenson *et al.*, 1999a, b, 2000a; Aquaterra Environmental and ESG, 2000; ESG 2000, 2001, 2002; ESG and Aquaterra Environmental, 2002, 2003; Becker-van Slooten *et al*., 2003, 2005; Stämpfli *et al*., 2005; EC, 2007a). These clean soils included one artificial soil and four natural soils (i.e., samples of sandy loam and silt loam agricultural soil from southern Ontario, a clay loam prairie soil from Alberta, and a forest loam soil from the Canadian Shield[23] in northern Ontario). These soils differed in composition with respect to the physicochemical characteristics that could potentially influence the fate and effects of contaminants. All of the field-collected soils originated from uncontaminated areas that had not been subjected to any direct application of pesticides in recent previous years; and therefore, were considered to be "*clean*". The origin and physicochemical characteristics of these natural soils are further described in Appendix G. The test validity criteria for *O. folsomi*, *F. candida*, and *F. fimetaria* described in Section 4.4 are based on the performance data for these springtails in negative control soil, that were generated for each of these five diverse soils (Aquaterra Environmental, 1998a; Stephenson *et al.*, 1999a, b, 2000a; Aquaterra Environmental and ESG, 2000; ESG 2000, 2001, 2002; ESG and Aquaterra Environmental, 2002, 2003; Becker-van Slooten *et al*., 2003, 2005; Stämpfli *et al*., 2005; EC, 2007a), among others (Krogh, 2004).

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[22] The Canadian Council of Ministers of the Environment (CCME) provides a comprehensive Web site on Canadian Environmental Quality Guidelines including those for soil (www.ccme.ca). This information is useful when reviewing analytical data (e.g., values for metals or PAHs) for samples of field-collected soil from a location under consideration as a source of natural soil suitable for use as negative control soil in toxicity tests. The summary table of these guidelines can be accessed at www.ccme.ca/assets/pdf/e1_06.pdf. These Web sites and associated links will assist the investigator(s) reviewing the physicochemical characteristics of presumably *clean* natural soils under consideration for use as negative control soil in soil toxicity tests. The CCME can also be contacted by toll-free phone (1-800-805-3025) or e-mail (info@ccme.ca).

[23] There is a suite of ectomycorrhizal fungi known to kill Collembola in soils having a high organic matter content (Klironomos and Hart, 2001). These fungi are likely most prevalent in the soils collected during the fall season; and therefore, caution should be taken when testing these types of soils with springtails. Storage of these forest soils at temperatures between 20 and 25 °C is ideal for the growth of fungi; therefore, storage should be maintained at lower temperatures until used for testing (G.L. Stephenson, personal communication, Aquaterra Environmental, Orton, Ontario, 2006).

3.3.1 Natural Soil

Negative control soil may be natural soil collected from a *clean* (uncontaminated) site which is known to have been free of pesticide or fertilizer applications for at least five years. The source of this *negative control soil* might be the same as that where springtails were collected to establish a culture (Section 2.2). Before using a sample of *clean* field-collected soil as negative control soil in a definitive toxicity test, the test laboratory must have previous experimental evidence showing that natural soil from this source can meet the criteria that must be achieved for the results of a toxicity test to be considered valid (see Section 4.4).

Accordingly, initial tests involving a sample of this soil must be performed using the intended Collembola test species, to confirm that the test organisms are able to meet the criteria for test validity that apply to the particular test species being used. Thereafter, and assuming that the preceding results for these initial *bioassays* are satisfactory, it is recommended that samples of natural soil selected for possible use as negative control soil in soil toxicity tests (as well as samples of candidate *reference soil*) be analyzed for the following physicochemical characteristics:

- pH,
- particle size distribution,
- *conductivity*,
- *texture*,
- *fertility*,
- *total organic carbon* content (%),
- *organic matter* content (%),
- cation exchange capacity,
- major cations,
- total nitrogen,
- total phosphorus,
- bulk density,
- WHC,
- metals,
- petroleum hydrocarbons (including PAHs),
- organophosphorus insecticides,
- organochlorine insecticides, and
- a suite of herbicides.

Pesticide and metal concentrations should not exceed CCME soil quality criteria, if available (see footnote 22). If indigenous organisms are present and/or problematic in the sample(s) of natural soil at any time (i.e., during storage or testing), their presence (e.g., physical description and estimated numbers) should be recorded, and they should be removed manually (e.g., by sieving), if possible. If the results of both the initial biological tests and the physicochemical analyses are satisfactory, a larger sample of this natural soil can be collected, air dried to a *moisture content* of between 10 and 20%, coarse-screened (4–6 mm), transferred to clean, thoroughly rinsed plastic pails, and stored in darkness at 4 ± 2 °C until required. Plastic pails should not be used for collection and storage of soils if there are concerns about chemical constituents of the plastic leaching into the soil.

3.3.2 Artificial Soil

Negative control soil may be *artificial soil* formulated in the laboratory. The use of artificial soil offers a consistent, standardized approach and is advantageous when testing the toxicity of chemicals or chemical products spiked in negative control soil (Section 6).

The formulation of artificial soil used internationally in various soil toxicity test method documents, using springtails, is very similar. Appendix F (Tables 4, 5, and 6) provides a summary of the ingredients and preparation of artificial soil recommended in various methods (Wiles and Krogh, 1998; ISO, 1999; OECD, 2005) for use as negative control soil in laboratory tests of the effects of contaminated soil on the survival and reproduction of springtails.

In keeping with the formulation of artificial soil used by Wiles and Krogh (1998), ISO (1999), OECD (2005) and in two other Environment

Canada soil toxicity test methods (EC 2004a, 2005a) the following ingredients should be used to prepare artificial soil to be used in the biological test method described herein:

- 10% *Sphagnum* sp. peat, air dried and sieved (e.g., through a 2-mm mesh screen)
- 20% kaolin clay with particles <40 µm
- 70% "grade 70" silica sand

The ingredients should be mixed thoroughly in their dry form using a mechanical stirrer and/or gloved hands.[24] Reagent-grade calcium carbonate should be added to the dry mixture in a quantity sufficient to attain a pH (measured using a calcium chloride slurry method; see Section 4.6) for the artificial soil ranging within 6.0–7.5, once it is hydrated.[25] Thereafter, the mixture should be hydrated gradually using *test water* (i.e., de-ionized or distilled water) until its moisture content is ~20% (which is ~28% of the *water-holding capacity*), while mixing further until the soil is visibly uniform in colour and texture. As necessary, reagent-grade calcium carbonate should be added to the hydrated mixture in a quantity sufficient to maintain a pH ranging within 6.0–7.5. Samples of pH-adjusted artificial soil should be stored in darkness at 20 ± 2 °C for a minimum of three days before being used in a toxicity test, to enable adequate time for pH equilibration.[25] Thereafter, artificial soil can be stored at 4 ± 2 °C. As, and when required for a soil toxicity test, a suitable quantity of stored artificial soil should be hydrated further using test water until its moisture content is ~70% of the water-holding capacity.

3.4 Positive Control Soil

The use of one or more samples of *positive control soil* is recommended for inclusion in each series of soil toxicity tests with springtails, to assist in interpreting the test results. In choosing a positive control soil, the intent is to select a *toxic* soil that will elicit a response in the test organisms which is predictable based on earlier toxicity tests with this material. The positive control soil might be a sample of negative

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[24] It is recommended that the dry ingredients initially be mixed (to incorporate the calcium carbonate) using a mechanical stirrer. Mixing should be completed using a gloved hand, to ensure that all of the soil from the corners of the container have been well mixed. Personnel must take the appropriate precautions for protection to prevent the inhalation of and contact with these ingredients.

[25] The amount of calcium carbonate ($CaCO_3$) required to adjust the pH of artificial soil to within this range depends on the nature (i.e., acidity) of the ingredients (and, in particular, that of the *Sphagnum* sp. peat). A quantity of 10–30 g of $CaCO_3$ for each kg of peat might prove adequate. A pH as low as 4.5 can occur when the soil is first formulated without the addition of $CaCO_3$. The initial pH adjustment should attempt to raise pH to range within 7.0 to 7.5, since the pH of artificial soil typically drops slightly (to 6.5 to 7.0) during the three-day equilibration period, before it stabilizes. The pH of stored samples of artificial soil should be checked regularly (e.g., once every two weeks) to ensure that it has not changed dramatically; adjustments should be made as necessary by adding additional quantities of $CaCO_3$ (Aquaterra Environmental, 1998a; G.L. Stephenson, personal communication, Aquaterra Environmental, Orton, Ontario, 2001).

A mixture of formulated artificial soil can also be stored dry, followed by partial hydration to ~20% moisture content, storage at 20 ± 2 °C for a minimum 3-day period, and subsequent hydration to ~70% WHC when required for use in a toxicity test. If storing formulated artificial soil dry, it is necessary to partially hydrate (to ~20% moisture) and equilibrate thereafter (for ≥3 days) to provide conditions for pH equilibrium similar to that recommended herein using artificial soil stored partially hydrated. Using this optional approach, the interim storage as partially hydrated artificial soil is necessary to enable the addition of more water (and, in certain instances, the addition of a chemical solution) as required when finalizing the pH and moisture content (i.e., adjusted to ~70% WHC) of artificial test soil. Storage of artificial soil that is partially hydrated, rather than dry, is considered a preferred approach since it enables laboratory personnel to more quickly hydrate to the desired moisture content (i.e., ~70% WHC) while ensuring pH equilibrium, and reduces any further delay in the time for pH stabilization associated with dry storage of artificial soil.

control soil that is spiked with a *reference toxicant* for which historic data are available on its toxicity to springtails using the specified test conditions and procedures. For the biological test method described herein, one or more reference toxicants must be used when appraising the sensitivity of the test organisms and the *precision* and reliability of results obtained by the laboratory for that material (see Section 4.9) . A test might also include a sample of negative control soil (natural or artificial; see Section 3.3) that has been spiked experimentally (Section 6) with one or more toxic chemicals or chemical products of particular concern when evaluating the sample(s) of *test soil*, at a concentration toxic to the Collembola species used, and according to the biological test method described herein. In some instances, a test might include a positive control soil that is comprised of a highly contaminated sample of field-collected soil or sludge shown previously to be consistently toxic to springtails according to the biological test method described herein.[26]

3.5 Reference Soil

One or more samples of *reference soil* might be included in a soil toxicity test using springtails. The type and nature of the sample(s) of soil used as reference soil in a particular study depend on the experimental design and the study's objectives. If the toxicity of samples of field-collected soil from a *contaminated* or potentially contaminated *site* is under investigation, the reference soil included in the study might be one or more samples of field-collected soil taken from a *clean* (uncontaminated) site where the physicochemical properties (e.g., organic carbon content, *organic matter* content, particle size distribution, *texture*, pH) represent the sample(s) of test (contaminated) soil as much as possible. Ideally, the reference soil is collected from the general vicinity of the site(s) where samples of *test soil* are collected, but is removed from the source(s) of contamination. One or more samples of field-collected *clean* reference soil from sites removed from the test site(s) might also be chosen due to their known lack of toxicity in previous tests with springtails, and their possession of physicochemical characteristics similar to the samples of test soil. The sample(s) of field-collected reference soil used in a study could be tested for toxic effects as undiluted soil only, or this soil could be mixed with the sample(s) of test soil to prepare a range of concentrations to be included in a multi-concentration test[27] (see Sections 3.6, 4.1, and 5.3). Samples of reference soil should not be collected from sites known to have received applications of pesticides or fertilizers within the past five years or more.

An investigator might choose to include one or more samples of artificial soil as *reference soil* in a particular test. For instance, these could be used in multi-concentration tests with site soils or chemical-spiked soils to investigate the influence of certain physicochemical characteristics (e.g., a number of artificial reference soils prepared to provide a range of differing values for texture and/or organic matter content (%); Sheppard and Evenden, 1998; Stephenson *et al.*, 2002) on the toxicity of a contaminated site soil or a chemical-spiked soil.

Multiple samples of *clean* field-collected soil collected from various sites, which differ markedly with respect to one or more

[26] If the *positive control soil* is comprised of a highly contaminated sample of field-collected soil, it is important that its toxic potential is stable over time (i.e., the sample is old enough that the bioavailability has been stabilized).

[27] Alternatively, the series of test concentrations used in a multi-concentration test could be prepared using negative control soil. The choice might be influenced by whether or not the candidate *reference soils* are known to likely be non-toxic in the test to which they are to be applied, or a desire to prepare a range of concentrations of test soil using a *clean* soil with characteristics (e.g., *texture*, organic matter content) that closely match that of the test soil.

physicochemical characteristics, might also be used for this purpose. For such a study, a portion of each reference soil used to prepare a series of concentrations of the the test soil should be included in the test without dilution (i.e., 100% reference soil).

Each test involving one or more samples of reference soil must include a sample of *negative control soil* (see Section 3.3). Conversely, certain tests (e.g., one involving a series of concentrations of chemical-spiked soil prepared using artificial or natural negative control soil) need not involve a sample of reference soil. For tests with field-collected site soil, the inclusion of one or more samples of reference soil from a neighbouring site is a preferred approach for comparative purposes (see Section 5.5); a decision to dilute site soil with reference soil (rather than negative control soil) when preparing multiple concentrations for testing depends on the study objectives.

3.6 Test Soil

This biological test method is intended to measure the toxicity of one or more samples or mixtures of contaminated or potentially contaminated soil (*test soil*), using springtails as test organisms. The sample(s) of test soil might be either field-collected soil from an industrial or other site of concern, or industrial or municipal biosolids (e.g., dredged material, municipal sludge from a sewage treatment plant, composted material, or manure) under consideration for possible land disposal. A sample of field-collected test soil might be tested at a single concentration (typically, 100%) or evaluated for toxicity in a multi-concentration test whereby a series of concentrations are prepared by mixing measured quantities with either *negative control soil* or *reference soil* (see Section 5). The test soil might also be one or more concentrations of a *chemical-spiked soil*, prepared in the laboratory by mixing one or more chemicals or chemical products with *negative control soil*, *reference soil,* or *site soil* (see Section 6).

Universal Test Procedures

General procedures and conditions described in this Section for toxicity tests with springtails apply when testing the toxicity of samples of soil, particulate waste, or chemical, and also apply to their associated reference toxicity tests. More specific procedures for conducting tests with field-collected samples of soil or other similar particulate material (e.g., sludge, de-watered mine tailings, drilling mud residue, compost, biosolids) are provided in Section 5. Guidance and specific procedures for conducting tests with *negative control soil* or other soils spiked (amended) experimentally with chemical(s) or chemical product(s) are given in Section 6.

All aspects of the test system described in Section 3 must be incorporated into these universal test procedures. Those conditions and procedures described in Section 2 for culturing *O. folsomi*, *F. candida*, and *F. fimetaria* in preparation for soil toxicity tests, also apply. Summary checklists in Table 2 describe recommended conditions and procedures to be universally applied to each test with samples of contaminated or potentially contaminated soil, as well as those for testing specific types of test materials or substances. These could include samples of *site soil*, biosolids (e.g., dredged material, sludge from a sewage treatment plant, composted material, or manure), or negative control soil (or other soil, *contaminated* or *clean*) spiked in the laboratory with one or more test chemicals or chemical products.

This biological test method measures the effects of exposure to contaminated soil on the survival and reproductive success of springtails. Test organisms must be chosen from three species options (*O. folsomi*, *F. candida*, or *F. fimetaria*; see Section 1.2). Test duration is 21 or 28 days, depending on the species chosen (i.e., 21 days for *F. fimetaria*, and 28 days for *F. candida* and *O. folsomi*),[28] and the test soils are hydrated during the test but not renewed.

This definitive test method was applied and validated by several participating laboratories in three rounds of concurrent tests using *F. candida* in artificial soil spiked with boric acid (EC, 2007b).[29]

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[28] The original test duration for *O. folsomi* was 35 days (Aquaterra, 1998a); however, with the development of synchronized *O. folsomi* cultures, and investigations into the effect of test duration on the endpoints, validity criteria could be met for adult survival and juvenile production for this species, regardless of test duration. It was therefore decided that the *O. folsomi* test would be standardized at 28 days (EC, 2007a).

[29] In the first Phase of the interlaboratory validation tests, eight laboratories participated in a 14-day reference toxicant test with *F. candida* exposed to boric acid in artificial soil. All laboratories met the proposed minimum acceptable control survival of ≥80%. The mean LC50 was 1149 mg H_3BO_3/kg soil dry wt., with values ranging from 800 to 1483 mg/kg. The interlaboratory variability, expressed as the co-efficient of variation, was 27%. This variability excluded the test data derived from one laboratory whereby toxicity was two times less, compared with other laboratories. Even though the laboratory produced valid test results, the data were questionable, because this laboratory did not use the subset of *F. candida* that was sent to all laboratories, and the test organisms used had been unusually less sensitive in previous toxicity tests using the laboratory's own culture. Six laboratories participated in Phase-2 of the interlaboratory validation tests. These were reproduction tests with *F. candida* exposed to boric acid in artificial soil for 28 days. Only four laboratories met the validity criteria; and therefore, were the only tests valid for this round. The mean LC50 was not calculable, based on the data presented. The mean IC50 for juvenile production was 349 mg H_3BO_3/kg soil dry weight, with values ranging from 320 to 414 mg/kg. The interlaboratory variability, expressed as the co-efficient of variation, was 12% (EC, 2007b).

Table 2 Checklist of Recommended Conditions and Procedures for Conducting Tests for Effects of Exposure to Contaminated Soil on the Survival and Reproduction of *Orthonychiurus folsomi*, *Folsomia candida*, and *Folsomia fimetaria*

Universal		
Test type	–	whole soil toxicity test; no renewal (static test)
Test duration	–	for ***O. folsomi*** and ***F. candida*****:** 28 days; for ***F. fimetaria*****:** 21 days
Test organisms	–	for ***O. folsomi*****:** age-synchronized laboratory cultures; 28 to 31 days after eclosion; for ***F. candida*****:** age-synchronized laboratory cultures; 10 to 12 days after eclosion; for ***F. fimetaria*****:** age-synchronized laboratory cultures; 23 to 26 days after eclosion
Number of replicates	–	≥3 replicates/treatment; ≥5 replicates/control soil; for ***O. folsomi*****:** each replicate consists of 15 individuals per test vessel; 10 females (> 2 mm with round abdomens) and 5 males (~1–1.5 mm, and more slender); for ***F. candida*****:** each replicate consists of 10 individuals per test vessel; for ***F. fimetaria*****:** each replicate consists of 20 individuals per test vessel; 10 females (larger with round abdomens) and 10 males (half the size of the females and more slender)
Negative control soil	–	depends on study design and objectives; *clean* field-collected soil or artificial soil if testing site soils; artificial soil recommended for tests with chemical(s) or chemical product(s) spiked in soil
Test vessel	–	100- to 125-mL glass jar (~5–8 cm diam.), covered; metal lid secured with a metal screw-top ring or plastic screw-top lid recommended as cover
Amount of soil/ test vessel	–	30 g wet weight
Moisture content, test soils	–	for soil preparation, hydrate to the optimal percentage of its WHC if field-collected soil (see Section 5.3), or to ~70% of WHC if artificial soil; during test, hydrate as necessary
Air temperature	–	daily average, 20 ± 2 °C; instantaneous, 20 ± 3 °C
Lighting	–	incandescent or fluorescent; intensity, 400–800 lux adjacent to surface test vessels; fixed photoperiod (e.g., 16h L:8h D or 12h L:12h D)
Feeding	–	granulated dry yeast (e.g., Fleischmann's ™); for ***O. folsomi*****:** ~5 mg per test vessel on Days 0, 7, 14, and 21; sprinkled onto the soil surface in each test vessel for ***F. candida*****:** ~10 mg per test vessel on Day 0 and ~20 mg on Day 14; sprinkled onto the soil surface in each test vessel; for ***F. fimetaria****:* ~10 mg per test vessel on Day 0 and on Day 14; sprinkled onto the soil surface in each test vessel

Aeration and hydration	–	open test vessels briefly, minimum once/week to aerate; hydrate if necessary
Measurements during test	–	air temperature in test facility, daily or continuously; percent moisture and pH of soil in each treatment/concentration, at start and end; any excessive growth of fungi, feeding activity, and presence and quantity of any uneaten food
Observations during test	–	total number of live adult springtails and total number of progeny in each test vessel at the end of the test (Day 21 for ***F. fimetaria*** and Day 28 for ***F. candida*** and ***O. folsomi***)
Biological endpoints	–	total number of live adult springtails in each replicate (i.e., in each test vessel) at test end; total number of live progeny in each replicate at test end (Day 21 for ***F. fimetaria*** and Day 28 for ***F. candida*** and ***O. folsomi***)
Statistical endpoints	–	mean (± SD) percent survival of adults in each treatment, at test end (Day 21 or Day 28); mean (± SD) number of live progeny in each treatment, at test end (Day 21 or Day 28); if multi-concentration test: 21- or 28-day LC50 for adult springtails and 21- or 28-day ICp for mean number of live progeny produced per concentration at test end
Test validity	–	invalid if mean survival of adults (first generation) in negative control soil at test end is <70% for ***F. candida*** in natural soil and <80% for ***F. candida*** in artificial soil; and <70% for ***O. folsomi***, and <70% for ***F. fimetaria***, regardless of soil type; invalid if mean reproduction rate for adults in negative control soil is <100 live progeny/vessel for all three species
Test with reference toxicant	–	must perform once every two months, or in conjunction with definitive test(s) with soil samples; use boric acid (H_3BO_3); prepare and test ≥5 concentrations plus a negative control, using artificial soil as substrate; ≥5 replicates/negative control and ≥3 replicates/test concentration; 10 springtails/replicate (5 males and 5 females for ***O. folsomi*** and ***F. fimetaria***); follow procedures and conditions for reference toxicity tests described in Section 4.9 (7-day for ***O. folsomi*** and ***F. fimetaria***, and 14-day for ***F. candida***); determine 7- or 14-day LC50 (including 95% confidence limits); express as mg boric acid/kg soil dry weight; invalid if mean survival of adults (first generation in negative control soil at test end is <80% for all three species; also recommend 21- and 28-day tests with boric acid, for ***F. fimetaria***, and for ***F. candida*** and ***O. folsomi*** respectively, performed according to Section 4 at least twice a year or in conjunction with definitive test

Field-collected Soil

Transport and storage	–	seal in plastic and minimize air space; transport in darkness (e.g., using an opaque cooler, plastic pail, or other light-tight container); do not freeze or overheat during transport; store in dark at 4 ± 2 °C; test should start within two weeks, and must start within six weeks unless soil contaminants are known to be stable

Negative control soil	–	either natural, uncontaminated field-collected soil or artificial soil, for which previous 21- or 28-day tests with ***F. fimetaria***, ***F. candida***, or ***O. folsomi,*** respectively, showed that all criteria for test validity could be regularly met
Reference soil	–	one or more samples for tests with field-collected soil; taken from site(s) presumed to be clean but near sites of test soil collection; characteristics including percent organic matter, particle size distribution, and pH similar to test soil(s)
Characterization of test soils	–	at least percent moisture, WHC, pH, conductivity, percent total organic carbon, percent organic matter, and particle sizes (% sand, % silt, % clay); optionally, contaminants of concern [e.g., metals, polycyclic aromatic hydrocarbons (PAHs), pesticides]
Preparation of test soils	–	if necessary, remove debris and indigenous macro-organisms using forceps; if necessary, press through a sieve of suitable mesh size (e.g., 4–6 mm); mix; determine percent moisture content; hydrate with test water (or, if and as necessary, dehydrate) to the optimal percentage of its WHC (see Section 5.3); mix; dilute with control or reference soil if multi-concentration test; ensure homogeneity

Soil Spiked with Chemical(s) or Chemical Product(s)

Negative control soil	–	artificial soil or a *clean* field-collected soil recommended
Characterization of chemical(s) or chemical product(s)	–	information on stability, water solubility, vapour pressure, purity, and biodegradability of chemical(s) or chemical product(s) spiked into negative control soil should be known beforehand
Solvent	–	de-ionized water is the preferred solvent; if an organic solvent is used, the test must include a solvent control
Preparation of mixtures	–	procedure depends on the nature of the test substance(s) and the test design and objectives; chemical/soil mixtures may be prepared manually or by mechanical agitation; test substance(s) may be added as measured quantities in solution (i.e., in water or an organic solvent) or as a solid material comprised partly or completely of the test substance(s); ensure homogeneity
Concentration within soil mixture of chemical(s) or chemical product(s) added	–	normally measure at beginning and end of test, in high, medium, and low concentrations as a minimum

4.1 Preparing Test Soils

Each test vessel (see Section 3.2.2) placed within the test facility must be clearly coded or labelled to enable identification of the sample and (if diluted), its *concentration.* The date and time when the test is started must be recorded, either directly on the labels or on separate data sheets dedicated to the test. The test vessels should be positioned such that observations and measurements can be made easily. Treatments should be positioned randomly within the test facility and the position of test vessels within the test facility should be changed during the test (i.e., once per week, randomly) (EC, 1997a, b, 2001, 2004a, 2005a).

The day that springtails are initially exposed to samples of test materials or substances is designated Day 0. On the day preceding the start of the test (i.e., Day -1), each sample or subsample of test soil or similar particulate material, including *negative control soil* and, if used, *reference soil*, should be mixed thoroughly[30] (see Sections 5.3 and 6.2) to provide a homogeneous mixture consistent in colour, texture, and moisture. If field-collected samples of *site soil* are being prepared for testing, large particles (stones, thatch, sticks, debris) should be removed before mixing, along with any vegetation or macroinvertebrates observed (see Section 5.3).

The quantity of each test soil mixed as a *batch* should be enough to establish the *replicates* of that *treatment* (see Table 2) plus an additional amount for the physicochemical analyses to be performed (Section 4.6) and a surplus to account for the unused soil that adheres to the sides of the mixing container. The *moisture content* (%) of each test soil should be known or determined, and adjustments made as necessary by mixing in *test water* (or, if and as necessary, by dehydrating the sample) until the desired moisture level is achieved (see Sections 5.3 and 6.2). Quantitative measures of the homogeneity of a *batch* might be made by taking aliquots of the mixture for measurements such as particle size analysis, *total organic carbon* content (%), *organic matter* content (%), *moisture content* (%), and concentration of one or more specific chemicals.

Immediately following the mixing of a *batch*, 30 g wet weight of test soil should be transferred to each replicate test vessel. The soil added to each test vessel should be smoothed (but not compressed) using a spoon or by gently shaking the vessel back and forth horizontally, or by gently tapping the glass jar ≥3 times on the benchtop or with a hand.

For a single-concentration test (e.g., *site soil* tested at 100% concentration only; a particular concentration of test soil; or a chemical-spiked soil tested at one concentration (e.g., Maximum Label Rate), a minimum of five replicate test vessels and five replicate negative control vessels must be set up by adding 30 g wet weight of the same *batch* to each replicate vessel. For a multi-concentration test, a minimum of five replicate test vessels per *negative control soil* and a minimum of three replicate test vessels per treatment must be set up. In the case of appreciable uncertainty about sample toxicity, a *range-finding* test might prove worthwhile for selecting, more closely, the concentrations to be used for the *definitive* test. For a range-finding test, the number of replicates used might be reduced (e.g., 2 replicates). For any test that is intended to estimate the inhibiting concentration for a specified percent effect (ICp) in a *definitive* multi-concentration test, at least seven concentrations plus the *control* treatment(s) must be set up, and more (i.e., ≥10 plus controls) are recommended to improve the likelihood of bracketing each endpoint sought.[31]

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[30] Any liquid that has separated from a sample or subsample of test soil during transport and/or storage must be remixed into the sample.

[31] The use of 10 or more concentrations (plus the controls) is advised. The large number of test treatments is needed to show the shape of the concentration-response

It is recommended that a minimum of two additional test vessels for each treatment (including any control or reference soils used) be included in the test for the purposes of conducting physicochemical analyses on Day 0 and at the end of the test (see Section 4.6).[32]

Concentrations should be chosen to span a wide range, including a low concentration that evokes no adverse effects (e.g., similar to that for the negative control treatment), and a high concentration that results in "complete" or severe effects. If the anticipated endpoint is bracketed with a closely spaced series of concentrations, all may turn out to be either too low or too high. To keep the wide range of concentrations, and also obtain the important mid-range effects, it might be necessary to use additional treatments in order to split the selected range more finely. In any case, a consistent geometric series should be used (see Appendix H). See EC (2005b) for additional guidance on selecting test concentrations that apply here.

Following the addition of a measured (30 g wet wt) aliquot of test soil to each test vessel, lids (Section 3.2.2) should be placed onto the test vessels and closed tightly to minimize loss of moisture. The test vessels should be held overnight under specified test temperature and lighting conditions (Section 4.3), for chemical equilibration (e.g., of chemical-spiked soil or site soil diluted with control soil) of the test soils.

4.2 Beginning the Test

Test organisms (see Section 2.3.8) are transferred to each test vessel the day after the soil is prepared (i.e., Day 0 of the toxicity test). A number of test organisms in excess of those required for the test should be available from a group of age-synchronized culture vessels established to yield the appropriate number of organisms required for a test (Section 2.3.8).

For tests using *O. folsomi*, 28- to 31-day-old individuals, from age-synchronized cultures (see Section 2.3.8) must be used. Fifteen individuals (10 females, larger with round abdomens and 5 males, smaller and more slender) are transferred into each test vessel.[33] For tests using *F. candida*, 10- to 12-day old juveniles, from age-synchronized cultures (see Section 2.3.8) must be used. Ten individuals are gently transferred from the age-synchronized cultures into each test vessel. For tests using *F. fimetaria*, 23- to 26-day-old organisms, from age-synchronized cultures (see Section 2.3.8) must be used and 20 individuals (10 females, larger with round abdomens and 10 males, more slender and half the size of the females) are transferred into each test vessel.[34]

For all three test species, organisms can be gently transferred from the age-synchronized culture to a piece of folded stiff black cardboard

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relationship and to choose the appropriate linear or nonlinear regression model (see Section 4.8.2.1). Use of 10 or more concentrations is particularly prudent if the investigators wish to determine a 21- or 28-day LC50 for the adult springtails, as well as an ICp for reproductive inhibition (see Section 4.8). In certain tests, the investigators might wish to focus on the sublethal endpoints and not derive an LC50, in which instance 7–9 test concentrations (plus the controls) might prove adequate.

[32] The flotation method, which is one of the methods used to enumerate Collembola at the end of the test, is destructive with respect to the physicochemical properties of the test soil. Therefore, an extra replicate (with or without test organisms, depending on the objectives) should be prepared for the sole purpose of conducting physicochemical measurements at test end (see Section 4.6).

[33] In 28- to 31-day old age-synchronized *O. folsomi* cultures, males (~1–1.5 mm; more slender) are easily distinguished from females (≥2 mm; with large rounded abdomens).

[34] Like *O. folsomi*, male and female *F. fimetaria* are easily distinguished from each other (i.e., sexually dimorphic) in age-synchronized cultures; the males have more slender bodies and are only half the size of the females (Wiles and Krogh, 1998).

(8.5 × 11 in. paper folded in half) or a weigh boat (previously washed and dried to remove a waxy film that coats the weigh boats), using a fine, moistened paintbrush and a probe or a low-suction exhaustor system (see Section 2.3.7). For *F. candida*, organisms can be transferred by tapping the individuals directly from the age-synchronized culture onto the piece of black cardboard. The latter method enables the transfer of the required amount of individuals with the least amount of loss due to the natural springing tendency of the *F. candida*.

For *O. folsomi* and *F. fimetaria*, the age-synchronized cultures should be carefully examined to determine which organisms are male and which are female. Organisms should be gently picked up one at a time until the desired number of males and females (i.e., 10 females and 5 males for *O. folsomi*; and 10 females and 10 males for *F. fimetaria*) has been collected. Final observation of springtails should be made to confirm that the correct number of organisms has been selected, and that their appearance is normal (i.e., organisms chosen should appear healthy and active, demonstrating movement, lack of visible defects or damaged bodies, and should be similar in colouration[35]). Any atypical Collembola should be discarded. Thereafter, the organisms should be carefully transferred to the surface of the soil in a test vessel, by gently tapping the cardboard or the weigh boat over the test vessel. The group of springtails transferred to each test vessel should be random across the replicates and treatments.

[35] Any individuals that appear damaged, undersized (relative the others chosen), or coloured differently (e.g., for *O. folsomi*, organisms that are slightly orange or with a black stripe running head to abdomen are immature) should not be used in the test (ESG and Aquaterra Environmental, 2003).

4.3 Test Conditions

- This is a 21- or 28-day soil toxicity test during which the soil in each test vessel is not renewed. The test duration for *F. fimetaria* is 21 days, and for *F. candida* and *O. folsomi*, the test duration is 28 days.

- The test vessel is a 100- to 125-mL wide-mouthed glass jar; its content (i.e., 30 g wet wt of test soil) is covered with an opaque lid (Section 3.2.2).

- For a single-concentration test, at least five replicate test vessels must be set up for each test soil (i.e., each treatment). For a multi-concentration test, a minimum of three replicate test vessels per test concentration and five replicate test vessels per control soil must be set up.

- For multi-concentration tests with all three species, at least seven concentrations plus the appropriate control treatment(s) must be used, and more concentrations (i.e., ≥10 plus controls) are recommended.

- The test must be conducted at a daily mean temperature of 20 ± 2 °C. Additionally, the instantaneous temperature must always be 20 ± 3 °C.

- Test vessels must be illuminated with a fixed daily *photoperiod* (e.g., 16-h light and 8-h dark, or 12-h light and 12-h dark), and should use incandescent or fluorescent lights. Light intensity adjacent to the surface of the soil in each test vessel should be 400–800 lux, and must be at least 400 lux as a minimum. This range is equivalent to a quantal flux of 5.6–11.2 μmol/(m^2 · s) for cool-white fluorescent, 6.4–12.8 μmol/(m^2 · s) for full-spectrum fluorescent, or 7.6–15.2 μmol/(m^2 · s) for incandescent.

4.4 Criteria for a Valid Test

For the results of this biological test method to be considered valid, each of the following criteria, specific to each species must be achieved[36]:

For *Folsomia fimetaria*:

- the mean survival rate for adult springtails held in *negative control soil* for 21 days must be ≥70% at the end of the test
- the reproduction rate for the adult springtails in *negative control soil* for 21 days must average ≥ 100 live juveniles per control vessel

For *Folsomia candida*:

- the mean survival rate for adult springtails held in *negative control soil* for 28 days must be ≥70% for tests conducted in natural soil, and 80% for tests conducted in artificial soil, at the end of the test
- the reproduction rate for the adult springtails in *negative control soil* for 28 days must average ≥100 live juveniles per control vessel

For *Orthonychiurus folsomi:*

- the mean survival rate for the adults held in negative control soil for 28 days must be ≥70% at the end of the test
- the reproduction rate for the adult springtails in *negative control soil* for 28 days must average ≥100 live juveniles per control vessel

4.5 Food and Feeding

During a toxicity test, *O. folsomi* in each test vessel are fed ~5 mg of granulated dry yeast, every 7 days, starting at Day 0 and continuing until, and including Day 21. For tests using *F. fimetaria*, ~10 mg of dry yeast is added to each test vessel on Days 0 and 14, and for *F. candida*, ~10 mg of dry yeast is added to each test vessel on Day 0 and ~20 mg on Day 14. The type of yeast used is a dried, activated yeast (e.g., Fleischman's™) and is prepared by distributing the yeast uniformly over the surface of the moist test soil, or over the dry soil and then spraying the soil 3 times to activate the yeast and moisten the soil. It is important that the same amount of yeast is available to organisms in each test vessel. If, when adding yeast to a test vessel, it is noticed that the yeast from a previous feeding period has not been consumed, the unconsumed yeast should not be removed and no further yeast is added to the test vessel at that time.[37]

4.6 Observations and Measurements During the Test

The biological endpoints for the test are the number of live adult springtails and the number of *progeny* produced in each test vessel at the end of the test (Day 21 for *F. fimetaria*, and Day 28 for *F. candida* and *O. folsomi*). The condition, appearance, and number of live springtails transferred to each test vessel on Day 0 must be observed and recorded. The lid must

[36] The test validity criteria presented here are based on control data generated for all three test species in many studies carried out during the development of the method (Aquaterra Environmental, 1998a; Stephenson *et al.*, 1999a, b, 2000a; Aquaterra Environmental and ESG, 2000; ESG 2000, 2001, 2002; and ESG and Aquaterra Environmental, 2002, 2003; Becker-van Slooten *et al*., 2003, 2005; Krogh, 2004; Stämpfli *et al*., 2005; C. Phillips, personal communication, US Army, RDECOM., Maryland, USA, 2006; EC, 2007a).

[37] If mycelium develops on the soil surface, simply disturb it by carefully breaking it up with a glass stir rod and/or incorporating it gently into the surface of the soil in the test vessel (G. Stephenson, personal communication, Stantec Consulting Ltd., Guelph, Ontario, 2004).

be removed from each test vessel for the purpose of aeration at least once/week or more frequently (i.e., ≥2 times per week) as necessary, or as the test progresses and the number of organisms per test vessel increases.[38] Observations and records should be made at this time regarding any excessive growth of bacteria or fungi, any feeding activity, and the presence and quantity of any uneaten food.

Air temperature in the test facility (Section 4.3) must be measured daily (e.g., using a maximum/minimum thermometer) or continuously (e.g., using a continuous chart recorder).

The contents of each replicate vessel should be examined weekly for apparent "wetness". If, for any treatment, the soil appears to be too dry at any time during the test, all replicates representing that treatment should be examined. The surface of the soil in each test vessel that appears to be too dry, should then be moistened with test water using a fine-spray mister that disperses about 1 mL of water per spray.[39] Alternatively, test vessels can be weighed to determine moisture loss (ISO, 1999). All vessels can be weighed at the beginning of the test. The weight of each test vessel can then be checked every two weeks and test water added to compensate for weight loss (i.e., due to water loss), if the loss is > 2% of the initial water content (ISO, 1999). For a large number of test vessels, the average amount of water lost can be calculated by weighing a random sample of 5 or 10 test vessels. This amount of test water can then be added to all of the test vessels.

The pH and moisture content of the test soil representing each treatment (including the *negative control soil* and, if used, *reference soil*) must be measured and recorded at the beginning and end of the test. Additionally, it is recommended that conductivity be measured at the beginning and end of the test in instances where the test soil is anticipated to have a high salt content. The initial (Day 0) measurements should be made using a composite sample made up of subsamples of each *batch* of test soil used to set up replicates of a particular treatment (see Section 4.3).[40] The final (i.e., Day 21 or Day 28, depending on the species used) measurements should be made using additional replicates set up for each treatment (see Section 4.1) which are analyzed at the end of the test.

Soil pH should be measured using a calcium chloride ($CaCl_2$) slurry method (modified from Hendershot *et al*., 1993).[41] For these analyses,

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[38] The lid should be removed slowly to allow any individuals hiding under the lid to fall back into the test vessel.

[39] The apparent "wetness" of a soil is affected by the nature of the soil and the amount of water lost from test vessels due to evaporation. However, weekly additions of water might result in the soil being too wet at test end. Any decision as to whether or not to spray water onto the surface of the soil in each test vessel should be made based on apparent "wetness" of the test soil, during each weekly period of observation. To assess this, a qualitative "squeeze test" (see Section 5.3) should be applied to a small quantity (i.e., a "pinch") of the surficial soil within the extra replicates set up for the purpose of physicochemical analyses. If no water appears, the soil is likely too dry. In this instance, the surface of the soil in the test vessel should be lightly misted.

[40] On the day before the start of the test (Day -1), one or more additional replicates of each test soil should be placed into a test vessel within the test facility. These replicates (with or without organisms added) should be reserved for physicochemical analyses of Day-0 conditions to which the springtails are exposed. A separate set of replicates should also be set up on Day -1, for physicochemical analyses of test end (Day 21 or Day 28) conditions.

[41] The method by Hendershot *et al.* (1993) includes a step that involves air drying the sample for 48 h before its analysis for pH. The experience of Environment Canada investigators is that this step is needlessly time consuming (K. Doe, personal communication, Atlantic Environmental Science Centre, Environment Canada, Moncton, New Brunswick; J. Princz, personal communication, Biological Methods Division, Environment Canada, Ottawa, Ontario, 2004), and does not appreciably modify the pH relative to that for hydrated (i.e., as per the toxicity test) soil

4 g of hydrated soil [42] is placed into a 30-mL glass beaker (~ 3 cm in diameter and ~7 cm high) with 20 mL of 0.01 *M* $CaCl_2$.[43] The suspension should be stirred intermittently for 30 min (e.g., once every 6 min). The slurry should then be left undisturbed for ~ 1 h. Thereafter, a pH probe is immersed into the supernatant and when the meter reading is constant, the pH is recorded.

The *moisture content* of each *test soil* is measured by placing a 3–5 g subsample of each test soil into a pre-weighed aluminum weighing pan, and measuring and recording the wet weight of the subsample. Each subsample should then be placed into a drying oven at 105 °C until a constant weight is achieved; this usually requires a minimum of 24 hours. The dry weight of each subsample should then be measured and recorded. Soil moisture content must be calculated (on a dry-weight basis) by expressing the moisture content as a percentage of the soil dry weight:

$$\text{Moisture content (\%)} = \frac{\text{wet weight (g) - dry weight (g)}}{\text{dry weight (g)}} \times 100$$

It is important that the calculation of moisture content (%) be based on a dry weight (**not** on wet weight), since the results of these calculations are used with calculations of water-holding capacity (also calculated based on dry weight) to express the optimal moisture content in test soils (see Section 5.3).

Depending on the nature of the test and the study design, concentrations of chemical(s) or chemical product(s) of concern might be measured for test soils or selected concentrations thereof, at the beginning and end of the test. For a test using a sample of field-collected *site soil*, the chemical(s) or chemical product(s) measured will depend on the contaminant(s) of concern (see Section 5.4). For a multi-concentration test with chemical-spiked soil, such measurements should be made for the high, medium, and low concentrations tested, as a minimum (see Section 6.3). Aliquots for these analyses should be taken as described previously for pH and moisture content; analyses should be according to proven and recognized (e.g., SAH, 1992; Carter, 1993) analytical techniques.

4.7 Ending the Test

The test is terminated after 21-days of exposure for *F. fimetaria*, and 28-days of exposure for *F. candida* and *O. folsomi*. At that time, the

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(Courchesne *et al.*, 1995; J. Princz, personal communication, Biological Methods Division, Environment Canada, Ottawa, Ontario, 2004).

Becker-van Slooten *et al*. (2004) assessed three different soil slurry methods for measuring pH. The need for this testing was identified during EC's soil toxicity workshop in Vancouver, British Columbia (February, 2003), where certain participants recommended that a commonly used and "universally standardized" method for measuring soil pH be incorporated into each of EC's soil toxicity test methods (EC, 2004b). The following three methods for measuring soil pH were compared: 1) 1 *M* KCl in water; 2) 0.01 *M* $CaCl_2$ in water; and 3) water only. Results showed advantages and disadvantages with each method for measuring pH. However, based on practical considerations and the recommendations of the workshop participants (i.e., that a widely used method for characterizing soil pH be applied), the 0.01 *M* $CaCl_2$ method was recommended as the most appropriate for EC's soil toxicity test methods (Becker-van Slooten *et al*., 2004).

[42] It might be necessary to use a lower soil:$CaCl_2$ solution ratio (e.g., 2 g of soil:20 mL of $CaCl_2$) for soils with a high organic matter content (i.e., for soils where the slurry does not yield a supernatant).

[43] To prepare 0.01 *M* $CaCl_2$, dissolve 2.940 g of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) with distilled water, in a 2000-mL volumetric flask. The conductivity of the $CaCl_2$ solution should be between 224 and 240 mS/m at 25 °C, and the pH should range within 5.5–6.5 at 25 °C (Henderson *et al.*, 1993). If the pH is outside this range, it should be adjusted to the range using a hydrogen chloride (HCl) or calcium hydroxide [$Ca(OH)_2$] solution. If the conductivity is not within the acceptable range, a new solution must be prepared.

number of live adult springtails and the number of live *progeny* in each test vessel must be observed and recorded. Before opening a test vessel, the lid should be tapped (e.g., 3 times) to dislodge any individuals from the underside. Two different options are recommended for extracting the Collembola from the test soil (i) the flotation method, and (ii) the heat extraction method.[44]

For the flotation method, test water is added to the test vessel, to about 2 cm above the surface of the soil and the slurry is stirred with a glass stir rod. The Collembola, both adult and progeny produced during the test, float to the surface of the water;[45] then, they can either be removed with a moistened paint brush and counted, or the supernatant can be poured into a wide Petri dish. The Collembola are distributed over the surface of the water in the Petri plate and can be easily counted systematically and their numbers recorded. Once the individuals have been counted, the water in the Petri dish is discarded. Water is then added again to the test vessel, the slurry is stirred vigorously to break up soil particles and dislodge Collembola, and the individuals enumerated by pouring the water (and the suspended or floating Collembola) into the Petri dish where they are counted and recorded. These procedures are repeated until Collembola no longer float to the surface when water is added to the soil remaining in the test vessel.

Alternatively, the soil from each test vessel is poured into 500 mL glass beakers (9-cm diam.) to which 150 mL of water is added. The test vessel is rinsed with water, which is then added to the slurry in the beaker. After gentle stirring with a spatula, approximately 250 mL of water and blue bromophenol (or any other dark-coloured dye that is not toxic to the Collembola) is added (the latter to improve the contrast between the whitish Collembola and the water). The mixture is stirred thoroughly. The beakers are then filled with water to 500 mL and the number of juveniles and adults are either counted manually on the surface of the water, or by taking a digital photograph or colour slide of the water surface. The image is then transferred to a computer screen or a paper hardcopy, or projected using a table projector for enumeration of adults and juveniles. If the test vessel contains a large number of organisms, the Collembola tend to clump together, thereby making it difficult to count individuals. In this case, the organisms can be poured out or split into several (e.g., three) aliquots in preparation for photography. The individuals in each digital image can then be more easily counted and the results of all of the aliquots tallied for a final vessel count. There are a number of other methods that can be used for enumerating adult and juvenile Collembola at the end of the test

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[44] Becker-van Slooten *et al.* (2005) evaluated the use of heat extraction on the adults and juveniles of two species of Collembola, *Folsomia candida* and *Folsomia fimetaria*. The source of heat was a constant light source (60 W lightbulbs) that increased the temperature at the soil surface by 5 °C up to 40 °C over 48 h. The temperature was increased at specific times by reducing the distance between the light and the soil surface (i.e., 20 °C at time zero; 25 °C at 6 h; 30 °C at 24 h; 35 °C at 30 h; and 40 °C at 48 h). Temperatures were measured using a soil thermometer with a thin wire probe. The flotation and heat extraction methods for the enumeration of *F. fimetaria* after 21-days of exposure to boric acid were also compared. Results showed that the recovery rate of *F. fimetaria* adults and juveniles from three different soil types (i.e., artificial, clay loam, and sandy loam) was greater with heat extraction than with extraction by flotation. The control results met all validity criteria proposed for *F. fimetaria* using heat extraction. It was also easier to differentiate between juveniles and adults, as well as juveniles and small particles of soil, using digital photography with heat extraction compared with flotation. A disadvantage of heat extraction is the processing time (over 48 h), whereas extraction by flotation takes ~ 4 h. In addition, the optimal distance between the heat source (light bulb) and the soil surface may differ for different soil types; therefore, it may need to be established prior to definitive testing (Becker-van Slooten *et al*., 2005).

[45] The springtails float to the surface, because of their hydrophobic outer integument.

including the use of image analysis software, and other image processing devices (Krogh *et al*., 1998). If springtails are enumerated using image analysis or any other automated counting method, the method must be previously verified using some form of a manual count to ensure that the numbers being produced by the automated system are accurate.

The heat extraction method described by Wiles and Krogh (1998) and OECD (2005) is based on principles of MacFayden and of Petersen, and involves a controlled temperature gradient extractor, where the organisms are collected over a 48-h period.[46] Becker-van Slooten *et al*. (2005) developed a simpler and more cost-effective heat extraction technique. This method, which was then further refined by Environment Canada (2006b) using equipment available in Canada, is recommended herein as an alternative to the flotation method for the extraction of springtails from test soil. The heat comes from a lamp fitted with a 60- or 100-watt lightbulb, and is regulated by the distance of the lightbulb from the surface of the soil in the heat extraction unit.[47] One heat extraction unit should be prepared for each test vessel. At test termination, the soil from each test vessel is transferred into a heat extraction unit.[48] The soil surface is smoothed out evenly over the mesh, using a spoon or a scoopula. The heat extraction units are placed underneath the lamps, limiting the number of units per lamp to no more than 5 or 6, so that the heat and light are kept consistent for each unit. The bottom of the lightbulb is adjusted to 30 cm above the top of the soil and a

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[46] In the heat extraction method described by Wiles and Krogh (1998), the heat comes from a heating element at the top of an extraction box (regulated through a thermistor placed on the surface of the soil sample). The temperature in the cooled liquid surrounding the collecting vessel is regulated through a thermistor situated at the surface of the collection box (placed below the soil). The thermistors are connected to a programmable controlling unit that raises the temperature according to a pre-programmed schedule (i.e., the soil is gradually heated from 25 °C to 40 °C at a rate of 5 °C every 12 h). The organisms are collected in a cooled collecting vessel (2 °C) with a Plaster of Paris™/charcoal layer at the bottom.

Addison and Bright (2002) assessed the flotation method and the high gradient heat extraction method for effectiveness in extracting *O. folsomi* and *F. candida* from test soils. Two desk lamps (40W) provided heat and light stimulus for the test organisms to leave the soil. The Collembola were collected into distilled water over a two-day period using the heat extraction method. For *O. folsomi*, both the flotation method and the heat extraction were equally effective (i.e., both recovered ~ 95% of the test organisms in the soil); however, for *F. candida*, the high gradient heat extraction was much more effective in recovering organisms (90–100 % recovery) than the flotation method (~70% recovery). In addition, the authors found the heat extraction method to be less labour intensive than the flotation method and allowed a larger number of samples to be processed at a time. Also, the heat extraction method allows for physicochemical analyses to be performed on the test replicates themselves, whereas additional replicates need to be set up for this purpose when the flotation method is used.

[47] The heat extraction unit consists of two plastic cups (e.g., Fisher cat # 11-838-17), one of which has ~1 cm cut off of the bottom, and the other which has ~ 1 cm of Plaster of Paris™ substrate (see Section 2.3.5) on the bottom. A piece of plastic canvas (used for needlework; 7 mesh) is cut to size and glued (with a hot glue gun and non-toxic glue sticks) into place ~ 1 cm below the top edge (not the cut edge) of the cup that has had the bottom removed. The heat extraction unit is assembled by placing the cut cup (i.e., with the mesh insert), upside down on top of the whole cup (i.e., with the Plaster of Paris™ substrate on the bottom) so that the two widest parts (i.e., the original top of each cup) of both cups meet (i.e., the cup with the mesh insert is inverted on top of the cup containing the Plaster of Paris™). A piece of Parafilm® should be wrapped around the seam between the two cups and secured with a piece of tape, if necessary (EC, 2006b).

[48] Soil from a test vessel can be transferred to a heat extraction unit by inverting an assembled heat extraction unit onto the opening of a test vessel. Both units are then inverted again, so that the soil from the test vessel falls into the heat extraction unit. The bottom of the test vessel should be tapped several times to dislodge any soil stuck to the sides and bottom, or a scoopula may be used to carefully scrape out the remaining soil particles into the heat extraction unit, taking care not to damage any springtails that may be in the remaining soil. Any live springtails attached to the empty test vessel should be recorded and combined with the final count data (EC, 2006b).

thermometer (e.g., electronic thermometer) is set up within one of the units to monitor temperature changes throughout the extraction. The temperature should be recorded every 12 hours or at the beginning and end of each work day (i.e., 9:00 am and 5:00 pm). For tests with *O. folsomi* and *F. fimetaria*, the lamp height does not need any adjustment and the temperature should reach ~32 °C after 48 hours. For *F. candida*, the lamp should be lowered so that the bottom of the lightbulb is 25 cm above the top of the soil after 24 hours of the extraction, and the temperature reaches ~36 °C after 48 h. At the end of the extraction period (i.e., 48 h), the lamps should be turned off, and the Parafilm® removed. The organisms that have dropped down through the mesh to the Plaster of Paris™ substrate can be counted immediately, either manually or through image analysis, as previously described, or they can be preserved (e.g., in 70% alcohol) for enumeration at a later date. Laboratories that are inexperienced with the heat extraction procedure described, must initially establish and document the efficiency of their heat extraction system (i.e., demonstrate and record data which show that a significant number of test organisms are not being left in the soil following heat extraction). This can be demonstrated by further processing the heat-extracted soil for test organisms using the flotation method as a check on the efficiency of the heat-extraction technique. This method requirement is the direct result of problems identified during the method interlaboratory studies. The heat extraction process is considered acceptable if <5% of the total number of test organisms extracted from the soil are removed using flotation, following heat extraction. Once laboratory personnel are experienced with heat extraction and have demonstrated the efficiency of their system, they should continue monitoring the efficiency periodically.

In general, adults can be easily distinguished from juveniles by their significantly greater size; however, male *F. fimetaria* can be mistaken for juveniles because their sizes are similar. Experience with this species will improve the ability to distinguish males from progeny (Stämpfli *et al*., 2005). The number of adult springtails and the number of progeny, alive or dead, must be counted and recorded. Live *O. folsomi*, *F. candida/fimetaria* are opaque/white, mobile on the water's surface, and are often curled up, taking 5 seconds to 1 minute to uncurl. A springtail is considered dead if there is complete cessation of movement of any type of body part including legs, abdomen, head, and antennae. Dead Collembola appear transparent and stretched out or elongated with legs fully extended, and can be distinguished from molted carapaces, as the latter are translucent and collapsed. Since the bodies of dead adult Collembola decompose rapidly and are usually not seen, any missing Collembola are considered as dead. Live juveniles must be distinguished from the adults and counted separately. If dead juveniles are observed, they should be noted.

Test vessels, irrespective of concentration levels, should be processed in a random manner since the perception of size tends to change over time, and discrimination between adults and juveniles and counting may become more or less accurate. Extra replicates of each test soil (including the *negative control soil* and, if included in the test, *reference soil*) set up for the purpose of physicochemical analyses should be analyzed to determine the pH and moisture content at the end of the test (Section 4.6). Analyses for other chemical constituents (i.e., concentrations of contaminants) should also be made at this time using additional replicates prepared for each test soil (Section 4.6).

4.8 Test Endpoints and Calculations

For each test, the percent survival for all replicate groups of adult springtails in each test vessel at the end of the test (i.e., Day 21 for *F. fimetaria*, and Day 28 for *F. candida* and *O.*

folsomi) must be calculated and reported. The mean (± SD) percent survival for all springtails exposed to each concentration must also be calculated and reported for the end of the test, using the survival data determined from all treatment replicates (e.g., the mean of the replicates within each treatment).

For a single-concentration test (Section 4.1), the mean (± SD) value for the percent survival of adults, as determined for each treatment at the end of the test, is compared with that for the sample(s) of *reference soil* or, as necessary and appropriate, compared with that for the *negative control soil*. Section 5.5 provides guidance in this regard. For a multi-concentration test (see Sections 4.1, 5.3, and 6.2), the 21-day or 28-day LC50 (depending on the species tested) for adult springtails must be calculated and reported, data permitting. Environment Canada (2005b) provides guidance for calculating LC50s, which should be followed; Section 4.8.1 gives further guidance in this regard.

The reproductive endpoint for this test is based on the number of surviving progeny produced in each replicate and each treatment during the test period. A significant reduction in this number is considered indicative of an adverse toxic effect of the treatment on the reproductive success of the adult Collembola. For a single-concentration test (see Sections 5.3 and 6.2), the mean (± SD) value for number of surviving progeny in the test soil on Day 21 for *F. fimetaria*, and Day 28 for *F. candida* and *O. folsomi* is determined and compared to that for the sample(s) of *reference soil* or, as necessary and appropriate, compared to that for the *negative control soil*. A *Student's t-test* or other appropriate statistic (EC, 2005b) should be used for this comparison. For a multi-concentration test (see Sections 5.3 and 6.2), the 21-day *ICp* for *F. fimetaria*, and the 28-day *ICp* for *F. candida* and *O. folsomi* for reproductive inhibition must be calculated and reported (data permitting).[49] EC (2005b) provides direction and advice for calculating ICps, which should be followed; Section 4.8.2 (including Appendix I) gives further guidance in this regard. Initially, regression techniques (see Section 4.8.2.1) must be applied to multi-concentration data intended for calculation of an ICp.[50] In the event that the data do not lend themselves to calculating the 21-day or 28-day ICp for reproductive inhibition using the appropriate regression analysis (see Appendix I), linear interpolation of these data using the program ICPIN should be applied in an attempt to derive an ICp (see Section 4.8.2.2). All statistical tests used to derive endpoints require that concentrations be entered as logarithms.

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[49] Historically, investigators have frequently analyzed *quantitative* sublethal data 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) (NERI, 1993; EC, 2005b). Given these disadvantages, ICp is the required statistical endpoint for reproductive data derived in a multi-concentration test using Collembola.

[50] Regression is the method of choice for estimating ICp. It involves fitting the data mathematically to a selected model and then calculating the statistical endpoint using the model that best describes the exposure-concentration response relationship. Nonlinear regression techniques were originally recommended by Stephenson *et al*. (2000b) for several reasons including: the relationship that exists between exposure concentration and springtail reproduction responses is typically nonlinear; the *heteroscedasticity* of the data is rarely reduced by transformation; the more standard bootstrap simulation technique has several limitations for these types of data; and nonlinear regression can describe distributions of the response variables showing *hormesis*. By using standard mathematical techniques, a regression can be well described in terms that convey useful information to others, effects at high and low concentrations can be predicted, and confidence intervals can be estimated. Deficiencies of the smoothing and interpolation method can be largely mitigated (EC, 2005b).

4.8.1 LC50

When a multi-concentration test with soil mixtures is conducted, the *quantal* mortality data for a specific period of exposure must be used to calculate (data permitting) the appropriate *median lethal concentration (LC50)*, together with its 95% confidence limits. For *F. fimetaria*, the 21-day LC50 for the adult (first generation) springtails must be calculated and reported, data permitting; and for *F. candida* and *O. folsomi*, the 28-day LC50 for the adult (first generation) springtails must be calculated and reported, data permitting[51] (see Section 4.8). To estimate a LC50, mortality data at the specified period of exposure are combined for all replicates at each concentration. If mortality is not ≥50% in at least one concentration, the LC50 cannot be estimated. If there are no mortalities at a specific concentration, that information is used as 0% effect of mortality. However, if successive concentrations yield a series of 0% mortalities, only the highest concentration of the series should be used in estimating the LC50 (i.e., the zero-effect that is "closest to the middle" of the distribution of data). Similarly, if there are a series of successive complete mortalities at the high concentrations in the test, only one value of 100% effect would be used, i.e., the one at the lowest concentration. Use of only one 0% and one 100% effect applies to any form of statistical analysis and to plotting on a graph.

The guidance provided by Environment Canada (2005b) on choosing statistical test methods to be applied to *quantal* (e.g., LC50) data, should be consulted when choosing the statistical test to be applied to such data for toxicity tests using springtails. Probit and/or Logit regressions are the "preferred" methods (EC, 2005b), provided that two or more concentrations showing partial effects are included in the data. The probit analysis also gives the slope of the line, which should be reported. If probit or logit do not work because of only one partial effect, use the Spearman-Kärber method with no trim. If no partial effect is evident, use the binomial method. The binomial estimate might differ somewhat from the others, and this estimate should only be used as a last resort. Formal confidence limits are not estimated using the binomial method; instead, outer limits of a range are provided, within which the LC50 and the true confidence limits would lie.

Various computer programs may be used to calculate the LC50. Stephan (1977) developed a program to estimate LC50s using probit, moving average, and binomial methods, and adapted it for the IBM-compatible personal computer. Use of this program, which was modified in 1989 to include estimates using the Spearman-Kärber method with no "trimming" (i.e., with no deletion of data from the calculations), is available on CD[52] from Environment Canada (address in Appendix B) and is recommended. Other satisfactory computer and manual methods may be used (e.g., SAS 1988 or version 3.5 of TOXSTAT 1996; see EC 2005b for additional information). Programs using the trimmed Spearman-Kärber method (e.g., Hamilton *et al.*, 1977) are available for personal computers; however, the use of this method (with trimming) should be applied cautiously to LC50 estimates according to EC (2005b), because divergent results might be obtained by operators who are unfamiliar with the implications of trimming ends of the concentration-response data. However, there are situations where application of the trimmed Spearman-Kärber method is warranted (see EC, 2005b for guidance).

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[51] Depending on the study objectives and the associated experimental design, a test for effects on the survival and reproduction of springtails might be focussed on sublethal effects. In this instance, the test might not include a sufficient number of high (lethal) concentrations to enable the calculation of the 21- or 28-day LC50.

[52] Through the courtesy of Dr. Charles E. Stephan (USEPA, Duluth, Minnesota).

Any computer-derived LC50 should be checked by examining a plot, on logarithmic-probability scales, of percent mortalities at a defined period of exposure for the various test concentrations (EC, 2005b). Any major disparity between the estimated LC50 derived from this plot and the computer-derived LC50 must be resolved. A hand-plotted graph is recommended for this check (EC, 2005b). A computer-generated plot (e.g., SigmaPlot™; Version 8.0.2 or later)[53] could be used if it were based on logarithmic-probability scales. If there has been an error in entering the data; however, a computer-generated plot would contain the same error as the mathematical analysis, and so the investigator should carefully check for correct placement of points (EC, 2005b).

A manual plot of mortality/concentration data to derive an estimated LC50 is illustrated in Figure 3. This (hypothetical) figure is based on test concentrations of 1.8, 3.2, 5.6, 10, and 18 mg chemical/kg soil (dry-weight basis) causing mortalities of 0, 20, 40, 90, and 100% of test organisms exposed to the respective concentrations for a specified period of time. The concentration expected to be lethal to 50% of the springtails can be read by following across from 50% (broken line) to the intersection with the best-fit line, then down to the horizontal axis for an estimated LC50 (5.6 mg/kg, dry weight).

In fitting a line such as that in Figure 3, more emphasis should be assigned to points that are near 50% mortality. Logarithmic-probability paper (*log-probit*, as in Figure 3) can be purchased in good technical bookstores, ordered through them, or photocopied (see blank graph in EC, 2005b).

For the regular set of data in Figure 3, computer programs gave very similar estimates to the graphic one. Some of the computed LC50s (and 95% confidence limits) were:

Stephan (1977) method:
- probit: 5.58 (4.24 and 7.37)
- moving average: 5.58 (4.24 and 7.33)
- binomial: 6.22 (between 1.8 and 10)

SAS (1988) probit analysis: 5.58 (4.26 and 7.40)

TOXSTAT (1996) method (version 3.5):
- probit: 5.58 (4.38 and 7.12)
- Spearman-Kärber, zero trim: 5.64 (4.40 and 7.23)
- logit: 5.63 (4.39 and 7.22)

Table 4.2 in EC (2005b) provides additional examples of computed data for quantal tests using various computer programs.

4.8.2 ICp

When a multi-concentration test for effects of exposure of springtails to field-collected or spiked-soil mixtures is conducted, the *quantitative* data representing reproductive inhibition must be used to calculate the *ICp (inhibiting concentration for a specified percent effect)* (see introductory paragraphs of 4.8 and Section 6.2). The ICp is a quantitative estimate of the concentration causing a fixed percent reduction in the mean number of *progeny* produced by the adult springtails during the test. The ICp is calculated as a specified percent reduction (e.g., the IC25 and/or IC20, which represent 25% and 20% inhibition, respectively). The desired value of *p* is selected by the investigator, and 25% or 20% is currently favoured. Any ICp that is calculated and reported must include the 95% confidence limits. In the analyses of reproductive performance, the number of progeny produced in each replicate is used to calculate the average number of surviving progeny produced per treatment

[53] Available for purchase from SYSTAT Software, Inc., 1735 Technology Drive, Ste 430, San Jose, California 95110, USA, phone: 1-800-797-7401; Web site www.systat.com/products/SigmaPlot/.

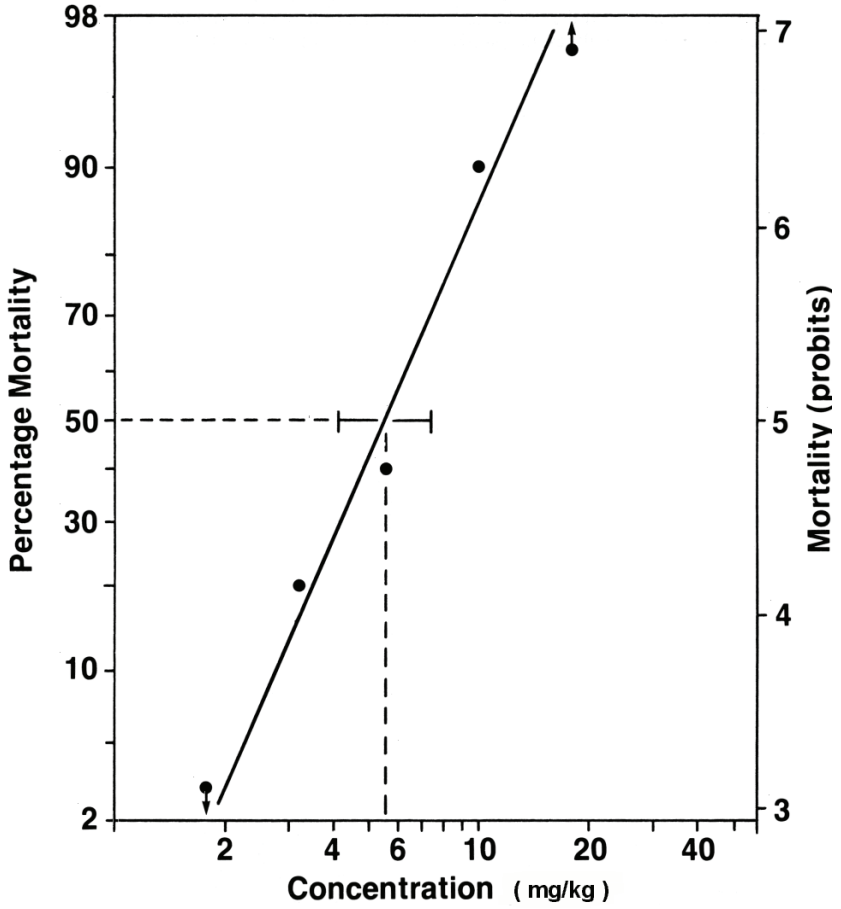

Figure 3 Estimating a Median Lethal Concentration by Plotting Mortalities on Logarithmic-Probability Paper

(concentration) in relation to the average number produced in the *negative control* replicates. A value of zero is assigned for the number of juveniles in a replicate, if all of the adult springtails in that replicate died before producing progeny. If any of the adult Collembola died during the test, after producing young, the number of progeny produced is still to be used in the analyses. If there are no surviving progeny in a replicate (test vessel), it contributes a value of zero to the calculation used to obtain the average number of survivors for that treatment (concentration). If there are no surviving progeny in all replicates at a given concentration, that concentration is still included in the analysis, using an average value of zero juveniles.

As previously indicated, an ICp for mean number of surviving progeny produced in each treatment must be calculated and reported (data permitting) upon completion of a 21-day multi-concentration test with *F. fimetaria*, and a 28-day multi-concentration test with *F. candida* and *O. folsomi*. These calculations must be made using the appropriate linear or nonlinear regression analyses (see the following Section 4.8.2.1). If, however, regression analyses fail to provide meaningful ICps for the mean number of live progeny produced, the ICPIN analyses described in Section 4.8.2.2 should be applied to the corresponding data.

4.8.2.1 Use of regression analysis. Upon completion of a definitive 21-day *(*for *F.*

fimetaria), or 28-day *(*for *F. candida* and *O. folsomi)* multi-concentration test, an ICp (including its 95% confidence limits) for the mean number of surviving progeny produced in each treatment must be calculated using linear and/or nonlinear regression procedures. These values may be calculated using a series of linear and nonlinear regression models (data permitting) proposed by Stephenson *et al*. (2000b) that have been re-parameterized, based on techniques applied by van Ewijk and Hoekstra (1993), to automatically generate the ICp and its 95% confidence limits for any value of 'p' (e.g., IC25 or IC50). The proposed models for application consist of one linear model, and the following four nonlinear regression models: exponential, Gompertz, logistic, and logistic adjusted to accommodate *hormesis*[54]. Further guidance on the use of these linear and nonlinear regression models for calculating ICps is provided by Stephenson (2003a) and Stephenson *et al*. (2000b). The reader is also strongly advised to consult EC (2005b) for additional guidance on the general application of linear and non-linear regression for the analysis of *quantitative* toxicity data. Instruction for the appropriate application of linear and non-linear regression, using Version 11.0 of the statistical program SYSTAT[55], is provided in Appendix I. However, any statistical software capable of linear and nonlinear regression may be used when calculating the respective ICps and their associated 95% confidence limits. Appendix I provides instruction on the use of regression models to derive the most appropriate ICps for reduced numbers of surviving progeny.

The five models recommended for application are as follows; further information on these specific models is presented in Appendix I:

Exponential model:

$$Y = a \times (1 - p)^{(C \div ICp)}$$

where:

- Y = number of progeny
- a = the y-intercept (i.e., the control response)
- p = desired value for 'p' (e.g., 0.25 for a 25% inhibition)
- C = the test concentration as a logarithm
- ICp = the ICp for the data set

Gompertz model:

$$Y = t \times \exp[\log(1 - p) \times (C \div ICp)^b]$$

where:

- Y = number of progeny
- t = the y-intercept (i.e., the control response)
- exp = the exponent of the base of the natural logarithm
- p = desired value for "p" (e.g., 0.25 for a 25% inhibition)
- C = the test concentration as a logarithm
- ICp = the ICp for the data set
- b = a scale parameter (estimated to be between 1 and 4) that defines the shape of the equation

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[54] A hormetic response (i.e., *hormesis*) might be observed at one or more of the lowest, sublethal concentration(s), i.e., performance at such concentration(s) is enhanced relative to that in the negative control. For instance, there might be more progeny produced in soil with low concentrations than in the control treatment. This is not a flaw in the testing. Rather, it is a real biological phenomenon. To calculate the ICp when this phenomenon occurs, the data should be analyzed using the hormesis model. The hormetic effects are included in the regression, but do not bias the estimate of the ICp. An estimated IC25 would still represent a 25% reduction in performance from that of the control.

[55] The latest (e.g., Version 11.0) version of SYSTAT™ is available for purchase by contacting SYSTAT Software, Inc., 1735 Technology Drive, Ste 430, San Jose, California 95110, USA, phone: 1- 800-797-7401; see Web site www.systat.com/products/Systat/.

Hormesis model:

$$Y = t \times [1 + (h \times C)] \div \{1 + [(p + (h \times C)) \div (1 - p)] \times (C \div ICp)^b\}$$

where:

- Y = number of progeny
- t = the y-intercept (i.e, the control response)
- h = describes the hormetic effect (estimated to be small, usually between 0.1 and 1)
- C = the test concentration as a logarithm
- p = desired value for 'p' (e.g., 0.25 for a 25% inhibition)
- ICp = the ICp for the data set
- b = a scale parameter (estimated to be between 1 and 4) that defines the shape of the equation

Linear model:

$$Y = [(-b \times p) \div ICp] \times C + b$$

where:

- Y = number of progeny
- b = the y-intercept (i.e., the control response)
- p = desired value for 'p' (e.g., 0.25 for a 25% inhibition)
- ICp = the ICp for the data set
- C = the test concentration as a logarithm

Logistic model:

$$Y = t \div \{1 + [p \div (1 - p)] \times (C \div ICp)^b\}$$

where:

- Y = number of progeny
- t = the y-intercept (i.e, the control response)
- p = desired value for 'p' (e.g., 0.25 for a 25% inhibition)
- C = the test concentration as a logarithm
- ICp = the ICp for the data set
- b = a scale parameter (estimated to be between 1 and 4) that defines the shape of the equation

The general process for the statistical analysis and selection of the most appropriate regression model (linear or non-linear) for quantitative toxicity data is outlined in Figure 4. The selection process begins with an examination of a scatter plot or line graph of the test data to determine the shape of the concentration-response curve. The shape of the curve is then compared to available models so that one or more appropriate model(s) that best suits the data is (are) selected for further examination (refer to Figure I.1, Appendix I, for an example of five potential models).

Once the appropriate model(s) is (are) selected for further consideration, assumptions of *normality* and *homoscedasticity* of the *residuals* are assessed. If the regression procedure for one or more of the examined models meets the assumptions, the data (and regression) are examined for the presence of outliers. If an outlier has been observed, the test records and experimental conditions should be scrutinized for human error. If there are one or more outliers present, the analysis should be performed with and without the outlier(s), and the results of the analyses compared to examine the effect of the outlier(s) on the regression. Thereafter, a decision must be made as to whether the outlier(s) should be removed from the final analysis. The decision should take into consideration natural biological variation, and biological reasons that might have caused the apparent anomaly. Additional guidance on the presence of outliers and unusual observations is provided in Appendix I (Section I.2.4) as well as in EC (2005b). If there are no outliers present or none are removed from the final analysis, the model that demonstrates the smallest residual mean square error is selected as the model of best choice. Additional guidance from a statistician familiar with dealing with outlier data is also advised.

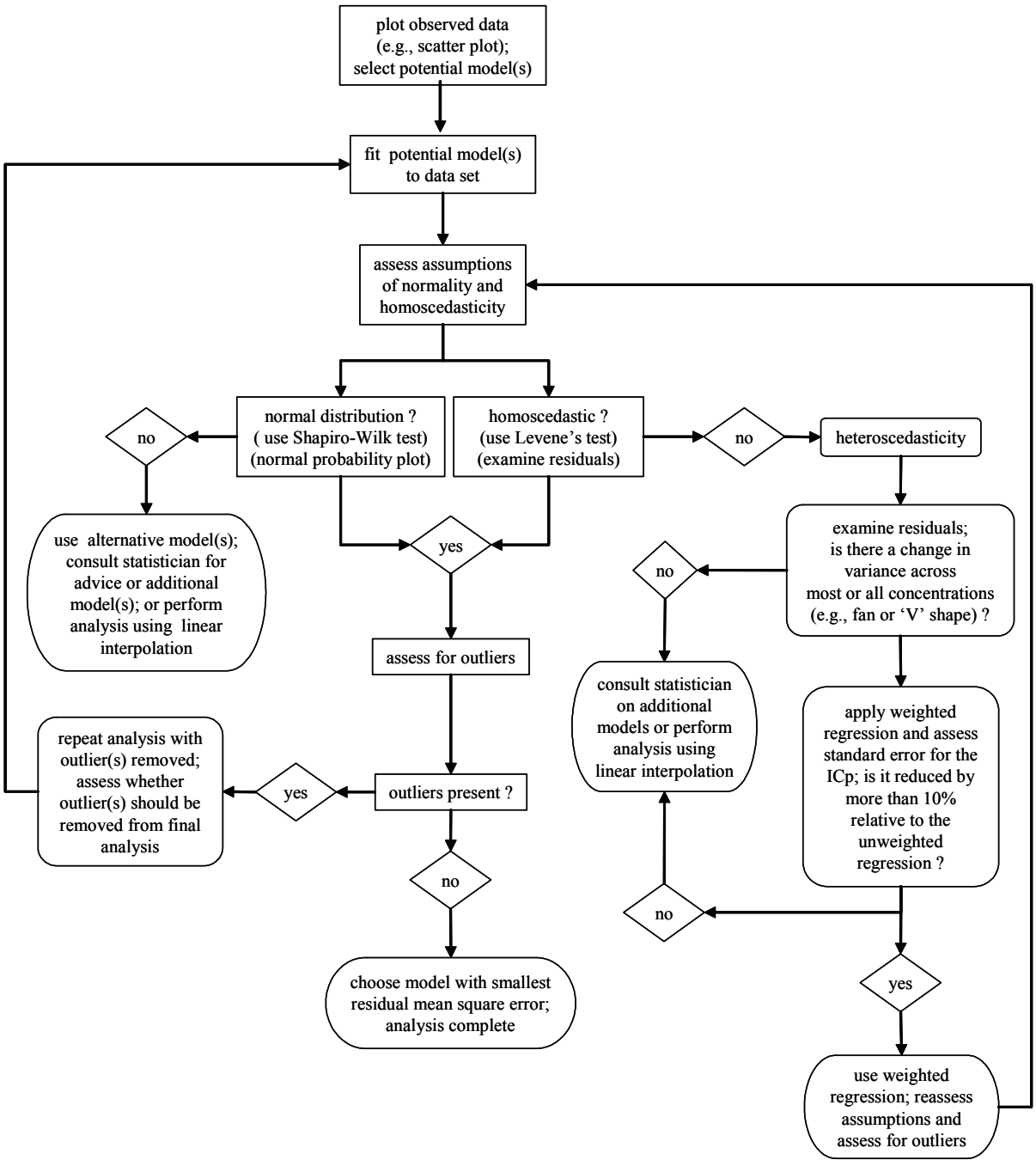

Figure 4 The General Process for the Statistical Analysis and Selection of the Most Appropriate Model for Quantitative Toxicity Data (adapted and modified from Stephenson *et al*., 2000b)

Normality should be assessed using the *Shapiro-Wilk's test* as described in EC (2005b). A normal probability plot of the residuals may also be used during the regression procedure, but is not recommended as a stand-alone test for normality as the detection of a 'normal' or 'non-normal' distribution is dependent upon the subjective assessment of the user. If the data are not normally distributed, then the user is advised to try another model, consult a statistician for further guidance on model selection, or to perform the less-desirable linear interpolation (using ICPIN, see Section 4.8.2.2) method of analysis.

Homoscedasticity of the *residuals* should be assessed using *Levene's test* as described in EC (2005b), and by examining the graphs of the residuals against the actual and predicted (estimated) values. Levene's test provides a definite indication of whether the data are homogeneous (e.g., as in Figure I.2A of Appendix I) or not. If the data (as indicated by Levene's test) are *heteroscedastic* (i.e., not homogeneous), then the graphs of the residuals should be examined. If there is a significant change in the variance and the graphs of the residuals produce a distinct fan or 'V' pattern (refer to Figure I.2B, Appendix I for an example), then the data analysis should be repeated using weighted regression. Traditionally, the data have been weighted by dividing by the inverse of the variance; however, other options are available. Before choosing the weighted regression, the standard error of the ICp is compared to that derived from the unweighted regression. If there is a difference of greater than 10% between the two standard errors[56], then the weighted regression is selected as the regression of best choice. However, if there is less than a 10% difference in the standard error between the weighted and unweighted regressions, then the user should consult a statistician for the application of additional models, given the test data, or the data could be re-analyzed using the less-desirable linear interpolation (using ICPIN, see Section 4.8.2.2) method of analysis. This comparison between weighted and unweighted regression is completed for each of the selected models while proceeding through the process of final model selection (i.e., model and regression of best choice). Some non-divergent patterns might be indicative of an inappropriate or incorrect model (refer to Figure I.2C, Appendix I, for an example), and the user is again urged to consult a statistician for further guidance on the application of additional models.

4.8.2.2 Linear interpolation using ICPIN. If regression analyses of the endpoint data (see preceding Section 4.8.2.1) fail to provide an acceptable ICp for reproductive inhibition, linear interpolation using the computer program called *ICPIN* should be applied. This program (Norberg-King, 1993; USEPA, 1994b, 1995) is not proprietary, is available from the USEPA, and is included in most computer software for *environmental toxicology,* including TOXSTAT. The original instructions for ICPIN from the USEPA are clearly written and make the program easy to use (Norberg-King, 1993).[57] An earlier version was called BOOTSTRP.

Analysis by ICPIN does not require equal numbers of replicates in different concentrations. The ICp is estimated by smoothing of the data as necessary, then using the two data-points

[56] The value of 10% is only a "rule-of-thumb" based upon experience. Objective tests for the improvement due to weighting are available, but beyond the scope of this document. Weighting should be used only when necessary, as the procedure can introduce additional complications to the modeling procedure. A statistician should be consulted when weighting is necessary.

[57] The instructions in Norberg-King (1993) are sometimes misleading on the identity of "replicates". The term is used in such a way that it would apply to numbers of individual organisms within the same vessel. This slip of wording does not affect the functioning of the program. Some commercial programs have been less user-friendly for entry of data and analysis.

adjacent to the selected ICp (USEPA, 1994b, Appendix L; USEPA, 1995, Appendix L). The ICp cannot be calculated unless there are test concentrations both lower and higher than the ICp; both those concentrations should have an effect reasonably close to the selected value of p, preferably within 20% of it. At present, the computer program does not use a logarithmic scale of concentration, and so Canadian users of the program must enter the concentrations as logarithms. Some commercial computer packages have the logarithmic transformation as a general option, but investigators should make sure that it is actually retained when proceeding to ICPIN. ICPIN estimates confidence limits by a special "bootstrap" technique because usual methods would not be valid. Bootstrapping performs many resamplings from the original measurements. The investigator must specify the number of resamplings, which can range from 80 to 1000. At least 400 is recommended here, and 1000 would be beneficial.[58]

If there are several adjacent high concentrations with no surviving juveniles, only the lowest of that string of concentrations should be used in analysis (i.e. the concentration closest to the middle of the series of concentrations used in the test). Normally, there is no particular benefit to including the additional concentrations, because they offer nothing to the analysis (i.e., the data consist only of zero progeny).

Besides determining and reporting the computer-derived ICps for Collembola reproduction at test end, a graph of percent reduction in number of live progeny produced should be plotted against the logarithm of concentration, to check the mathematical estimations and to provide visual assessments of the nature of the data (EC, 2005b).

If the ICPIN program is used when there is a hormetic effect, an inherent smoothing procedure could change the control value and bias the estimate of ICp. Accordingly, before statistical analysis, hormetic values at low concentration(s) should be arbitrarily replaced by the control value. This is considered a temporary expedient until a superior approach is established (EC 2005b). The correction is applied for any test concentration in which the average effect (i.e., the geometric average of the replicate means) is higher ("better") than the average for the control. To apply this correction, replace the observed mean numbers of progeny of the replicates in the hormetic concentration(s), with the means of replicates in the control. The geometric average for that/those concentration(s) will then be the same as that for the control.

4.9 Tests with a Reference Toxicant

Table 14 of Appendix F summarizes the guidance for performing reference toxicity tests given in other documents describing procedures and conditions for conducting tests of soil toxicity using springtails. Described herein are the procedures and conditions to be followed when performing reference toxicity tests in conjunction with a 21-day (for *F. fimetaria*), or 28-day (for *F. candida* and *O. folsomi*) test of soil toxicity using springtails. These procedures also apply to tests for assessing the acceptability and suitability of cultures of *F. fimetaria*, *F. candida*, or *O. folsomi* to be used in soil toxicity tests. They should be applied to assess intralaboratory precision when a laboratory is inexperienced with the biological test method defined in this document and during initial test setup (see Sections 2.3.1, 2.3.9).

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[58] ICPIN has some deficiencies, which is why it is recommended herein only in cases where the use of regression fails to provide an acceptable ICp. Its interpolation method is an inefficient use of data, sensitive to peculiarities of the two concentrations used. The program fails to adopt logarithm of concentration, which would introduce a slight bias towards a higher value of ICp. A modification of the bootstrap method has now remedied a problem of overly narrow confidence limits; however, regression analyses provide more accurate methods of estimating the ICp and its 95% confidence limits (EC, 2005b) (see Section 4.8.2.1)

The routine use of a *reference toxicant* is necessary to assess, under standardized test conditions, the relative sensitivity of a portion of the population of adult springtails within a particular culture (Section 2.3.9) from which test organisms are selected for use in one or more definitive soil toxicity tests. Tests with a reference toxicant also serve to demonstrate the precision and reliability of data produced by the laboratory personnel for that reference toxicant, under standardized test conditions. A reference toxicity test, conducted according to the procedures and conditions described herein must be performed according to one of the following regimes:

(1) at least once every two months[59] using organisms taken from the population of springtails that is being cultured for use in the definitive test(s) (Section 2.3).

(2) at the same time as the definitive soil toxicity test(s), using organisms taken from the same population as those used for the definitive test(s) (see Sections 2.3.8 and 2.3.9).

A laboratory that cultures springtails and frequently performs soil toxicity tests using these organisms might choose to monitor the sensitivity of their culture(s) to one or more reference toxicants on a routine (e.g., every two months) schedule, while including a reference toxicity test using a portion of the springtails used to start a definitive soil toxicity test. Alternatively, a laboratory might choose to monitor the sensitivity of their culture(s) to a reference toxicant less frequently (e.g., two or three times a year), and perform a reference toxicity test at the time that each definitive soil toxicity test is performed.

Each reference toxicity test performed in conjunction with a *definitive* test for soil toxicity must be conducted as a *static* multi-concentration *acute* lethality test. The reference toxicity test must be 7 days in length for *O. folsomi* and *F. fimetaria*, and 14 days in length for *F. candida*.[60] The test conditions and procedures described herein (Section 4.9) for performing an acute (7- or 14-day) lethality test must be applied to each reference toxicity test. Additional conditions and procedures described in Section 4 for performing a multi-concentration test with samples of test soil apply equally to each reference toxicity test. Procedures given in Section 6 for the preparation and testing of chemicals spiked in *negative control soil* also apply here, and should be referred to for further information. Environment Canada's guidance document on using negative control sediment spiked with a reference toxicant (EC, 1995) provides useful information that is also applicable when performing reference toxicity tests with negative control soil spiked with a reference toxicant.

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[59] Environment Canada typically includes monthly reference toxicity tests as the option for routine testing (EC 2004a); however, due to the age-synchronization process required for two of the three Collembola species described in this test method, the number of organisms for testing that are available each month is limited.

[60] A research study carried out by Becker-van Slooten *et al*. (2003) recommended that the acute-lethality reference toxicity test, using boric acid with *F. candida*, as described in Section 4.9 should be 14 days in duration, rather than 7 days, as described for *O. folsomi* and *F. fimetaria*. Two reasons for this recommendation include: (1) the 14-day acute lethality test was twice as sensitive as the 7-day test (i.e., LC50s of 800 and 1521 mg boric acid/kg (dry wt) artificial soil, respectively); and (2) the *F. candida* were too small after 7 days to count the surviving individuals and to differentiate between those that were alive and those that were dead (Becker van-Slooten *et al*., 2003). In a separate study by Stämpfli *et al*. (2005) the toxic effect of boric acid to *F. fimetaria* was greater at 14 days than at 7 days in both artificial and natural soils. Because *F. fimetaria* are older and larger than *F. candida* at the start of the test (23 days instead of 10 days), enumeration at the end of a 7-day exposure for *F. fimetaria* was not compromised by organisms that were too small. With *O. folsomi*, ESG and Aquaterra Environmental (2003) determined that there was little difference in 7-day and 14-day LC50s for boric acid; and therefore, a 7-day reference toxicity test with boric acid was recommended.

The reference toxicity test should be performed using 100- to 125-mL glass jars as test vessels (Section 3.2.2) and a 30-g wet wt aliquot of test soil representing each treatment (concentration) in each test vessel. The number of replicate test vessels per reference toxicant concentration must be ≥3; and ≥5 for *negative control soil*. The number of springtails per test vessel is 10 for *F. candida*, as described in Section 4.2. For reference toxicity tests using *O. folsomi*, and *F. fimetaria*, 10 organisms (5 males and 5 females) per test vessel are also required; however, this differs from the definitive test design that requires 15 organisms (5 males and 10 females) per test vessel for *O. folsomi* and 20 organisms (10 males and 10 females) per test vessel for *F. fimetaria* (see Section 4.2).

Procedures for starting and ending a reference toxicity test should be consistent with those described in Sections 4.2 and 4.7 with the exception of the shorter (7 days for *O. folsomi*, and *F. fimetaria*, and 14 days for *F. candida*) test duration. Test conditions described in Section 4.3 apply. Test observations and measurements given in Section 4.6 should be followed.

To be valid, the mean adult survival rate at the end of the test (Day 7 or Day 14) for springtails held in the aliquots of negative control soil used in a particular reference toxicity test must be at least 80%. Test endpoints to be calculated and reported include the mean percent survival in each treatment at test end (Day 7 or Day 14), and the 7-day or 14-day LC50 (including its 95% confidence limits), depending on the species used. Results for a reference toxicity test should be expressed as mg reference chemical/kg soil, dry weight.

Appropriate criteria for selecting the reference toxicant to be used in conjunction with a definitive test for soil toxicity using Collembola include the following (EC, 1995):

- chemical readily available in pure form;
- stable (long) shelf life of chemical;
- can be interspersed evenly throughout *clean* substrate;
- good concentration-response curve for test organism;
- stable in aqueous solution and in soil;
- minimal hazard posed to user; and
- concentration easily analyzed with precision.

The 7- or 14-day reference toxicity test requires a minimum of six treatments (i.e., *negative control soil* and five concentrations of *reference toxicant*). Reagent-grade boric acid (H_3BO_3) is recommended for use as the reference toxicant(s) when performing soil toxicity tests with springtails, although other chemicals may be used if they prove suitable.[61] Each test concentration should be made up according to the guidance in Sections 4.1 and 6.2, using *artificial soil* (Section 3.3.2) as the substrate.

Routine reference toxicity tests (e.g., those performed once every two months or in conjunction with each definitive test for soil toxicity) using boric acid (or another suitable reference chemical) spiked in *negative control soil* should consistently apply the same test conditions and procedures described herein. A series of test concentrations should be chosen based on preliminary tests, to provide partial mortalities in two or more concentrations and

[61] Aquaterra Environmental (1998a) initially evaluated the performance of various chemicals as candidate reference toxicants for use in conjunction with acute lethality tests for measuring soil toxicity to *O. folsomi*. Subsequent studies by ESG and Aquaterra Environmental (2003) and Becker-van Slooten *et al*. (2003) using boric acid spiked in negative control soil confirmed the usefulness of this chemical as a reference toxicant in soil toxicity tests with *O. folsomi* and *F. candida*, respectively. Boric acid is a commonly used chemical that is water-soluble and relatively innocuous to humans. Boric acid is inexpensive to analyze at concentrations that elicit biological effects and as a non-volatile inorganic compound, boric acid is easily incorporated into test soil, relatively stable, and persistent throughout the test (ESG and Aquaterra, 2003).

enable calculation of a 7-day (for *O. folsomi*[62] or *F. fimetaria*[63]) or 14-day (for *F. candida*)[64] LC50 (see Section 6.4).

Once sufficient data are available (EC, 1995), all comparable LC50s for a particular reference toxicant derived from these toxicity tests must be plotted successively on a *warning chart*. Each new LC50 for the same reference toxicant should be examined to determine whether it falls within ± 2 SD of values obtained in previous comparable tests using the same reference toxicant and test procedure (EC, 1997a, b, 2001, 2004a, 2005a). A separate warning chart must be prepared and updated for each dissimilar procedure (e.g., differing Collembola species or differing reference toxicant). The warning chart should plot logarithm of concentration on the vertical axis against date of the test or test number on the horizontal axis. Each new LC50 for the reference toxicant should be compared with established limits of the chart; the LC50 is acceptable if it falls within the *warning limits*.

The logarithm of concentration (including LC50) should be used in all calculations of mean and standard deviation, and in all plotting procedures. This simply represents continued adherence to the assumption by which each LC50 was estimated based on logarithms of concentrations. The warning chart may be constructed by plotting the logarithmic values of the mean and ±2 SD on arithmetic paper, or by converting them to arithmetic values and plotting those on the logarithmic scale of semi-log paper. If it were demonstrated that the LC50s failed to fit a log-normal distribution, an arithmetic mean and SD might prove more suitable.

The mean of the available values of log(LC50), together with the upper and lower warning limits (±2 SD), should be recalculated with each successive LC50 for the reference toxicant until the statistics stabilize (EC, 1995, 1997a, b, 2001, 2004a, 2005a). If a particular LC50 fell outside the warning limits, the sensitivity of the test organisms and the performance and precision of the test would be suspect. Since this might occur 5% of the time due to chance alone, an outlying LC50 would not necessarily indicate abnormal sensitivity of the culture of Collembola, nor unsatisfactory precision of toxicity data. Rather, it would provide a warning that there might be a problem. A thorough check of all culturing and

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[62] Results for 7-day tests with boric acid, as performed by ESG and Aquaterra Environmental (2003) using *O. folsomi* and the test method for a reference toxicity test described herein, demonstrated 7-day LC50s for artificial soil and a *clean* field-collected clay-loam soil of 3730 and 1807 mg boric acid/kg soil (dry wt), respectively. For these tests, test concentrations of 0, 2000, 4000, 6000, 8000, and 10 000 mg boric acid/kg soil (dry wt) were used. See Appendix H for guidance in selecting an appropriate series of test concentrations (assuming a log-concentration response) for use in toxicity tests with this or other chemicals to be used in a reference toxicity test.

[63] Stämpfli *et al*. (2005) performed 7-day reference toxicity tests with boric acid using *F. fimetaria* and the test method for a reference toxicity test described herein. They demonstrated 7-day LC50s for artificial soil and a *clean* field-collected clay-loam soil of 958 and 905 mg boric acid/kg soil (dry wt), respectively. Test concentrations of 0, 270, 370, 520, 730, 1020, 1430, 2000, and 2800 mg boric acid/kg soil (dry wt) and 0, 248, 372, 540, 826, 1242, 1864, 2792, and 4195 mg boric acid/kg soil were used for the artificial soil and clay-loam soil tests, respectively. See Appendix H for guidance in selecting an appropriate series of test concentrations (assuming a log-concentration response) for use in toxicity tests with this or other chemicals to be used in a reference toxicity test.

[64] Results for 14-day tests with boric acid, as performed by Becker-van Slooten *et al*. (2003) using *F. candida* and the test method for a reference toxicity test described herein, demonstrated 14-day LC50s for artificial soil and a *clean* field-collected clay-loam soil of 800 and 663 mg boric acid/kg soil (dry wt), respectively. These results were almost 2-times lower than 7-day LC50s for the same species and the same soils (1521 and 1590 mg boric acid/kg soil (dry wt) for artificial soil and clay-loam soils, respectively). For the 14-day tests, test concentrations of 0, 190, 270, 370, 520, 730, 1020, 1430, and 2000 mg boric acid/kg soil (dry wt) were used for tests in artificial soil, and 0, 90, 130, 200, 300, 440, 670, 1000, and 1500 mg boric acid/kg soil (dry wt) were used for tests in clay-loam soil. See Appendix H for guidance in selecting an appropriate series of test concentrations (assuming a log-concentration response) for use in toxicity tests with this or other chemicals to be used in a reference toxicity test.

test conditions and procedures should be carried out. Depending on the findings, it might be necessary to repeat the reference toxicity test, establish a new culture, select springtails from an alternate culture, or obtain a new population of test organisms from an outside source, before undertaking further soil toxicity tests.

Results that remained within the warning limits might not necessarily indicate that a laboratory was generating consistent results. Extremely variable historic data for a reference toxicant would produce wide warning limits; a new data point could be within the warning limits but still represent undesirable variation in test results. A coefficient of variation (CV) of no more than 30%, and preferably 20% or less, has been suggested as a reasonable limit by Environment Canada (EC, 1995, 2005b) for the mean of the available values of log(LC50) (see preceding paragraph). For this biological test method, the CV for mean historic data derived for reference toxicity tests performed using boric acid should not exceed 30%.

Concentrations of *reference toxicant* in all *stock solutions* can be measured chemically using appropriate methods (e.g., analytical methods involving AES with ICAP scan, for concentration of boron). Test concentrations of reference toxicant in soil are prepared by adding a measured quantity of the stock solution to negative control soil[65], and mixing thoroughly.[66]

Upon preparation of the mixtures of the reference toxicant in soil, aliquots should be taken from at least the negative control soil as well as the low, middle, and high concentrations.[67] Each aliquot should either be analyzed directly, or stored for future analysis (i.e., at the end of the test) if the 7- or 14-day LC50 based on nominal concentrations was found to be outside the warning limits. If stored, sample aliquots must be held in the dark at 4 ± 2 °C. Stored aliquots requiring chemical measurement should be analyzed promptly upon completion of the reference toxicity test. The 7- or 14-day LC50 should be calculated based on the measured concentrations if they are appreciably (i.e., ≥20%) different from nominal ones and if the accuracy of the chemical analyses is satisfactory.

If boric acid is used as a reference toxicant, the following analytical method applies (OMEE, 1996). A 1–5 g subsample of soil spiked with boric acid is dried at 105 °C to constant weight. A 1-g aliquot is then extracted using an 0.01 *M* solution of $CaCl_2$, by boiling a slurry of soil in 50 mL of this extraction solution and then re-adjusting the final volume to 50 mL using more extraction solution. The 50-mL extract is then filtered through a #4 Whatman™ filter, and

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[65] Section 6.2 "*Preparing Test Mixtures*" includes an example showing the amounts of test water and boric acid to be added to artificial soil, to prepare a given treatment for a reference toxicity test with a specific concentration of boric acid in artificial soil. The calculations in this example show the amount of water necessary to adjust the moisture content of the artificial soil to a fixed percentage (i.e., 70%) of the soil's water-holding capacity, while taking into account the volume of the *stock solution* of boric acid as part of the overall adjustment for soil moisture content.

[66] An accepted procedure is to add a precalculated volume of stock solution (using volumetric and/or graduated pipets) to a glass Erlenmeyer™ flask, diluting to a graduated mark using de-ionized water, and then adding a measured volume of this mixture to the soil. The flask is then rinsed three times with de-ionized water, and the rinsate is added to the soil. The mixture of soil and stock solution is then mixed thoroughly (for approximately three minutes) with a mechanical mixer (e.g., a hand-held mixer with revolving stainless steel beaters) until the soil appears homogeneous in colour, texture, and moisture content. During the mixing process, the soil in the mixing bowl should also be stirred intermittently using a large stainless steel spoon for facilitate homogenization.

[67] If the LC50 for each reference toxicity test is to be based on measured concentrations, it is recommended that one or more aliquots of the chemical-in-soil mixture representing each test concentration be collected and analyzed. If the LC50 for each test is based on nominal concentrations, however, sampling and analysis of aliquots from at least the low, middle, and high test concentrations is recommended.

diluted to a final volume of 100 mL. A blank sample is prepared in a similar manner. The filtrate is analyzed for elemental boron using ICAP/AES. The boric acid concentration in the soil is then calculated using the following equation:

$$\text{boric acid (mg/kg, dry wt)} = \frac{\mu\text{g B/mL (measured)} \times \text{final volume (mL)} \times MW_{\text{boric acid}}/MW_{\text{boron}}}{1000\ (\mu\text{g}) \times \text{weight of sample (mg dry wt)}} \times 10^6$$

The analytical limit of detection for boric acid in soil is reportedly 1 mg boric acid/kg soil dry wt in most instances (Stephenson, 2003b).

Besides performing *acute* lethality tests with a reference toxicant, it is recommended that any laboratory performing 21- or 28-day tests with samples of contaminated (field-collected or chemical-spiked) soil also conduct one or more 21- or 28-day test(s) with their culture(s) of *F. fimetaria*, or *F. candida* and *O. folsomi*, respectively, using a reference toxicant. In keeping with the guidance in EC (2004a, 2005a), these tests should either be performed at least twice a year or, where the testing of contaminated soil is carried out at a lesser frequency, in parallel with each definitive soil toxicity test. The procedures and conditions to be applied to these 21- or 28-day toxicity tests should be consistent with those described in Section 4 herein. Any endpoint data (i.e., 21- or 28-day LC50 and/or ICp; see Section 4.8) should be compared with values obtained in the past for the same species, by the same laboratory and for the same reference toxicant. This testing and comparison is useful to provide assurance that the laboratory's test conditions and procedures when performing a 21- or 28-day test are adequate, and to verify that the long-term response of the springtails to the reference toxicant has not changed appreciably from that of earlier long-term tests with this chemical performed at the testing facility. Boric acid spiked in artificial soil is the recommended reference toxicant for this 21- or 28-day test.[68]

[68] Results for three studies involving 21-day (for *F. fimetaria*), 28-day (for *F. candida*) and 35-day (for *O. folsomi*) reference toxicity tests with boric acid, performed by Stämpfli *et al*. (2005), Becker-van Slooten *et al*. (2003) and ESG and Aquaterra Environmental (2003), respectively, according to the biological test method described herein in Section 4, demonstrated similar findings. Data for the number of live progeny generated during three tests yielded IC50s of 179 and 188 mg boric acid/kg dry wt of artificial soil for *F. fimetaria*, and 147 and 503 mg boric acid/kg dry wt of artificial soil for *F. candida* and *O. folsomi*, respectively. Three additional tests using a clay-loam negative control soil yielded the following results. Data for the number of live progeny generated during these three tests yielded IC50s of 77, 169, and 113 mg boric acid/kg dry wt of clay-loam soil for *F. fimetaria*, *F. candida*, and *O. folsomi*, respectively. The following concentrations of boric acid were used by Stämpfli *et al*. (2005) and Becker-van Slooten *et al*. (2003) to calculate sublethal endpoints during 21-, and 28-day tests with this reference toxicant for *F. fimetaria* and *F. candida*: 0, 20, 35, 50, 80, 120, 180, 270, and 400 mg/kg soil (dry wt). An expanded range (based on a logarithmic series of concentrations; see Appendix H) that includes one or two higher test concentrations is recommended for future tests intended to calculate both lethal and sublethal endpoints. For tests conducted by ESG and Aquaterra Environmental (2003) to calculate both lethal and sublethal endpoints during 35-day exposure tests with this reference toxicant and *O. folsomi*, the following concentrations of boric acid were used: 0, 50, 75, 125, 250, 500, 650, 850, 1000, and 1500 mg/kg soil (ESG and Aquaterra Environmental, 2003).

Specific Procedures for Testing Field-Collected Soil or Similar Particulate Material

This section provides specific instructions for preparing and testing samples of field-collected (site) soil or similar particulate material, in addition to the procedures discussed in Section 4.

Detailed guidance for the collection, handling, transport, storage, and analyses of field-collected soil is given in a number of reports specific to these subjects (e.g., van Ee *et al.*, 1990; Webster and Oliver, 1990; USEPA, 1991; Keith, 1992; Klute, 1986; Carter, 1993; OMAFRA, 1999). In the absence of guidance specific to these subjects from Environment Canada, such reports should be consulted and followed (in addition to the guidance provided here), when collecting samples of field-collected soil and preparing them for toxicity tests with springtails using the biological test method described herein.

5.1 Sample Collection

Crépin and Johnson (1993) provide a useful summary of field-sampling design and appropriate techniques for sample collection. Field surveys of soil toxicity using biological tests with springtails and/or other suitable, soil-associated test organisms (e.g., EC, 2004a, 2005a) are frequently part of more comprehensive surveys. Such surveys could include a *battery of toxicity tests* to evaluate the toxicity of soil together with tests for bioaccumulation of contaminants, chemical analyses, biological surveys of epifaunal and/or infaunal organisms, and perhaps the compilation of geological and hydrographic data. Statistical correlation can be improved and costs reduced if the samples are taken concurrently for these tests, analyses, and data acquisitions.

Samples of soil to be used in the biological test method described herein (Section 4), might be collected quarterly, semiannually, or annually from a number of contaminated or potentially contaminated sites for *monitoring* and *compliance* purposes. Samples of soil might also be collected on one or more occasions during field surveys of sites for spatial (i.e., horizontal or vertical) or temporal definition of soil quality. One or more sites should be sampled for *reference* (presumably clean) *soil* during each field collection.[69]

The number of stations to be sampled at a study site and the number of *replicate samples* per station will be specific to each study. This will involve, in most cases, a compromise between logistical and practical constraints (e.g., time and cost) and statistical considerations. Webster and Oliver (1990), Crépin and Johnson (1993), and OMAFRA (1999) provide guidance on the sampling design; van Ee *et al.* (1990) and USEPA (1991) address issues related to *quality assurance* and *quality control*.

[69] Ideally, a *reference soil* is collected near the site(s) of concern. It possesses geochemical characteristics (e.g., *texture*, total *organic carbon* content, *organic matter* content, pH) similar to those of the field-collected test soil(s) but without anthropogenic contaminants. It is not unusual for nearby reference sites to have some degree of contamination due to anthropogenic chemicals. In some instances, reference soil might be toxic or otherwise unacceptable for use in a soil toxicity test, because of naturally occurring physical, chemical, or biological properties.

For certain *monitoring* and regulatory purposes, multiple replicates (i.e., separate samples from different grabs or cores taken at the same site) should be taken at each *sampling station*, including one or more reference stations. Each of these field replicates should be tested for its toxicity to springtails using five or more test vessels per replicate sample (see Section 4.1).[70] The use of power analysis (see Section 5.5.2) with endpoint data obtained in previous tests of the same type, performed with previous samples from the same or similar sites, will assist in determining if additional laboratory replicates need to be tested with each field replicate. Also, some of the statistical tests have requirements for a minimum number of replicates. For certain other purposes (e.g., preliminary or extensive surveys of the spatial distribution of toxicity), the survey design might include only one sample from each station, in which case the sample would normally be homogenized and split between 5 replicate test vessels. The latter approach precludes any determination of mean toxicity at a given sampling location (station), and completely prevents any conclusion on whether a station is different from the control or reference, or from another location. It does, however, allow a statistical comparison of the toxicity of that particular sample with the reference or control, or with one or more samples from other locations. It is important to realize that any conclusion(s) about differences, which arise from testing single field samples lacking replication, cannot be extended to make any conclusion(s) about the sampling locations.

Sites for collecting *reference soil* should be sought where the geochemical properties of the soil are similar to soil characteristics encountered at the test sites. Matching of *total organic carbon* content (%) or organic matter content (%) might not be warranted in cases where *pollution* (e.g., from or within sewage or industrial sludge) is responsible for the high organic carbon content of test soils. Preliminary surveys to assess the toxicity and geochemical properties of soil within the region(s) of concern and at neighbouring sites are useful for selecting appropriate sites at which to collect reference soil.

Samples of municipal or industrial sludge (e.g., sewage sludge, dewatered mine tailings, or biosolids from an industrial clarifier or settling pond) might be collected for the assessment of their *toxic* effect(s) on springtails, and for geochemical and contaminant analyses. Other

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[70] More replicates may be set up to meet specific study objectives, such as those defined for Phase I (i.e., site soil screening tests) in the recommended framework for toxicity assessments in support of the development of site-specific remediation objectives for petroleum hydrocarbons in soil (ECASG, 2006). This framework for toxicity assessment of contaminated lands is divided into two phases, the first of which includes site soil screening tests using undiluted soil samples representative of the study site. The purpose of the screening tests are to: 1) quickly determine if there is toxicity associated with short-term *(acute)* exposure of the test organisms to the site soil; and 2) if there is no *acute toxicity,* continue the test to assess for chronic toxicity associated with prolonged exposure to the site soil. An investigator, therefore, may choose to expand the test design for the single-concentration tests described in this test method document by setting up extra replicates to look for potential acute responses (i.e., mortality) early in the test (i.e., at 7 or 14 days). If an acute response is observed early in the test, the experiment can be ended after 7 or 14 days; however, if there is no acute mortality observed, the remaining replicates (i.e., a minimum of 5) are left intact to assess for *chronic toxicity* associated with prolonged exposure to the site soil. This approach serves only to judge the potential of an acute response, but is not suitable for defining remedial or cleanup objectives. Phase II of the proposed framework uses multi-concentration tests to determine the magnitude of the toxicity. As described in Section 4.1 of this test method document, a range-finding test can be useful, and is recommended in the framework, for determining the range of effect concentrations (i.e., narrow the range of concentrations to be used in a definitive *chronic* test). As described for the single-concentration tests, extra replicates may be set-up in a definitive multi-concentration test to judge the potential of an acute response (i.e., mortality) early in the test. As with the screening test, the definitive tests can be short (i.e., terminated early due to the presence of an acute toxic response), or long (i.e., thereby generating quantitative data) in duration (ECASG, 2006).

particulate wastes being considered for disposal to land might also be collected for toxicity and physicochemical evaluation.

Guidance for various soil sampling plans and procedures is available in the technical literature (e.g., Petersen and Calvin, 1986; Keith, 1992; Crépin and Johnson, 1993). Procedures used for sample collection (i.e., core, grab, or composite) will depend on the study objectives and the nature of the soil or other particulate material being collected. A shovel, auger, or soil corer (preferably stainless steel) is frequently used for collecting soil samples (ASTM, 1999b).

The surface of the location where each sample is to be collected should be cleared of debris such as twigs, leaves, stones, thatch, and litter. If the location is an area of grass or other herbaceous plant material, the plants should be cut to ground level and removed before the sample is collected. Removal of the vegetation should be done such that removal of soil particles with the roots is minimal. Dense root masses (e.g., grasses) should be removed and then shaken vigorously to remove soil particles adhering to the roots. The soil sample to be collected for toxicity evaluation and chemistry should be collected from one or more depths that represent the layer(s) of concern (e.g., a surficial layer of soil, or one or more deeper layers of soil or subsoil if there are concerns about historical deposition of contaminants).

The required volume of soil per sample should be calculated, before commencing a sampling program. This calculation should take into account the quantity of soil required to prepare laboratory replicates for soil toxicity tests, as well as that required for particle size characterization, *total organic carbon* content (%), *organic matter* content (%), *moisture content* (%), and specific chemical analyses. At least 2–3 kg of soil per sample is normally required, although this will depend on the study objectives/design (e.g., single-concentration or multi-concentration test) and the nature of the chemical analyses to be performed, and possibly also on the nature of the soil (e.g., need for removal of excess water and/or debris in the laboratory, which can reduce the sample volume). To obtain the required sample volume, it is frequently necessary to combine subsample retrieved using the sampling device. The same collection procedure should be used at all field sites sampled.

5.2 Sample Labelling, Transport, Storage, and Analyses

Containers for transport and storage of samples of field-collected soil or similar particulate material must be made of nontoxic material. The choice of container for transporting and storing samples depends on both sample volume and the potential end uses of the sample. The containers must either be new, thoroughly cleaned, or lined with high-quality plastic. Thick (e.g., 4 mil) plastic bags are routinely used for sample transport and storage. If plastic bags are used, it is recommended that each be placed into a second clean, opaque sample container (e.g., a cooler or a plastic pail with a lid) to prevent tearing and to support the weight of the sample and to maintain darkened conditions during sample transport (ASTM, 1999b). Plastic containers or liners should not be used if there is concern about the plastic affecting the characteristics of the soil (e.g., compounds from plastic leaching into the soil).

Following sample addition, the air space in each container used for sample transport and storage should be minimized (e.g., by collapsing and taping a filled or partially filled plastic bag). Immediately after filling, each sample container must be sealed, and labelled or coded. Labelling and accompanying records made at this time must include at least a code or description that identifies sample type (e.g., grab, core, composite), source, precise location, land use information, replicate number, and date of

collection; and should include the name and signature of sampler(s). Persons collecting samples of soil should also keep records that describe details of:

- the nature, appearance, and volume of each sample;
- the sampling procedure and apparatus;
- any procedure used to composite or subsample grabs or cores in the field;
- the number of replicate samples taken at each *sampling station*;
- the sampling schedule;
- the types and numbers of containers used for transporting samples;
- any field measurements (e.g., temperature, pH, soil moisture content) of the soil at the collection site;
- procedures and conditions for cooling and transporting the samples;
- observations of environmental conditions at the time of sampling (e.g., raining); and
- observations of soil fauna and vegetation at the collection site.

Soil samples should not freeze or become overheated during transport or storage. It is recommended that samples be kept in darkness (i.e., held in light-tight, opaque transfer containers such as coolers or plastic pails with lids) during transport, especially if they might contain PAHs or other chemicals or chemical products that could be photoactivated or otherwise altered due to exposure to sunlight. As necessary, gel packs, regular ice, or other means of refrigeration should be used to ensure that the temperature of the sample(s) remains cool (e.g., 7 ± 3 °C) during transit.

The date the sample(s) is received at the laboratory must be recorded. Sample temperature upon receipt at the laboratory should also be measured and recorded. Samples to be stored for future use must be held in airtight containers. If volatile contaminants are in the soil or of particular concern, any air "headspace" in the storage container should be purged with nitrogen gas, before being capped tightly. Samples must not freeze or partially freeze during transport or storage (unless they are frozen when collected), and must not be allowed to dehydrate. If, however, one or more samples are saturated with excess water upon arrival at the laboratory (e.g., sampling occurred during a significant rainfall event), the sample(s) may be transferred to plastic sheeting for a brief period (e.g., one or more hours) to enable the excess water to run off or evaporate. Thereafter, the sample(s) should be returned to the transport container(s) or transferred to one or more airtight containers for storage.

It is recommended that samples be stored in darkness at 4 ± 2 °C. These storage conditions must be applied in instances where PAHs or other light-sensitive contaminants are present; or if the samples are known to contain unstable volatiles of concern. It is recommended that samples of soil or similar particulate material be tested as soon as possible after collection. The soil toxicity test(s) should begin within two weeks of sampling, and preferably within one week. The test must begin within six weeks, unless it is known that the soil contaminants are aged and/or weathered; and therefore, considered stable.

Dry sieving (i.e., press sieving; not wet sieving) of samples through a coarse-mesh sieve (~ 6 mm) is desirable to remove large-sized particles (see Section 5.3); this procedure may be performed in the field. Undesirable coarse material (e.g., large gravel or stones, large debris, large indigenous macroinvertebrates, or large plant material) may also be removed in the field before sample transport. In the laboratory, each sample of field-collected soil should be thoroughly mixed (Section 5.3), and representative subsamples collected for physicochemical characterization. Each sample (including all samples of *negative control soil* and *reference soil*) must be characterized by

analyzing subsample for at least the following:

- particle size distribution (% sand, % silt, and % clay),
- total organic carbon content (%),
- organic matter content (%),
- moisture content (%),
- water-holding capacity (%, based on dry wt of soil),
- pH, and
- conductivity.

Additionally, the following analyses should be performed:

- texture,
- cation exchange capacity,
- major cations,
- organophosphorus insecticides, and
- organochlorine insecticides.

Other analyses could include:

- bulk density,
- fertility,
- C:N ratio,
- total inorganic carbon,
- total volatile solids,
- biochemical oxygen demand,
- chemical oxygen demand,
- oxidation-reduction potential,
- total nitrogen,
- total phosphorus,
- metals,
- petroleum hydrocarbons (including PAHs), and
- a suite of pesticides.

Unless indicated otherwise, identical chemical, physical, and toxicological analyses should be performed with subsample representative of each replicate sample of field-collected soil (including *reference soil*) taken for a particular survey of soil quality, together with one or more subsamples of *negative control soil*.

5.3 Preparing Sample for Testing

Field-collected soil or similar particulate waste material must not be sieved with water, as this would remove contaminants present in the interstitial water or loosely sorbed to particulate material. Large gravel or stones, debris, indigenous macroinvertebrates, or plant material should normally be removed using forceps or a gloved hand. If a sample contains a large quantity of debris (e.g., plant material, wood chips, glass, plastic, large gravel) or large macroinvertebrates, these may be removed by pressing the soil through a coarse sieve (e.g., mesh size of ~ 6 mm).

Qualitative descriptions of each sample of field-collected *test soil* should be made and recorded at the testing laboratory, including information on sample colour, texture, and the presence and description of roots, leaves, and macroscopic soil organisms. Unless research or special study objectives dictate otherwise, each sample of field-collected test material should be homogenized in the laboratory before use (USEPA, 1989).[71] Mixing can affect the concentration and bioavailability of contaminants in the soil, and sample homogenization might not be desirable for all purposes.

As indicated in Section 3.6, one or more samples of field-collected test soil might either be tested at a single concentration only (typically, 100%), or evaluated for toxicity in a multi-concentration test whereby a series of concentrations are prepared by mixing measured quantities with either negative control soil or reference soil. When performing a multi-concentration test, the following series of concentrations of test soil (mixed in negative control soil or reference soil),

[71] One of the reasons for routinely homogenizing samples is to mix into the soil, any pore water which rises to the surface during sample shipment and storage. Homogenization is also necessary to redistribute the sample constituents that have compacted and layered according to particle size during transport and storage.

which spans the range of 100–1% test soil using eight concentrations, might prove suitable: 100%, 80%, 50%, 30%, 15%, 7.5%, 3%, 1%, and 0%. Guidance on other concentration series that might prove as or more suitable is found in Section 6.2, along with that for preparing test mixtures which might apply equally when performing a multi-concentration test with one or more samples of field-collected soil. Refer to Section 4.1, for additional guidance when selecting test concentrations. In each instance, the test must include a treatment comprised solely of negative control soil (see Section 3.3).

To achieve a homogeneous sample, transfer it to a clean, rigid mixing container (e.g., a large stainless steel or plastic bowl) or for larger volumes of soil, to clean plastic sheets spread out on the floor. The sample should be mixed manually (using a gloved hand or a nontoxic device such as a stainless steel spoon) or mechanically (e.g., using a domestic hand-held mixer with beaters at low speed, or a hand-held wire egg beater) until its texture and colour are homogeneous. While mixing, care should be taken to ensure that the impact of mixing on soil structure is minimal and that the structure is not destroyed entirely. As soon as the texture and colour of the sample appears to be homogeneous, mixing should be discontinued.

For each sample included in a test, mixing conditions including duration and temperature should be as similar as possible. If there is concern about the effectiveness of sample mixing, subsamples of the soil should be taken after mixing, and analyzed separately to determine the homogeneity of particle sizes, chemical(s) of interest, etc. Any moisture that separates from a sample during its transport and/or storage must be remixed into it, if possible.

Soil structure is an important factor that influences the survival and reproduction of springtails, and moisture content plays an important role in the determination of soil structure. A qualitative procedure, informally known as a "squeeze test", is useful when determining if the optimal moisture content of a sample of test soil has been achieved. Investigators might find it useful to apply this procedure when adjusting the moisture content of each sample of test soil to a particular percentage of the sample's water-holding capacity (see following paragraphs), in preparation for a toxicity test. To perform this test, a small, representative subsample of the test soil (e.g., a "pinch" of soil) is randomly taken using a gloved hand, and gently compressed between the thumb and forefinger. If a small quantity of water can be squeezed from the soil with gentle pressure, then the soil's moisture content is acceptable. If, however, no water appears, the soil is likely too dry. Conversely, if a substantial amount of water can be squeezed from the subsample of soil, it is likely too wet. The squeeze test can also be applied as a test proceeds[72], or test vessels can be weighed to determine water loss (see Section 4.6).

The *moisture content* of a given sample of field-collected test soil should be standardized during its preparation by determining its *water-holding capacity* (WHC) and then hydrating the soil to an optimal moisture content based on a percentage of this value. The optimal percentage of the WHC for each sample of field-collected soil must be determined prior to sample preparation and test initiation. In order to do so, the moisture content of each homogenized sample (i.e., each sample of test soil, including the negative control soil) must be determined (Sections 4.1 and 4.6). Thereafter, the WHC of each sample must be

[72] The "squeeze test" is useful when making weekly observations of soil "wetness" during a 21- or 28-day test for effects of exposure to samples of test soil. The squeeze test should be applied to the additional replicates prepared for the purpose of physicochemical analyses, thereby ensuring that no test organisms are injured in the process (see Section 4.6).

determined using a recognized standard procedure (see following three paragraphs). A subsample of each soil sample is then hydrated to a homogeneous, crumbly consistency with clumps approximately 3–5 mm in diameter.[73] Based on the initial moisture content of the sample, the WHC of the sample, and the amount of water added to achieve the desired soil consistency, the sample's optimal moisture content can be calculated and expressed as a percentage of the WHC for each soil.[74] Once this target (or optimal) percentage of the WHC has been determined, the moisture content of each sample of test soil (including the negative control soil) can be standardized to the selected (sample-specific) moisture content. *Test water* (i.e., de-ionized or distilled water) should be added to each sample with a moisture content that is less than the pre-determined optimal percentage of its WHC, until this moisture content is achieved [75] (Aquaterra Environmental, 1998a). If a sample is too wet, it should be spread as a thin layer on a clean sheet of plastic (e.g., a new plastic garbage bag or vapour-barrier plastic) or a clean, non-reactive (e.g., stainless steel or plastic) tray, and allowed to dry by evaporation at ambient (~20 °C) room temperature[76]; rehydration to the pre-determined optimal percentage of its WHC might be necessary. Upon completion of adjustment of a sample's moisture content to the desired percentage of its WHC, the moisture content (%) of the hydrated soil must be determined and the percent WHC and percent moisture content recorded and reported.

The WHC (and the percent WHC that is optimal for biological testing) of a particular soil is generally unique to each soil type, and is ultimately the result of the interaction of many variables associated with soil structure (e.g., micro/macro-aggregation, pore space, bulk density, texture, organic matter content). There are a number of methods that can be used to determine WHC; however, most of these methods require measurements to be made on an intact soil sample (e.g., soil core) where characteristics (structural aggregations, pore

[73] An unpublished study, carried out by Environment Canada (J. Princz, personal communication, Biological Methods Division, Environmental Science and Technology Centre, Ottawa, Ontario, 2004), determined the optimal moisture content for each of the diverse types of soil used while developing the biological test method described herein (see Section 3.3 and Appendix G), based on a percentage of each sample's WHC. The optimal percentage of the WHC of these soils ranged from approximately 45–50% for the silt and sandy loam soils to 60% for the clay loam soil. These values were considered optimal since, at these levels of saturation, the soil mixed well, had an adequate moisture content according to the "squeeze test", and formed an acceptable structure (i.e., the resulting macro-aggregation of soil particles was conducive to healthy springtails). Experience indicates that the actual moisture content of the test soils hydrated to optimal conditions can vary greatly (e.g., 20% for sandy loam soil to 50% for clay loam soil), depending on the bulk density and the WHC of the sample(s) of field-collected soil being tested (ESG and Aquaterra Environmental, 2002; Becker-van Slooten *et al*., 2003).

[74] For soils with high peat content (i.e., extremely high water holding capacity), the method for determining the percent WHC described herein can be inaccurate and the results misleading. In such cases, the optimal moisture content can be estimated by eye (i.e., sample hydrated to a homogeneous, crumbly consistency with clumps approximately 3–5 mm in diameter) and the moisture content determined thereafter, reported as such (i.e., as moisture content instead of percent WHC).

[75] An alternate approach sometimes used by certain investigators is to standardize (and adjust) the moisture content of each sample of field-collected soil to a fixed concentration, such as 35–45% of its dry weight (ASTM, 1999b). However, a disadvantage of this approach is that certain samples of field-collected soil can appear to be very wet and have standing water on the surface after hydration to only 35–45% of their dry weight; whereas other site soils can appear considerably dryer after the same level of hydration (ASTM, 1999b). Accordingly, the use of this alternate approach is not recommended here.

[76] If there is concern about volatilization of potential toxicants and/or changes in the nature of the toxicant of concern due to the drying process, alternative methods of drying the soil and/or the effects of drying the soil on the toxicity of the soil can be investigated.

space, bulk density, texture, and organic matter content) are preserved during collection. The USEPA (1989) has described an appropriate method for toxicity testing using unconsolidated materials (such as samples of field-collected soils that have been dried, sieved, and homogenized; or samples of soil formulated in the laboratory from constituents).[77] This method is outlined here.

For this method, ~130 g (wet wt) of sample is placed in an aluminum pan or petri dish (15 × 1 cm), and dried at 105 °C until a constant weight is achieved (this usually takes a minimum of 24 h). Thereafter, 100 g of the oven-dried soil is placed into a 250-mL glass beaker with 100 mL of distilled or de-ionized water. The resulting slurry is mixed thoroughly with a glass stir rod. A folded filter paper (e.g., 185-mm diameter Fisherbrand P8 coarse porosity, qualitative creped filter paper; catalogue no. 09-790-12G) is placed into a glass funnel (with a top inside diameter of 100 cm and a stem length of 95 cm). The folded filter paper should be level with the top of the glass funnel. Using a pipette, up to 9 mL of distilled or de-ionized water is slowly added to the filter paper to wet the entire surface. The funnel and hydrated filter paper are then weighed. To obtain the initial weight for the mass of the funnel plus hydrated filter paper plus dried soil (see "I" in Equation 1), the weight of the dried soil (100 g) is added to the weight of the funnel and the wet filter paper.

The funnel is then placed into a 500-mL Erlenmeyer flask and the soil slurry is slowly poured onto the hydrated filter paper held in the funnel. Any soil remaining on the beaker and stir rod is rinsed into the funnel with the least amount of water necessary to ensure that all of the solid material has been washed onto the filter. The funnel is then tightly covered with aluminum foil and allowed to drain for three hours at room temperature. After three hours, the funnel containing the hydrated filter paper and wet soil is weighed. This weighing represents the final weight for the mass of the funnel plus hydrated filter paper plus (wet) soil (see "F" in Equation 1).

The water-holding capacity for the subsample of soil in the funnel, expressed as percentage of soil dry mass, is then calculated using the following equation:

$$WHC = \frac{F - I}{D} \times 100 \quad \text{(Equation 1)}$$

where:

WHC = water-holding capacity (%)
F = mass of funnel + hydrated filter paper + wet mass of soil
I = mass of funnel + hydrated filter paper + dry mass of soil
D = 100 g (i.e., dry mass of soil)

The WHC of each sample of *test soil* should be determined in triplicate, using three subsamples.

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[77] Some participants at the soil toxicity testing workshop sponsored by Environment Canada (EC) in Vancouver, BC (February, 2003) considered the determination of WHC and a percentage of that capacity to be the most appropriate way of expressing soil moisture content (EC, 2004b). This led to a testing program to compare two different methods for estimating the WHC of soil (i.e., as per Annex C in ISO, 1999 or according to USEPA, 1989) as well as a somewhat different method for expressing soil moisture content, as a percentage of the soil's water-filled pore space (WFPS). The results of this investigation showed that each method had distinct advantages and disadvantages; however, the USEPA (1989) method for measuring WHC was recommended for use in EC's soil toxicity test methods when adjusting (if and as necessary) the moisture content of soil samples (Becker-van Slooten, *et al*., 2004).

The percentage of water (i.e., P_w) that is added to a sample of field-collected soil to achieve the desired hydration (i.e., the optimal percentage of the WHC) can be calculated as follows:[78]

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[78] The following example provides calculations that pertain to the hydration of samples of a contaminated field-collected soil and a negative control soil, when preparing a test concentration of 25% for use in a survival and reproduction test with springtails involving three replicates per treatment.

Assumptions:

Soil #1: Negative Control (nc) Soil		**Soil #2: Contaminated (C) Soil**	
W_{nc}	= 2.3934 g	W_c	= 7.0575 g
D_{nc}	= 1.9108 g	D_c	= 5.6174 g
WHC_{nc}	= 80.30 %	WHC_c	= 67.10 %
P_{WHCnc}	= 60.00 %	P_{WHCc}	= 40.00 %
MC_{nc}	= 25.26%	MC_c	= 25.64 %
P_{Wnc}	= 22.92 %	P_{Wc}	= 1.20 %
M_{Dnc}	= 63.75 g dry wt	M_{Dc}	= 21.25 g dry wt
V_{Wnc}	= 14.61 mL	V_{Wc}	= 0.26 mL
M_{Wnc}	= 79.85 g wet wt	M_{Wc}	= 26.70 g wet wt

MC	$= [(W - D) / D] \times 100$	[Equation 1]
P_W	$= [WHC \times (P_{WHC} / 100)] - MC$	[Equation 2]
V_W	$= (P_W \times M) / 100$	[Equation 3]
M_W	$= (M_D \times W) / D$	

W = wet mass of substrate (g)
D = dry mass of substrate (g)
WHC = water-holding capacity (% of dry mass)
P_{WHC} = percentage of WHC desired (%)
MC = initial moisture content of substrate (%)
P_W = percentage of water to add to soil (%)
M_D = total mass of soil required for experiment (expressed as dry wt)
V_W = volume of water to add to soil (mL)
M_W = total mass of soil required for experiment (expressed as wet wt based on initial MC)

Calculations for a 25% concentration of a contaminated soil in negative control soil:

For a collembolan test using this example, it is assumed that a total mass of 85.00 g dry weight (wt) of soil is sufficient to satisfy the requirement for each treatment (i.e., 20.00 g dry wt per replicate × 3 replicates + 25.00 g dry wt extra soil for pH and conductivity etc.). To simplify the calculations, this example assumes that 20 g (dry wt) of either type of soil is sufficient to provide the 30-g wet wt aliquot of soil to be added to each test vessel.

For a 25% concentration of contaminated soil in negative control soil, 25% of the total mass of soil, on a dry-wt basis, must consist of the contaminated soil:

= 85.00 g dry wt × (25/100)
= 21.25 g dry wt of contaminated soil

The remainder of the test soil required to prepare this treatment (i.e., 75 %) will consist of the negative control soil:

= 85.00 g dry wt × (75/100) [or 85.00 g dry wt – 21.25 g dry wt]
= 63.75 g dry wt of negative control soil

Therefore, the final total mass of soil required, based on wet weight, is 94.46 g [79.85 g wet wt at the soil's initial moisture content (i.e., M_{Wnc}) + 14.61 mL of water] for the negative control soil, and 26.96 g [26.70 g wet wt at the soil's initial moisture content (i.e., M_{Wc}) + 0.26 mL of water] for the contaminated soil.

The final moisture content for each soil would be 48.17 % {[(94.46 – 63.75) / 63.75] × 100} for the negative control soil, and 26.87 % {[(26.96 – 21.25) / 21.25] × 100} for the contaminated soil.

The final moisture content of the negative control soil (i.e., 48.17%) represents 60% of that soil's water-holding capacity (48.17 ÷ 80.30 = 0.60). The final moisture content of the contaminated soil (i.e., 26.87%) represents 40% of that soil's water-holding capacity (26.87 ÷ 67.10 = 0.40).

$P_W = [WHC \times (P_{WHC}/100)] - MC$ (Equation 2)

where:

P_W = percentage of water to add to the soil (%)
WHC = water-holding capacity (%)
MC_i = initial moisture content of the soil

The volume of water (i.e., V_w) that should be added to a sample of field-collected soil to achieve the desired hydration (i.e., the optimal percentage of the sample's water-holding capacity) can be calculated as follows:[78]

$V_W = (P_W \times M)/100$ (Equation 3)

where:

V_W = volume of water to add to the soil (mL)
P_W = percentage of water to add to the soil (%)
M = total mass of soil required for test (expressed as dry weight)[79]

[79] For tests with samples of field-collected soil, the amount of soil added to each test vessel is based on the wet weight of soil (i.e., 30 g wet wt). However, "M" (i.e., the total mass of soil required for the test) is expressed as dry weight in the formula used to calculate the volume of water to be added to a sample of field-collected soil to achieve the desired hydration (see Equation 3). To calculate the amount of soil required per test vessel on a dry-weight basis, a simple calculation is carried out. For example, assume that (for a given sample) the wet and dry weights of a subsample of this soil, previously determined for the purpose of calculating the sample's water-holding capacity, are 4.1507 g and 2.7813 g, respectively. The dry weight equivalent to a 30-g wet weight of this sample of soil can be calculated as follows:

$(30 \text{ g} \times 2.7813 \text{ g}) \div 4.1507 \text{ g} = 20.1 \text{ g}$

This mass of soil can be rounded up to 21 g dry weight, thereby providing a little extra soil, if necessary. Therefore, for the example provided here, the mass of this sample of soil required for each replicate (expressed as dry wt) is 21 g. The total mass ("M") can then be calculated simply by multiplying the dry mass required for each replicate (in this instance, 21 g dry wt) by the number of replicates to be used in the test (i.e., for this example, three replicates).

Except for research-oriented toxicity tests intended to determine the influence of pH on sample toxicity, the pH of samples of field-collected soil must not be adjusted. Studies intending to investigate the effect of pH on sample toxicity should conduct two side-by-side tests, whereby one or more sets of treatments is adjusted to a fixed pH value using calcium carbonate or a suitable acid or base, and the pH of one or more duplicate sets of treatments is not adjusted.

Immediately following sample hydration (or dehydration) and mixing, subsamples of test material required for the toxicity test and for physicochemical analyses must be removed and placed into labelled test vessels (see Section 4.1), and into the labelled containers required for the storage of subsample for subsequent physicochemical analyses. Any remaining portions of the homogenized sample that might be required for additional toxicity tests using springtails or other test organisms (e.g., according to EC 2004a, 2005a) should also be transferred to labelled containers at this time. Subsamples to be stored for future toxicity testing should be held in sealed containers with minimal air space, in darkness at 4 ± 2 °C (Section 5.2) until tested. These storage conditions must be applied for subsamples collected for physicochemical analysis. Just before it is analyzed or used in the toxicity test, each subsample must be brought to room temperature and thoroughly remixed to ensure that it is homogeneous.

5.4 Test Observations and Measurements

A qualitative description of each field-collected test material should be made at the time that the test is being set up. This might include observations of sample colour, texture, and homogeneity; and the presence of plants or macroinvertebrates. Any changes in the appearance of the test material observed during

the test or upon its termination, should be noted and reported.

Section 4.6 provides guidance and requirements for the observations and measurements to be made during or at the end of each test. These observations and measurements apply and must be made when performing the soil toxicity tests described herein using one or more samples of field-collected (site) soil.

Depending on the test objectives and experimental design, additional test vessels might be set up at the beginning of the test (Sections 4.1) to monitor soil chemistry. These would be destructively sampled during and at the end of the test. Test organisms might or might not be added to these extra test vessels, depending on the study's objectives. Measurements of chemical concentrations in the soil within these vessels may be made by removing aliquots of the soil for the appropriate analyses (see Section 5.2).

5.5 Test Endpoints and Calculations

The common theme for interpreting the results of tests with one or more samples of field-collected *test soil*, is a comparison of the biological effects for the test (site) soil(s) with the effects found in a *reference soil*. The reference sample should be used for comparative purposes whenever possible or appropriate, because this provides a site-specific evaluation of toxicity (EC, 1997a, b, 2001, 2004a, 2005a). Sometimes the reference soil might be unsuitable for comparison because of toxicity or atypical physicochemical characteristics. In such cases, it would be necessary to compare the test soils with the *negative control soil*. Results for the *negative control soil* will assist in distinguishing contaminant effects from non-contaminant effects caused by soil physicochemical properties such as particle size, *total organic carbon* content (%), and *organic matter* content (%). Regardless of whether the *reference soil* or *negative control soil* is used for the statistical comparisons, the results from *negative control soil* must be used to judge the validity and acceptability of the test (see Section 4.4).

Analysis of the results will differ according to the purposes and particular designs of the test. This section covers the analytical procedures, starting with the simplest design and proceeding to the more complex designs. Standard statistical procedures are generally all that is needed for analysing the results. Investigators should consult EC (2005b) for guidance on the appropriate statistical endpoints and their calculation. As always, the advice of a statistician familiar with *toxicology* should be sought for the design and analysis of tests.

Analysis of variance (ANOVA) involving multiple comparisons of endpoint data derived for single-concentration tests involving field replicates of field-collected soil from more than one sampling location is commonly used for statistical interpretation of the significance of findings from soil toxicity tests. This hypothesis-testing approach is subject to appreciable weaknesses. Notably, any increased variability within the test will weaken its power to distinguish *toxic* effects (i.e., less toxicity is concluded). Similarly, use of only a few replicates instead of many replicates will weaken the discrimination of a test and will lead to a conclusion of less apparent toxicity, other things being equal (see Section 5.5.2). There is no alternative to hypothesis testing, when comparing toxicity data for multiple samples of field-collected soil (i.e., field replicates of soil from more than one sampling location) that use only one concentration (usually full strength, i.e., 100% sample). There are alternatives for comparing point estimates of toxicity if various concentrations of each sample of field-collected soil are tested and multiple endpoint values for ICp or LC50 are determined (see Section 6.4). Section 9 in EC (2005b) should be consulted for guidance when comparing multiple ICps or LC50s.

The parametric analyses involving ANOVA for comparative quantitative data from single-concentration tests with multiple samples of field-collected soil (i.e., field replicates of soil from more than one sampling location) assume that the data are normally distributed, that the treatments are independent, and that the variance is homogeneous among the different treatments. As the first step in analysis, these assumptions should be tested using the *Shapiro-Wilk's Test* for *normality* and *Levene's Test* for Homogeneity of Variance (EC, 2005b). If the data satisfy these assumptions, analysis may proceed. If not, a more sophisticated parametric method appropriate for the data could be used, or data could be transformed (e.g., as square roots, logarithms, or as arcsine square root for *quantal* data which are to be used in statistical analysis; EC, 2005b). The tests for normality and homogeneity might then show conformance with normality and homogeneity and, in fact, that is a likely outcome of a transformation. Assumptions should be re-tested following any transformation of data. Parametric tests are reasonably robust in the face of moderate deviations from normality and equality of variance. Therefore, parametric analysis (e.g., ANOVA and multiple comparison) should proceed, even if moderate nonconformity continues after transformation. Excluding a data set for minor irregularities might lose a satisfactory and sensitive analysis and forgo the detection of real effects of toxicity.[80] Analysis by nonparametric statistical procedures should also proceed in parallel, with the more sensitive of the two analyses providing the final estimates of toxicity. Section 3 in EC (2005b) should be consulted for guidance when comparing the findings for single-concentration tests involving field replicates of samples from multiple locations, using parametric or non-parametric tests.

Guidance in Section 6 (including that in Section 6.2 for performing range-finding tests, and that in Section 6.4 and 4.8 for calculating test endpoints) should be followed if a multi-concentration test is performed using one or more samples of field-collected soil diluted with *negative control soil* or *clean* reference soil. Section 9 in EC (2005b) should be consulted when comparing such point estimates of toxicity for multiple samples of field-collected soil.

5.5.1 Variations in Design and Analysis

A very preliminary survey might have only one sample of *test soil* (i.e., contaminated or potentially contaminated *site soil*) and one sample of *reference soil*, without replication. Simple inspection of the results might provide guidance for designing more extensive studies.

If there is a single test sample and a single reference sample, and data are *quantitative*, a standard *Student's t-test* should be used for analysis (Paine and McPherson, 1991; EC, 1997a, b, 2001, 2005b). The t-test is fairly robust, and handles unequal numbers of replicates in the test and reference samples, as well as moderately unequal variances in the two groups (Newman, 1995; USEPA, 1995).

A preliminary evaluation might conceivably be conducted with samples from many stations, but without either field replicates or laboratory (within-sample) replicates. The objective might be to identify a reduced number of *sampling stations* deserving of more detailed and further study. Opportunities for statistical analysis would be limited. The non-replicated test data could be compared with the reference data using outlier detection methods (USEPA, 1994a;

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[80] Tests for normality and homogeneity become less meaningful with the small number of samples from individual *sampling stations* typically collected in studies of *environmental toxicology*. Plotting and examining the general nature of the distribution of toxicity and its apparent deviations can be more revealing and is recommended (EC, 2005b). Equality in sample sizes and the magnitude of variation are probably more important factors for the outcome of parametric analysis, but they have received scant attention in toxicology. The robustness of ANOVA is shown by its ability to produce realistic probabilities if the distribution of data is reasonably symmetrical, and if treatment variances are within threefold of each other (Newman, 1995).

Newman, 1995; EC, 1997a, b, 2001, 2004a, 2005a, b). A sample would be considered *toxic* if its result was rejected as an extreme value when considered as part of the data for the *reference soil* and/or the *negative control soil*.

A more usual survey of soils would involve the collection of replicate samples from several places by the same procedures, and their comparison with replicate samples of a single *reference soil* and/or *negative control soil*. There are several pathways for analysis, depending on the type and quality of data, but often there would be an analysis of variance (ANOVA) followed by one of the multiple-comparison tests. In the ANOVA, the *reference soil* would also be treated as that from a "location".

In these multi-location surveys, the type of replication would influence the interpretation of results. If field replicates were collected at each of the sampling locations, and no laboratory replicates were used, a one-way ANOVA would evaluate the overall difference in test results with respect to sampling location, over and above the combined variability of sampling the location and running the test. It would be unusual but much more powerful, to have field replicates for all sampling locations and also laboratory replicates of each field replicate. If that were done, the laboratory replicates would become the replicates in a nested one-way ANOVA, and would be the basis of variability for comparing differences in the samples. The ANOVA could be used to see (a) if there was an overall difference in test results for samples with respect to their sampling location, and (b) whether there was an overall difference in replicates taken at the various locations. After an ANOVA, the analysis would proceed to one or more types of multiple-comparison test, as described in the following text.

If only laboratory replicates and no field replicates were tested, it is difficult to make statistically robust conclusions regarding differences between sampling sites (see also Section 5.1). The laboratory replicates would only show any differences in the samples that were greater than the baseline variability in the within-laboratory procedures for setting up and running the test. Sample variability due to location would not really be assessed in the statistical analysis, except that it would contribute to any difference in test results associated with sampling location.

If it were desired to compare the test results for the replicate samples from each sampling location with those for the reference soil, a number of tests are recommended, depending on whether the samples show a gradient and depending on whether there are an even or uneven number of replicates. If it is clear that there is a gradient of effects and there are an equal number of replicates, *Williams' test* should be used. If no gradient exists and there are an equal number of replicates, *Dunnett's test* should be used. If there are an unequal number of replicates, *Dunnett's test* (modified for unequal replication), the *Dunn-Sidak test*, or the *Bonferroni-adjusted t-test* should be used (p. 189 in Newman, 1995; Appendix D in USEPA, 1995; Section 7.5.1 in EC, 2005b).

In a multi-location survey, an investigator might wish to know which of the samples from various sampling locations showed results that differed statistically from the others, as well as knowing which ones were different from the reference and/or negative control sample(s). Such a situation might involve sampling from a number of locations at progressively greater distances from a point source of contamination, in which instance the investigator might want to know which sampling locations provided samples that had significantly higher toxicity than others, and thus which locations were particularly deserving of cleanup. *Tukey's test*, *Fisher's Least Significant Difference* or the *Student-Newman-Keuls test* are recommended for this purpose (Section 7.5.1 in EC, 2005b).
If it were desired to compare the toxicity of the samples from each sampling location with that

for the reference sample(s) (or compare samples from different sites with each other), but the data do not conform to requirements of normality and equal variance, the ANOVA and subsequent tests would be replaced by nonparametric tests. First, a nonparametric ANOVA analogue should be run to test the null hypothesis of no difference among treatments, followed by a multiples comparison test if the null hypothesis is rejected (Section 7.5.2 in EC, 2005b).[81] If a gradient is expected and pairwise comparisons are desired, the *Jonckheere-Terpstra test* is recommended, followed by a multiple comparison test using the *Hayter-Stone test* or the *Edwards-Berry test*. If no gradient is expected and comparisons with the control are desired, the *Kruskal-Wallis test* or the *Fligner-Wolfe test* is recommended, followed by one of the following multiple comparison tests: *Nemenyi-Damico-Wolfe test*, *Wilcoxon Rank Sum test*, *Steel's Many-One Rank test* or the *Edwards-Berry test*. If no gradient is expected and pairwise comparisons are desired, the *Kruskal-Wallis test* is recommended, followed by one of the following multiple comparison tests: *Critchlow-Fligner-Steel-Dwass test*, *Steel's Pairwise test*, *Kruskal-Wallis test* (here used for multiple comparison), or the *Edwards-Berry test*. See Section 7.5.2 in EC, 2005b for further details.

5.5.2 Power Analysis

An important factor to consider in the analysis of the results for toxicity tests with soil is the potential for declaring false positives (i.e., calling a *clean* site *contaminated*; Type I error) or false negatives (i.e., calling a contaminated site clean; Type II error). Scientists are usually cautious in choosing the level of significance (α) for tolerating false positive results (Type I error), and usually set it at P = 0.05 or 0.01. Recently, toxicologists have been urged to report both α and statistical power (1 - β), i.e., the probability of correctly rejecting the null hypothesis (H_0) and not making a Type II error. There are several factors that influence statistical power, including:

- variability of replicate samples representing the same treatment;
- α (i.e., the probability of making a Type I error);
- *effect size (ES)*, (i.e., the magnitude of the true effect for which you are testing); and
- *n* (i.e., the number of samples or replicates used in a test).

Environment Canada's guidance document on statistical methods for environmental toxicity tests (EC, 2005b) provides further information and guidance on errors of Types I and II.

Power analysis can be used *a priori* to determine the magnitude of the Type II error and the probability of false positive results. It can also be used to ascertain the appropriate number of field and laboratory replicates for subsequent surveys involving this test, or to assist in the selection of future sampling sites. It is always prudent to include as many replicates in the test design as is economically and logistically warranted (see Section 5.1); power analysis will assist in this determination. A good explanation of the power of a test, and how to assess it, can be found in USEPA (1994a). Guidance on power analysis is provided in EC (2005b).

Many investigators have difficulty with power analysis, and do not apply it due to its perceived complexity and the differing formulae specific to various statistical tests. In view of this complexity, the *Minimum Significant Difference* may be applied as an alternative approach (i.e., as an "index of power"; see EC, 2005b for guidance).

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[81] The exception is *Shirley's test*, which is not preceded by an ANOVA analogue. *Shirley's test* is recommended if a gradient is expected, and comparisons with the control are desired.

Specific Procedures for Testing Chemical-Spiked Soil

This section gives guidance and instructions for preparing and testing *negative control soil* spiked experimentally with chemical(s) or chemical product(s). These recommendations and instructions apply to the biological test method described. Guidance in EC (1995) for *spiking* negative control sediment with chemical(s) and conducting toxicity tests with chemical/sediment mixtures is also relevant here, for *chemical-spiked soil*. Further evaluation and standardization of procedures for preparing chemical-spiked soil provided herein (Section 6.2) might be required before soil toxicity tests with springtails or other appropriate soil organisms are applied to evaluate specific chemical/soil mixtures for regulatory purposes.

The cause(s) of soil toxicity and the interactive toxic effects of chemical(s), or chemical product(s) in association with otherwise *clean* soil can be examined experimentally by spiking *negative control soil* (Section 3.3) with these substances. The *spiking* might be done with one or more chemicals or chemical products. Other options for toxicity tests with springtails, performed using the procedures described herein, include the spiking of chemical(s) or chemical product(s) in *reference soil* (Section 3.5) or *test soil* (Section 3.6). Toxicity tests using soil spiked with a range of concentrations of test chemical(s) or chemical product(s) can be used to generate data that estimate LC50s (see Section 4.8.1), and can determine other statistical endpoints based on threshold concentrations causing specific *sublethal effects* (see Section 4.8.2).

In Section 6.2, procedures are described for preparing test mixtures of chemical-spiked soil. Section 6.3 described making observations and measurements during and at the end of the toxicity test. Section 6.4 (and Section 4.8) provides procedures for estimating test endpoints for multi-concentration tests. These procedures also apply to the mixing of multiple concentrations of field-collected *test soil* (including particulate waste material such as sludge or other dredged material intended for land disposal) in *negative control soil* or *reference soil*, and to performing multi-concentration tests and determining statistical endpoints for these mixtures (see Section 5, and especially 5.5). Multi-concentration tests with *positive control soil* (Section 3.4) or one or more reference toxicants spiked in *negative control soil* (Section 4.9) are also performed using the procedures and statistical guidance described in this section. Additionally, the influence of the physicochemical characteristics of natural or artificial negative control soil on chemical toxicity can be determined with spiked-soil toxicity tests according to the procedures and statistical guidance described in this section.

6.1 Sample Properties, Labelling, and Storage

Information should be obtained on the properties of the chemical(s) or chemical product(s) to be spiked experimentally in the *negative control soil*.[82] Information should also be obtained for

[82] Some studies might require the *spiking* (mixing) of one or more concentrations of chemical(s), chemical product(s), or *test soils* (e.g., contaminated or potentially contaminated field-collected soil or waste sludge) in either *negative control soil* or *reference soil*. Other applications could include the spiking of chemical(s) or chemical product(s) in one or more samples of test soil. For such studies involving samples of contaminated soil or similar particulate material (e.g., domestic or industrial sludge), instructions on sample characterization given in Section 5.2 should be followed. Sample(s) of field-collected negative

individual chemicals or chemical products (e.g., pesticides or other commercial formulations), on their concentration of major "active" ingredients and impurities, water solubility, vapour pressure, chemical stability, dissociation constants, adsorption coefficients, toxicity to humans and terrestrial organisms, and biodegradability. 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. If an acceptable procedure for solubilizing the test chemical(s) in water is not available, preliminary testing for its solubility in test water or a non-aqueous solvent should be conducted and confirmed analytically. Other available information such as the structural formulae, nature and percentage of significant impurities, presence and amounts of additives, and n-octanol:water partition coefficient, should be obtained and recorded. Any pertinent Material Safety Data Sheets (MSDSs) should be obtained and reviewed.

Chemical(s) to be tested should be at least reagent grade, unless a test on a formulated commercial product or technical grade chemical(s) is required. Chemical containers must be sealed and coded or labelled upon receipt. Required information (chemical name, supplier, date received, person responsible for testing, etc.) should be indicated on the label and/or recorded on a separate datasheet dedicated to the sample, as appropriate. Storage conditions (e.g., temperature, protection from light) are frequently dictated by the nature of the chemical.

6.2 Preparing Test Mixtures

On the day preceding the start of the toxicity test (i.e., Day -1), the mixture(s) of chemical(s) or chemical product(s) spiked into *negative control soil* should be prepared, transferred to test vessels, and held overnight before adding the test organisms the next day (i.e., Day 0) (see Section 4.1). Each *batch* of *test soil* representing a particular treatment (concentration) should be prepared in a quantity sufficient to enable all test replicates of that treatment (concentration) to be set up along with any additional replicates or quantities required for physicochemical analyses (Sections 4.6 and 6.3) or the performance of other soil toxicity tests using springtails or other soil organisms (e.g., those performed according to EC, 2004a or EC, 2005a).

The use of *artificial soil* (Section 3.3.2) to prepare each test mixture is recommended since it offers a consistent, standardized approach for comparing results for other chemicals or chemical products tested similarly in the same laboratory or by others (e.g., according to USEPA, 1989; Wiles and Krogh, 1998; ISO, 1999; OECD, 2005). If used, the formulation for artificial soil provided in Section 3.3.2 should be followed. The quantity of artificial soil required for the test(s) should be prepared, hydrated to ~20% moisture content, adjusted if and as necessary to a pH within the range of 6.0 to 7.5[83], aged for a minimum three-day period, and stored until required (see Section 3.3.2). The final

control soil, reference soil, contaminated soil, or particulate waste to be evaluated in spiked-soil toxicity tests should be collected, labelled, transported, stored, and analyzed according to instructions provided in Sections 5.1 and 5.2.

[83] If, however, the test chemical(s) or chemical product(s) are anticipated to modify soil pH and the intent of the study is to nullify this influence, the (aqueous) pH of each *batch* (concentration) should be adjusted to a standard value (e.g., pH 6.5) after the chemical(s) or chemical product(s) has been added. Studies wishing to determine the extent to which an acidic or basic test substance modifies the toxicity of soil spiked with a range of concentrations of this substance, due to the influence of pH *per se*, should conduct two side-by-side tests whereby one test adjusts the pH of each test concentration to a standard value (e.g., pH 6.5) using the required (differing, depending on concentration) quantity of calcium carbonate, and the other test uses an identical quantity of calcium carbonate for each treatment sufficient to attain the "standard" pH (e.g., pH 6.5) in the *negative control* treatment.

moisture content (including that due to the addition of a measured aliquot of a test chemical or chemical product dissolved in *test water*, with or without an organic solvent) of any *chemical-spiked soil* prepared using artificial soil should be ~70% of the water-holding capacity of the final mixture (Section 3.3.2), for each treatment (concentration).[84]

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[84] The following example provides calculations that show the volume of both water (de-ionized or distilled) and a stock solution of a reference toxicant (boric acid) to be added to a sample of artificial soil with an existing moisture content, to create a treatment with a moisture content that is 70% of the WHC for the artificial soil. The calculations take into account the volume of a stock solution of boric acid added when preparing the treatment, as part of the overall adjustment for soil moisture content. To simplify the calculations, this example assumes that 20 g (dry wt) of artificial soil (AS) is sufficient to provide the 30-g wet wt aliquot of soil to be added to each test vessel when performing a collembolan toxicity test involving three replicate test vessels per treatment.

The equations shown in Section 5.3 for calculating WHC and adjusting soil moisture content to a certain percentage of this value apply equally here. For this example, assume that the following assumptions apply (see Section 5.3 for equations and associated definitions of these terms).

Assumptions:

Wet mass of artificial soil (AS)	= 3.2486 g
Dry mass of AS	= 2.6924 g
Moisture content (MC) of AS	= [(3.2486 – 2.6924)/2.6924] × 100 = 20.66% (initial moisture content)
Water-holding capacity (WHC) of AS	= 72.10%
Percentage of WHC desired (P_{WHC})	= 70.00%
Dry mass of AS required for test (M_D)	= [20.00 g per rep × 3 reps] + 25.00 g extra = 85.00 g dry wt
Wet mass of AS required for test (M_W)	= (85.00 × 3.2486)/2.6924 = 102.56 g wet wt

Calculations to prepare a treatment comprised of 2000 mg boric acid per kg artificial soil (dry wt):
The stock solution consists of 25 g of H_3BO_3 in 1 L of de-ionized water.

The amount of boric acid required, on a dry-mass basis is:
H_3BO_3 = (2 g H_3BO_3/1000 g soil dry wt) × 85.00 g dry wt
= 0.17 g H_3BO_3

The amount of stock solution required, on a volume basis, is:
H_3BO_3 = 0.17 g H_3BO_3 / (25 g H_3BO_3/1000 mL of water)
= 6.80 mL stock solution

The percentage of water (P_W) required for addition to this treatment to achieve the desired percentage of WHC (70%) is:
P_W = [WHC × (P_{WHC}/100)] – MC
= [72.10 × (70.00/100)] – 20.66
= 29.81 %

The volume of water (V_W) required for addition to this treatment to achieve the desired percentage of WHC (70%) is:
V_W = (P_W × M_D)/100
= (29.81 × 85.00 g dry wt)/100
= 25.34 mL of water required

However, as part of this required volume, 6.80 mL of the stock solution is to be added for dosing; therefore, an additional volume of water of only 18.54 mL will be required (25.34 mL of water – 6.80 mL of stock solution).

Accordingly, the final total mass of soil required, based on wet weight, would be 127.90 g [102.56 g wet wt at the soil's initial moisture content (i.e., M_W) + 18.54 mL of water + 6.80 mL of stock solution], and the final moisture content of the soil, based on dry weight, would be 50.47 % {[(127.90 – 85.00)/85.00] × 100}.

The final moisture content of this test treatment (i.e., 50.47% moisture) represents 70% of the test soil's water-holding capacity (50.47 ÷ 72.10 = 0.70).

The final moisture content of each mixture (treatment) included in a test should be as similar as possible.

Investigators may choose to use natural control soil (Section 3.3.1) rather than artificial control soil (Section 3.3.2) as the *negative control soil* to be spiked with chemical(s) or chemical product(s) and for the corresponding replicates of control soil to be included in the test. Procedures described herein for artificial soil apply equally if natural soil is used. An exception is that the final moisture content of each *batch* of chemical-spiked soil (including control batches) prepared using field-collected soil should be adjusted to the optimal percentage of its WHC (by hydrating or dehydrating the sample, as the case may be) using guidance in Section 5.3. For natural soils, the volume of soil in each test vessel might also differ, due to differences in bulk density of the various soils that might be used.

The procedure to be used for experimentally *spiking* soil is contingent on the study objectives and the nature of the test substance to be mixed with negative control soil or other soil. In many instances, a chemical/soil mixture is prepared by making up a *stock solution* of the test chemical(s) or chemical product(s) and then mixing one or more measured volumes into *hydration water* which is then added to artificial or natural negative control soil (Section 3.3)[85]. The preferred solvent for preparing stock solutions is *test water* (i.e., de-ionized or distilled water); use of a solvent other than 100% test water should be avoided unless it is absolutely necessary. For test chemical(s) or chemical product(s) that do not dissolve readily in test water, a suitable water-miscible organic solvent of low toxicity (e.g., acetone, methanol, or ethanol) may be used in small quantities to help disperse the test substance(s) in water (OECD, 2005). Surfactants should not be used.

If an organic solvent is used, the test must be conducted using a series of replicate test vessels containing only *negative control soil* (i.e., 100% artificial or natural *clean* soil containing no solvent and no test substance), as well as a series of replicate test vessels containing only *solvent control soil* (ISO, 1999; OECD, 2005). For this purpose, a *batch* of solvent control soil must be prepared that contains the concentration of solubilizing agent that is present in the highest concentration of the test chemical(s) or chemical product(s) in soil. Solvent from the same *batch* used to make the stock solution of test substance(s) must be used. Solvents should be used sparingly, since they might contribute to the toxicity of the prepared test soil. The maximum concentration of solvent in the soil should be at a concentration that does not affect the survival or reproduction of springtails during the test. If this information is unknown, a preliminary *solvent only* test, using various concentrations of solvent in negative control soil, should be conducted to determine the threshold-effect concentration of the particular solvent being considered for use in the definitive test.

For tests involving the preparation of concentrations of chemical spiked in *artificial soil*, in which the chemical is insoluble in water but soluble in an organic solvent, the quantity of test substance needed to prepare a required volume of a particular test concentration should be dissolved in a small volume of a suitable organic solvent (e.g., acetone). This chemical-in-solvent mixture should then be sprayed onto or mixed into a small portion of the final quantity of fine quartz sand that is required when preparing each test concentration comprised of a measured amount of a particular chemical-in-solvent mixture spiked in artificial soil (see Section 3.3.2). The solvent is then removed by evaporation by placing the container under a fume hood for at least one hour, and until no

[85] Adding the stock solution to the hydration water and then to the soil assists with homogenization and decreases the risk of having the contaminant bind to a very small area of soil.

residual odour of the solvent can be detected. Thereafter, the chemical-in-sand mixture (with solvent evaporated) is mixed thoroughly with the remaining quantity of pre-moistened sand and other ingredients required to make up artificial soil (Section 3.3.2). An amount of test water necessary to achieve a final moisture content of approximately 70% of the maximum water-holding capacity for this artificial soil is then added and mixed with the soil/sand/peat mixture. The chemical-spiked soil can then be added to the test vessel (OECD, 2005).

For tests involving the spiking of natural soil, in which the chemical is insoluble in water, the following procedure can be used (R. Kuperman, personal communication, US Army Edgewood Chemical Biological Center, Maryland, USA, 2004). The chemical is dissolved in a solvent (e.g., acetone) and pipetted onto a 2.5 cm thick layer of soil to establish each chemical concentration in soil, ensuring that the volume of solution added at any one time does not exceed 15% (v:m) of the dry mass soil. The same total chemical:solvent solution volume at different concentrations is added to every treatment, equalling the volume required to dissolve the chemical at the highest concentration tested. The solvent is allowed to volatilize (usually requires a minimum of 18 h) in a dark chemical fumehood to prevent photolysis. Each amended soil sample is mixed until homogeneous (e.g., transferred into a fluorocarbon-coated high-density polyethylene container and mixed for 18 h on a three-dimensional rotary mixer).

The sample of *solvent control soil* to be included in the test must be prepared using the same procedure but without the addition of the test chemical. Additionally, the solvent control soil must contain a concentration of solvent that is as high as that in any of the concentrations of chemical-spiked soil included in a test.

If the test chemical to be spiked in artificial soil is insoluble in both water and any suitable (non-toxic) organic solvent, a mixture should be prepared that is comprised of 2.5 g of finely ground industrial quartz sand and the quantity of the test chemical necessary to achieve the desired test concentration in the soil. This mixture should then be mixed thoroughly with the remaining constituents of the pre-moistened artificial soil. An amount of de-ionized water necessary to achieve a final *moisture content* of ~70% of the maximum water-holding capacity is then added and mixed in. The resulting mixture of chemical-spiked soil can then be added to the test vessels (OECD, 2005).

Concentrations of chemical(s) or chemical product(s) in soil are usually calculated, measured, and expressed as mg test substance/kg soil (or µg substance/g soil) on a dry-weight basis (ISO, 1999; OECD, 2005). The assessment endpoints (e.g., ICps) are similarly expressed on a dry-weight basis (Sections 4.8 and 6.4).

Mixing conditions, including solution:soil ratio, mixing and holding time, and mixing and holding temperature, must be standardized for each treatment included in a test. Time for mixing a *spiked soil* should be adequate to ensure homogeneous distribution of the chemical, and may be from minutes up to 24 h. During mixing, temperature should be kept low to minimize microbial activity and changes in the mixture's physicochemical characteristics. Analyses of subsamples of the mixture are advisable to determine the degree of mixing and homogeneity achieved.

For some studies, it might be necessary to prepare only one concentration of a particular mixture of negative control (or other) soil and chemical(s) or chemical product(s), or a mixture of only one concentration of *contaminated* soil or particulate waste in negative control or other soil. For instance, a single-concentration test might be conducted to determine whether a specific concentration of chemical or chemical product in *clean* soil is *toxic* to the test organisms. Such an application could be used for research or regulatory purposes (e.g., “Limit test”).

A multi-concentration test, using a range of concentrations of chemical added to *negative control soil* (or other soil) under standardized conditions, should be used to determine the desired endpoint(s) (i.e., LC50 and ICp; see Sections 4.8 and 6.4) for the chemical/soil mixtures. A multi-concentration test using negative control soil spiked with a specific particulate waste might also be appropriate. At least seven test concentrations plus the appropriate control treatment(s) must be prepared for each multi-concentration test, and more (i.e., ≥10 plus controls) are recommended (see Sections 4.1 and 4.8). When selecting the test concentrations, an appropriate geometric dilution series may be used in which each successive concentration of chemical(s) or chemical product(s) in soil is at least 50% of the previous one (e.g., 40, 20, 10, 5, 2.5, 1.25, 0.63 mg/kg). Test concentrations may also be selected from other appropriate logarithmic dilution series (see Appendix H); or may be derived based on the findings of preliminary "range-finding" toxicity tests. The reader is referred to Section 4.1 for additional guidance when selecting test concentrations.

To select a suitable range of concentrations, a preliminary or range-finding test covering a broader range of test concentrations might prove worthwhile. The number of replicates per treatment (see Section 4.1) could be reduced or eliminated altogether for range-finding tests and, depending on the expected or demonstrated (based on earlier studies with the same or a similar test substance) variance among test vessels within a treatment, might also be reduced for nonregulatory screening *bioassays* or research studies.

Based on the objectives of the test, it might be desirable to determine the effect of substrate characteristics (e.g., particle size or organic matter content) on the toxicity of chemical/soil mixtures. For instance, the influence of soil particle size on chemical toxicity could be measured by conducting concurrent multi-concentration tests with a series of mixtures comprised of the test chemical(s) or chemical product(s) mixed in differing fractions (i.e., segregated particle sizes) or types of natural or artificial *negative control soil* (Section 3.3). Similarly, the degree to which the total organic carbon content (%) or organic matter content (%) of soil can modify chemical toxicity could be examined by performing concurrent multi-concentration tests using different chemical/soil mixtures prepared with a series of organically enriched negative control soils. Each fraction or formulation of natural or artificial *negative control soil* used to prepare these mixtures should be included as a separate control in the test.

Depending on the study objectives and design, certain soil toxicity tests using springtails might be performed with samples of negative control soil or reference soil to which chemical(s) or chemical product(s) are applied to the soil surface, rather than mixing it with the soil. Surface applications can be applied in the field or the laboratory. Procedures for chemical application include the use of a calibrated track sprayer to achieve a uniform distribution of the chemical over a specific area. Concentration of chemical(s) or chemical product(s) in the soil can be determined based on the penetration depth, the surface area or swathe width, the nozzle size, the pressure, and the speed of coverage of the sprayer (G.L. Stephenson, personal communication, Aquaterra Environmental, Orton, Ontario, 2001). The OECD (2005) provides some guidelines for applying test substances to the soil surface in preparation for reproduction tests with springtails.

6.3 Test Observations and Measurements

A qualitative description of each mixture of *chemical-spiked soil* should be made when the test is being established. This might include

observations of the colour, texture, and visual homogeneity of each mixture of chemical-spiked soil. Any change in appearance of the test mixture during the test, or upon its termination, should be recorded.

Section 4.6 provides guidance and requirements for the observations and measurements to be made at the beginning, during, and at the end of the test. These observations and measurements apply and must be made when performing the soil toxicity test described herein using one or more samples of chemical-spiked soil.

Depending on the test objectives and experimental design, additional test vessels might be set up on Day -1 of the test (see Section 4.1) to monitor soil chemistry. These would be destructively sampled during (i.e., on Day 0 and, in certain instances, other days as the test progresses) or at the end of the test (i.e., Day 21 or Day 28, depending on the test species used). Test organisms might or might not be added to these extra test vessels, depending on study objectives. Measurements of chemical concentrations in the soil within these test vessels could be made by removing aliquots of soil for the appropriate analyses, at the beginning of the test, as it progresses, and/or at its end, depending on the nature of the toxicant and the objectives of the test.

Measurements of the quality (including soil pH and moisture content) of each mixture of *spiked soil* being tested (including the *negative control soil*) must be made and recorded at the beginning and end of the test, as described in Section 4.6. If analytical capabilities permit, it is recommended that the *stock solution*(s) be analyzed together with one or more subsamples of each spiked-soil mixture, to determine the chemical concentrations, and to assess whether the soil has been spiked satisfactorily. These should be preserved, stored, and analyzed according to suitable, validated procedures.

Unless there is good reason to believe that the chemical measurements are not accurate, toxicity results for any test in which concentrations are measured for each spiked-soil mixture included in the test should be calculated and expressed in terms of these measured values. As a minimum, sample aliquots should be taken from the high, medium, and low test concentrations at the beginning and end of the test[86]; in which instance, the endpoint values calculated (Sections 4.8 and 6.4) would be based on nominal ones. Any such measurements of concentrations of the test chemical(s) or chemical product(s) should be compared, reported, and discussed in terms of their degree of difference from nominal strengths. If nominal concentrations are used to express toxicity results, this must be explicitly stated in the test-specific report (see Section 7.1.6).

6.4 Test Endpoints and Calculations

Multi-concentration tests with mixtures of *spiked soil* are characterized by test-specific statistical endpoints (see Section 4.8). Guidance for calculating the LC50 is provided in the Section 4.8.1, whereas that for calculating an ICp (based on data showing reproductive inhibition) is given in Section 4.8.2. Section 5.5 provides guidance for calculating and comparing endpoints for single-concentration tests using samples of field-collected soil. This guidance applies equally to single-concentration tests performed with mixtures of spiked soil. For further information on these or other appropriate parametric (or nonparametric) statistics to apply to the endpoint data, the investigator should consult the Environment Canada report on statistics for the determination of toxicity endpoints (EC, 2005b).

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[86] Certain chemicals might be known to be stable under the defined test conditions, and unlikely to change their concentration over the test duration. In this instance, an investigator might choose to restrict their analyses to samples taken only at the beginning of the test.

For any test that includes *solvent control soil* (see Section 6.2), the test results for springtails held in that soil must be compared statistically with that for test organisms held in *negative control soil*. If any of the endpoints for these two control soils used to establish test validity (see Section 4.4) differ significantly according to *Student's t-test*, only the *solvent control soil* may be used as the basis for comparison and calculation of results. If the results for the two controls are the same, the data from both controls should be combined before using it to calculate results or to assess test validity.

Reporting Requirements

Each test-specific report must indicate if there has been any deviation from any of the *must* requirements delineated in Sections 2 to 6 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 7.1 provides a list of items that must be included in each test-specific report. A list of items that must either be included in the test-specific report, provided separately in a general report, or held on file for a minimum of five years, is found in Section 7.2. Specific *monitoring* programs, related test *protocols*, or regulations might require selected test-specific items listed in Section 7.2 (e.g., details about the test material and/or explicit procedures and conditions during sample collection, handling, transport, and storage) to be included in the test-specific report, or might relegate certain test-specific information as *data to be held on file*.

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

Details on the procedures, conditions, 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 field-collected or other samples tested for regulatory or monitoring purposes;
- a copy of the record of acquisition for the sample(s);
- chemical analytical data on the sample(s) 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/or holding and acclimation 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.

7.1 Minimum Requirements for a Test-Specific Report

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

7.1.1 Test Substance or Material

- brief description of sample type (e.g., waste sludge, reference or contaminated field-collected soil, negative control soil) or coding, as provided to the laboratory personnel;

- information on labelling or coding of each sample; and
- date of sample collection; date and time sample(s) received at test facility.

7.1.2 Test Organisms

- species and source of brood stock and test organisms;
- age-range of *O. folsomi*, *F. candida*, or *F. fimetaria* at start of test; and
- any unusual appearance, behaviour, or treatment of the organisms, before their use in the test.

7.1.3 Test Facilities

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

7.1.4 Test Method

- citation of biological test method used (i.e., as per this document);
- design and description if specialized procedure(s) (e.g., preparation of mixtures of *spiked soil*; preparation and use of solvent and, if so, solvent control) or modification(s) of the standard test method described herein;
- brief description of frequency and type of all measurements and all observations made during test; and
- name and citation of program(s) and methods used for calculating statistical endpoints.

7.1.5 Test Conditions and Procedures

- design and description of any deviation(s) from, or exclusion of, any of the procedures and conditions specified in this document;
- number of discrete samples per treatment; number of replicate test vessels for each treatment; number and description of treatments in each test including the control(s); test concentrations (if applicable);
- volume and/or mass of soil in each test vessel;
- number of organisms per test vessel and treatment;
- dates when test was started and ended;
- feeding regime and ration during the test;
- for each soil sample — any measurements of soil particle size, moisture content, water-holding capacity, pH, and conductivity; and
- for each composite sample of subsample taken at the same time from all replicates of each treatment — all measurements of temperature (air and soil), pH, moisture content, and water-holding capacity.

7.1.6 Test Results

- mean (± SD) percent survival of adult Collembola in each treatment, including controls on Day 21 for *F. fimetaria*, and Day 28 for *F. candida* and *O. folsomi*; mean (± SD) number of surviving juveniles in each treatment, including controls on Day 21 for *F. fimetaria*, and Day 28 for *F. candida* and *O. folsomi*;
- any LC50 (including the associated 95% confidence limits and, if calculated, the slope) determined; any additional LCx (e.g., LC25) calculated;
- any ICp (together with its 95% confidence limits) determined for the data on reproductive success (i.e., number of surviving juvenile springtails in each treatment at test end); details regarding any transformation of data, and

indication of quantitative statistical method used or procedures applied to the data;

- for a multi-concentration test with chemical-spiked soil, indication as to whether results are based on nominal or measured concentrations of chemical(s) or chemical product(s); all values for measured concentrations;

- results for any 7-day LC50 for *O. folsomi*, or *F. fimetaria*, or 14-day LC50 for *F. candida* (including its 95% confidence limits) performed with the reference toxicant in conjunction with the definitive soil toxicity test; *geometric mean* value (± 2 SD) for the same reference toxicant and test species, as derived at the test facility in previous 7- or 14-day LC50 tests using the procedures and conditions for reference toxicity tests described herein; and

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

7.2 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.

7.2.1 Test Substance or Material

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

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

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

7.2.2 Test Organisms

- name of person(s) who identified the organisms and the taxonomic guidelines used to confirm species;

- history and age of brood stock, for any culture used to provide test organisms;

- description of culture conditions and procedures, including temperature, lighting, type and amount of substrate and details on its periodic renewal, measurements of substrate quality, density of springtails, feeding regime and quantity, records of health and performance indices;

- procedures used to count, handle, sort, and transfer animals; and those to determine their mortality, condition, appearance, and behaviour; and

- source and composition of food, procedures used to prepare and store food, feeding method(s), feeding frequency and ration.

7.2.3 Test Facilities and Apparatus

- all results for initial tests with negative control soil and reference toxicant, undertaken by the laboratory previously inexperienced with performing the biological test method described herein in advance of any reporting of definitive test results (see Section 3.2.1);

- description of systems for providing lighting and for regulating temperature within test facility;

- description of test vessels and covers; and

- description of procedures used to clean or rinse test apparatus.

7.2.4 Negative Control Soil or Reference Soil

- procedures for the preparation (if *artificial soil*) or *pretreatment* (if natural soil) of *negative control soil*;
- source of natural soil; history of past use and records of analysis for pesticides or other contaminants;
- formulation of artificial soil, including sources for the constituents and conditions and procedures for hydration and pH adjustment; and
- storage conditions and duration before use.

7.2.5 Test Method

- procedures used for mixing or otherwise manipulating *test soils* before use; time interval between preparation and testing;
- procedure used in preparing stock and/or test solutions of chemicals; description and concentration(s) of any solvent used;
- details concerning aliquot sampling, preparation, and storage before physicochemical analysis, together with available information regarding the analytical methods used (with citations); and
- use and description of preliminary or range-finding test.

7.2.6 Test Conditions and Procedures

- measurements of light intensity adjacent to surface of soil in test vessels;
- procedure for adding test organisms to test vessels;
- appearance of each sample (or mixture thereof) in test vessels; changes in appearance noted during test;
- records of the addition of test water on the surface of the soil in each test vessel throughout the test, for increasing moisture content;
- any other physicochemical measurements (e.g., analyses of aliquots from the same *batch* to determine homogeneity; contaminant concentration, total volatile solids, biochemical oxygen demand, chemical oxygen demand, total inorganic carbon, cation exchange capacity, oxidation-reduction potential, total nitrogen) made before and during the test on test material (including *negative control soil* and *reference soil*) and contents of test vessels; including analyses of whole soil and pore water;
- any other observations or analyses made on the test material (including samples of *negative control soil* or *reference soil*); e.g., qualitative and/or quantitative data regarding indigenous macrofauna or detritus, or results of geochemical analyses; and
- any chemical analyses of the concentration of chemical in stock solution(s) of reference toxicant and, if measured, in test concentrations.

7.2.7 Test Results

- results for any range-finding test(s) conducted;
- number of surviving adult springtails in each test vessel at test end (i.e., Day 21 for *F. fimetaria*, and Day 28 for *F. candida* and *O. folsomi*); number of surviving progeny in each test vessel at test end (i.e., Day 21 or Day 28); for regression analyses, information indicating sample size (e.g., number of replicates per treatment), parameter estimates with variance, any ANOVA table(s) generated, plots of fitted and observed values of any models used, and the output provided by the statistical program (e.g., SYSTAT);

- warning chart showing the most recent and historic results for acute toxicity tests with the reference toxicant and the species of test organisms used in these tests;
- results for any 21- or 28-day chronic test(s) performed with a reference toxicant;
- graphical presentation of 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

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

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 1st Edition	February 1992	November 1997
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 the Freshwater Alga *Selenastrum capricornutum*	EPS 1/RM/25 1st Edition	November 1992	November 1997
Growth Inhibition Test Using a Freshwater Algae	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

* These documents are available for purchase from Environmental Protection Publications, Environment Canada, Ottawa, Ontario, K1A 0H3, Canada. Printed copies can also be requested by e-mail at: epspubs@ec.gc.ca. These documents are available for free in PDF at the following Web site: http://www.etc-cte.ec.gc.ca/organization/bmd/bmd_publist_e.html. For further information or comments, contact the Chief, Biological Methods Division, Environmental Science and Technology Centre, Science and Technology Branch, 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.)			
Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout)	EPS 1/RM/28 2nd Edition	July 1998	—
Test for Survival and Growth in Sediment Using the Larvae of Freshwater Midges *(Chironomus tentans* or *Chironomus riparius)*	EPS 1/RM/32	December 1997	—
Test for Survival and Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca*	EPS 1/RM/33	December 1997	—
Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*	EPS 1/RM/37 1st Edition	March 1999	—
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	September 2007	—
B. Reference Methods **			
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	—

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

Appendix B

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* A computer program for calculating LC50 is available from the Environmental Toxicology Section, Pacific Environmental Science Centre, 2645 Dollarton Highway, North Vancouver, British Columbia, V7H 1B1, by providing a CD.

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Appendix E

Procedural Variations for Culturing Springtails, as Described in International Guides and Test Methods for Measuring Soil Toxicity Using Various Species of Springtail

Source documents are listed chronologically, by originating agency of author(s).

W& K 1998 (Wiles and Krogh 1998) – this publication describes protocols for measuring survival, growth, and reproduction effects in three species of springtails including *Folsomia candida* (Willem, 1902), *Isotoma viridis* (Bourlet, 1839), and *Folsomia fimetaria* (Linné, 1758); published in 1998 in the "*Handbook of Soil Invertebrate Toxicity Tests,*" Løkke and van Gestel (eds.), West Sussex, England.

ISO 1999 – an international standard test method for measuring soil toxicity using a test for effects on reproduction of *Folsomia candida*, published in 1999 by the International Standard Organization in Geneva, Switzerland.

OECD 2005 – a draft proposal released by the National Environmental Research Institute in Denmark, to the Organization for Economic Cooperation and Development (OECD), for a new test guideline that assesses the effects of chemical-spiked soils on the reproduction of two species of Collembola (*Folsomia fimetaria* and *Folsomia candida*); under consideration for publication by the OECD in Paris, France.

1. Source of Brood Stock for Culture

Document[1]	Test Species	Initial Source
W & K 1998	*Folsomia candida* (Willem, 1902)	starter cultures may be obtained from numerous existing laboratory cultures
	Folsomia fimetaria (Linné, 1758)	may be obtained from soil samples collected from fields, meadows, or grasslands, after a heat/dry extraction of the soil
	Isotoma viridis (Bourlet, 1839)	may be collected from field sites (grassy habitats) by suction sampling[2]
ISO 1999	*Folsomia candida* (Willem, 1902)	NI[3]
OECD 2005	*Folsomia candida* (Willem, 1902)[4]	commercially available
	Folsomia fimetaria (Linné, 1758)[4]	commercially available

[1] See preceding page.
[2] April to May and September to October are optimum times of the year for collection in temperate climates.
[3] NI = not indicated.
[4] Other collembolan species may also be suitable, e.g., *I. viridis* or *O. folsomi*. If other species of Collembola are used, they must be clearly identified and the rationale for the selection of the species should be reported.

2. Culture Vessels and Capacity

Document	Vessel Type and Size	Number of Units and Capacity for Substrate or Springtail Production
W & K 1998		
F. candida and *I. viridis*	28 × 16 × 9 cm perspex container[1]	1 cm of breeding substrate moistened with distilled water
F. fimetaria	90 × 13 mm Petri dishes	0.5 cm breeding substrate
ISO 1999	400-mL commercial plastic containers, covered tightly	1 cm of breeding substrate with deionized water to almost saturation
OECD 2005	90 × 13 mm Petri dishes	0.5 cm breeding substrate

[1] Larger stocks may be held in large plastic food containers (30 × 30 × 20 cm); note: some types of plastic may emit toxic organic compounds, which could influence the culturing and testing.

3. Temperature and Lighting During Culturing

Document	Temperature (°C)	Lighting Conditions[1]	Humidity
W & K 1998			
F. candida and *I. viridis*	15 ± 0.5 [2]	16h L:8h D at <1000 lux	NI [3, 4]
F. fimetaria	20 ± 1	12h L:12h D	NI
ISO 1999	20 to 22	continuous lighting at 400 to 800 lx[5]	70–80% RH[6]
OECD 2005	20 ± 1 or 20 ± 0.5	12h L:12h D or 16h L:8h D at <1000 lux[7]	NI

[1] L = light; D = dark.
[2] May be kept at 20 °C, if required.
[3] NI = not indicated.
[4] Cultures are to be kept moist at all times; however, for *I. viridis*, it is advisable to avoid a layer of moisture being formed on the surface of the plaster of Paris as this tends to increase hatchling mortality.
[5] A light:dark cycle of 16h:8h is also suitable.
[6] RH = relative humidity.
[7] Containers are kept moist at all times.

4. Culturing Substrate

Document	Culturing Substrate	pH	Renewal Conditions
W & K 1998			
F. candida and *I. viridis*	plaster of Paris and charcoal, hydrated[1]	NI [2]	stock cultures periodically moved to fresh plaster of Paris (e.g., every 2–3 months)
F. fimetaria	plaster of Paris and charcoal, hydrated[1]	NI	transfer to fresh breeding containers every 4–8 weeks
ISO 1999	8:1 mixture of plaster of Paris and activated charcoal, hydrated[3, 4]	6.0–7.0	transfer to fresh breeding containers after 8 weeks[5]
OECD 2005	plaster of Paris and activated charcoal[6]	NI	transfer to fresh breeding containers every 4–8 weeks[7, 8]

[1] 260–300 mL of water is added to 450 g of the plaster charcoal mixture.
[2] NI = not indicated.
[3] Higher ratios (9:1 or 10:1) of plaster and charcoal may also be used.
[4] 60 to 100 g of water is added to 100 g of the plaster and charcoal mixture; however, the amount of water added depends on the type of plaster. The presence of water on the saturated substrate surface is essential for breeding springtails.
[5] Transfer Collembola by tapping or blowing individuals off the plaster of Paris onto fresh substrate.
[6] 20 mL of activated charcoal, 200 g of plaster of Paris and 200 mL of distilled water or 50 g of activated pulverized charcoal, 400 g plaster of Paris, and 260–300 mL of distilled water.
[7] Stock cultures periodically moved (e.g., every 2–3 months) to fresh plaster of Paris.
[8] Dead individuals and stale food are removed from container.

5. Feeding During Culturing

Document	Description of Food Used	Quantity and Feeding Procedure	Feeding Frequency
W & K 1998			
F. candida and *I. viridis*	dried baker's yeast[1, 2]	10–30 mg placed on filter paper disks	at least once or twice per week
F. fimetaria	dried baker's yeast[1]	15 mg	once per week
ISO 1999	granulated dry yeast	small amounts at frequent intervals	at least once or twice per week
OECD 2005	granulated dry baker's yeast	10–30 mg or mass needed	once or twice per week

[1] Alternative food stuffs include green algae (*Pleurococcus* spp.), Tetramin fish food, shredded carrots mixed with oatmeal, *Drosophila* food flakes, and potato chunks.

[2] For *I. viridis* a small amount of defaunated field soil (5 g sieved and heated to 70 °C) may be placed at one end of the containers.

6. Culture Maintenance and Developmental Rate

Document	Maintenance of age-synchronized cultures
W & K 1998	
F. candida	transfer several hundred adult Collembola from stock cultures into fresh breeding containers, and supply with baker's yeast; remove adults after 24–48 h; incubate eggs at 15 °C (or 20 °C, as required); observe daily and record date of hatching; feed
I. viridis	prepare hatching tubes (2.5 × 5 cm) containing 1 cm depth of breeding substrate; transfer egg batches from stock cultures into tubes with dampened fine paint brush; incubate eggs at 15 °C (or 20 °C, as required); observe daily; brush eggs showing fungal contamination with distilled water; transfer any hatchlings to screw top jar (120 mL capacity) containing 1 cm breeding substrate; feed
F. fimetaria	transfer 150–300 adults from a 4–8 week-old substrate to fresh breeding containers and feed; after 9 days carefully collect eggs with a needle and spatula and moved to an egg-paper (small piece of filter paper dipped in breeding substrate); place in fresh breeding container and maintain humidity; most eggs will hatch in 3 days; remove egg-paper from Petri dish to obtain age-synchronized cultures; feed
ISO 1999	avoid overcrowding[1]; transfer egg clusters[2] from breeding containers to a freshly prepared breeding substrate using a fine spatula or hairbrush; after 48 h remove the egg clusters and feed the instars hatched from the eggs[3]
OECD 2005	
F. candida	transfer several hundred adults from stock cultures into fresh breeding containers with a 1 cm layer of substrate, and supply with baker's yeast; remove adults after 24–48 h; observe daily and record date of hatching; feed
F. fimetaria	transfer 150–300 adults from a 4–8 week-old substrate to fresh breeding containers with a 0.5 cm layer of breeding substrate and feed with 15 mg of baker's yeast; after 9 days carefully collect eggs with a needle and spatula and moved to an egg-paper (small piece of filter paper dipped in breeding substrate); place in fresh breeding container and maintain humidity; most eggs will hatch in 3 days; remove egg-paper from Petri dish to obtain age-synchronized cultures; feed

[1] Overcrowding the containers may lead to reduced growth and as a consequence the 10- to 12-day old organisms used for the test may be too small and not yet able to produce a sufficient number of eggs to meet the requirements of the test.

[2] The egg clusters can be easily removed if placed on small pieces of breeding substrate or cover glasses are laid on the breeding substrate.

[3] An alternative method of obtaining age-synchronized test organisms is to place a number of adult springtails in a small container with plaster of Paris in the base and allow them to lay eggs over a two-day period. The adults are then removed and the juveniles used 12 days after they first emerge from the eggs. It is advisable to check the containers for egg production before removing the adults since in some cases the adults do not start laying eggs immediately. If only a few eggs are produced in two days, the adults are kept in the containers for an additional day or more.

7. Indices of Culture Health and Acceptability; Age of Springtails Used in Toxicity Tests; Transfer of Organisms to Test Vessels

Document	Indices of Culture Health and Acceptability	Age of Springtails Used in Toxicity Tests	Transfer of Springtails to Test Vessels
W & K 1998			
F. candida	NI[1]	10- to 12-day old juveniles	hand-held air aspirator
I. viridis	NI	5- to 7-day old juveniles	
F. fimetaria	NI	23 to 26 days old	
ISO 1999	NI	10- to 12-day old juveniles	by tapping or with an exhaustor [2]
OECD 2005	NI	adults	low-suction air-flow device

[1] NI = not indicated.

[2] Springtails are sucked individually through a pipette tip to a small covered container. Care shall be taken to ensure that the suction of the pump is low to avoid damage to the organisms. After removing the cover, springtails provided for one test container are transferred onto the substrate surface of the test chamber. A manual exhaustor may also be used.

Appendix F

Procedural Variations for Tests of Effects of Contaminated Soil on the Survival and Reproduction of Springtails, as Described in International Methodology Documents

Source documents are listed chronologically, by originating agency or by author(s).

W& K 1998 (Wiles and Krogh 1998) – this publication describes protocols for measuring survival, growth, and reproduction effects in three species of springtails including *Folsomia candida* (Willem, 1902), *Isotoma viridis* (Bourlet, 1839), and *Folsomia fimetaria* (Linné, 1758); published in 1998 in the "*Handbook of Soil Invertebrate Toxicity Tests*," Løkke and van Gestel (eds.), West Sussex, England.

ISO 1999 – an international standard test method for measuring soil toxicity using a test for effects on reproduction of *Folsomia candida*, published in 1999 by the International Standard Organization in Geneva, Switzerland.

OECD 2005 – a draft proposal released by the National Environmental Research Institute in Denmark, to the Organization for Economic Cooperation and Development (OECD), for a new test guideline that assesses the effects of chemical-spiked soils on the reproduction of two species of Collembola (*Folsomia fimetaria* and *Folsomia candida*); under consideration for publication by the OECD in Paris, France.

1. Test Species, Test Type, and Test Duration

Document	Species	Test Type	Test Duration
W & K 1998	*Folsomia candida* (Willem, 1902)	static-renewal[1]	8 weeks[2]
	Isotoma viridis (Bourlet, 1839)	static-renewal[1]	8 weeks
	Folsomia fimetaria (Linné, 1758)	static	3 weeks
ISO 1999	*Folsomia candida* (Willem, 1902)	static	28 days
OECD 2005	*Folsomia fimetaria* (Linné, 1758)	static	3 weeks
	Folsomia candida (Willem, 1902)	static	4 weeks

[1] Collembola are placed in fresh soil at weekly intervals biological observations have been made.
[2] It is also feasible to carry out the test for 4 weeks at 20 °C.

2. Specifics on Test Organisms at Start

Document	Description of Organisms Used to Start Test	Acclimation Conditions	Age of Organisms at Test Start
W & K 1998			
F. candida	cultured juveniles, age-synchronized	NI[1]	10 to 12 days
I. viridis	cultured juveniles, age-synchronized	NI	5 to 7 days
F. fimetaria	cultured juveniles, age-synchronized	NI	23 to 26 days
ISO 1999	cultured juveniles, age-synchronized	NI	10 to 12 days
OECD 2005	cultured adults, age-synchronized	NI	adults

[1] NI = not indicated

3. Test Vessels and Materials

Document	Test Vessel	Cover	Type of Test Soil[1]	Amount of Soil/Container
W & K 1998				
F. candida	5 × 2 cm glass tubes, closed tightly	tight fitting lid	AS, StS	4 ± 0.1 g per tube, dry wt (~ 5 g wet wt)
I. viridis	5 × 2.5 cm glass tubes, closed tightly	tight fitting lid	AS, StS	4 ± 0.1 g per tube, dry wt (~ 5 g wet wt)[2]
F. fimetaria	cylinder; 6 cm in diameter, 5.5 cm high; 1 mm mesh bottom; closed	lids at top and bottom	AS, StS	30 g per container, wet wt
ISO 1999	100 mL glass container; 5 cm in in diameter; closed tightly	plastic or glass disk, or parafilm	AS	30 g per container, wet wt
OECD 2005	partly transparent; transparent lids which reduce water evaporation but allow gas exchange	glass or inert plastic	AS, StS	amount corresponding to 25 g dry wt; 3–4 cm deep

[1] AS = artificial soil; StS = standard soil.

[2] Approximately 150 g dry weight of soil substrate is required for each concentration tested, to allow for eight weeks of assessment and extra soil (~50 g) to determine soil moisture and pH at the start and end of the test. At the onset of the study, soils for each test concentration are prepared in sufficient mass for the complete test. If, however, the test chemical is non-persistent, it is advisable to prepare a freshly treated portion of soil at each weekly assessment period.

4. Description of Test Soils, Including Composition of Artificial Soil

Document	Description of Test Soil(s)	Composition of Artificial Soil[1]
W & K 1998		
F. candida	standard soil[2] with added test substance (e.g., chemical in deionized water or organic solvent; if insoluble, test substance mixed in fine quartz sand)[3] and distilled or deionized water	10% sphagnum peat[4], 20% kaolinite clay with ≥30% kaolinite, and 70% industrial sand with >50% particles 50–200 µm, adjust to pH 6.0 ± 0.5 using $CaCO_3$
I. viridis	standard soil[2, 5] with added test substance (e.g., chemical in deionized water or organic solvent; if insoluble, test substance mixed in sand with fine quartz sand)[3] and distilled or deionized water	10% sphagnum peat[4], 20% kaolinite clay with ≥30% kaolinite, and 70% industrial sand with >50% particles 50–200 µm, adjust to pH 6.0 ± 0.5 using $CaCO_3$
F. fimetaria	standard soil[2] with added test substance (e.g., chemical in deionized water or organic solvent; if insoluble, test substance mixed in sand with fine quartz sand)[3] and distilled or deionized water	10% sphagnum peat[7], 20% kaolinite clay with ≥30% kaolinite, and 70% industrial sand with >50% particles 50–200 µm, adjust to pH 6.0 ± 0.5 using $CaCO_3$
ISO 1999	artificial soil with added test substance (e.g., chemical in deionized water or organic solvent; if insoluble, test substance mixed in fine quartz sand)[6]	10% sphagnum peat[7], 20% kaolinite clay with ≥30% kaolinite, and 70% industrial sand with >50% particles 50–200 µm, adjust to pH 6.0 ± 0.5 using $CaCO_3$
OECD 2005	artificial soil or standard soil[8] with added test substances (e.g., chemical in de-ionized water[9] or in organic solvent; if insoluble, test substance is mixed with fine quartz sand[10]	5 or 10% sphagnum peat[11], 20% kaolin clay with >30% kaolinite, and 69–70%[12] air-dried industrial sand with >50% particles 50–200 µm, adjust to pH 6.0 ± 0.5 using $CaCO_3$

[1] Percentages are expressed on a dry mass basis.

[2] Test soils may be artificial soil or LUFA 2.2 soil, which is a sandy soil with a pH of 6.0 ± 0.5 and a particle composition of 4% organic matter and 5.1% clay.

[3] Substances insoluble in water but soluble in organic solvents are dissolved in a volatile solvent (acetone or hexane) and mixed with a portion of the quartz sand or dry soil substrate. After evaporating the solvent (fume hood for 1 h), the remainder of the test substrate and the water are added and mixed thoroughly before introducing it into the test containers. Substances insoluble in water or organic solvents are mixed with 10 g of finely ground industrial quartz sand or 10 g of the dry soil substrate prior to adding the remainder of the soil substrate and water.

[4] The sphagnum peat is air dried, finely ground and sieved (mesh width of 1 mm).

[5] Test may be adapted for testing site soils (e.g., from remediated sites).

[6] Substances insoluble in water but soluble in organic solvents are dissolved in a volatile solvent (acetone or hexane) and mixed with a portion of the quartz sand required for the artificial soil formulation. After evaporating the solvent, the remainder of the artificial soil constituents and the water are added and mixed thoroughly before introducing it into the test containers. Substances insoluble in water or organic solvents are mixed with 10 g of finely ground industrial quartz sand prior to adding the remainder of the artificial soil constituents and water.

[7] The sphagnum peat is air dried, finely ground and with no visible plant remains.

[8] A natural standard soil such as LUFA Speyer is recommended.

[9] The test substance is applied by mixing it into the soil (this procedure is recommended in general) or by application to the soil surface after the Collembola have been added.

[10] Substances insoluble in water but soluble in organic solvents are dissolved in a volatile solvent (acetone) and mixed with 2.5 g of the quartz sand required for the artificial soil formulation. After evaporating the solvent, the remainder of the

artificial soil constituents and the water are added and mixed thoroughly before introducing it into the test containers. Substances insoluble in water or organic solvents are mixed with 2.5 g of finely ground quartz sand prior to adding the remainder of the artificial soil constituents and water.

[11] The sphagnum peat is air-dried and finely ground (particle size of 2 ± 1 mm); check that soil prepared with a fresh batch of peat is suitable for Collembola before use in a test; recommend measuring the C/N ratio, pH, and CEC of the peat.

[12] The amount of sand (69–70%) depends on the amount of calcium carbonate ($CaCO_3$) needed (i.e., 0.3–1 % $CaCO_3$ is used depending on the quality and nature of the peat).

5. Manipulation of Artificial Soil Before Use in Test

Document	Mixing	Hydration	pH Adjustment
W & K 1998			
F. candida	blend dry constituents in correct proportions and mix with some deionized water; hydrate	hydrate to crumbly structure; normally to 40–60% of total water-holding capacity	none when acidic or basic substances are tested
I. viridis	blend dry constituents in correct proportions and mix with some deionized water; hydrate	hydrate to crumbly structure; normally to 40–60% of total water-holding capacity	none when acidic or basic substances are tested
F. fimetaria	blend dry constituents in correct proportions and mix with some deionized or distilled water; hydrate	hydrate to porous soil texture one week before initiating test	none when acidic or basic substances are tested
ISO 1999	blend dry constituents in correct proportions and mix with some deionized water; hydrate	hydrate to crumbly structure; normally to 40–60% of total water-holding capacity[1]	NI[2]
OECD 2005	blend dry constituents[3]	hydrate to ~50% of the maximum water-holding capacity[4] (corresponding to 50 ± 10% moisture dry mass) moisture content should be optimized to loose porous texture	NI

[1] The final water content (~40–60%) of the test substance is achieved either by adding the test substance in a sufficient amount of deionized water, or, if the test substance is added in a dry form, by adding deionized water.

[2] NI = not indicated.

[3] Artificial soil should be stored for two weeks in order to equilibrate/stabilise the acidity. If the soil is too acidic, more $CaCO_3$ is added; if it is too alkaline, the soil can be adjusted by adding more artificial soil mixture without the $CaCO_3$.

[4] Maximum water-holding capacity determined as described in the method.

6. Negative Control Soil

Document	Description of Negative Control Soil	Number of Control Vessels (replicates)
W & K 1998		
F. candida	prepare in the same way as that for test soils, but without the test substance; use additional controls if auxillary substances other than water are used to dissolve test substance (e.g., organic solvent, quartz sand)	≥3
I. viridis	prepare in the same way as that for test soils, but without the test substance; use additional controls if auxillary substances other than water are used to dissolve test substance (e.g., organic solvent, quartz sand)	≥3
F. fimetaria	prepare in the same way as that for test soils, but without the test substance; use additional controls if auxillary substances other than water are used to dissolve test substance (e.g., organic solvent, quartz sand)	≥4
ISO 1999	prepare in the same way as that for test soils, but without the test substance; use additional controls if auxillary substances other than water are used to dissolve test substance (e.g., organic solvent, quartz sand)	5
OECD 2005	prepare in the same way as for test soils, but without the test substance; apply organic solvents, quartz sand or other vehicles to additional controls in amounts consistent with those used in treatments	≥4

7. Storage and Characterization of Test Soil

Document	Storage Conditions	Soil Characterization[1]
W & K 1998		
F. candida	store mixed test soils in closed glass vessels at 15 °C until required	moisture content and pH after hydrating
I. viridis	store mixed test soils in closed glass vessels at 15 °C until required	moisture content and pH after hydrating
F. fimetaria	NI[2]	moisture content and pH after hydrating
ISO 1999	NI, NA[3]	moisture content and pH after hydrating
OECD 2005	NI, NA	water-holding capacity and pH

[1] pH = hydrogen ion concentration
[2] NI = not indicated.
[3] NA = not applicable (artificial soil prepared, hydrated, and then used).

8. Preliminary Test — Number of Organisms per Vessel, Number of Replicates per Treatment, Number of Concentrations per Sample, and Recommended Dilution Factor

Document	Number of Organisms per Vessel	Number of Replicates per Treatment or Concentration	Number of Concentrations per Sample or Test Material	Recommended Dilution Factor
W & K 1998				
F. candida[1, 2]	10	NI[3]	5	geometric series[4]
I. viridis[1, 2]	20	NI	5	geometric series[4]
F. fimetaria[1, 5]	20[6]	1	4 + control	geometric series[7]
ISO 1999[1, 8]	10	1	4 + control	geometric series[7]
OECD 2005[1, 9]				
F. fimetaria	20[6]	2	5 + control	geometric series[10]
F. candida	10	2	5 + control	geometric series[10]

1 A preliminary (range-finding) test is optional.
2 The preliminary acute mortality test may use the same methodology and equipment as the sublethal test but may be of shorter duration (e.g., one week exposure); only a single assessment of adult survival at the end of the test is required.
3 NI = not indicated.
4 It is recommended that the concentrations be spaced by a factor of 10.
5 If a preliminary test is necessary to determine the range of concentrations for use in the final test, a preliminary one week acute test is performed.
6 10 males and 10 females are added to each container.
7 For example, 0, 1, 10, 100, and 1000 mg/kg, dry mass.
8 If a preliminary test is necessary to determine the range of concentrations for a definitive test, perform an acute lethality test of two weeks duration. The test period can be extended to four weeks to obtain additional information for determining the concentration range of the final test (i.e., qualitative determination of concentrations at which effects on reproduction could be expected).
9 The range-finding test is used in cases where sufficient information is not available for determining definitive test concentrations; mortality is the main endpoint accessed after two weeks of exposure.
10 For example, 0.1, 1, 10, 100, and 1000 mg/kg, dry mass.

9. Definitive Test — Number of Organisms per Vessel, Number of Replicates per Treatment, Number of Concentrations per Sample, and Recommended Dilution Factor

Document	Number of Organisms per Vessel	Number of Replicates per Treatment or Concentration	Number of Concentrations per Sample or Test Material	Recommended Dilution Factor
W & K 1998				
F. candida	10	≥3	≥5 + control	NI[1, 2]
I. viridis	20	≥3	≥5 + control	NI[2, 3]
F. fimetaria	20[4]	≥4	NI	NI[5, 6]
ISO 1999[7]	10	for NOEC: 5 for ECx: ≥2 for treatments and 5 for controls	≥5 + control 12 + control	geometric series[8] geometric series[9]
OECD 2005				
F. fimetaria	20[4]	≥4	NI	NI[5, 6]
F. candida	10	≥4	≥4 + control[10]	NI[1, 11]

1 NI = not indicated
2 The highest test concentration must be ≤1000 mg/kg dry wt of the test chemical.
3 The range of test concentrations should be selected to obtain a concentration-response relationship for growth (i.e., adult mortality should be similar in the control and test treatments).
4 10 males and 10 females are added to each container.
5 The concentrations selected to provide the EC10 are based on the results of the preliminary test (see Table 8, this appendix).
6 Substances do not need to be tested at concentrations higher than 1000 mg/kg dry wt of the test substance.
7 Two experimental designs are proposed. Depending on that chosen, the statistical endpoints would differ as would the recommended number of test concentrations and the recommended number of replicates/treatment (including the number of control vessels).
8 The concentrations should be spaced by a factor not exceeding 2.0 (e.g., $\sqrt[4]{10} = \sim 1.8$).
9 The spacing factor may vary, i.e., smaller at low concentrations and larger at high concentrations.
10 For determination of the ECx, an adequate number of concentrations to cause at least four statistically significant different mean responses at these concentrations is recommended. The spacing factor should ensure that the majority of test concentrations are on the slope of the ECx curve.
11 If no effects are observed at the highest concentration in the range-finding test (i.e., 1000 mg/kg), the reproduction test could be performed as a limit test, using a test concentration of 1000 mg/kg, in which case eight replicates should be used for both the treated soil and the control.

10. Feeding and Aeration During Test

Document	Type of Food Recommended	Feeding Quantity, Procedure, and Frequency	Aeration of Test Vessels
W & K 1998			
F. candida	granules of baker's yeast	add 3 granules to each test tube at the start of the test and at each weekly transferral	NI[1]
I. viridis	granules of baker's yeast	add 3 granules to each test tube at the start of the test and at each weekly transferral	NI
F. fimetaria	dried baker's yeast	add 15 mg at the beginning of the test and after 14 days	NI
ISO 1999	granulated dry yeast	add ~2 mg of granulated dry yeast to each test vessel at the beginning of the test and after a period of 14 days; cover vessels tightly after feeding	test containers are opened briefly two times per week to allow for aeration
OECD 2005	granulated dry baker's yeast	add 15 mg at the beginning of the test and after each 14-day interval	NI

[1] NI = not indicated.

11. Temperature and Lighting During Test

Document	Temperature (°C)	Lighting Conditions[1]
W & K 1998		
F. candida	15 ± 0.5	12h L:12h D or 16h L:8h D; intensity <1000 lux
I. viridis	15 ± 0.5 [2]	16h L:8h D; intensity <1000 lux
F. fimetaria	20 ± 1	12h L:12h D; intensity 400–800 lux
ISO 1999	20 ± 2	12h L:12h D or 16h L:8h D; intensity 400–800 lux at substrate surface
OECD 2005	20 ± 2	16h L:8h D preferred; intensity of 400–800 lux in the area of the test vessels

[1] L = light; D = dark.
[2] Test may be carried out at 20 °C if required.

12. Measurements and Biological Observations During Test

Document	Measurements[1]	Biological Observations
W & K 1998		
F. candida	W and pH, each treatment, at start and end of test [2, 3]	number of live adults per test vessel, measurements of individual body lengths of surviving adults[4], and number of juveniles hatching from eggs collected from soil[5] weekly and at the end of the test
I. viridis	W and pH, each treatment at start and end of test[2, 3]	number of live adults per test vessel, measurements of individual body lengths of surviving adults[4], weekly and at the end of the test
F. fimetaria	W and pH at start and end of test [3, 6]	number of live adults per test vessel, number of offspring per test container at the end of the test
ISO 1999	W and pH, each treatment, at start and end of test; reweigh test containers periodically throughout test[7, 8]	number of live adult and juvenile springtails per test vessel at the end of the exposure
OECD 2005	W and pH[9] each treatment at start and end of test; weight of test vessels at start and weekly thereafter[10]	

1 W = percent water (moisture content); pH = hydrogen-ion concentration.

2 Actual concentrations of the test chemical can be confirmed at the beginning and end of the test, where analytical techniques are available.

3 An additional 2 replicates are set up for each concentration and control(s) to allow measurements of pH, moisture content, and actual test chemical concentrations. Water content and pH are determined in the presence of 1*M* potassium chloride (KCl) of the test substrate.

4 Measurements of individual body lengths are made using a computerized image analysis system calibrated to measure lengths of between 0.5 and 3.5 mm.

5 The soil from which individuals have been removed is collected and placed into a tube. It is then incubated at 15 °C for 21 days or at 20 °C for 14 days until egg hatch is complete. After the incubation period the number of juveniles present is assessed by flotation in water.

6 The water content of the soil substrate is maintained during the test period by reweighing the test containers periodically and if necessary replenishing lost water. At the end of the test, the water content should not differ by more than 10% from the water content at the beginning of the test.

7 After two weeks, the water content is checked by reweighing. If water loss exceeds 2% of the initial water content, then water is added to compensate for loss.

8 To facilitate checking of pH and moisture content of the test substrate, it is recommended that an additional vessel be set up for each concentration.

9 pH is measured in a 1*M* KCl solution.

10 Vessels are weighed to check soil humidity; weight loss is replenished by the addition of an appropriate amount of deionized water (note: weight loss can be reduced by maintaining a high air-humidity, >80%).

13. Terminating Test, Biological Endpoints, and Statistical Endpoints

Document	Terminating Test	Biological Endpoints	Statistical Endpoints
W & K 1998			
F. candida	Collembola are extracted from soil in the tubes at weekly intervals during the exposure and at the end of the test; the soil from each tube is put into a larger vessel and Collembola are collected with a hand-held air aspirator	mean percent survival, mean body size of individuals, and mean number of juveniles produced at each concentration and each assessment interval	EC10 and EC50 for growth and reproduction (mg/kg dry wt of soil)
I. viridis	Collembola are extracted from soil in the tubes at weekly intervals during the exposure and at the end of the test; the soil from each tube is put into a larger vessel and Collembola are collected with a hand-held air aspirator	mean percent survival and mean body size of individuals produced at each concentration and each assessment interval	EC10 and EC50 for growth (mg/kg dry wt of soil)
F. fimetaria	At the end of 3 weeks, the organisms are extracted from each test container with a high gradient extractor[1]	mortality, percent loss or increase of adult *biomass*, and number of offspring produced	EC10, EC50, and LC50 for chronic mortality and growth (mg/kg dry wt of soil)
ISO 1999	4 weeks after introducing parental springtails onto the test and control substrates, the test substrate is poured into a 500–600 mL container and water is added; the suspension is gently stirred; the adults and juveniles floating on the water surface are counted[2]	mean number of adults and juveniles for each concentration; percent mortality of adults per concentration; mean number of offspring produced for each concentration after 28 days	NOEC and ECx[3]
OECD 2005	Collembola are extracted from soil with a high gradient heat extractor[1] or by floatation[4]	mortality and number of offspring produced	LC50, ECx, and NOEC

[1] A controlled temperature gradient extractor is used to collect Collembola from the soil. The heat coming from a heating element at the top of the extraction box is regulated through a thermistor placed on the surface of a soil sample. The temperature in the cooled liquid surrounding the collecting vessel is regulated through a thermistor situated at the surface of the collecting vessel. The thermistors are connected to a programmable controlling unit which raises the temperature according to pre-programmed schedules. At the end of the test, the animals are collected either in saturated aqueous benzoic acid with a few drops of detergent added, or at 2 °C on a smooth plaster/charcoal surface. Heat development during extraction may be increased every 12 h beginning with 25 °C and ending with 40 °C. Extraction onto plaster/charcoal surface makes chemical analysis and enumeration using digital image processing possible.

[2] Juveniles may be counted manually with a counting grid or with image analysis. When using an estimation technique, the average error of counting should not exceed 10%.

[3] NOEC and LOEC (expressed in mg/kg dry mass soil); EC10 and EC50 are optional additional endpoints.

[4] Test soil is emptied into a 250 mL vessel to which 200 mL of distilled water is added. The soil is gently agitated with a fine paintbrush to allow Collembola to float to the water surface. A small amount, approximately 0.5 mL of black Kentmere photographic dye (nontoxic) may be added to the water to aid counting by increasing the contrast between the water and the white Collembola. Counts may be carried out by eye, under a light microscope, or by photographing the surface of each vessel and later counting the Collembola on the enlarged prints or projected slides. Counts may also be performed using digital image processing.

14. Requirements for Valid Test; Use of Reference Toxicity Test

Document	Requirements for Valid Test	Reference Toxicant(s)	Procedures and Conditions for Reference Toxicity Test
W & K 1998			
F. candida	≥10 juveniles/surviving parental adult in control soil each week; percent mortality of adults in control(s) < 20%	none recommended at present	NI[4]
I. viridis	percent mortality of adults in control(s) <50%	none recommended at present	NI
F. fimetaria	>200 juveniles/control vessel; percent mortality of adults in control(s) <20%	dimethoate[1]	determine effects on survival and reproduction
ISO 1999	≥100 instars/control vessel; CV for control reproduction ≤30%; percent mortality of adults in control(s) ≤20%	Betanal plus; and E 605 forte[2, 3]	determine effects on survival and reproduction once or twice per year
OECD 2005			
F. fimetaria	>200 juveniles/control vessel; percent mortality of adults in control(s) <20%; CV of juveniles in controls <25%	to be determined	determine effects on survival and reproduction
F. candida	≥100 juveniles/control vessel; percent mortality of adults in control(s) < 20%; CV of juveniles in controls <25%	to be determined	determine effects on survival and reproduction

[1] A concentration series from 0 to 4 mg dimethoate (a.i.)/kg LUFA 2.2 soil will produce a decreasing survival and reproduction. EC50 for reproduction should range within 1.5 to 2.5 mg/kg in the LUFA 2.2 soil.

[2] Betanal plus (a.i. 160 g/L Phenmedipham); E 605 forte (a.i. 507.5 g/L Parathion).

[3] For Betanal plus, effects on reproduction ($\alpha = 0.05$) were observed at concentration of between 100 mg and 200 mg of the product per kg dry mass of the substrate. For E 605 forte, effects on mortality and reproduction were observed at concentrations of between 0.18 mg and 0.32 mg product and between 0.1 mg and 0.18 mg product per kg dry mass of the substrate, respectively.

[4] NI = not indicated.

Appendix G

Natural and Artificial Negative Control Soils Used for Method Development and the Establishment of Test Validity Criteria

Negative control soil must be included as one of the experimental treatments in each soil toxicity test. This treatment requires a soil that is essentially free of any contaminants that could adversely affect the performance of test organisms during the test (see Section 3.3). Before applying the test method described in this document as a standardized test to be conducted according to Environment Canada, it was necessary to first assess the performance of test organisms in different types of negative control soil representative of an array of *clean* soils found within Canada. Five types of negative control soils were used to develop the biological test method described herein and to further assess its robustness with samples of soil that varied considerably in their physical and chemical characteristics. These soils were also used to establish reasonable criteria for valid test results, based on control performance. The five soils tested include an artificial soil (see Section 3.3.2) and four natural soils (see Section 3.3.1) (Aquaterra Environmental, 1998a; Stephenson *et al.*, 1999a, b, 2000a; Aquaterra Environmental and ESG, 2000; ESG, 2000, 2001, 2002; ESG and Aquaterra Environmental, 2002, 2003; Becker-van Slooten *et al*., 2003, 2005; Stämpfli *et al*., 2005; EC, 2007a). The artificial soil was formulated in the laboratory from natural ingredients. The four natural soils included two agricultural soils from southern Ontario, a prairie soil from Alberta, and a forest soil from northern Ontario. The physicochemical characteristics of all five soils are summarized in Table G-1 of this appendix.

The artificial control soil (AS) used in this series of performance evaluation studies with diverse soil types was the same as that recommended for use herein (see Section 3.3.2). It consists of 70% silica sand, 20% kaolin clay, 10% *Sphagnum* sp. peat, and calcium carbonate (10–30 g $CaCO_3$/kg peat). The soil was formulated by mixing the ingredients in their dry form thoroughly, then gradually hydrating with de-ionized water, and mixing further until the soil was visibly uniform in colour, texture, and degree of wetness. This artificial soil is much the same as that described by ISO (1999) and OECD (2005).

The four natural soils used as negative control soil while developing this biological test method and establishing the test validity criteria herein (see Section 4.4) do not represent all Canadian soil types. However, they do vary greatly in their physicochemical characteristics and include agricultural soils with diverse textures as well as a forest soil (see Table G-1). The soils originated from areas that had not been subjected to any direct application of pesticides in recent years. They were collected with either a shovel or a backhoe, depending on the location and the amount of soil collected. Sampling depth depended upon the nature of the soil and the site itself.

The sample of clay loam soil, classified as a Delacour Orthic Black Chernozem, was collected in May 1995 from an undeveloped road allowance east of Calgary, Alberta. The soil beneath the sod was air dried to about 10–20% moisture content, sieved (4 or 9 mm), placed into 20-L plastic pails, and shipped to the University of Guelph (Guelph, Ontario) where it was kept in cold storage (4 °C) until needed. The soil was determined to be virtually free of any contaminants (Komex International, 1995).

Table G-1. Physicochemical Characteristics of Candidate Artificial and Natural Negative Control Soils [1]

Parameter	Artificial Soil	Clay Loam	Sandy Loam	Silt Loam	Forest Soil	Analytical Method
Source	formulated from constituents	field-collected from Alberta	field-collected from Ontario	field-collected from Ontario	field-collected from Ontario	—
Soil Texture	Fine Sandy Loam	Clay Loam	Fine Sandy Loam	Silt Loam	Loam	as per Hausenbuiller (1985); based on grain size distribution
Sand (%)	77.3	26.6	60.8	36.6	48.6	gravimetric grain size distribution
Silt (%)	7.8	43.3	27.8	50.1	36.9	gravimetric grain size distribution
Clay (%)	14.9	30.1	11.4	13.3	14.5	gravimetric grain size distribution
Gravel (%)	—[2]	—	0	0	0	gravimetric grain size distribution
Very Coarse Sand (%)	—	—	1.5	1.2	0.6	gravimetric grain size distribution
Coarse Sand (%)	—	—	3.2	2.3	2.2	gravimetric grain size distribution
Medium Sand (%)	—	—	10.1	5.4	9	gravimetric grain size distribution
Fine Sand (%)	—	—	25.9	13.4	20.4	gravimetric grain size distribution
Very Fine Sand (%)	—	—	20.2	14.3	16.4	gravimetric grain size distribution
Water-holding Capacity (%)	71.5	80.3	44	56.5	75.6	gravimetric analysis[3]
pH (units)	6	5.9	7.3	7.4	4.2	0.01 *M* $CaCl_2$ method[4]
Conductivity (mS/cm)	0.3	1.52	0.092	0.373	0.39	saturated paste method
Bulk Density (g/cm^3)	0.98	0.83	—	—	0.51	clod method
Total Carbon (%)	4.46	6.83	1.88	2.57	11.9	Leco furnace method
Inorganic Carbon (%)	—	—	0.18	0.58	< 0.05	Leco furnace method
Organic Carbon (%)	—	—	1.7	1.99	11.9	Leco furnace method

Parameter	Artificial Soil	Clay Loam	Sandy Loam	Silt Loam	Forest Soil	Analytical Method
Organic Matter (%)	9	12.8	2.9	3.5	19.9	dichromate oxidation
Cation Exchange Capacity (Cmol+/kg)	18.5	34.5	16.1	21.9	20	barium chloride method
Total Nitrogen (%)	0.05	0.59	0.115	0.166	0.74	Kjeldahl method
NH_4-N (mg/kg)	—	—	0.53	10.25	260	Kjeldahl method
NO_3-N (mg/kg)	—	—	6.94	5.44	2.26	Kjeldahl method
NO_2-N (mg/kg)	—	—	0.94	< 0.1	< 0.1	Kjeldahl method
Phosphorus (mg/kg)	23	12	6	10	35	nitric/perchloric acid digestion
Potassium (mg/kg)	22	748	61	75	250	NH_4 acetate extraction, colourimetric analysis
Magnesium (mg/kg)	149	553	261	256	192	NH_4 acetate extraction, colourimetric analysis
Calcium (mg/kg)	1848	5127	1846	4380	963	NH_4 acetate extraction, colourimetric analysis
Chloride (mg/kg)	—	—	69	42	113	H_2O extraction, colourimetric analysis
Sodium (mg/kg)	67	57	33	19	38	NH_4 acetate extraction, colourimetric analysis

[1] Characteristics of the artificial and various negative control soils that have been used to develop the definitive biological test method and associated criteria for test validity described herein (Aquaterra Environmental, 1998a; Stephenson *et al.*, 1999a, b, 2000a; Aquaterra Environmental and ESG, 2000; ESG, 2000, 2001, 2002; ESG and Aquaterra Environmental, 2002, 2003; Becker-van Slooten *et al*., 2003, 2005; Stämpfli *et al*., 2005; EC, 2007a).

[2] Not determined.

[3] Determined according to USEPA (1989) using a Fisherbrand P8 creped filter paper (see Section 5.3).

[4] Determined by Becker-van Slooten *et al.* (2004) according to Hendershot *et al.* (1993) (see Section 4.6).

The physicochemical characteristics of the soil show that it is a moderate-to-fine clay loam, with a relatively high organic content and cation exchange capacity compared to the other *clean* soils used during the development of this biological test method and the establishment of test validity criteria (see Table G-1).

A large (~3000 L) sample of sandy loam soil was collected in June 1999 from Beauchamp Farms, Eramosa, Ontario, from a site that had been cultivated regularly for crop production but not subjected to pesticide application. The soil was air-dried and sieved (2 or 5 mm), placed into 20-L plastic buckets, and kept in cold storage (4 °C) until needed. This soil was analyzed for common organic and inorganic contaminants, and its physicochemical characteristics established to determine if any unusual soil characteristics (e.g., high conductivity or anomalous nutrient levels) were present. The sample was found to be virtually free of both contaminants and anomalies. This soil is a fine sandy loam with a moderate organic content and a moderate cation exchange capacity compared to the other *clean* soils included in these studies (see Table G-1).

The sample of silt loam soil was collected in June 1999 from the University of Guelph Elora Research Station, in Nichol Township, Ontario. The topsoil had been removed several years ago when the research facility was built, and had been stockpiled beside a field. Soil collected for these method development studies was removed from the interior of the pile to avoid collecting soil that might have been inadvertently contaminated with pesticide or fertilizer spray drift from the adjacent field. The soil was air-dried and sieved (2 or 5 mm), placed into 20-L plastic buckets, and kept in cold storage (4 °C) until needed. The soil was also analyzed and found to be free of both organic and inorganic contaminants and anomalies. The measured physicochemical characteristics of this silt loam soil showed that it had a moderate organic content and a moderate cation exchange capacity, compared to the other four soils included in these method development studies (see Table G-1).

A 400-L sample of forest soil, classified as Orthic Humo-Ferric Podzols, was collected in June 2001 from a forested area located on the Canadian Shield, in Sudbury, Ontario. The leaf litter was gently raked away and a hand trowel was used to remove soil to a depth ranging from 5–10 cm. The soil was placed without sieving into 20-L plastic-lined buckets, and transported to ESG International at Guelph, Ontario. It was air-dried for 48 hours to no less than ~10% moisture content, homogenized, and then sieved through 6-mm mesh. Once the sample was sieved, it was thoroughly homogenized and stored in the same 20-L plastic buckets until used. This soil was stored at room temperature (20 °C) until used. The physicochemical characteristics of the forest soil show that it is a loam with a moderate cation exchange capacity, and the highest total organic carbon content (11.9%) and highest percentage of organic matter (19.9%) of the five soils used in the method development studies (see Table G-1).

Appendix H

Logarithmic Series of Concentrations Suitable for Toxicity Tests*

Column (Number of concentrations between 10.0 and 1.00, or between 1.00 and 0.10)**

1	2	3	4	5	6	7
10.0	10.0	10.0	10.0	10.0	10.0	10.0
3.2	4.6	5.6	6.3	6.8	7.2	7.5
1.00	2.2	3.2	4.0	4.6	5.2	5.6
0.32	1.00	1.8	2.5	3.2	3.7	4.2
0.10	0.46	1.00	1.6	2.2	2.7	3.2
	0.22	0.56	1.00	1.5	1.9	2.4
	0.10	0.32	0.63	1.00	1.4	1.8
		0.18	0.40	0.68	1.00	1.3
		0.10	0.25	0.46	0.72	1.00
			0.16	0.32	0.52	0.75
			0.10	0.22	0.37	0.56
				0.15	0.27	0.42
				0.10	0.19	0.32
					0.14	0.24
					0.10	0.18
						0.13
						0.10

* Modified from Rocchini *et al.* (1982).

** A series of seven (or more) successive concentrations should be chosen from a column. Midpoints between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed on a weight-to-weight (e.g., mg/kg) or weight-to-volume (e.g., mg/L) basis. 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, since such usage gives poor resolution of the confidence limits surrounding any threshold-effect value calculated.

Appendix I

Instruction on the Derivation of ICps Using Linear and Nonlinear Regression Analyses

I.1 Introduction

This appendix provides instruction for the use of linear and nonlinear regression analyses to derive, based on the concentration-response relationships for quantitative endpoint data (in this instance, number of surviving progeny), the most appropriate ICps. It represents an adaptation and modification of the approach described by Stephenson *et al.* (2000b). Instructions herein are provided using Version 11.0 of SYSTAT*; however, any suitable software may be used. The regression techniques described in this appendix are most appropriately applied to continuous data from tests designed with 10 or more concentrations or treatment levels (including the negative control treatment). The test design for measuring the effects of prolonged exposure on *F. candida* , *F. fimetaria*, and *O. folsomi* is summarized in Table I.1.

An overview of the general process used to select the most appropriate regression model for each data set under consideration is presented in Figure 4; Section 4.8.2.1.

The reader is encouraged to refer to the appropriate sections within this biological test method, as well as the sections on regression analyses within the "Guidance Document on Statistical Methods for Environmental Toxicity Tests" (EC, 2005b), before data analyses. Environment Canada (2005b) also contains several additional references for the statistical analysis of quantitative test data using linear and nonlinear regression procedures. Some of the related guidance from these documents has been provided in this appendix, where appropriate.

* The latest (e.g., Version 11.0) version of SYSTAT™ is available for purchase by contacting SYSTAT Software, Inc., 1735 Technology Drive, Ste 430 San Jose, California 95110, USA, phone: 1-800-797-7401; Web site www.systat.com/products/Systat/.

Table I.1 Summary of Test Design for Environment Canada's Biological Test Method for Measuring Survival and Reproduction of Springtails Exposed to Contaminants in Soil (see Section 4).

Parameter	Description
Test type	– whole soil toxicity test; no renewal (static test)
Test duration	– 21 days for *Folsomia fimetaria*; 28 days for *Folsomia candida* and *Orthonychiurus folsomi*
Test species	– *Orthonychiurus folsomi*; age-synchronized; 28 to 31 days after eclosion; 10 females (rounder, ≥2 mm) and 5 males (more slender, ~1–1.5 mm) per test vessel; – *Folsomia candida*; age-synchronized; 10 to 12 days after eclosion; 10 organisms per test vessel; or – *Folsomia fimetaria*; age-synchronized; 23 to 26 days after eclosion; 10 females (rounder, twice as large as males) and 10 males (smaller, more slender) per test vessel
Number of replicates	– ≥3 replicates per treatment; ≥5 replicates per control treatment
Number of treatments	– negative control soil and ≥7 test concentrations as a minimum; however, ≥10 concentrations plus a negative control are strongly recommended
Statistical endpoints	Quantal: – mean percent survival of adults in each treatment, on Day 21 for *F. fimetaria*, and on Day 28 for *F. candida* and *O. folsomi* – 21- or 28-day LC50 calculated if dose-response observed (using appropriate statistical procedures for quantal data; the procedures outlined in this appendix are not appropriate for quantal data) Quantitative: – mean number of live juveniles in each treatment, on Day 21 for *F. fimetaria*, or on Day 28 for *F. candida* and *O. folsomi* – ICp (e.g., IC50 and/or IC25) for the number of live juveniles produced

I.2 Linear and Nonlinear Regression Analyses

I.2.1 Creating Data Tables

Note: The statistical analysis must encompass the transformation of the concentrations logarithmically (e.g., $\log_{10}$ or $\log_e$). If the concentrations fall below one (1) (e.g., 0.25), then the data can be transformed by transforming the units (e.g., from mg/kg to µg/g) with a multiplication factor (e.g., 1000); the modified data are then transformed logarithmically. The transformation can be done either in the original electronic spreadsheet, or when the original data are transferred to the SYSTAT data file.

1) Open the appropriate file containing the data set in an electronic spreadsheet.

2) Open the SYSTAT program. In the main screen, go to **File**, **New**, and then **Data**. This will open up an empty data table. Insert the variable names into the column heading by double-clicking on a variable name, which opens the '**Variable Properties**' window. Insert an appropriate name for the variable of interest within the '**Variable name**' box, and select the variable type; additional comments can be inserted within the '**Comments:**' box. For example, the following variable names might be used:

conc	=	concentration or treatment level
logconc	=	$\log_{10}$ transformation of concentration or treatment level
rep	=	replicate within a treatment level
juveniles	=	number of juveniles produced

3) The data can now be transferred. To transfer the data, copy and paste each column from the electronic spreadsheet containing the concentrations, the replicates, and associated mean values, to the SYSTAT data table.

4) Save the data by going to **File**, then **Save As**; a **'Save As**' window will appear. Use appropriate coding to save the data file. Select **Save** when the file name has been entered.

5) Record the file name of the SYSTAT data file in the electronic spreadsheet containing the original data.

6) If the data (i.e., the test concentrations) require transformation, the data can be transformed by selecting **Data**, **Transform**, and then **Let...**. Once in the **Let...** function, select the column heading containing the appropriate header for the transformed data (e.g., logconc), and then select **Variable** within the '**Add to**' box to insert the variable into the '**Variable:**' box. Select the appropriate transformation (e.g., L10 for $\log_{10}$ transformation or LOG for the natural logarithm) in the '**Functions:**' box (the '**Function Type:**' box should be **Mathematical**), and then select **Add** to insert the function into the '**Expression:**' box. Select the column heading containing the original untransformed data (i.e., 'conc' for concentration or treatment level), followed by **Expression** within the '**Add to**' box to insert the variable into the '**Expression:**' box. If a multiplication factor is required to adjust the concentration before the log-transformation, this step can be completed within the '**Expression:**' box (e.g., L10[conc*1000]).

Select **OK** when all of the desired transformations are complete. The transformed data will appear in the appropriate column. *Save the data* (i.e., select **File**, followed by **Save**).

Note: The $\log_{10}$ of the negative control treatment cannot be determined (i.e., the $\log_{10}$ of zero is undefined); therefore, assign the negative control treatment level a very small number (e.g., 0.001) known or assumed to be a no-effect level, to include this treatment in the analysis and differentiate it from the other transformed treatment levels.

7) From the data table, calculate and record the mean of the negative controls for the variable under study; each measurement endpoint is statistically analyzed independently. The mean value of these control data will be required when estimating the model parameters. In addition, determine the maximum value within the data set for that particular variable and round up to the nearest whole number. This number is used as the maximum value of the y-axis (i.e., 'ymax') when creating a graph of the regressed data.

I.2.2 Creating a Scatter Plot or Line Graph

The scatter plots and line graphs provide an indication of the shape of the concentration-response curve for the data set. The shape of the concentration-response curve can then be compared to each model (Figure I.1) so that the appropriate model(s) likely to best suit the data is (are) selected. Each of the selected models should be used to analyze the data. Subsequently, each model is reviewed, and the model that demonstrates the best fit is selected.

1) Select **Graph**, **Summary Charts**, and then **Line...**. Select the independent variable (e.g., logconc), followed by **Add** to insert the variable into the '**X-variable(s):**' box. Select the dependent variable under examination, followed by **Add** to insert the variable into the '**Y-variable(s):**' box. Select **OK**. A graph will be displayed within the '**Output Pane**' of the main SYSTAT screen containing the mean values for every treatment level; to view a larger version of the graph, simply select the '**Graph Editor**' tab located below the central window. A scatter plot of the data can also be viewed by selecting **Graph**, **Plots**, and then **Scatterplot...** and following the same instructions for inserting the x- and y-variables. The graphs will provide an indication as to the general concentration-response trend allowing the selection of the potential model(s) of best fit to be chosen, in addition to an estimation of the ICp of interest.

Note: The main SYSTAT screen is divided into three parts. The left-hand side of the screen (i.e., '**Output Organizer**' tab) provides a list of all of the functions completed (e.g., graphs) – each function can be viewed by simply selecting the desired icon. The right-hand side of the screen forms the central window in which the general output of all of the functions completed (e.g., regression, graphs) can be viewed. The tabs below this central window allow the user to toggle between the data file (i.e., '**Data Editor**'), individual graphs (i.e., '**Graph Editor**') and the output (i.e., '**Output Pane**'). The various graphs produced can be viewed individually within the '**Graph Editor**' tab by selecting the graph of interest within the left-hand side of the screen (i.e., '**Output Organizer**' tab). The bottom portion of the screen displays the command codes used to derive the desired functions (e.g., regression and graphing codes). The '**Log**' tab within this command screen displays a history of all of the functions completed.

2) Visually estimate and record an estimate of the ICp of interest (e.g., IC50) for the data set. For example, for an IC50, divide the average of the controls by two, and find this value on the y-axis. Estimate a horizontal line from the y-axis until the line intercepts the data points. At this intersection point, extend a vertical line down towards the x-axis and record this concentration value as an estimate of the IC50.

3) Using the scatter plots or line graphs, select the potential model(s) that will best describe the concentration-response trend (refer to Figure I.1 for an example of each model).

I.2.3 Estimating the Model Parameters

1) Select **File**, **Open**, and then **Command**.

2) Open the file containing the command codes for the particular model chosen from Section I.2.2 (i.e., select the appropriate file, followed by **Open**):

nonline.syc	=	exponential model
nonling.syc	=	gompertz model
nonlinh.syc	=	logistic with hormesis model
linear.syc	=	linear model
nonlinl.syc	=	logistic model

The file will provide the command codes for the selected model within the appropriate tab of the command editor box at the bottom of the main screen. All of the command codes for deriving IC50s and IC25s are provided in Table I.2; however, the equations can be formatted to derive any ICp. For example, the command codes for the logistic model to derive an IC50 would be:

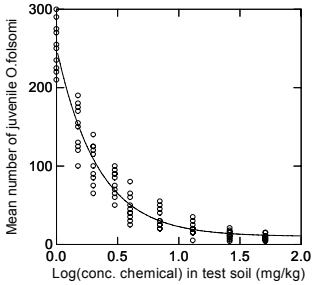
```
nonlin
print=long
model juveniles = t/(1+logconc/x)^b)
save resid1/ resid
estimate/ start = 170, 0.7, 2 iter=200
use resid1
pplot residual
plot residual*logconc
plot residual*estimate
```

3) Type in the header of the column in the data table containing the variable of interest to be analyzed within the line entitled ‘model y=’ (where ‘y’ is the dependent variable, e.g., juveniles).

Exponential Model

IC50: juveniles = a*exp(log((a-a*0.5-b*0.5)/a)*(logconc/x))+b
IC25: juveniles = a*exp(log((a-a*0.25-b*0.75)/a)*(logconc/x))+b

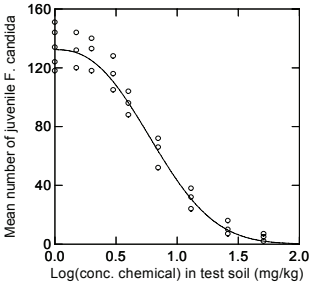
Where:
a = the y-intercept (the control response)
x = ICp for the data set
logconc = the logarithmic value of the exposure concentration
b = a scale parameter (estimated between 1 and 4)



Gompertz Model

IC50: juveniles = g*exp((log(0.5))*(logconc/x)^b)
IC25: juveniles = g*exp((log(0.75))*(logconc/x)^b)

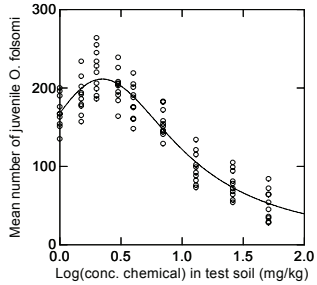
Where:
g = the y-intercept (the control response)
x = ICp for the data set
logconc = the logarithmic value of the exposure concentration
b = a scale parameter (estimated between 1 and 4)



Hormesis Model

IC50: juveniles = (t*(1+h*logconc))/(1+((0.5+h*logconc)/0.5)*(logconc/x)^b)
IC25: juveniles = (t*(1+h*logconc))/(1+((0.25+h*logconc)/0.75)*(logconc/x)^b)

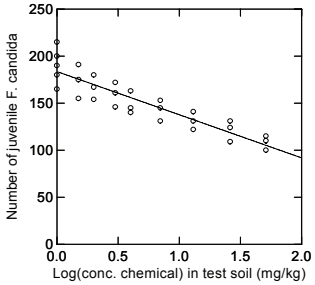
Where:
t = the y-intercept (the control response)
h = the hormetic effect (estimated between 0.1 and 1)
x = ICp for the data set
logconc = the logarithmic value of the exposure concentration
b = a scale parameter (estimated between 1 and 4)



Linear Model

IC50: juveniles = ((-b*0.5)/x)*logconc+b
IC25: juveniles = ((-b*0.25)/x)*logconc+b

Where:
b = the y-intercept (the control response)
x = ICp for the data set
logconc = the logarithmic value of the exposure concentration



Logistic Model

IC50: juveniles = t/(1+(logconc/x)^b)
IC25: juveniles = t/(1+(0.25/0.75)*(logconc/x)^b)

Where:
t = the y-intercept (the control response)
x = ICp for the data set
logconc = the logarithmic value of the exposure concentration
b = a scale parameter (estimated between 1 and 4)

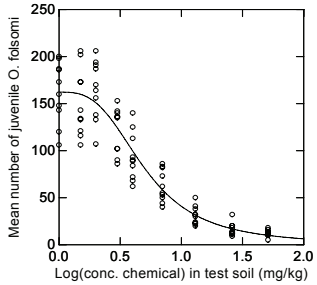


Figure I.1 SYSTAT Version 11.0 Equations for Linear and Nonlinear Regression Models and Example Graphs of the Observed Trends for Each Model

4) The fourth line of the text should read 'save resida/ resid', where 'a' indicates a number to which the residual file is assigned. Substitute this same number into the sixth line (i.e., 'use resida') so that the same file is used to generate a normal probability plot and graphs of the residuals. The command lines that follow provide instruction for the generation of a probability plot (i.e., 'pplot residual'), the generation of a graph of residuals against the concentration or treatment level (i.e., 'plot residual*logconc'), and a graph of the residuals against the predicted and fitted values (i.e., 'plot residual*estimate'). These graphs are used to aid in the assessment of the assumptions of normality (e.g., probability plot) and homogeneity of the residuals (e.g., graphs of the residuals) when evaluating for the model of best fit (Section I.2.4).

5) Substitute the mean of the controls and the estimated ICp (e.g., IC50 and/or IC25) within the fifth line entitled 'estimate/ start=' (refer to Table I.2 for details on the substitution for each model). These values were initially derived from examination of the scatter plot or line graph. The model, once it converges, will provide a set of parameters from which the ICp, and its 95% confidence limits, are reported (i.e., parameter 'x'). It is essential to provide accurate estimates for each parameter before running the model, or the iterative procedure used to derive the reported parameters might not converge. The scale parameter (Table I.2) is typically estimated to range from one to four. The number of iterations can be changed, but for this example, has been set to 200 (i.e., 'iter=200'). Typically, 200 iterations are sufficient for a model to converge; if more iterations are required, it is likely that the most appropriate model is not being applied.

6) Select **File**, and then **Submit Window** to run the commands; alternatively, right-click the mouse and select **Submit Window**. This will generate a printout of the iterations, the estimated parameters, and a list of the actual data points with the corresponding predicted values and residuals. A preliminary graph of the estimated regression line will also be presented; this preliminary graph should be deleted. The graph can be deleted by selecting the graph in the left-hand window within the main screen. A normal probability plot and graphs of the residuals will also be presented.

I.2.4 Examining the Residuals and Test Assumptions

An examination of the residuals for each model tested helps to determine whether assumptions of normality and homoscedasticity have been met. If any of the assumptions cannot be met, regardless of the model examined, a statistician should be consulted for further guidance on assessing additional models or the data should be re-analyzed using the less desirable linear interpolation method of analysis (using ICPIN; see Section 4.8.2.2 in the main text).

I.2.4.1 Assumptions of normality. Normality should be assessed using *Shapiro-Wilk's test* as described in EC (2005b); Section I.2.4.3 provides instructions for conducting this test. The normal probability plot, displayed in the '**Output Pane**', can also be used to evaluate whether the assumption of normality is met. The residuals should form a fairly straight line diagonally across the graph; the presence of a curved line represents deviation from normality. The normal probability plot should not, however, be used as a stand-alone test for normality, since the detection of a 'normal' (e.g., straight) or 'non-normal' (e.g., curved) line is dependent upon the subjective assessment of the user. If the data are not normally distributed, then the user should try another model, consult a statistician for further guidance, or the data should be analyzed using the less desirable linear interpolation method of analysis.

Table I.2 SYSTAT Command Codes for Linear and Nonlinear Regression Models

Model	Command Codes	
Exponential	nonlin print=long model juveniles=a*exp(log((a-a*0.5-b*0.5)/a)*(logconc/x))+b save resid1/ resid estimate/ start=250[a], 1[b], 0.3[c] iter=200 use resid1 pplot residual plot residual*logconc plot residual*estimate	where: [a] Represents the estimate of the y-intercept (i.e., 'a') (the control response) [b] Represents the scale parameter (i.e., 'b') (estimated between 1 and 4) [c] Represents the estimate of the ICp for the data set (i.e., 'x')
Gompertz	nonlin print=long model juveniles=g*exp((log(0.5))*(logconc/x)^b) save resid2/ resid estimate/ start=130[a], 0.8[b], 1[c] iter=200 use resid2 pplot residual plot residual*logconc plot residual*estimate	where: [a] Represents the estimate of the y-intercept (i.e., 'g') (the control response) [b] Represents the estimate of the ICp for the data set (i.e., 'x') [c] Represents the scale parameter (i.e., 'b') (estimated between 1 and 4)
Hormesis	nonlin print=long model juveniles=(t*(1+h*logconc))/(1+((0.5+h*logconc)/0.5)*(logconc/x)^b) save resid3/ resid estimate/ start=170[a], 0.01[b], 1.2[c], 1[d] iter=200 use resid3 pplot residual plot residual*logconc plot residual*estimate	where: [a] Represents the estimate of the y-intercept (i.e., 't') (the control response) [b] Represents the hormetic effect (i.e., 'h') (estimated between 0.1 and 1) [c] Represents the estimate of the ICp for the data set (i.e., 'x') [d] Represents the scale parameter (i.e., 'b') (estimated between 1 and 4)
Linear	nonlin print=long model juveniles=((-b*0.5/x)*logconc+b save resid4/ resid estimate/ start=180[a], 2.1[b] iter=200 use resid4 pplot residual plot residual*logconc plot residual*estimate	where: [a] Represents the estimate of the y-intercept (i.e., 'b')(the control (the control [b] Represents the estimate of the ICp for the data set (i.e., 'x')
Logistic	nonlin print=long model juveniles=t/(1+(logconc/x)^b) save resid5/ resid estimate/ start=170[a], 0.7[b], 2[c] iter=200 use resid5 pplot residual plot residual*logconc plot residual*estimate	where: [a] Represents the estimate of the y-intercept (i.e., 't') (the control response) [b] Represents the estimate of the ICp for the data set (i.e., 'x') [c] Represents the scale parameter (i.e., 'b') (estimated between 1 and 4)

I.2.4.2 Homogeneity of residuals. Homoscedasticity (or homogeneity) of the residuals should be assessed using *Levene's test* as described in EC (2005b) (Section I.2.4.3 provides instructions for conducting this test), and by examining the graphs of residuals against the actual and predicted (estimated) values. Homogeneity of the residuals is described by an equal distribution of the variance of the residuals across the independent variable (i.e., concentration or treatment level) (Figure I.2A). Levene's test, if significant, will indicate that the data are not homogeneous. If the data (as indicated by Levene's test) are heteroscedastic (i.e., not homogeneous), then the graphs of the residuals should be examined. If there is a significant change in the variance and the graphs of the residuals produce a distinct fan or 'V' pattern (refer to Figure I.2B for a plot of the 'residual*estmate'; a corresponding 'V' pattern in the opposite direction also occurs in the plot of the 'residual*logconc'), then the data analysis should be repeated using weighted regression. Alternatively, a non-divergent pattern suggestive of a systematic lack of fit (Figure I.2C) will indicate that an inappropriate or incorrect model was selected.

I.2.4.3 Assessing assumptions of normality and homogeneity of residuals. SYSTAT Version 11.0 can perform both Shapiro-Wilk's and Levene's tests to assess the assumptions of normality and homogeneity of residuals. Levene's test can only be performed by conducting an analysis of variance (ANOVA) on the absolute values of the residuals derived in Section I.2.3.

1) Select **File**, **Open**, and then **Data** to open the data file containing the residuals created in Section I.2.3 (e.g., resid1.syd).

2) Insert a new variable name into an empty column by double-clicking on the variable name, which opens the '**Variable Properties**' window. In this window, insert an appropriate name for the transformed residuals (e.g., absresiduals) into the '**Variable name:**' box. Transform the residuals by selecting **Data**, **Transform**, and then **Let...**. Once in the **Let...** function, select the column heading containing the appropriate header for the transformed data (e.g., absresiduals), and then select **Variable** within the '**Add to**' box to insert the variable into the '**Variable:**' box. Select the appropriate transformation (e.g., ABS for the transformation of data into its absolute form) in the '**Functions:**' box (the '**Function Type:**' box should be **Mathematical**), and then select **Add** to insert the function into the '**Expression:**' box. Select the column heading containing the original untransformed data (i.e., residuals), followed by **Expression** within the '**Add to**' box to insert the variable into the '**Expression:**' box. Select **OK**; the transformed data will appear in the appropriate column. Save the data.

3) To perform Shapiro-Wilk's test, select **Analysis**, **Descriptive Statistics**, and then **Basic Statistics...**. A '**Column Statistics**' window will appear. Select the residuals from the '**Available variable(s):**' box, followed by **Add** to insert this variable into the '**Selected variable(s):**' box. Within the '**Options**' box, select the **Shapiro-Wilk normality test**, followed by **OK**. A small table will appear within the SYSTAT Output Organizer window, where the Shapiro-Wilk critical value (i.e., 'SW Statistic') and probability value (i.e., SW P-Value') will be displayed. A probability value greater than the usual criterion of $P > 0.05$ indicates that the data are normally distributed.

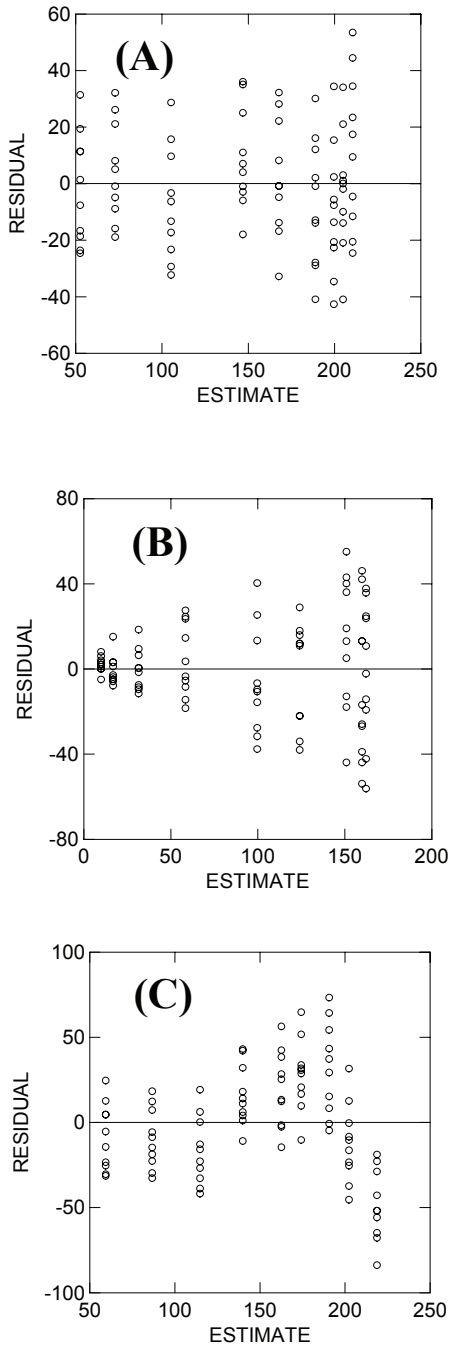


Figure I.2 **Graph of the Residuals Against the Predicted (Estimated) Values (i.e., 'residuals*estimate') Indicating Homoscedasticity (A), and Two Types of Heteroscedasticity; One Demonstrating a Fan or 'V' Shape (B) Requiring Further Examination Using Weighted Regression, and a Second Demonstrating a Systematic Lack of Fit (C) as a Result of the Selection of an Incorrect Model**

4) To perform Levene's test, select **Analysis**, **Analysis of Variance (ANOVA)**, and then **Estimate Model...**, an '**Analysis of Variance: Estimate Model**' window will appear.

5) Select the variable within which the data are to be grouped (e.g., logconc), and place this variable into the '**Factor(s):**' box by selecting **Add**.

6) Select the transformed residuals (i.e., absresiduals), followed by **Add**, to insert the variable into the '**Dependent(s):**' box. Select **OK**. A graph of the data and a printout of the output will appear within the '**Output Pane**' tab. A probability value greater than the usual criterion of $P > 0.05$ indicates that the data are homogeneous.

I.2.5 Weighting the Data

If the residuals are heteroscedastic, as indicated by Levene's test, and there is a significant change in variance across treatment levels (i.e., the presence of a distinct fan or 'V' shape; refer to Figure I.2B), the data should be re-analyzed using weighted regression. Weighted regression involves using the inverse of the variance of observations within each concentration or treatment level as the weights. When performing the weighted regression, the standard error for the ICp (presented in SYSTAT as the asymptotic standard error ('A.S.E.'; refer to Figure I.3) is compared to that derived from the unweighted regression. If there is a difference of greater than 10% between the two standard errors, then the weighted regression is selected as the regression of best choice. However, if there is a significant change in variance across all treatment levels, and there is less than a 10% difference in the standard error between the weighted and unweighted regressions,[**] then the user should consult a statistician for further guidance and the application of additional models, or the data could be re-analyzed using the less desirable linear interpolation method of analysis. The comparison between weighted and unweighted regression is completed for each of the selected models while proceeding through the process of final model selection (i.e., model and regression of best choice). Alternatively, if Levene's test demonstrates that the data are not homogeneous, and the graphs of the residuals demonstrate a non-divergent pattern (e.g., Figure I.2C), an inappropriate or incorrect model might have been selected. The user is then advised to consult a statistician for further guidance on the use and application of alternate models.

1) Select **File**, **Open**, and then **Data**. Select the file containing the data set to be weighted. Insert the two new variable names into the column heading by double-clicking on a variable name, which opens the '**Variable Properties**' window. In this window, insert an appropriate name for the variable of interest, select the variable type, and specify comments if desired. The two new column headings should indicate the variance of a particular variable (e.g., varjuv), and the inverse of the variance for that variable (e.g., varinvsjuv). Save the data file by selecting **File**, and then **Save**.

[**] The value of 10% is only a "rule-of-thumb" based upon experience. Objective tests for the improvement due to weighting are available, but beyond the scope of this document. Weighting should be used only when necessary, since the procedure can introduce additional complications to the modeling procedure. A statistician should be consulted when weighting is necessary, but the parameter estimates are nonsensical.

```
SYSTAT Rectangular file C:\SYSTAT\STATAPP.syd,
created Fri Jun 25, 2004 at 08:44:18, contains variables:
               CONC            REP            LOGCONC        JUVENILES

 Iteration
 No.      Loss       T            X            B
   0 0.120173D+06 0.134000D+03 0.600000D+00 0.200000D+01
   1 0.513085D+05 0.157213D+03 0.774654D+00 0.307352D+01
   2 0.483896D+05 0.162406D+03 0.694415D+00 0.297019D+01
   3 0.483088D+05 0.162048D+03 0.701888D+00 0.307781D+01
   4 0.483078D+05 0.162223D+03 0.700681D+00 0.306500D+01
   5 0.483078D+05 0.162205D+03 0.700802D+00 0.306703D+01
   6 0.483078D+05 0.162207D+03 0.700783D+00 0.306676D+01
   7 0.483078D+05 0.162207D+03 0.700786D+00 0.306680D+01

Dependent variable is JUVENILES

    Source   Sum-of-Squares    df  Mean-Square
 Regression     1047602.177     3 349200.726
   Residual       48307.823    87    555.262 ------------>        residual mean square error

      Total     1095910.000    90
Mean corrected   357157.600    89

       Raw  R-square (1-Residual/Total)        =        0.956
Mean corrected R-square (1-Residual/Corrected) =        0.865
          R(observed vs predicted) square      =        0.865

                                                  Wald Confidence Interval
Parameter         Estimate       A.S.E.    Param/ASE        Lower < 95%> Upper
 T                 162.207        5.169       31.380      151.933      172.481
 X                   0.701        0.032       21.873        0.637        0.764 ------------>
 B                   3.067        0.343        8.945        2.385        3.748

           JUVENILES    JUVENILES
   Case     Observed    Predicted     Residual
      1      173.000      162.207       10.793
      2      148.000      162.207      -14.207
      3      106.000      162.207      -56.207
      4      120.000      162.207      -42.207
      5      186.000      162.207       23.793
      6      200.000      162.207       37.793
      7      187.000      162.207       24.793
      .      .......      .......      .......
      .      .......      .......      .......
      .      .......      .......      .......
     86       14.000        9.918        4.082
     87       10.000        9.918        0.082
     88       12.000        9.918        2.082
     89        5.000        9.918       -4.918
     90       18.000        9.918        8.082

Asymptotic Correlation Matrix of Parameters

              T            X            B

T             1.000
X            -0.638        1.000
B            -0.479        0.363        1.000
```

ICp, asymptotic standard error, and lower and upper 95% confidence limits

Figure I.3 Example of the Initial Output Derived using the Logistic Model in SYSTAT Version 11.0. The initial output provides the residual mean square error used to select the model of best choice, as well as the ICps, the standard error for the estimate, and the upper and lower 95% confidence limits. The number of cases displayed has been shortened for the purpose of this diagram; however, the output within SYSTAT displays all cases including the actual variable measurement and the corresponding predicted estimate and residual.

2) Select **Data**, followed by **By Groups...**. Select the independent variable (i.e., logconc), followed by **Add**, to insert this variable into the '**Selected variable(s):**' box; this will enable the determination of the variance of the variable of interest by concentration or treatment level (i.e., "group"). Select **OK**.

3) Select **Analysis**, **Descriptive Statistics**, and then **Basic Statistics...**. Select the variable of interest to be weighted (e.g., juveniles), followed by **Add** to insert this variable into the '**Selected variable(s):**' box. Select **Variance** within the '**Options**' box, followed by **OK**. This function will display the variance for the variable of interest, grouped by concentration or treatment level within the '**Output Pane**' tab of the main screen.

4) Select **Data**, **By Groups...**, and then click on the box beside **Turn off**, and select **OK** so that any analysis that follow will not be analyzed according to each individual concentration or treatment level; the analysis should consider the entire data set as a whole.

5) Return to the data file by selecting the '**Data Editor**' tab within the main screen. Transfer the variances for each concentration or treatment level to the corresponding concentration within the variance column (e.g., varjuv). Note that the variance is the same among replicates within a treatment.

6) Select **Data**, **Transform**, and then **Let...**, and select the column heading containing the inverse of the variance (e.g., varinvsjuv) for the variable of interest, followed by **Variable** within the **'Add to**' box to insert the variable into the '**Variable:**' box. Select the '**Expression:**' box and type in '1/', and then select the column heading containing the variances (e.g., varjuv) of the variable of interest for each replicate and concentration, followed by **Expression** within the '**Add to**' box to insert the variable into the '**Expression:**' box. Select **OK**. The inverse of the variance for each replicate and concentration will be displayed in the appropriate column. Save the data by selecting **File**, and then **Save**.

7) Select **File**, **Open**, and then **Command**; open the file containing the command codes for estimating the equation parameters (e.g., Section I.2.3, step 2) for the same model selected for the *unweighted* analysis.

8) Insert an additional row after the third line by typing 'weight=varinvsy', where 'y' is the dependent variable to be weighted (e.g., weight=varinvsjuv), as per the shaded area below:

```
nonlin
print=long
model juveniles = t/(1+(logconc/x)^b)
weight=varinvsjuv
save resid2/ resid
estimate/ start = 170, 0.7, 2 iter=200
use resid2
pplot residual
plot residual*logconc
plot residual*estimate
```

9) Assign a new number for the residuals within the line entitled 'save resida' (where 'a' represents the assigned number).

10) Substitute the mean of the controls and the estimated ICp within the line entitled 'estimate/ start . . .' (refer to Table I.2 for details on the substitution for each model). These estimates will be the same as those used for the unweighted analysis.

11) Select **File**, and then **Submit Window** to run the commands. This will generate output of the iterations, the estimated parameters, and a list of the data points with the corresponding predicted data points and residuals within the '**Output Pane**' tab of the main screen. A preliminary graph of the estimated regression line will also be presented; this should be deleted. A normal probability plot and graphs of the residuals will also be presented.

12) Proceed with the analysis as described in Section I.2.4 to ensure that all model assumptions have been met.

13) Compare the weighted regression analysis with the unweighted regression analysis. Select the weighted regression if weighting reduced the standard error for the ICp by 10%, relative to the unweighted regression analysis.

I.2.6 The Presence of Outlier(s) and Unusual Observations

Outliers are indicative of a measurement that does not seem to fit the other values derived from the test. Outliers and unusual observations can be identified by examining the fit of the concentration-response curve relative to all data points, and by examining the graphs of the residuals. If an outlier has been observed, the test records (e.g., hand-recorded and electronic data sheets and experimental conditions) should be scrutinized for human error. If the outlier is a data point that has been obtained through a transcription error that cannot be corrected, or through a faulty procedure, then the data point should be removed from the analysis. If an outlier has been identified, the analysis should be completed with and without the presence of the outlier. The decision on whether or not to remove the outlier should also take into consideration natural biological variation, and biological reasons that might have caused the apparent anomaly. Regardless of whether or not the outlier is removed, a description of the data, outliers, analyses with and without the outlier, and interpretive conclusions, must accompany the final analysis. If it appears as if there is more than one outlier present, the selected model should be re-assessed for appropriateness and alternative models considered. Additional guidance on the presence of outliers and unusual observations is provided in EC (2005b) and should be consulted for further details.

The Analysis of Variance (ANOVA) function within SYSTAT can be performed to determine whether or not the data contain outliers. However, ANOVA assumes that the residuals are normally distributed, and therefore, assumptions of normality must be met prior to using the ANOVA to detect outliers. The presence of outliers can also be determined from the graphs of residuals.

1) Perform an Analysis of Variance (ANOVA) as described in Section I.4 of this appendix, to determine whether any outliers exist. Any outlier(s) will be identified as a case number that corresponds with the row number in the SYSTAT data file. The program uses the studentized residuals as an indication of outliers; values greater than 3 indicate the possibility of an outlier. This should be confirmed with the graphs of the residuals.

2) If a decision is made to remove the outlier(s), delete the value from the original data table (file), and re-save the file under a *new* name (i.e., select **File**, and then **Save As...**). For example, the new file name might contain the letter 'o' (for outlier(s) removed) at the end of the file's original name.

3) Repeat the regression analysis with the outlier(s) removed, using the same model and estimated parameters that were used before the outlier(s) were removed. Alternatively, additional models may be used for analysis if the alternative model results in a better fit and smaller residual mean square error. If the removal of the outlier(s) does not result in a significant change to both the residual mean square error and the ICp (including its corresponding confidence intervals), then the individual performing the analysis must make a subjective decision (i.e., professional judgement) as to whether or not to include the outlier(s). Justification for the removal or inclusion of the outlier(s) must be recorded along with the final analysis.

I.2.7 Selection of the Most Appropriate Model

Once all of the contending models have been fit, each one should be assessed for normality, homogeneity of the residuals, and the residual mean square error. The model which meets all of the assumptions and has the smallest residual mean square error (refer to Figure I.3) should be selected as the most appropriate model. However, in the case where more than one model has the same residual mean square error, and all other factors are equivalent, the simplest model should be selected as the model of best choice. If a weighted regression was performed, the weighted and unweighted analyses should be compared and the weighted analysis selected if weighting reduced the standard error for the ICp by more than 10%. The residual mean square error is presented in the '**Output Pane**' tab just following the iterations, and preceding the parameter estimates. However, if none of the models adequately fit the data, then the user is advised to consult a statistician for the application of additional models, or the data should be re-analyzed using the less desirable linear interpolation method of analysis (see Section 4.8.2.2 in the main text).

Note: Since the concentration or treatment levels were logarithms in the calculations, the ICps and the corresponding confidence limits should be transformed to arithmetic values for the purpose of reporting them.

I.2.8 Creating the Concentration-Response Curve

Once an appropriate model has been selected, the concentration-response curve for that particular model must be generated.

1) Within the command editor window at the bottom of the screen, copy the model equation (i.e., the equation after the '=' sign, third line of the command codes depicted in Table I.2) from the command codes used to derive the estimates for the selected model; the equation should consist of the original alphabetic characters (e.g., t, b, h, etc.). The equation can be copied by highlighting the equation and selecting **Edit**, followed by **Copy** (or right-clicking the mouse and selecting **Copy**).

2) Select **File**, **Open**, and then **Command** and open an existing graph command file (i.e., any file with '*.cmd') similar to the following example (or, if and as necessary, create a new one), using

the logistic model. The first plot (i.e., 'plot') is a scatter plot of the dependent variable against the log concentration series. The second plot (i.e., 'fplot') is the regression equation, which is superimposed upon the scatter plot.

```
graph
begin
plot juveniles*logconc/ title='Number of Juvenile O. folsomi', xlab='Log(ug chemical/kg
soil d.wt)', ylab='Mean number of juveniles O. folsomi',
xmax=2.0, xmin=0, ymax=250, ymin=0
fplot y=162.207/(1+(logconc/0.701)^3.067); xmin=0,
xmax=2.0, xlab='' ymin=0, ylab='', ymax=250
end
```

3) Paste the previously copied equation in place of the pre-existing equation (as seen in the shaded area above) by highlighting the previous equation, and then selecting **Edit**, followed by **Paste** (or right-clicking the mouse and selecting **Paste**). Replace all of the alphabetical characters (e.g., t, b, h, x, a, etc.), together with the respective estimates, provided in the '**Output Pane**' tab generated by the application of the selected model.

4) Type in the correct information within the line entitled 'plot y*logconc . . .', where 'y' is the dependent variable under study (e.g., juveniles). Adjust the 'xmax' (i.e., the maximum log-concentration used) and 'ymax' (refer to Section I.2.1, Step 7) numerical values accordingly. Ensure that all 'xlab' and 'ylab' (i.e., axis labels) entries are correct, if not, then adjust accordingly. Ensure that all quotation marks and commas are placed within the command program as depicted in the previous example; SYSTAT is case- and space- insensitive.

Note:		
	'title'	refers to the title of the graph
	'xlab'	refers to the x-axis label
	'xmin'	refers to the minimum value requested for the x-axis
	'xmax'	refers to the maximum value requested for the x-axis
	'ylab'	refers the y-axis label
	'ymax'	refers to the maximum value requested for the y-axis
	'ymin'	refers to the minimum value requested for the y-axis

The 'xmin', 'xmax', 'ymin', and 'ymax' must be the same for both plots to superimpose the regression line accurately on the scatter plot of the data. An example of the final regression graph is provided in Figure I.1 for each of the five proposed models.

5) Select **File**, then **Save As** to save the graph command codes in an appropriate working folder using the same coding used to generate the data file, with indication as to which model the regression corresponds to. Select **Save** to save the file.

6) Select **File**, then **Submit Window** to process the command codes. A graph of the regression, using the model estimate parameters for the selected model, will appear.

I.3 Determining Additional ICps

In some cases, it might be desirable to estimate another value for 'p' (besides or instead of an IC50). The models proposed by Stephenson *et al*. (2000b) enable the selection and determination of any ICp. The following section, as well as Figure I.1, provide guidance on determining an IC25, however, the models can be changed to suit any 'p' value (e.g., IC20).

1) Select **File**, **Open**, and then **Command** and open the file corresponding to the command codes used to generate the estimate parameters (refer to Table I.2 for the command codes for each model). Change the model equation such that it will calculate the desired ICp (e.g., IC25); Figure I.1 provides guidance on adjusting the models to calculate the IC25. However, any ICp can be determined by modifying the fractions used in each model. For example, to calculate an IC20 using the logistic model, the equation would change from 't/(1+(logconc/x)^b)' (for calculating an IC50) to 't/(1(0.20/0.80)*(logconc/x)^b)' (for calculating an IC20).

2) Once the equation has been adjusted for the ICp of interest, follow each step outlined in Section I.2.3 of this appendix. However, substitute the estimated ICp (e.g., IC25) within the fifth line entitled 'estimate/ start=' (refer to Figure I.1 for details on the substitution for each model). These values were initially derived from an examination of the scatter plot or line graph. The model, once it converges, will provide a set of parameters from which the ICp, and its corresponding 95% confidence limits, are reported (i.e., parameter 'x').

3) Proceed with the analysis as described in Sections I.2.4 to I.2.8 herein.

I.4 Analysis of Variance (ANOVA)

1) Select **File**, **Open**, and then **Data** to open the data file containing all of the observations for the data set under examination.

2) Select **Analysis**, **Analysis of Variance (ANOVA)**, and then **Estimate Model...**.

3) Select the variable within which the data are to be grouped (e.g., logconc), and place this variable into the '**Factor(s):**' box by selecting **Add**.

4) Select the variable of interest (e.g.,juveniles), followed by **Add**, to insert the variable into the '**Dependent(s):**' box.

5) Select the box beside '**Save**' (bottom left-hand corner of the 'Analysis of Variance: Estimate Model' window) and scroll down the accompanying selections to choose **Residuals/Data**. Type in an appropriate file name within the adjacent empty box to save the residuals (e.g., anova1). Select **OK**. A graph of the data and the generate output will appear within the '**Output Pane**' tab. At this point, any outlier(s), based on the studentized residuals, will also be identified (refer to Section I.2.6 of this appendix for guidance on assessing outlier(s)).

6) Assess the assumptions of normality and homogeneity of the residuals as per Section I.2.4 using the data file that was created to save the Residuals/Data prior to conducting the ANOVA (i.e., anova1). After assessing normality and homogeneity of the residuals using Shapiro-Wilk's and Levene's tests, respectively, the following coding may be used to examine the graphs of the residuals:

```
graph
use anova1
plot residual*logconc
plot residual*estimate
```