TEST SPECIFIC CHECKLIST [1]

Prepared: July 2014

Tests for Toxicity of Contaminated Soil to Earthworms

Parameter	Specification	Met Specifics Y	N	NA
Sample Handling				
Containers	Non-toxic material for transport and storage **(Must)**.....	...	...	...
	New or thoroughly cleaned, or lined with high-quality plastic **(Must)**.....	...	...	...
Labelling	Sample containers sealed and labelled or coded immediately after filling **(Must)**	...	...	...
	Labelling and accompanying records include 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 **(Must)**; name and signature of sampler(s) should also be included	...	...	...
Transport	Samples to be kept from overheating during transport or storage.....	...	...	...
	Samples must not freeze or partially freeze during transport or storage (unless they are frozen when collected) **(Must)**	...	...	...
	Samples must not be allowed to dehydrate during transport or storage (unless samples are saturated with excess water upon arrival at the lab)**(Must)**	...	...	...
	Samples should be kept in the dark (i.e., light-tight or opaque containers)	...	...	...
	Samples should remain cool (e.g., 7 ± 3°C) during transit	...	...	...
	Date sample(s) received at the laboratory recorded **(Must)**.....	...	...	...
	Sample temperature upon receipt at laboratory measured and recorded	...	...	...
Holding Time	Test should start within 2 weeks, and must start within 6 weeks unless soil contaminants are known to be stable **(Must)**.....	...	...	...
Holding Conditions	Samples to be stored for future use must be held in airtight containers **(Must)** ..	...	...	...
	Store samples 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 **(Must)**.....	...	...	...
Sample Preparation a) Field-Collected Test Soil				
Sieving.....	Sample sieved (e.g. 4 - 6 mm mesh) without water to remove oversize material, if necessary (e.g., debris and indigenous macro-organisms).....	...	...	...
Homogenization.....	Soil and/or solid particulate waste for testing should be homogenized, unless inappropriate (e.g., affects concentration or bioavailability of contaminants)	...	...	...
	Any moisture that separates from a sample during its transport and/or storage must be remixed into it if possible **(Must)**.....	...	...	...
T° Adjustment and Soil Equilibrium.....	Test soil prepared on day preceding test (Day -1)and held under test conditions (i.e., 20 ± 2°C) overnight, prior to testing.....	...	...	...
Characterization	Each soil (including negative control and reference soil) is analysed for particle size distribution (%sand, silt, and clay), total organic carbon content (%), organic matter content (%), moisture content (%), WHC (%), pH and conductivity, as a minimum **(Must)**.....	...	...	...
	Optional analyses of contaminants of concern (e.g., metals, polycyclic aromatic hydrocarbons (PAHs), pesticides)	...	...	...
Moisture Content	Water Holding Capacity (WHC) of soils (artificial and site) are known **(Must)**	...	...	...
	Optimal moisture content of test soils (artificial and site) determined and expressed as % WHC **(Must)**.....	...	...	...
	WHC determined gravimetrically by drying subsample for ~24h at 105°C	...	...	...
	Test soil hydrated to optimal % of WHC after preparing test conc.	...	...	...

[1] Checklist based on Environment Canada's "Test for Toxicity of Contaminated Soil to Earthworms (*Eisenia andrei, Eisenia fetida,* or *Lumbricus terrestris*)". See Endnote for references.

Parameter	Specification	Met Specifics Y	N	NA
Sample Preparation (continued)				
Test Concentrations ..	Each batch (i.e., treatment) is prepared in sufficient quantity for all replicates and physicochemical analyses	...	...	...
	Mix homogenized test soil with negative control soil or reference soil, if multi-concentration test to prepare each treatment/concentration using geometric series; ensure homogeneity (i.e., mix until texture, colour, and moisture are homogeneous) and divide into replicates	...	...	...
b) Chemical-Spiked Test Soil				
Chemical Characterization	Information on chemical or chemical product(s) obtained before test starts includes: stability, water solubility, vapour pressure, purity, estimated toxicity to test species and humans and biodegradability	...	...	...
	Concentration of test chemical in soil measured at beginning and end of test, in high, medium and low concentrations, as a minimum	...	...	...
Preparation of Mixtures	Procedure depends on nature of test substance(s), test design, and objectives; test substance(s) 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.....	...	...	...
	For each treatment, mixing conditions (solution:soil ratio, mixing and holding time and T°) must be standardized **(Must**)	...	...	...
	Each batch (i.e., treatment) is prepared in sufficient quantity for all replicates and physicochemical analyses	...	...	...
Solvent.....	Solvent control included in test (in addition to negative control) if organic solvent used for test substance(s) that are not soluble in water **(Must)**	...	...	...
	Solvent control from same batch used to make the stock solution of test substance and contains the same concentration of solubilizing agent that is present in the highest concentration of test chemical **(Must)**	...	...	...
Test Soil Equilibrium..	All test soil batches are hydrated, homogenized and placed in replicate test chambers on the day prior to testing (Day -1).....	...	...	...
	Replicate chambers covered (unperforated lids) and incubated overnight under test conditions (i.e., 20 ± 2°C), prior to testing.....	...	...	...
Test Conditions				
Test Facility	Isolated areas with temperature & lighting control (e.g., environ. chambers, or equivalent); well ventilated & free of fumes; isolated from areas for organism culturing/holding/acclimating, and for sample preparation/storage.....	...	...	...
	Equipment, apparatus and construction materials made of non toxic material (e.g., borosilicate glass, nylon, Teflon™, high-density polyethylene, high density polystyrene, polypropylene, polycarbonate, fluorocarbon plastics, type 316 stainless steel, fibre glass) **(Must)**.....	...	...	...
	Instruments for routine measurements (e.g., pH, temp.) are available **(Must)**...	...	...	...
	Laboratory equipped for analysis of soil moisture content.....	...	...	...
	Other equipment includes: drying oven (capable of 90°C & 105°C), a weighing balance (accurate to 0.1 mg), and a pH meter **(Must)**.....	...	...	...
	Safety apparatus used when preparing mixtures and test soils **(Must)**.....	...	...	...
	All test chambers, equipment, and supplies that might contact site soils, test soils, test (hydration) water, stock solutions, or test solutions, are clean and rinsed with test water before being used **(Must)**	...	...	...

Parameter	Specification	Met Specifics Y	N	NA
Test Conditions (continued)				
Initial Tests	≥5 control performance tests and ≥5 reference toxicity tests with candidate samples of artificial or natural negative control soil intended for routine use, should be undertaken by laboratory personnel to confirm acceptable performance of test species using procedures & conditions in test method	...	...	...
	Conditions and procedures for initial control performance test should follow those for the acute lethality test, the avoidance test, or the survival, reproduction & growth test, depending on the test to be used in the laboratory	...	...	...
	Conditions and procedures for initial reference toxicity tests should be identical to those described for routine reference toxicant tests	...	...	...
	Each test should be performed using a different lot (group) of test organisms of the same species, from the same source	...	...	...
	Data from initial control performance test shows that criteria for test validity can be met **(Must)**	...	...	...
	Data from initial reference toxicity tests should be compared by calculating and appraising the magnitude of the coefficient of variation (CV) of the derived LC50s	...	...	...
Negative Control Soil	Natural clean field-collected soil or artificial soil for which previous tests with the chosen test species demonstrated that the test validity criteria could be regularly met; recommend artificial soil for tests with chemicals or chemical products spiked in soil	...	...	...
	Negative control soil included as a treatment in every toxicity test **(Must)**	...	...	...
Clean Field-Collected Soil	Natural soil collected from a clean (uncontaminated) site; free of pesticide or fertilizer for ≥5 years	...	...	...
	Laboratory demonstrates experimental evidence that natural soil from a given source has met test validity criteria before being used as negative control soil in a definitive test **(Must)**	...	...	...
	Soil analysed for recommended physicochemical characteristics (see Section 3.2.1 in EPS 1/RM/43)	...	...	...
	Natural soil can be air-dried (10 - 20% moisture content), coarse-screened (4 - 6 mm), transferred to clean plastic pails, and stored in darkness at 4 ± 2°C	...	...	...
Artificial Soil	10% *Sphagnum* sp. peat, air dried and sieved (2-mm mesh); 20% kaolin clay (with particle size < 40μm); and 70% silica sand (grade 70); mixed dry	...	...	...
	Add reagent-grade calcium carbonate to dry mixture to adjust pH to 6.0 - 7.5	...	...	...
	Hydrate using test water to ~28% of WHC and adjust pH as necessary with more calcium carbonate	...	...	...
	Artificial soil stored in the dark at 20 ± 2°C for ≥3 days before use in toxicity test; thereafter soil can be stored at 4 ± 2°C	...	...	...
Positive Control Soil	Should be included in each series of soil toxicity tests; may be a negative control soil spiked with a reference toxicant or with one or more toxic chemicals of concern; or a highly contaminated sample of field-collected soil	...	...	...
Reference Soil	One or more samples for tests with field-collected soil, ideally 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 are similar to test soils	...	...	...
	Tests involving samples of reference soil must also include a sample of negative control soil **(Must)**	...	...	...
Initial Hydration of Test Soils	Field-collected soils are hydrated with test water to the optimal percentage of its WHC (i.e., soil is a homogenous, crumbly consistency; clumps 3 - 5 mm); artificial soils are hydrated to ~70% of WHC	...	...	...
Test Water	Deionized or distilled water or better, such as reagent-grade water produced by a system of reverse osmosis, carbon and ion exchange cartridges **(Must)**	...	...	...

Parameter	Specification	Met Specifics Y	N	NA
Test Conditions (continued)				
Glassware Cleaning ..	Soak; detergent wash; 2 tap water rinses; acid wash (e.g., 10% nitric or hydrochloric acid, metal-free grade) to remove scale, metals and bases; 2 rinses with test water; pesticide free acetone wash to remove organic compounds and HPLC-grade hexane wash for oily residues; allow organic solvent to volatilize and rewash with detergent if necessary; 3 rinses with test water	...	...	...
Measurements During Test				
Moisture Content	Soil moisture content in each treatment/concentration at test start and end **(Must)**	...	...	...
	Moisture content determined gravimetrically (see EPS 1/RM/43)	...	...	...
	Moisture content calculated on a dry wt. basis **(Must)**	...	...	...
pH	Soil pH in each treatment/concentration at test start and end **(Must)**	...	...	...
	Soil pH measured using a modified $CaCl_2$ Slurry Method (see EPS 1/RM/43) ...	...	...	...
Temperature	Temperature in test facility, daily or continuously **(Must)**	...	...	...
Conductivity	Conductivity measured at test start and end when test soil is suspected of having a high salt content	...	...	...
Chemical Analyses	Normally measure at beginning and end of test, in high, medium, and low strengths as a minimum.....	...	...	...
Reference Toxicity Tests	Static 7-day multi-concentration test **(Must)**	...	...	...
	Conditions the same as those for a multi-concentration, 14-day acute lethality except the test duration is 7-days **(Must)**	...	...	...
	Use a portion of the population of earthworms that are being cultured for use in definitive tests	...	...	...
	Perform once per month, or in conjunction with definitive test(s) with soil samples **(Must)**; use boric acid.....	...	...	...
	Prepare and test ≥5 concentrations plus a negative control **(Must)**, using artificial soil	...	...	...
	Calculate mean (± SD) % survival in each treatment on Day 7 **(Must)**	...	...	...
	Determine 7-day LC50 and 95% confidence limits **(Must)**; express as mg boric acid/kg dry wt.	...	...	...
	For survival, growth & reproduction test: recommend ≥8-week tests with boric acid be performed according to Section 4.3 of EPS 1/RM/43, at least twice a year or in conjunction with definitive test	...	...	...
Warning Chart	Prepared and updated with all comparable LC50s for each species and reference toxicant (i.e., all comparable LC50s plotted successively on a warning chart) **(Must)**	...	...	...
	Separate warning chart prepared and updated for each dissimilar procedure (e.g., species of test organisms, reference toxicant, test duration) **(Must)**.....	...	...	...

Parameter	Specification	Met Specifics Y	N	NA
Acute Lethality Test				
Test Type	Static; whole soil **(Must)**	...	...	...
Test Duration	14 days **(Must)**	...	...	...
Test T°	20 ± 2°C daily average **(Must)**; 20 ± 3°C instantaneous **(Must)**	...	...	...
Light Quality	Incandescent or fluorescent **(Must)**	...	...	...
Light Intensity	400 to 800 lux; ≥400 lux **(Must)**	...	...	...
Photoperiod	Fixed daily photoperiod **(Must)** (i.e., 16 h light: 8 h dark or 12 h light:12 h dark for lethality and prolonged exposure tests); same photoperiod as that to which worms are acclimated before the test	...	...	...
Chamber Size & Type	500-mL wide-mouthed glass jars **(Must)**; covered (perforated translucent or transparent lid, secured with rubber band, recommended)	...	...	...
	All test units are cleaned thoroughly and rinsed with test water immediately before use **(Must)**	...	...	...
Soil Mass	Identical wet weight of test soil equivalent to a volume of ~350 mL;~200 g dry weight if artificial soil	...	...	...
Chamber Labelling	Clearly labelled/coded: test substance, conc., and replicate # **(Must)**	...	...	...
	Date and time of test initiation on labels or data sheets (**Must)**	...	...	...
Chamber Position	Test chambers are positioned randomly within test facility	...	...	...
# Replicates/Conc.	≥5 replicates/treatment if single-concentration test **(Must)**	...	...	...
	≥3 replicates/treatment if multi-concentration test using *E. andrei/fetida* **(Must)**	...	...	...
	≥5 replicates/treatment if multi-concentration test using *L. terrestris* **(Must)**	...	...	...
# Test Conc.	1, plus controls for single-concentration test	...	...	...
	≥5, plus controls for multi-concentration test (LC50 calculation)**(Must)**; more recommended (6 - 10, plus controls); geometric series	...	...	...
# Worms/Chamber	5 per chamber if *E. andrei/fetida*; 3 per chamber if *L. terrestris* **(Must)**	...	...	...
Organism Selection	Worms transferred to test chambers on the day after soil equilibration (Day 0)	...	...	...
	Worms are handled by gloved hand or using the blunt arm(s) of rounded forceps	...	...	...
	Excess number of worms (similar in size and colour and active) than those required for testing are removed from culture/acclimation chamber and rinsed in clean test water	...	...	...
	For each replicate, worms are moved to a transfer container and transferred individually to the soil surface in each test chamber	...	...	...
	The group of worms transferred to each chamber are randomly allocated with respect to treatment	...	...	...
	≥10 worms, taken randomly as surplus from those selected for use in the test are weighed individually to determine size variability for the sample; individual wet weights and mean (± SD) wet weights are recorded **(Must)**	...	...	...
Feeding Regime	None **(Must)**	...	...	...
Test Soil Hydration	Soil moistened with de-ionized water on Day 7, as necessary	...	...	...
Biological Observations	Condition, appearance, and # of live worms placed in each test chamber on Day 0 **(Must)**	...	...	...
	# of live worms in each test chamber on Days 7 (optional) and 14 **(Must)**	...	...	...
	# worms seen on soil surface in each test chamber on Days 0 (i.e., at 1 hr), 7 (optional) and 14	...	...	...
	Appearance and behaviour of surviving worms on Days 7 (optional) and 14	...	...	...
	Dead worms are removed **(Must)**	...	...	...
	Missing worms are counted as dead **(Must)**	...	...	...
Test Validity Criteria	Test invalid if mean 14-day survival in negative control soil is <90% **(Must)**	...	...	...
Biological Endpoint	# live worms in each replicate on Day 7 (optional) and Day 14 **(Must)**	...	...	...
Statistical Endpoint	% survival (% mortality) in each test chamber and each treatment/concentration, on Days 7 (optional) and 14 **(Must)**	...	...	...
	Mean (± SD) % survival for all worms exposed to each treatment for Days 7 (optional) and 14, using survival data for all replicates **(Must)**	...	...	...
	7-d LC50 (optional) and 14-d LC50 if multi-concentration test **(Must)**	...	...	...

Parameter	Specification	Met Specifics Y	N	NA
Avoidance Test				
Test Type	Static; whole soil **(Must)**	...	...	...
Test Duration	48 hours (*E. andrei* or *E. fetida*)or 72 hours (*L. terrestris*) **(Must)**	...	...	...
Test T°	20 ± 2°C daily average **(Must)**; 20 ± 3°C instantaneous **(Must)**	...	...	...
Photoperiod	Continuous dark **(Must)**	...	...	...
Test Unit Size & Type	Circular container with central chamber and six pie-shaped interconnecting compartments with fitted lid; constructed of high-quality stainless steel or Plexiglass™; holes in bottom of central chamber and sides of compartments for worm movement (as described in Section 3.1.3 of EPS 1/RM/43)	...	...	...
	All test units are cleaned thoroughly and rinsed with test water immediately before use **(Must)**	...	...	...
Soil Mass per Test Compartment	Identical wet weight of test soil equivalent to a volume of ~350 mL;~200 g dry weight if artificial soil; none in central chamber	...	...	...
Test Unit Labelling	Test units and compartments are clearly labelled/coded: test substance, concentration, and replicate # **(Must)**	...	...	...
	Date and time of test initiation on labels or data sheets (**Must)**	...	...	...
Test Unit Position	Test units are positioned randomly within test facility	...	...	...
# Replicates/Conc.	≥5 test units per test for single concentration test; ≥1 test unit per test concentration for multi-concentration **(Must)**	...	...	...
# Test Conc.	1, plus controls for single-concentration test	...	...	...
	≥5, plus controls for multi-concentration test (EC50 calculation)**(Must)**; more recommended (6 - 10, plus controls); geometric series	...	...	...
	2 treatments per test unit (negative control soil or reference soil plus a single sample or concentration of a test soil) **(Must)**	...	...	...
	Alternate treatment in each neighbouring compartment **(Must)**	...	...	...
	3 compartments per test unit with the same treatment **(Must)**	...	...	...
# Worms/Unit	10 per test unit **(Must)**	...	...	...
Organism Selection	Worms transferred to test chambers on the day after soil equilibration (Day 0)	...	...	...
	Worms are handled by gloved hand or using the blunt arm(s) of rounded forceps	...	...	...
	Excess number of worms (similar in size and colour and active) than those required for testing are removed from culture/acclimation chamber and rinsed in clean test water	...	...	...
	For each replicate, worms are moved to a transfer container and then transferred individually to the central chamber of the test unit; a second worm is not added until the first worm has moved from the central chamber into a compartment containing soil; this procedure is repeated for all 10 worms	...	...	...
	The group of worms transferred to each chamber are randomly allocated with respect to treatment	...	...	...
	Once placed in test facility avoid any further movement or disturbance of test units until after the side partitions have been inserted at test end	...	...	...
	≥10 worms, taken randomly as surplus from those selected for use in the test are weighed individually to determine size variability for the sample; individual wet weights and mean (± SD) wet weights are recorded **(Must)**	...	...	...
Feeding Regime	None **(Must)**	...	...	...
Test Soil Hydration	None	...	...	...
Biological Observations	Compartment (treatment) entered by each worm at start of test	...	...	...
	# live/dead worms in each compartment at test end following insertion of side partitions (i.e., confining test organisms to each compartment)**(Must)**	...	...	...
	# live/dead worms on soil surface of each compartment at test end **(Must)**	...	...	...
	Appearance and behaviour of surviving worms in each compartment at test end	...	...	...
	Missing worms are counted as dead **(Must)**	...	...	...

Parameter	Specification	Met Specifics Y	N	NA
Avoidance Test (continued)				
Test Validity Criteria ..	Test invalid if % survival of worms in any test unit is <90% at test end **(Must)** ...	...	...	...
Biological Endpoint	# live worms per treatment/concentration in each test unit (i.e., total # of live worms in the three compartments containing the same test soil, for each of the two treatments) at test end **(Must)**.....	...	...	...
Statistical Endpoint	% of live worms/treatment in each unit at test end	...	...	...
	% avoidance, calculated based on # worms in each treatment **(Must)**	...	...	...
	For a single-concentration test: mean (± SD) # live worms recovered from the test soil and the clean soil in each of the five (or more) replicate test units **(Must)**	...	...	...
	For a multi-concentration test: EC50 (or other ECp) causing avoidance, based on percent avoidance determined for each test concentration	..	...	...

Parameter	Specification	Met Specifics Y	N	NA
Survival, Reproduction & Growth Test				
Test Type	Static; whole soil **(Must)**	...	...	...
Test Duration	≥56 days = ≥8 weeks for prolonged exposure test for survival, reproduction and growth **(Must)**; may be extended to 63 days	...	...	...
Test T°	20 ± 2°C daily average **(Must)**; 20 ± 3°C instantaneous **(Must)**	...	...	...
Light Quality	Incandescent or fluorescent **(Must)**	...	...	...
Light Intensity	400 to 800 lux; ≥400 lux **(Must)**	...	...	...
Photoperiod	Fixed daily photoperiod **(Must)** (i.e., 16 h light: 8 h dark or 12 h light:12 h dark for lethality and prolonged exposure tests); same photoperiod as that to which worms are acclimated before the test	...	...	...
Chamber Size & Type	500-mL wide-mouthed glass jars **(Must)**; covered (perforated translucent or transparent lid, secured with rubber band, recommended)	...	...	...
	All test units are cleaned thoroughly and rinsed with test water immediately before use **(Must)**	...	...	...
Soil Mass	Identical wet weight of test soil equivalent to a volume of ~350 mL;~200 g dry weight if artificial soil	...	...	...
Chamber Labelling	Clearly labelled/coded: test substance, concentration, and replicate **# (Must)**	...	...	...
	Date and time of test initiation on labels or data sheets (**Must)**	...	...	...
Chamber Position	Test containers are positioned randomly within test facility	...	...	...
# Replicates/Conc.	10 replicates/treatment **(Must)**	...	...	...
	≥1 additional replicate for each of negative control soil and reference soil and/or the lowest concentration of test soil is recommended to assess whether acceptable production of young in these treatments has occurred on Day 28	...	...	...
# Test Conc.	1, plus controls for single-concentration test	...	...	...
	≥7, plus controls for multi-concentration test **(Must)**; more recommended (≥10, plus controls); geometric series	...	...	...
# Worms/Chamber	2 per test chamber **(Must)**	...	...	...
Organism Selection	Worms transferred to test chambers on the day after the soil equilibration period (Day 0)	...	...	...
	Worms are handled by gloved hand or using the blunt arm(s) of rounded forceps	...	...	...
	Excess number of worms (similar in size and colour and active) than those required for testing are removed from culture/acclimation chamber and rinsed in clean test water	...	...	...
	For each replicate, worms are moved to a transfer container and transferred individually to the soil surface in each test chamber	...	...	...
	The order of adding earthworms to each chamber are randomly allocated with respect to treatment	...	...	...
	≥20 worms are weighed individually to determine size variability for the sample; weights may be from worms used in the test or from the surplus worms that are chosen for use in the test; individual wet weights and mean (± SD) wet weights are recorded **(Must)**	...	...	...
Feeding Regime	Identical quantity of cooked oatmeal (i.e., 5 mL per test chamber) on Days 0, 14, 28, and 42 only **(Must)**	...	...	...
Test Soil Hydration	Soil moistened with de-ionized water on feeding occasions, as necessary	...	...	...
Test Validity Criteria	Test invalid if mean 28- or 35-day survival of adults in negative control soil is <90% **(Must)**	...	...	...
	Test invalid if mean reproduction rate for adults in negative control soil <3 live juveniles/adult **(Must)**	...	...	...
	Test invalid if mean dry wt. of individual live juveniles in negative control soil at test end is <2.0 mg **(Must)**	...	...	...

Parameter	Specification	Met Specifics Y	N	NA
Survival, Reproduction & Growth Test (continued)				
Biological Observations	Condition, appearance, and # live adult worms placed in each test chamber on Day 0 **(Must)**.....	...	...	...
	# live adult worms in each test chamber on Day 28 or 35 **(Must)**	...	...	...
	# of live/dead adult worms on soil surface at test start (i.e., at 1 hr) and on each feeding occasion	...	...	...
	Appearance and behaviour of worms in each test chamber on Days 28 or 35, and at test end	...	...	...
	Presence of uneaten food on each feeding occasion.....	...	...	...
	# progeny produced in "extra" replicates on Day 28 (i.e., to determine if progeny production is acceptable; if no Cocoons or juveniles are observed the definitive test chambers are left undisturbed for an additional 7-days before removal of adults)	...	...	...
	Missing adults are counted as dead **(Must)**.....	...	...	...
	All adults are discarded on Day 28 (or Day 35) and test soil is returned to jar with any cocoons and juvenile worms until test end (i.e., Day 56 or, in the case of an extra 7-days added at Day 28, Day 63) **(Must)**	...	...	...
Biological Endpoint....	# live juvenile worms in each test chamber at test end **(Must)**.....	...	...	...
	# hatched or unhatched cocoons at test end	...	...	...
	Surviving juveniles in each test chamber are rinsed, dried (at 90°C) until constant weight, and weighed **(Must)**.....	...	...	...
	# live adult worms in each replicate on Day 28 (or Day 35 if test extended); total dry wt. and # live juvenile worms in each replicate on Day 56 (or Day 63, if test extended) **(Must)**	...	...	...
Statistical Endpoint....	Mean (± SD) % survival of adults in each treatment on Day 28 or 35 **(Must)**	...	...	...
	Mean (± SD) # live juveniles in each treatment on Day 56 or 63 **(Must)**.....	...	...	...
	Mean (± SD) dry wt of live juveniles in each treatment on Day 56 or 63 **(Must)** .	...	...	...
	For multi-concentration test: 28- or 35-day LC50 for adult worms, 56- or 63-day ICp for reproductive inhibition based on numbers of live juveniles produced in each concentration during 56- or 63-day test, and 56- or 63-day ICp for growth inhibition based on mean dry wt. of individual worms surviving in each treatment/concentration at test end **(Must)**.....	...	...	...
Calculation of ICp	Linear and/or nonlinear regression procedures used for calculation of ICps and 95% confidence limits **(Must)**	...	...	...
	ICPIN analyses used only if regression analyses fail to provide meaningful ICps.....	...	...	...

Parameter	Specification	Met Specifics Y	N	NA
Test Organisms				
Species	*Eisenia andrei, Eisenia fetida,* or *Lumbricus terrestris* for acute lethality and acute avoidance tests **(Must)**	...	...	...
	Only laboratory cultured *E. andrei* for survival, reproduction, and growth test **(Must)**	...	...	...
	Species identification confirmed and documented by qualified personnel **(Must)**	...	...	...
	Cultures of *Eisenia* sp. held in a testing laboratory should be identified to species every 2 years, as a minimum	...	...	...
Source	Government or private laboratories, or commercial suppliers culturing *E. andrei/fetida* or holding *L. terrestris*; natural populations of *L. terrestris* may be collected from clean sites	...	...	...
	E. andrei/fetida for use in acute lethality and avoidance tests may be cultured in the testing laboratory or obtained from outside cultures (i.e., purchased for use in tests)	...	...	...
	E. andrei for use in survival, reproduction and growth test must be cultured in the testing laboratory **(Must)**	...	...	...
	All organisms used in a test are derived from the same population **(Must)**	...	...	...
Age	Acute lethality test: sub-adult or sexually mature adults with clitellum; individual wet weights at test start: 250 - 600 mg if *E. andrei*/*fetida* or 3 - 10 g if *L. terrestris* **(Must)**	...	...	...
	Acute avoidance test: clitellated adults; individual wet weights at test start: 250 - 600 mg if *E. andrei*, 250 - 800 mg if *E. fetida*, or 3 - 10 g if *L. terrestris* **(Must)**	...	...	...
	Survival, reproduction and growth test: clitellated adults; individual wet weights at test start: 250 - 600 mg **(Must)**	...	...	...
Culture Conditions for *E. andrei/fetida*				
Source of Brood Stock for Culture	Cocoons, juveniles, or adults from a government, private, or commercial culture	...	...	...
Facilities	Controlled-temperature laboratory facility **(Must)**	...	...	...
	Culture area isolated from testing, sample storage, or sample-preparation areas; designed and constructed to prevent culture contamination **(Must)**	...	...	...
Apparatus	All containers and accessories that might contact organisms, test water or substrate is clean, rinsed and made of non-toxic material (e.g., glass, Teflon™, type 316 stainless steel, nylon, Nalgen™, porcelain, polyethylene, polypropylene, fibreglass)**(Must)**	...	...	...
	Breeding boxes of 10 - 50-L capacity with transparent or translucent sides and/or lid are recommended; minimum substrate depth of 10 cm; perforated lids; wood is not recommended	...	...	...
	Copper, zinc, brass, galvanized metal, lead, and natural rubber must not be used **(Must)**	...	...	...
Bedding	Optional; e.g., mixture of potting soil, artificial soil, and peat moss; or mixture of shredded un-inked paper, artificial soil, and peat moss	...	...	...
Hydration	Hydrate with distilled or de-ionized water; maintain moisture such that surface of bedding is moist but there is no standing water in culture chambers (i.e., soil particles should not adhere to worms)	...	...	...
Temperature of Substrate	Daily average, 20 ± 2°C; instantaneous, 20 ± 3°C	...	...	...

Parameter	Specification	Met Specifics Y	N	NA
Culture Conditions for *E. andrei/fetida* (continued)				
pH	Adjusted to 6.0 - 7.5 using reagent-grade calcium carbonate	...	...	...
Lighting	Incandescent or fluorescent; 400 to 800 lux at substrate surface; fixed daily photoperiod (e.g., 16 h light: 8 h dark or 12 h light:12 h dark); avoid overheating cultures	...	...	...
Acclimation	Gradually (recommend ≤3°C/day) for temperature differences upon arrival	...	...	...
	For acute lethality test: worms are to be acclimated to test temperature and lighting conditions for ≥7 days	...	...	...
	For avoidance test: worms are to be acclimated to test temperature conditions only for ≥7 days (i.e., worms are not acclimated to constant dark test conditions)	...	...	...
	For survival, reproduction and growth test: laboratory-cultured *E. Andrei* are acclimated in the lab to test conditions (i.e., negative control soil, light, temperature, and food) for ≥7 days if culturing conditions differ from those to be used in the test	...	...	...
Culture Maintenance	Examine substrate in culture chambers at least once/week; rehydrate and/or gently turn manually (i.e., if excess water at bottom of substrate), as necessary; remove injured or atypical worms (if many dead or stressed worms found, tray should be discarded); record condition of culture; maintain loading density of worms at ≤0.03 g/cm^3	...	...	...
	Remove dead worms from cultures **(Must**)	...	...	...
Substrate Renewal	As required, and at least once every 2 - 3 months, regardless of loading densities	...	...	...
	Sort and transfer worms and cocoons manually; alternatively, prepare new tray of bedding, cover with contents of old tray, leave undisturbed under constant light for two days, then removed and discard old bedding	...	...	...
Substrate Monitoring	Temp., pH, and moisture content measured once per week, each chamber **)**; adjust as necessary	...	...	...
Feeding	Either cooked oatmeal, or alfalfa pellets saturated with water; once/week place food in a shallow depression of the substrate and then cover it with a thin layer of substrate, after removing excess (unused) food and scraping off any visible mould or mites nearby; supplement weekly with small quantities of composted vegetable matter.	...	...	...
Indices of Culture Health	Considered healthy if: (1) worms move actively through the substrate, do not try to leave it, and reproduce continuously, and (2) results for reference toxicity tests using worms from the culture fall within historic warning limits; discard culture if >20% of juvenile or adult worms are dead, inactive, or unhealthy at any time **(Must**)	...	...	...
Selecting Worms	Selected randomly; excess number than required removed from culture and rinsed; then appropriate number for a replicate are placed into a transfer containers and moved individually to a replicate; worms are randomly allocated among treatments	...	...	...
Handling	Handling minimized; worms are transferred individually using gloved hand or the blunt arm of rounded forceps	...	...	...
	Worms that are dropped, injured, or appear stressed should be discarded and must not be used in a test **(Must)**	...	...	...

Parameter	Specification	Met Specifics Y	N	NA
Holding/Acclimating Conditions for *E. andrei/fetida* or *L. terrestris*				
Source of Worms	Government or private laboratory or commercial supplier, or collected from grassland known to have not been treated with pesticides or fertilizer for ≥5 years	...	...	...
Life Stage and Size on Receipt	Depending on timing of toxicity test, may be obtained as juveniles or as sexually mature worms with clitellum; individual wet weight within the indicated range	...	...	...
Facility	Controlled-temperature laboratory facility **(Must)**	...	...	...
	Culture area isolated from testing, sample storage, or sample-preparation areas; designed and constructed to prevent culture contamination **(Must)**	...	...	...
Apparatus	All containers and accessories that might contact organisms, test water or substrate is clean, rinsed and made of non-toxic material (e.g., glass, Teflon™, type 316 stainless steel, nylon, Nalgen™, porcelain, polyethylene, polypropylene, fibreglass)**(Must)**	...	...	...
	Breeding boxes of 10 - 50-L capacity with transparent or translucent sides and/or lid are recommended; minimum substrate depth of 10 cm; perforated lids; wood not recommended	...	...	...
	Copper, zinc, brass, galvanized metal, lead, and natural rubber must not be used **(Must)**	...	...	...
Bedding	Options include: negative control soil (natural or artificial); mixture of potting soil, artificial soil, and peat moss; or mixture of shredded un-inked paper, artificial soil, and peat moss	...	...	...
Hydration	Hydrate with distilled or de-ionized water; maintain moisture such that surface of bedding moist but no standing water in trays (i.e., soil particles should not adhere to worms)	...	...	...
Temperature of Substrate	Adjust gradually (e.g., ≤3 °C/day) for temperature differences upon arrival; thereafter, maintain *Eisenia* sp. at a daily avg. temp. of 20 ± 2°C and instantaneous temp. of 20 ± 3°C	...	...	...
	Adjust field-collected *L. terrestris* to a daily average temperature of 20 ± 2°C for ≥7 days before testing; alternatively, adjust *L. terrestris* to a cooler temperature (e.g., ≤15 ± 2 °C) and hold for several weeks or months followed by adjustment to the test temperature over a minimum 6-h period immediately preceding the test	...	...	...
pH	≥6.0; no adjustment if natural (field-collected) negative control soil; adjusted to range within 6.0 - 7.5 using reagent-grade calcium carbonate if artificial substrate	...	...	...
Lighting	Incandescent or fluorescent; 400 to 800 lux at substrate surface; fixed daily photoperiod (e.g., 16 h light: 8 h dark or 12 h light:12 h dark); acclimate to these conditions for a minimum seven-day period immediately preceding the test	...	...	...
Duration of Acclimation	For survival, reproduction, and growth test: ≥7 days during the period immediately preceding the test, to laboratory conditions **(Must)**; earthworms obtained from a commercial supplier should be acclimated to laboratory conditions for a minimum period of 14 days immediately preceding the test	...	...	...
	For acute lethality test: worms are to be acclimated to test temperature and lighting conditions for ≥7 days	...	...	...
	For avoidance test: worms are to be acclimated to test temperature conditions only for ≥7 days (i.e., worms are not acclimated to constant dark test conditions)	...	...	...

Parameter	Specification	Met Specifics Y	N	NA
Holding/Acclimating Conditions for *E. andrei/fetida* or *L. terrestris* (continued)				
Weekly Maintenance .	Examine substrate in chambers at least once/week; rehydrate and/or gently turn substrate manually as necessary; remove injured or atypical worms (if many dead or stressed worms found, tray should be discarded); record condition of substrate and worms; maintain loading density of worms at ≤0.03 g/cm^3	...	...	...
	Remove dead worms from cultures **(Must)**	...	...	...
Substrate Renewal	As required, and at least once every 2 - 3 months, if worms held for an extended period before use in soil toxicity test	...	...	...
	Sort and transfer worms and cocoons manually; alternatively, prepare new tray of bedding, cover with contents of old tray, leave undisturbed under constant light for two days, then removed and discard old bedding	...	...	...
Substrate Monitoring .	Temp. and moisture content measured ≥once per week, each holding/acclimation chamber	...	...	...
Feeding.....	Either cooked oatmeal, or alfalfa pellets saturated with water; feed only cooked oatmeal for ≥7-day period immediately preceding test if acclimating *E. Andrei* for use in eight-week test;	...	...	...
	Feed worms once/week following guidance for culturing if held for more than one week.....	...	...	...
Indices of Culture Health	Considered healthy if: (1) worms appear to be active when observed, and do not try to leave it, and (2) results for reference toxicity tests using worms from the holding/acclimation chamber(s) fall within historic warning limits; discard group if >20% of juvenile or adult worms are dead, inactive, or unhealthy at any time **(Must)**.....	...	...	...
Selecting Worms	Selected randomly; excess number than required removed from culture and rinsed; then appropriate number for a replicate are placed into a transfer containers and moved individually to a replicate; worms are randomly allocated among treatments	...	...	...
Handling	Handling minimized; worms are transferred individually using gloved hand or the blunt arm of rounded forceps	...	...	...
	Worms that are dropped, should be discarded and must not be used in a test **(Must)**	...	...	...

Parameter	Specification	Met Specifics Y	N	NA
Test Report				
Test Substance.....	Sample type or coding as provided to laboratory personnel **(Must)**	...	...	...
	Information on labelling or coding of each sample **(Must)**	...	...	...
	Date of sample collection **(Must)**	...	...	...
	Date and time sample(s) received at test facility **(Must)**	...	...	...
Test Organisms	Species and source of brood stock and test organisms **(Must)**	...	...	...
	Wet weight (mean ± SD), at start of test **(Must)**	...	...	...
	Any unusual appearance, behaviour, or treatment of the organisms before the test **(Must)**	...	...	...
Test Facilities.....	Name and address of test laboratory **(Must)**	...	...	...
	Name of person(s) performing the test (or each component of the test)**(Must)**	...	...	...
Test Method.....	Citation of biological test method used (i.e., as per EPS 1/RM/43) **(Must)**	...	...	...
	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 **(Must)**	...	...	...
	Brief description of frequency and type of all measurements and all observations made during test **(Must)**	...	...	...
	Name and citation of program(s) and methods used for calculating statistical endpoints **(Must)**	...	...	...
Test Conditions.....	Design and description of any deviation(s) from, or exclusion of, any of the procedure and conditions specified in EPS 1/RM/43 **(Must)**	...	...	...
	Number of discrete samples per treatment **(Must)**	...	...	...
	Number of replicate test chambers for each treatment **(Must)**	...	...	...
	Number and description of treatments in each test including the control(s); test concentrations (if applicable) **(Must)**	...	...	...
	Volume of soil in each test chamber **(Must)**	...	...	...
	Number of organisms per test chamber and treatment **(Must)**	...	...	...
	Dates when test was started and ended **(Must)**	...	...	...
	Feeding regime and ration, for 56-day test **(Must)**)	...	...	...
	Date when adults were removed from test chambers, for 56-day (or longer) test **(Must)**	...	...	...
	For each soil sample: any measurements of soil particle size, moisture content, water holding capacity, and pH **(Must)**	...	...	...
	For each composite sample of subsamples taken at the same time from all replicates of each treatment: all measurements of temperature, pH, moisture content, and water holding capacity **(Must)**	...	...	...
Test Results.....	For an acute lethality test: mean (± SD) percent survival in each treatment on Days 0, 7 (if determined), and 14 **(Must)**	...	...	...
	For an acute avoidance test: mean (± SD) number of surviving worms in replicates of each treatment representing clean soil and test soil, at 48 h if *E. andrei/fetida* or at 72 h if *L. terrestris* **(Must)**	...	...	...
	For survival, reproduction, and growth test:			
	- Mean (± SD) percent survival of adult worms in each treatment on Day 28 or 35 **(Must)**	...	...	...
	- Mean (± SD) number of surviving juveniles in each treatment on Day 56 or 63 **(Must)**	...	...	...
	- Mean (± SD) dry weight of individual juveniles surviving in each treatment on Day 56 or 63 **(Must)**	...	...	...
	- Mean (± SD) number of surviving juveniles produced by each adult worm in negative control soil (and in positive control soil and/or solvent control soil, if used), on Day 56 or 63 **(Must)**	...	...	...
	Any LC50 or EC50 (including the associated 95% confidence limits and, if calculated, the slope) determined **(Must)**	...	...	...
	Any additional LCx or ECx (e.g., LC20 or EC20) calculated **(Must)**	...	...	...

Parameter	Specification	Met Specifics Y	N	NA
Test Report (continued)				
Test Results (continued).....	Any ICp (with its 95% confidence limits) determined for the data on reproductive success (i.e., number os surviving juvenile worms in each treatment at test end) **(Must)**	...	...	...
	Any ICp (with its 95% confidence limits) determined for the data on growth (i.e., dry weight of individual juveniles surviving at test end) **(Must)**	...	...	...
	Details regarding any transformation of data, and indication of quantitative statistical method used or procedures applied to the data **(Must)**.....	...	...	...
	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)**(Must)**	...	...	...
	All values for measured concentrations **(Must)**	...	...	...
	Results for any seven-day LC50 (including its 95% confidence limits) performed with the reference toxicant in conjunction with the definitive soil toxicity test, using the same lot (group) of test organisms**(Must)**	...	...	...
	Geometric mean value (± 2 SD) for the same reference toxicant and test species, as derived at the test facility in previous seven-day LC50 tests using the procedures and conditions for reference toxicity tests described in EPS 1/RM/43 **(Must)**.....	...	...	...
	Anything unusual about the test, any problems encountered, and any remedial measures taken **(Must)**.....	...	...	...
Original Data Sheets .	Original data sheets must be signed or initialled, and dated by the laboratory personnel conducting the tests **(Must)**	...	...	...
Info. Kept on-File	Do lab SOPs indicate that the information on Section 7.2 of the EPS 1/RM/43 method must be kept on file for ≥5 years? **(Must)**	...	...	...
	For details of this information, see Section 7.2 of EPS 1/RM/43.			

Environment Canada, "Biological Test Method: Tests for Toxicity of Contaminated Soil to Earthworms (*Eisenia andrei, Eisenia fetida,* or *Lumbricus terrestris*)", Method Development and Applications Section, Environment Canada, Ottawa, ON, Report EPS 1/RM/43, 156 p. (2004).