

This checklist is a summary of the requirements and recommendations in the Environment and Climate Change Canada test method. As a summary, it will not contain all supplementary information. If there is a discrepancy between the checklist and the Environment and Climate Change Canada test method, the test method is taken as the definitive source. **Green shaded text** reflects changes in the 2nd edition (published in 2022).

Y= Yes, meets requirements; N= No, does not meet requirements; NA= not applicable.

DO = dissolved oxygen; temp = temperature; conc = concentration(s); SD = standard deviation; TOC = total organic carbon content; PAH = polycyclic aromatic hydrocarbons; WHC = water-holding capacity; CEC = cation exchange capacity; OM = organic matter content

TEST SPECIFIC CHECKLIST							
Tests for Measuring Avoidance Behaviour or Reproduction of Earthworms (<i>Eisenia andrei</i> or <i>Dendrodrilus rubidus</i>) Exposed to Contaminants in Soil							
Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Sample Collection and Handling							
Sample Collection	Soil collection procedures follow the guidance provided in EPS 1/RM/53 (EC, 2012), including procedures for soils contaminated with volatile or unstable compounds						
	Reference soil from sites with similar geochemical properties (especially: particle size distribution, total organic carbon content (%), organic matter content (%), pH, and electrical conductivity, but also: CEC, total inorganic carbon, redox potential, and water-holding capacity) to the test soil collected during each field collection						
	Collected soils classified to the subgroup level according to the Canadian System of Soil Classification						
	Soils from boreal or taiga ecozones or any soils exhibiting distinct horizons collected as separate soil horizons where possible (must)						
	If collecting by horizon, soil profiled first as described in EPS 1/RM/53 (must)						
	If collecting by horizon, care taken not to dilute the potential soil contamination						
	If collecting by horizon, each horizon stored in separate containers (must)						
	Soils without distinct horizons are collected by depth						
	Required volume of soil per sample calculated before commencing a sampling program						
	Guidance provided in EPS 1/RM/53 regarding compositing subsamples is followed						
Containers	Non-toxic, inert material for transport and storage (must)						
	Clean and sealable (must); plastic not used if there is a possibility of leaching						
Labelling	Sample containers sealed and labelled or coded immediately after filling for field-collected soils and/or upon receipt in the lab for chemicals (must); air space is minimized						

TEST SPECIFIC CHECKLIST

Tests for Measuring Avoidance Behaviour or Reproduction of Earthworms (*Eisenia andrei* or *Dendrodrilus rubidus*) Exposed to Contaminants in Soil

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Labelling cont.	Labelling and accompanying records include a code or description that identifies sample type (e.g., point, bulk, composite), sample date and time, sample site, precise location of sampling, sample condition, sample identification number (including replicate number, where applicable), and sample volume (must); name and signature of sampler(s)						
Transport	Samples do not freeze or partially freeze (unless they are frozen when collected) or become overheated						
	Samples are not allowed to dehydrate (unless they are saturated with excess water upon arrival at the lab) during transport or storage (must)						
	Samples are kept in the dark (i.e., light-tight or opaque containers)						
	Samples remain cool (e.g., 7 ± 3 °C) during transit						
	Date, temperature and moisture content measurements are recorded upon receipt of sample(s) at laboratory (must)						
Holding Time	Test is initiated within 6 weeks after sampling (must) unless soil contaminants are known to be stable; recommend testing within 2 weeks and preferably 1 week after sampling						
Holding Conditions	Samples stored for future use are held under conditions that maintain the characteristics and quality of the soil for its intended use (must)						
	Samples stored in the dark at 4 ± 2 °C if they contain PAHs, unstable volatiles, or other light-sensitive toxicants (must)						
	Sample brought to room temperature and thoroughly re-mixed just before analysis or testing (must)						
Sample Preparation: Field-Collected Soil							
Sieving	Water not used during sieving (must)						
	Debris and indigenous organisms removed by hand, by passing through coarse sieve (mesh size 4–10 mm), or by using freeze-thaw; grinding is avoided						
Homogenization	Soil and/or solid particulate waste for testing are homogenized, unless inappropriate (e.g., affects concentration or bioavailability of contaminants)						
	Any moisture that separates from a sample during its transport and/or storage is remixed into it if possible (must)						
	For each sample or soil horizon, mixing conditions (e.g., duration and temperature) are as similar as possible (must)						
	Mixed manually or mechanically until texture and colour are homogeneous						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Temp & pH Adjustment and Soil Equilibrium	Test soil prepared on day preceding test (Day -1) and placed in covered replicate test vessels (unperforated lids) or covered test units, and held under test conditions (i.e., 20 ± 2 °C) overnight, prior to testing						
	Samples of field-collected soil are not adjusted or manipulated (e.g., washing, aging/weathering, pH adjustment, conditioning, etc.) (must)						
	When soils are manipulated or adjusted, side-by-side tests with adjusted and non-adjusted samples are used						
	Documentation of soil manipulation procedures are reported (must)						
Characterization	Each soil/horizon (including negative control and reference soil) is analyzed for: moisture content (%), WHC (%), pH, electrical conductivity, TOC (%), OM (%), particle sizes (% sand, % silt, % clay), and CEC (must); analyses for forms of nitrogen, phosphorus, potassium, C:N ratio, and major cations and anions recommended						
	Optional analyses of contaminants of concern (e.g., metals, PAHs, pesticides)						
Moisture Content	WHC of soils (artificial and site) are known and determined using a recognized standard procedure for each sample or horizon (must)						
	Optimal moisture content of test soils (artificial and site) or each horizon determined and expressed as % WHC (must)						
	High peat content soils: optimal moisture content can be estimated by eye (appropriate consistency) instead of as % WHC						
	WHC is determined gravimetrically by drying subsample for ~24 h at 105 °C, saturating the subsample with water, and using wet weight and dry weight of soil following formula in Section 5.3						
	Test soil hydrated to optimal % of WHC (i.e., soil is a homogenous, crumbly consistency; clumps 3–5 mm) after preparing test conc.						
Test Concentrations	Each batch (i.e., treatment) is prepared in sufficient quantity for all replicates and physicochemical analyses						
	If multi-concentration test, mix homogenized test soil with negative control soil or reference soil to prepare each treatment/concentration using geometric series; ensure homogeneity						
	For test and/or reference soils collected as separate horizons, each horizon is treated as a separate sample and is prepared and tested separately (must); each horizon of test soil mixed with same horizon of negative control or reference soil						

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Tests for Measuring Avoidance Behaviour or Reproduction of Earthworms (<i>Eisenia andrei</i> or <i>Dendrodrilus rubidus</i>) Exposed to Contaminants in Soil								
Parameter	Specification	Document Review			Implementation			
		Y	N	NA	Y	N	NA	
Sample Preparation: Chemical-Spiked Soil								
Chemical Characterization	Information on chemical or chemical product(s) obtained before test starts includes: stability, water solubility, vapour pressure, purity, dissociation constants, adsorption coefficients, estimated toxicity to test species and humans, and biodegradability							
	Chemicals are reagent-grade							
	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 temp) are standardized (must)							
	Each batch (i.e., treatment) is prepared in sufficient quantity for all replicates and physicochemical analyses							
	Soils are hydrated (if artificial soil, to ~ 70% WHC), homogenized, and placed in covered replicate test vessels/units on the day prior to testing (Day -1)							
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, contains the same concentration of solubilizing agent that is present in the highest concentration of test chemical, and is prepared using the same procedure (must)							
	Results from both solvent control and negative control soils are examined independently to determine if they meet test validity criteria (must); if either fail, the test is considered invalid (must)							
	If both solvent and negative control soils meet validity criteria, results for the test soil are compared statistically with those for solvent control soil; investigate potential solvent interference if results from solvent and negative control soils differ significantly							
Test Conditions								
Test Facility	Facility with temperature & lighting control (e.g., environ. chambers, or equivalent) (must)							
	Isolated from areas for organism culturing and holding/acclimation and sample preparation/storage; well-ventilated & free of fumes							

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Test Facility cont.	Equipment, apparatus and construction materials made of non-toxic material (must) and minimize chemical sorption and leaching (e.g., borosilicate glass, nylon, high-density polyethylene, high-density polystyrene, polycarbonate, fluorocarbon plastics, Teflon™, Nalgene™, porcelain, fibreglass, type 316 stainless steel)						
	Copper, zinc, brass, galvanized metal, lead, and natural rubber are not used (must)						
	Instruments for measuring pH, temp, weights (accurate to 0.1 mg), and a drying oven (capable of 105 °C) are available (must)						
	Safety apparatus used when preparing mixtures and test soils (must)						
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)						
Equipment Cleaning	All test vessels/units, equipment, and supplies that might contact site soils, test soils, control soils, test (hydration) water, stock solutions, or test solutions, are clean and rinsed with test water before being used (must)						
	Soak; detergent wash; 2 tap water rinses; acid wash (e.g., 10% v/v nitric or hydrochloric acid, metal-free grade); 2 rinses with test water; pesticide-grade acetone wash to remove organic compounds, and HPLC-grade hexane wash for oily residues; allow organic solvent to volatilize, and rewash with detergent; 3 rinses with test water						
Initial Tests	≥5 control performance tests and ≥5 reference toxicity tests using artificial or natural negative control soil intended for routine use are performed to confirm acceptable performance						
	Conditions and procedures for initial control performance tests follow those described for conducting definitive tests						
	Conditions and procedures for initial reference toxicity tests be identical to those described for routine reference toxicity tests						
	Each test is performed using a different lot (group) of test organisms of the same species from the same source						
	Data from initial control performance tests show that criteria for test validity can be met (must)						
	Data from initial reference toxicity tests is evaluated using the magnitude of the coefficient of variation (CV) of the derived endpoint values						
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 (must) ; artificial soil is used for tests with chemicals or chemical products spiked in soil; uncontaminated natural soil is used for definitive tests with field-collected boreal forest and taiga soils						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Negative Control Soil cont.	Negative control soil included as a treatment in every toxicity test (must)						
Negative Control Soil: Natural Soil	Natural soil collected from a clean (uncontaminated) site; free of pesticide or fertilizer for ≥5 years						
	Soil is analyzed for: particle size distribution (% sand, % silt, % clay); total organic carbon content (%); organic matter content (%); pH; electrical conductivity; MC (%); WHC (%); and CEC (must)						
	Soil is analyzed for recommended cations and anions , forms of nitrogen, phosphorus, potassium, C:N ratio, and contaminants (see Section 3.3.1 in STB 1/RM/43 2nd ed.)						
	Natural soil can be air-dried (10–20% moisture content), coarse-screened (4–10 mm), transferred to clean plastic pails, and stored in darkness at 4 ± 2 °C						
	If present, indigenous organisms are recorded and removed or killed						
Negative Control Soil: Artificial Soil	10% <i>Sphagnum</i> sp. peat, air dried and sieved (2-mm mesh); 20% kaolin clay (with particle size < 40 µm); 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 to 6.0–7.5 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						
	Soil is analyzed for: particle size distribution (% sand, % silt, % clay); total organic carbon content (%); organic matter content (%); pH; electrical conductivity; MC (%); WHC (%); and CEC (must)						
	Additional analyses are performed as necessary (see Section 3.3.2 in STB 1/RM/43 2nd ed.)						
Positive Control Soil	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						
	Physicochemical characteristics including e.g., organic carbon, organic matter, particle size distribution, texture, pH, and electrical conductivity are similar to test soils						
	Tests using reference soil also include a sample of negative control soil (must)						
Measurements During Test							
Moisture Content	Soil moisture content in each treatment/concentration at test start and end (must)						
	Moisture content determined gravimetrically (see STB 1/RM/43 2nd ed.)						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Moisture Content cont.	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 STB 1/RM/43 2 nd ed.)						
Temperature	Air temperature in test facility, daily or continuously (must)						
Conductivity	Electrical conductivity measured at test start and end when test soil is suspected of having a high salt content						
Chemical Analyses	Normally measured at beginning and end of test, in high, medium, and low strengths as a minimum						
Reference Toxicity Tests	Choose between positive control concentration or multi-concentration reference toxicity test (must)						
	For avoidance test: not required if used for screening or range-finding purposes only						
	Using the results of the reproduction reference toxicity test or positive control for avoidance toxicity testing requirements is optional						
	Use worms derived from the same population (i.e., culture) of worms used for the definitive tests (must)						
	Reproduction reference toxicity test invalid if mean survival of adult worms in negative control soil is <90% at Day 28 (or Day 35), or if mean reproduction for adult worms in negative control soil is <3 live juveniles/adult at test end (must)						
	Avoidance reference toxicity test invalid if mean % survival of worms per test unit is <90% for each test concentration at test end (must)						
Reference Toxicity Test: Positive Control	Include with every definitive test (must)						
	≥5 replicates (for reproduction test) or ≥3 replicates (for avoidance test) of one concentration of a toxicant previously shown to elicit a consistent partial response, plus negative control (must) , using artificial soil; use boric acid						
	For reproduction test: conditions the same as those for definitive tests (must) ; calculate % reduction of progeny production in positive control relative to negative control at test end (Day 56, or 63) (must)						
	For avoidance test: conditions the same as those for definitive avoidance test (recommended) (must) ; calculate % avoidance in positive control relative to negative control at test end for avoidance (must)						
	Positive control response and acceptability limits are defined (must)						
	Identified outliers or extreme variability trigger investigations (must)						
Reference Toxicity Test: Multi-concentration Test	Perform at least twice/year (must) ; once every six months						
	Prepare and test ≥5 concentrations plus a negative control (must) , using artificial soil; use boric acid						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Reference Toxicity Test: Multi-concentration Test cont.	For reproduction test: conditions the same as those for a multi-concentration definitive test, except for # of test concentrations (must) ; determine 56-day (or 63-day) IC50 for inhibition of number of juvenile worms (including 95% confidence limits) (must) ; express as mg boric acid/kg soil dry wt						
	For avoidance test: conditions the same as those for a multi-concentration definitive avoidance test (recommended (must) ; determine 48-hour EC50 for avoidance (must) ; express as mg boric acid/kg soil dry wt						
Warning Chart	Prepared and updated with all comparable endpoints (i.e., EC50s or IC50s derived from multi-concentration reference toxicity tests, or % reduction of juvenile worm production or % avoidance relative to control for a single concentration of reference toxicant tested as positive controls) plotted successively on a warning chart (must)						
	Separate warning chart prepared and updated for each dissimilar procedure (e.g., different species, test type, reference toxicant) and endpoint (must)						
	For multi-concentration reference toxicity test, log concentration is used in all calculations (must)						
	If a particular data point is outside warning limits, quality checks are performed (must)						
Recommendations and options for warning charts are in Section 4.4 of test method							
Reproduction Test							
Test Type	Static; whole soil (must)						
Test Duration	≥56 days (must) ; may be extended to 63 days						
Test Temp	Air temperature: 20 ± 2 °C daily average (must) ; 20 ± 3 °C instantaneous (must)						
Light Quality	Incandescent, fluorescent, or LED						
Light Intensity	≥400 lux (must) ; preferably 400 – 800 lux at surface of soil in test vessel						
Photoperiod	Fixed daily photoperiod (must) (i.e., 16 h light:8 h dark or 12 h light:12 h dark); same photoperiod as that to which worms are acclimated before the test						
Vessel Size & Type	Glass jars, 500 mL for <i>E. andrei</i> or 250 mL for <i>D. rubidus</i> (must) ; covered (for <i>E. andrei</i> : perforated lid or 50-µm Nitex mesh, secured with a rubber band or screw ring; for <i>D. rubidus</i> : 50-µm Nitex mesh, secured with a screw ring and loosely covered)						
	All test vessels are cleaned thoroughly and rinsed with test water before use (must)						
Soil Mass	Identical wet weight in each test vessel (must) ; ~350 mL for <i>E. andrei</i> or ~200 mL for <i>D. rubidus</i> (must) ; smoothed but not compressed						
Moisture Content of Test Soils	Hydrate to the optimal percentage of its WHC if field-collected soil (see Section 5.3), or to ~70% of WHC if artificial soil (see Sections 3.3.2 and 6.2)						
Vessel Labelling	Clearly labelled/coded: test substance, concentration, and replicate # (must)						
	Date and time of test initiation on labels or data sheets (must)						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Vessel Position	Test containers are positioned randomly within test facility and moved during test						
# Replicates/Conc.	For a multi-concentration test, ≥5 replicates/treatment (must)						
	For a single-concentration test, depending on the target effect size: • a minimum of 13–21 replicates/treatment for <i>E. andrei</i> (must); and • a minimum of 5–13 replicates/treatment for <i>D. rubidus</i> (must) (see Section 5.6.2 in STB 1/RM/43 2 nd ed.)						
	For site soils, use replicate samples (i.e., field replicates) collected individually from a given sample location (see Section 5.1 in STB 1/RM/43 2 nd ed.)						
	≥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						
	If a range-finding test is conducted prior to the definitive multi-concentration test, the number of concentrations may be reduced in the definitive test						
# Worms/Vessel	4 adult organisms/vessel (must)						
Organism Selection	Worms are transferred to test vessels 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 vessel and rinsed in clean test water						
	Individuals that appear atypical are not used in the test (must)						
	For each replicate, worms are selected and moved to a transfer container and transferred individually to the soil surface in each test vessel						
	The order of adding worms to each vessel are randomly allocated with respect to replicate and 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	An identical measured quantity of Magic® Worm Food or organic mixed grains on Days 0, 14, 28, and 42 only (must); 2 g/vessel for <i>E. andrei</i> or 1 g/vessel for <i>D. rubidus</i> each feeding; if food not consumed from previous feeding, a reduced amount of food is added; food not removed if unconsumed						

TEST SPECIFIC CHECKLIST Tests for Measuring Avoidance Behaviour or Reproduction of Earthworms (<i>Eisenia andrei</i> or <i>Dendrodrilus rubidus</i>) Exposed to Contaminants in Soil							
Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Test Soil Hydration	Soil moistened with test water weekly, as necessary (i.e., if > 10% loss)						
Test Validity Criteria	Test invalid if mean 28- or 35-day survival of adult worms in negative control soil is <90%, or if mean reproduction for adult worms in negative control soil is <3 live juveniles/adult at test end (must)						
	Negative control soil used to judge validity of test regardless of whether the reference or negative control soils are used for statistical comparisons (must)						
Biological Observations	Condition, appearance, and # live adult worms in each test vessel on Day 0 (must)						
	# live adult worms in each test vessel on Day 28 or 35 (must)						
	# live/dead adult worms on soil surface in each test vessel at start of test (t = 1 h) and on each feeding occasion						
	Appearance and behaviour of worms in each test vessel on Day 28 or 35, and at test end						
	# live juvenile worms produced in "extra" replicates on Day 28 (i.e., to determine if juvenile worm production is acceptable; if no cocoons or juvenile worms are observed, the definitive test vessels 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)						
	# live juvenile worms in each test vessel on Day 56 (or Day 63) (must) ; counted using manual sorting or heat-extraction; heat-extraction is not used for recovery of cocoons; test vessels processed in random manner						
	Dead juvenile worms are not included in juvenile count (must) ; # dead juvenile worms recorded, if observed						
	Juvenile weight at test end is optional						
	Observations of any excessive growth of mould or fungi, and the presence and quantity of any uneaten food, on each feeding occasion						
	Examine ≥1 vessel from each treatment for apparent "wetness" of soil every two weeks (must)						
Biological Endpoint	# live adult worms in each replicate on Day 28 (or Day 35 if test extended); # live juvenile worms in each replicate on Day 56 (or Day 63, if test extended) (must)						
	# hatched or unhatched cocoons at test end						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Statistical Endpoint	% survival of adults in each test vessel on Day 28 or 35 (must)						
	Mean (\pm SD) % survival of adults in each treatment (including reference and negative control soils) on Day 28 or 35 (must)						
	Mean (\pm SD) # live juveniles in each treatment (including reference and negative control soils) on Day 56 or 63 (must)						
	For multi-concentration test: the 28- or 35-day LC50 for adult worms and 56- or 63-day ICp for reproductive inhibition based on numbers of live juveniles produced in each concentration during the 56- or 63-day test (must)						
Calculation of ICp	Calculation of endpoints by entering concentrations as logarithms (must)						
	Linear and/or non-linear regression procedures used for calculation of ICps and 95% confidence limits (must)						
	Initial plot of raw data against log concentration						
	All requirements for regression analysis outlined in Section 4.8.1 of STB 1/RM/43 2 nd ed. are met (must)						
	Endpoints generated by regression analysis are bracketed by test concentrations (i.e., extrapolation of endpoints beyond the highest test concentration is not acceptable) (must)						
	ICPIN analyses used only if regression analyses fail to provide meaningful ICps						
Avoidance Test							
Test Type	Static; whole soil (must)						
Test Duration	48 hours (must)						
Test Temp	Air temperature: 20 ± 2 °C daily average (must); 20 ± 3 °C instantaneous (must)						
Lighting/Photoperiod	Continuous darkness (test units wrapped in aluminum foil if made of transparent or translucent Plexiglas™) (must)						
Vessel Size & Type	Test unit is a circular container with a central chamber and six pie-shaped interconnecting compartments, with a fitted lid; constructed of high-quality stainless steel or Plexiglas™; holes in bottom of central chamber and sides of compartments for worm movement; modified with false back plates for use with <i>D. rubidus</i> (as described in Section 3.2.3 in STB 1/RM/43 2 nd ed.) (must)						
	All test units are cleaned thoroughly and rinsed with test water before use (must)						
Amount of Soil per Test Compartment	Identical wet weight and volume in each test compartment (must); ~350 mL for <i>E. andrei</i> or ~200 mL for <i>D. rubidus</i> (must); none in central chamber (must); soil is smoothed but not compressed						
Moisture Content of Test Soils	Hydrate to the optimal percentage of its WHC if field-collected soil (see Section 5.3), or to ~70% of WHC if artificial soil (see Sections 3.3.2 and 6.2)						

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Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Number of Compartments per Test Unit with Same Treatment	Three (negative control soil or reference soil in each of three compartments, with a single test soil or concentration thereof in each of three alternate compartments) (must)						
Vessel 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)						
Vessel Position	Test units are positioned randomly within test facility and not disturbed during test						
# Replicates/Conc.	For a multi-concentration test, ≥2 replicates (test units) per concentration (must) ; for screening or range-finding tests, 1 replicate (test unit) per concentration						
	For a single-concentration test, ≥5 replicates (test units) (must)						
	For site soils, use replicate samples (i.e., field replicates) collected individually from a given sample location (see Section 5.1 in STB 1/RM/43 2 nd ed.)						
# Test Conc.	1, plus controls for single-concentration test						
	≥5, plus controls for multi-concentration test (ECp calculation) (must) ; more recommended (≥7, 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 unit with the same treatment (must)						
	If a range-finding test is conducted prior to the definitive multi-concentration test, fewer concentrations and 3 replicates/concentration may be used in the definitive test						
# Worms/Vessel	10 adult organisms/test unit (must)						
Organism Selection	Worms are transferred individually to test units 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 vessels and rinsed in clean test water						
	Individuals that appear atypical are not used in the test (must)						
	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 test unit are randomly allocated with respect to test soil or concentration						

TEST SPECIFIC CHECKLIST							
Tests for Measuring Avoidance Behaviour or Reproduction of Earthworms (<i>Eisenia andrei</i> or <i>Dendrodrilus rubidus</i>) Exposed to Contaminants in Soil							
Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Organism Selection cont.	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						
Test Validity Criteria	Test invalid if % survival of worms in any test unit is <90% at test end; or where more than one replicate test unit is used per test soil/concentration, mean % survival of worms per test unit is <90% for each test soil or test concentration at test end (must)						
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)						
Biological Endpoint	# live worms per treatment in each test unit (i.e., total number of live worms in the three compartments containing the same test soil, for each of the two treatments) at test end (must)						
Statistical Endpoint	Percent survival of all worms in each test unit at test end; for more than one replicate test unit, mean percent survival of all earthworms per test unit for each test soil or test concentration at test end (must)						
	For a single-concentration test: mean (± SD) # live worms recovered from the test soil and the clean soil in each of the replicate test units (must)						
	For a multi-concentration test: % avoidance per treatment in each test unit at test end (must) ; 48-h EC50 (or other ECp) for avoidance, based on percent avoidance determined for each test concentration (must)						
Test Organisms							
Species	Laboratory-cultured <i>Eisenia andrei</i> or <i>Dendrodrilus rubidus</i> (must)						
	Species identification confirmed and documented upon establishment of a new culture, and/or with each new batch of <i>E. andrei</i> or <i>D. rubidus</i> introduced to the laboratory culture (must)						
	DNA-based taxonomic identification is highly recommended; identification by qualified personnel using taxonomic keys is also acceptable						

TEST SPECIFIC CHECKLIST

Tests for Measuring Avoidance Behaviour or Reproduction of Earthworms (*Eisenia andrei* or *Dendrodrilus rubidus*) Exposed to Contaminants in Soil

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Species cont.	Cultures held in a testing laboratory are identified to species every 2 years, as a minimum						
Source	Test organisms are cultured in testing laboratory or obtained from outside cultures (government or private laboratories culturing for toxicity testing) (must)						
	All organisms used in a test are derived from the same population (must)						
Source of Breeding Culture	Juveniles, adults, and/or cocoons from government or private laboratories						
Age/Size at Test Start	Clitellated adult earthworms (must) ; individual wet wt at test start within 250–600 mg for <i>E. andrei</i> and 50–200 mg for <i>D. rubidus</i> (must)						
Culture Conditions							
Facilities	Controlled-temperature laboratory facility						
	Culture area isolated from testing, sample storage, or sample-preparation areas; designed and constructed to prevent culture contamination						
Culture Vessels	Breeding boxes with 6–50-L capacity; covered with perforated lid; transparent or translucent sides and/or lid; minimum substrate depth, 10 cm; wood not recommended						
Culture Substrate	Optional (e.g., mixture of potting soil, artificial soil, and peat moss)						
Hydration	Hydrate with test water; maintain moisture such that surface of bedding is moist but there is no standing water in culture vessels; soil particles do not adhere to worms						
Air Temp	Daily average, 20 ± 2 °C; instantaneous, 20 ± 3 °C						
pH	6.0–7.5; adjusted to range if necessary using reagent-grade calcium carbonate						
Lighting	Incandescent, fluorescent, or LED ; 400 to 800 lux at surface of culture vessel; continuous dark or fixed daily photoperiod (e.g., 16 h:8 h or 12 h:12 h, light:dark); avoid overheating cultures						
Culture Maintenance	Examine contents of culture vessels at regular intervals (e.g., biweekly, just before feeding) ; rehydrate and/or gently turn manually (i.e., if excess water at bottom of substrate), as necessary; record condition of culture (organisms and substrate) ; if health of culture deteriorates, increase monitoring frequency; maintain loading density of worms at ≤ 0.03 g/cm ³						
	Remove and discard any dead (must) , injured, or atypical (lethargic) worms						
Substrate Renewal	As required; sort and transfer worms and cocoons manually to maintain loading density; renew substrate as needed ; use constant light to move worms out of top layer of old bedding into fresh bedding placed underneath or into bottom half of old bedding; remove and discard top layer of old bedding, and replace with fresh bedding						
Substrate Monitoring	Temp, pH, and moisture content of bedding monitored in each culture vessel at regular intervals; adjust as necessary						

TEST SPECIFIC CHECKLIST							
Tests for Measuring Avoidance Behaviour or Reproduction of Earthworms (<i>Eisenia andrei</i> or <i>Dendrodrilus rubidus</i>) Exposed to Contaminants in Soil							
Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Feeding	Magic® Worm Food and ground and sieved organic mixed grains recommended; oatmeal, alfalfa pellets, and/or other optional food supplements; quantity based on previous food consumption; biweekly at time of aeration and re-hydration; place food in shallow depression of substrate, hydrate with deionized water, and cover with thin layer of substrate; remove excess (uneaten) food and any visible mould, fungi, or mites before feeding						
Indices of Culture Health	Cultures have low mortalities, appear healthy, and behave and feed normally (must)						
	Considered healthy if: (1) worms are moving actively through the substrate, do not try to leave it, and reproduce continuously, and (2) results for reference toxicity tests or positive controls using worms derived from the culture fall within historic warning limits (must)						
	Discard contents of culture vessel if >20% of juvenile or adult worms are dead, inactive, or unhealthy at any time						
Handling of Worms	Handling minimized; worms are transferred individually using gloved hand and/or the arm(s) of rounded forceps						
	Worms that are dropped, injured, or appear stressed are not used in a test (must)						
Acclimation							
Source of Worms	Government or private toxicity testing laboratories with existing culture of the species; all from same source						
Life Stage and Size on Receipt	Juveniles or sexually mature worms with clitellum; individual wet weight within the indicated range for testing						
Facility	Controlled-temperature laboratory facility						
	Culture area isolated from testing, sample storage, or sample-preparation areas; designed and constructed to prevent culture contamination						
Vessels	Breeding boxes of 6–50-L capacity; covered with perforated lid; transparent or translucent sides and/or lid; minimum substrate depth of 10 cm; wood not recommended						
Substrate	Negative control soil (natural or artificial) or a mixture of potting soil, artificial soil, and peat moss; worms are transported to the lab using a portion of soil/substrate to which they are adapted; adjust substrate gradually upon arrival						
Hydration	Hydrate with test water; maintain moisture such that surface of substrate is moist but with no standing water in the bottom of the acclimation vessel; soil particles do not adhere to worms						

TEST SPECIFIC CHECKLIST

Tests for Measuring Avoidance Behaviour or Reproduction of Earthworms (*Eisenia andrei* or *Dendrodrilus rubidus*) Exposed to Contaminants in Soil

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Temp	Adjust gradually ($\leq 3^{\circ}\text{C}/\text{day}$) upon arrival; thereafter, maintain at daily average of $20 \pm 2^{\circ}\text{C}$ and instantaneous of $20 \pm 3^{\circ}\text{C}$						
pH	Near neutral; no adjustment if natural (field-collected) negative control soil; adjust to 6.0-7.5 using reagent-grade calcium carbonate if necessary						
Lighting	Incandescent, fluorescent, or LED; 400 to 800 lux at surface of acclimation vessel; fixed daily photoperiod (e.g., 16 h:8 h or 12 h:12 h, light:dark); for acclimation to avoidance test, continuous dark is optional						
Duration of Acclimation	For worms obtained from an outside source for use in tests: ≥ 7 days during the period immediately preceding the test, to laboratory conditions (must); ≥ 14 days highly recommended for earthworms for use in reproduction test						
	For reproduction test: ≥ 7 days (or ≥ 14 days, if worms are from an outside source) immediately before test start; organisms are acclimated in the lab to food (must), substrate, lighting, and temperature to be used in test if culturing conditions differ						
	For avoidance test: ≥ 7 days immediately before test start; organisms are acclimated in the lab to substrate and temperature to be used in test; acclimation to continuous darkness is optional						
Maintenance	Examine contents of holding/acclimation vessels at regular intervals (e.g., biweekly); rehydrate and gently turn manually (i.e., if excess water at bottom of substrate), as necessary; record condition of organisms and substrate; if health of culture deteriorates, increase monitoring frequency; maintain loading density of worms at $\leq 0.03 \text{ g}/\text{cm}^3$						
	Remove and discard any dead (must), injured, or atypical (lethargic) worms						
Substrate Renewal	As required; if worms held for an extended period before use in soil toxicity test; sort and transfer worms manually to maintain loading density; renew substrate as needed: use constant light to move worms out of top layer of old bedding into fresh bedding placed underneath or into bottom half of old bedding; remove and discard top layer of old bedding, and replace with fresh bedding						
Substrate Monitoring	Temp, pH, and moisture content measured in each holding/acclimation vessel at start of acclimation period, and at regular intervals if holding > 2 weeks; adjust as necessary						
Feeding	Magic® Worm Food or ground and sieved organic mixed grains (must); feed upon establishment of acclimation vessel and then at least once biweekly (must); place food in shallow depression of substrate, hydrate with deionized water, and cover with thin layer of substrate; remove any excess (uneaten) food and any visible mould, fungi, or mites before feeding						

TEST SPECIFIC CHECKLIST							
Tests for Measuring Avoidance Behaviour or Reproduction of Earthworms (<i>Eisenia andrei</i> or <i>Dendrodrilus rubidus</i>) Exposed to Contaminants in Soil							
Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Indices of Culture Health	Considered healthy if: (1) worms are moving actively through the substrate and do not try to leave it, and (2) results for reference toxicity tests or positive controls using worms from the original culture fall within historic warning limits (must)						
	Discard contents of acclimation vessel if >20% of juvenile or adult worms are dead, inactive, or unhealthy at any time (must)						
Handling	Handling minimized; worms are transferred individually using gloved hand and/or the arm(s) of rounded forceps						
	Worms that are dropped, injured, or appear stressed are not used in a test (must)						
Test Report							
Test Substance	Brief description of sample type or coding as provided to laboratory personnel (must)						
	Information on labelling or coding of each sample (must)						
	Brief description of soil sampling, storage, and preparation (i.e., pretreatment) procedures (must)						
	Information on sample horizons as they were collected (i.e., number, relative depth of each soil horizon), for test, reference, and negative control soils, if applicable (must)						
	Type of negative control soil (natural or artificial) and, if applicable, reference soil (must)						
	Date of sample collection (must)						
	Date and time sample(s) received at test facility (must)						
	Sample temperature and moisture content upon receipt at the test facility (must)						
Test Organisms	Species and source of breeding stock and test organisms (must)						
	Wet weight (mean \pm SD) of organisms 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) and verifying results (must)						
Test Method	Citation of biological test method used (i.e., as per STB 1/RM/43 2 nd ed.) (must)						
	Design and description of specialized procedure(s) (e.g., soil manipulation; 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)						

TEST SPECIFIC CHECKLIST							
Tests for Measuring Avoidance Behaviour or Reproduction of Earthworms (<i>Eisenia andrei</i> or <i>Dendrodrilus rubidus</i>) Exposed to Contaminants in Soil							
Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Test Method cont.	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 procedures and conditions specified in STB 1/RM/43 2 nd ed. (must)						
	Number of discrete samples per treatment (must)						
	Target effect size for single-concentration reproduction tests (if applicable) (must)						
	Number of replicate test vessels/units for each treatment (must)						
	Number and description of treatments in each test including the control(s); test concentrations (if applicable) (must)						
	Volume and/or mass of soil in each test vessel or compartment of avoidance test unit (must)						
	Number of organisms per test vessel/unit and treatment (must)						
	Dates and times when test and control soils were prepared, and when test was started and ended (must)						
	Feeding regime and ration during reproduction test (must)						
	Indication of assessment of soil moisture during the reproduction test (must)						
	Dates when adults were removed from test vessels, for the reproduction test (must)						
	For each soil sample: any measurements of soil particle size, moisture content, water-holding capacity, pH, TOC (%), OM (%), CEC, and electrical conductivity (must)						
	For each composite sample of subsamples 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 (must)						
	Test Results	For reproduction test: mean (\pm SD) percent survival of adult worms in each treatment, on Day 28 or 35 (must) ; mean (\pm SD) number of surviving juveniles in each treatment on Day 56 or 63 (must) ; and mean (\pm SD) number of surviving juveniles produced by each adult worm in control(s) on Day 56 or 63 (must)					
For avoidance test: % survival of all worms in each test unit at test end; mean % survival of worms per test unit where more than one replicate test unit is used for each test soil or test concentration; mean (\pm SD) number of surviving worms in replicates of each treatment representing clean soil and test soil at 48 hours for single-concentration tests (must) ; % avoidance for each treatment for multi concentration tests (must) ; % avoidance for single-concentration tests, if calculated (must)							
Any LC50 or EC50 (with its 95% confidence limits, and if calculated, the slope) determined (must) ; any additional Lc _p or EC _p (e.g., LC25 or EC25) calculated (must)							

TEST SPECIFIC CHECKLIST							
Tests for Measuring Avoidance Behaviour or Reproduction of Earthworms (<i>Eisenia andrei</i> or <i>Dendrodrilus rubidus</i>) Exposed to Contaminants in Soil							
Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Test Results cont.	Any ICp (with its 95% confidence limits) determined for the data on reproductive success (i.e., number of surviving juvenile worms in each treatment 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 and degree of difference from nominal strength (must)						
	If using multi-concentration reference toxicity test, any 56-day (or 63-day, if applicable) IC50 for inhibition of reproduction or 48-hour EC50 for avoidance (including its 95% confidence limits) performed with the reference toxicant in conjunction with the definitive soil toxicity test (must)						
	If using positive control concentration, any % reduction in juvenile production relative to the negative control or % avoidance for positive controls performed with the reference toxicant in conjunction with the definitive soil toxicity test (must)						
	Geometric mean value (± 2 SD) for the same test type, reference toxicant, and test species, as derived at the test facility in previous tests with the reference toxicant using the procedures and conditions for testing with a reference toxicant described in STB 1/RM/43 2 nd ed. (must)						
	Anything unusual about the test, any problems encountered, and any remedial measures taken (must)						
Original Data Sheets	Original data sheets are signed or initialled, and dated by the laboratory personnel conducting the tests (must)						
Information to be Kept On-file							
	Do lab SOPs indicate that the information on Section 7.2 of the STB 1/RM/43 2 nd ed. method must be kept on file for ≥ 5 years? (must)						
	For details of this information, see Section 7.2 of STB 1/RM/43 2 nd ed.						

Notes: