

TEST SPECIFIC CHECKLIST

Revised: March 2007

Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte *Lemna minor*

N.B. Shaded text reflects January 2007 2nd edition changes

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Parameter	Specification	Met Specifics?		
		Y	N	NA
Sample Preparation				
T° Measurement.....	T° to be measured in sample/subsample on arrival at lab (Must)
Filtering.....	None for wastewater samples unless algae present ; if indigenous organisms or algae present , filter through glass fibre filter (~1 µm) (Must) ; additional filtration through 0.22 µm filters is optional.....
T° Adjustment.....	Sample/subsample adjusted to 25 ± 2°C before starting the test (Must) No use of immersion heaters (Must) ; water bath recommended.....
pH Measurement.....	pH measured in each sample/subsample prior to its use (Must)
pH Adjustment.....	No adjustment if pH of test solution is within 6.5 - 9.5 for tests with APHA, 6.0 to 8.0 for tests with SIS, and 5.0 to 8.0 for tests with Steinberg ; a second (pH adjusted) test might be required if pH is beyond the specified range.....
Pre-aeration.....	Spiked sample to be gently aerated for 20 min before test initiation or renewal of test solutions (Must) Rate of pre-aeration not exceeding 100 bubbles/min.....
Nutrient spiking.....	Wastewater/receiving water to be spiked with the nutrients used to prepare the modified APHA test medium (Must) ; samples spiked following filtration, if filtration required
Test Conditions				
Facility.....	Constant-temperature room, incubator, environmental chamber or equivalent facility with good temperature control and acceptable lighting (Must) Instruments available to measure basic water quality variables (T°, pH, conductivity) and lab prepared for other analysis (ie: hardness, alkalinity, ammonia and residual chlorine) (Must)
Test Type.....	Static or static-renewal.....
Duration.....	7 days (Must)
Temperature.....	Daily mean of 25 ± 2 °C throughout the test (Must)
Lighting.....	Continuous, full-spectrum (fluorescent or equivalent); 64 to 90 µmol/(m²·s) at surface of culture media (Must); within ± 15% of selected light fluence rate throughout test area
In-test pH.....	No adjustment if pH of test solution is between 6.5 - 9.5 for tests with APHA, 6.0 to 8.0 for tests with SIS, and 5.0 to 8.0 for tests with Steinberg
Aeration.....	No aeration during test.....
Vessel Size & Type.....	Disposable polystyrene cups or Erlenmeyer flasks recommended; may be glass beakers, crystallizing dishes, petri dishes; vessels covered (polystyrene lids that fit plastic test cups or petri dish lids for Erlenmeyer flasks are recommended) Glass vessels are used for chemical tests Wide enough for no overlap of <i>Lemna</i> fronds in controls at test end (Must) ... All test vessels and covers as well as solution depth and volume be identical for a given test (Must)
Test Volume.....	≥ 100 mL (Must) ; preferably 150 mL; water depth ≥ 4 cm.....
Test Surface.....	Vessels are place on non-reflective dark background during test
Renewal of Solution.....	None for static option (Must) At least every 3 days for static-renewal option (Must) <i>Lemna</i> colonies to be aseptically transferred to fresh test solutions (Must) ... Transfer done in random order across the replicates within a conc. (Must) ... Test medium recommended (which is deionized or glass-distilled water to which reagent-grade chemicals (nutrients for <i>Lemna</i>) have been added)..... Adjusted to 25 ± 2°C prior to use (Must) Same water to be used to prepare sample dilutions and controls (Must) If upstream water is used as control/dilution water, a separate control solution is to be prepared using the modified APHA medium that is normally used for testing <i>Lemna</i> (Must) Receiving water used as control/dilution water is to be filter (~1 µm) (Must) ; with optional further filtration (0.22 µm).....
Dilution/Control Water.....	Test medium recommended (which is deionized or glass-distilled water to which reagent-grade chemicals (nutrients for <i>Lemna</i>) have been added)..... Adjusted to 25 ± 2°C prior to use (Must) Same water to be used to prepare sample dilutions and controls (Must) If upstream water is used as control/dilution water, a separate control solution is to be prepared using the modified APHA medium that is normally used for testing <i>Lemna</i> (Must) Receiving water used as control/dilution water is to be filter (~1 µm) (Must) ; with optional further filtration (0.22 µm).....

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Test Medium.....	For wastewaters & receiving waters, modified APHA growth medium is used (Must) For chemicals, either the SIS or modified Steinberg growth media should be used; APHA used if testing metals.....
Vessel Identification. . .	All test vessels clearly coded or labelled to enable proper identification of the sample and its concentration (Must) Date & time of test initiation recorded on data sheets (Must)
# Test Conc.....	≥7 plus control to calculate ICp (Must) ; > 8 are recommended.. 1 plus control for single conc. test. Test vessels are left at room temperature for 1h to allow equilibration of medium and toxicant (Must)
# Replicates/Conc.....	Nominal concentrations of the solutions are corrected for the volume of nutrient stock are adopted and reported as the test concentrations (Must) For single-conc. test: ≥3 replicates per test conc. and control(s) (Must) For multi-conc. test with equal replication among treatments: ≥4 replicates per test conc. and control(s) (Must) For multi-conc. test with unequal replicate test design: 6 replicates for control, 4 replicates for lowest 3-5 test concentrations and 3 replicates for highest 4-5 test concentrations.
# Organisms/Vessel. . .	Two 3-frond <i>Lemna</i> plant are randomly assigned/transferred to each test vessel (Must) Care is to be taken to not contaminate the <i>Lemna</i> while transferring to their individual test vessel (Must) Care is to be taken to ensure that the plant does not adhere to the side of the test vessel and that the roots are inside the test vessel (Must) Any plants that break apart during the transfer are to be replaced (Must)
Vessel Randomization.	Each group of replicate vessels representing a particular treatment (eg: a specific test conc.) is to be placed in randomized positions in the environmental chamber or test area (Must)
Vessel Cleaning.	All test vessels, measurement, stirring devices and accessories thoroughly cleaned and rinsed (Must) New and previously used glassware is to be chemically cleaned and sterilized before use (Must)
Chemical Testing.	Solubilizing agent control solution is to be run, if used (Must) Test conc. should be measured at beginning and end of exposure, in high, medium and low strengths and in the control for static option; additional measurements at beginning and end of each renewal period for static-renewal option.
Biological Endpoints. . .	Agent concentration should not exceed 0.1mL/L. Growth based on: 1) the reduction of the increase in the number of fronds during the test (compared to controls); increase in frond number is calculated by subtracting the initial number of fronds in a given vessel from the final number of fronds in the same vessel (Must) ; and. 2) the decrease in the final dry weight of the fronds at the end of the test (compared to controls); frond dry weight measures the total dry weight of <i>Lemna</i> fronds compared to the control at test end (i.e., no determination of initial weight made) (Must)
Statistical Endpoints. . .	Mean (± SD) increase in frond number in each treatment, including control(s) as determined at test end (Must) Mean (± SD) dry weight of fronds in each treatment, including control(s) as determined at test end (Must) For multi-conc. test, ICp for both growth endpoints (with their 95% confidence limits) are to be calculated (i.e., separate ICp for each endpoint) (Must)

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<u>Culture/Holding Conditions</u>				
Facilities.....	<i>Lemna</i> cultured in facilities with controlled temperature and lighting (Must) ... Culture area well ventilated, isolated from the test facility and from regions of the laboratory where stock or test solutions are prepared, effluent or other material is stored or equipment is cleaned..... Lab is clean, employs good sterile technique and properly uses laminar flow hood, all essential for axenic culturing of <i>Lemna minor</i> (see Appendix F in 2nd ed. EPS 1/RM/37).
Apparatus.....	Vessels and accessories in contact with the <i>Lemna</i> cultures and culture media are to be made of nontoxic, chemically-inert material (Must) Materials such as copper, brass, galvanized metal, lead, and natural rubber are not to come in contact with culture vessels or media, nor with test samples, test vessels, dilution water, or test solutions (Must) New and previously used glassware are to be chemically cleaned and sterilized before use (Must)
Culture Medium.....	Sterile modified Hoagland's E + medium (new formula as per Table 2 in 2nd ed. of EPS 1/RM/37) for cultures to be used for wastewater or receiving water tests (Must) Other nutrient-rich media (i.e., SIS or Steinberg) can be used for culturing <i>Lemna</i> for chemical testing only, as long as cultures meet health criteria
Stock Cultures.....	Axenic stock cultures are maintained by weekly subculture of 1 or 2 plants (transferred under aseptic conditions) into 25 mL of sterile modified Hoagland's E + medium in 25 X 150 mm test tubes with caps, and incubated on an angle..... Multiple subcultures of axenic <i>Lemna</i> culture made to ensure the availability of at least one sterile culture in case of contamination. <i>Lemna</i> that has not been subcultured on a weekly basis is to be subcultured in fresh medium at least twice during the 14 days immediately preceding the test, to allow the recovery of its fast growth rate (Must) Contaminated <i>Lemna</i> cultures (with algae, protozoa, fungi or bacteria) are to be discarded or sterilized (Must) It is strongly recommended that cultures showing signs of contamination be discarded rather than treated..... If the use of cultures having undergone sterilization cannot be avoided, a minimum 8-week period is to follow sterilization prior to use in tests (Must)
Temperature.....	Within the range 25 ± 2 °C..... Culture temperature to be adjusted gradually (≤ 3 °C/day) and maintained at test temperature for ≥2 weeks before tests initiated (Must)
pH.....	4.4 to 4.8
Aeration.....	No aeration since axenic culture.....
Lighting.....	Continuous, full-spectrum (fluorescent or equivalent); 64 to 90 μmol/(m²·s) at surface of culture media; within ±15% of selected light fluence rate throughout culture area.....
Test Culture.....	Under aseptic conditions, 5 to 10 plants transferred from a week-old test tube culture to sterile modified Hoagland's E + medium; incubated for 7- 10 days under test conditions; culture is not crowded (i.e., <i>Lemna</i> are not overlapping and do not cover more than two thirds of the medium surface) when used.
Acclimation.....	If the medium becomes cloudy (contamination) the <i>Lemna</i> cannot be used and is to be replaced with an uncontaminated culture (Must) 7 to 10-day old plants from test culture transferred to fresh test medium (APHA, SIS or Steinberg) and incubated under test conditions for 18 to 24 hours prior to testing..... Organisms obtained from an outside culture collection are to be cultured in the lab for ≥3 weeks before used in test (Must)

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QA/QC				
Validity Criterion.	Mean number of fronds in the controls have increased to ≥ 8-times the original number of fronds (i.e., the mean number of fronds per control vessel is ≥48) at the end of the 7-day test period (Must)
Reference Toxicant. . .	Nickel (i.e., nickel sulphate) or potassium chloride are recommended. Fresh stock solutions prepared for each reference toxicity test. Start within 14 days of the toxicity test period (Must) Following the same procedure as the effluent test (Must) Standard test with ICp endpoint for frond number only (Must) The same test culture (7 to 10 days old) may be used for tests with both the reference toxicant and sample(s) when simultaneous tests. The control/dilution water is appropriate for the reference toxicant used (i.e., APHA for Ni; and APHA, SIS, or modified Steinberg medium for KCl)
Warning Chart.	Prepared for each reference toxicant and continually updated (Must) A separate warning chart is to be prepared for each <i>Lemna minor</i> clone and/or each medium used in reference toxicant testing (Must) ICp is acceptable if within warning limits (± 2 SD on log scale). Log concentration used for mean & SD calculations and plotting (Must)
Verification of Test System.	Any new test system (e.g., vessel, cover, lighting, background colour) is tested by conducting a non-toxicant test (see Section 3.3 in 2nd ed. EPS 1/RM/37); CV for frond number and dry weight at test end is <20%.
Sample Handling				
Sample Collection. . . .	Static option: a single sample of wastewater is to be collected and used to prepare the test solutions at the beginning of the test (Must) Static-renewal option: samples are to be collected using one of the two following procedures and approaches (Must) 1) A single sample of wastewater may be used throughout the test, provided that it is divided into 3 separate containers (3 subsamples) upon collection. 2) Fresh samples are to be collected on at least 3 separate occasions using sampling intervals of 2 to 3 days or less. These samples must be used consecutively during the test.
Containers.	Non-toxic materials for sample and transport containers (Must) New containers or thoroughly cleaned/rinsed if used containers (Must) Collapsible polyethylene or polypropylene containers recommended.
Volumes.	Volumes of 4L.
Labelling.	Upon collection, sample containers filled, sealed and labelled/coded (Must) Include at least sample type, source, date and time of collection and name of sample collectors.
Holding Time.	Test to be initiated within 3 days after sampling (Must) Recommend test initiation within 1 day after sampling.
Holding Conditions. . . .	Make effort to keep samples cool throughout their period of transport (Must) at 1 - 7°C (preferably $4 \pm 2^\circ\text{C}$) using regular ice or frozen gel packs. Upon collection, if sample $> 7^\circ\text{C}$, cool to 1 - 7°C with regular ice or frozen gel packs (not dry ice) (Must) Sample be kept from freezing during transport or storage (Must) The portion of sample/subsamples required for solution renewals be stored in darkness in sealed containers without air headspace at $4 \pm 2^\circ\text{C}$ (Must)
Sample Aliquots.	Each sample or subsample in a collection container be agitated thoroughly just before pouring (Must)

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Test Results (continued)	<p>Any ICp (together with its 95% confidence limits) determined for the growth (i.e., increase in frond number and dry weight) using concentrations corrected for the volume of nutrient stock; details regarding any weighting techniques applied to the data; and indication of quantitative statistic used (Must).</p> <p>Any outliers and the justification for their removal (Must).</p> <p>Results/duration of any toxicity tests with reference toxicants started within 14 days of the test period, together with the geometric mean value (\pm SD) for the same reference toxicant(s), test species, clone, and test medium as derived at the test facility in previous tests (Must).</p> <p>Any findings of significant growth stimulation, expressed as % stimulation, at any concentration(s) (Must).</p> <p>Anything unusual about the test, any problems encountered, any remedial measures taken (Must).</p>
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	<p>Do lab SOPs indicate that the information on Section 8.2 of the 2nd edition of EPS 1/RM/37 method must be kept on file for 5 years? (Must).</p> <p>For details of this information, see the 2nd edition of EPS 1/RM/37, Section 8.2.</p>

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