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

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 wasterwater 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/(m^2·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 *Lemna* 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)**	☐	☐	☐
	Lemna colonies to be aseptically transferred to fresh test solutions **(Must)**	☐	☐	☐
	Transfer done in random order across the replicates within a conc. **(Must)**	☐	☐	☐
Dilution/Control Water	Test medium recommended (which is deionized or glass-distilled water to which reagent-grade chemicals (nutrients for *Lemna*) 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 *Lemna* **(Must)**	☐	☐	☐
	Receiving water used as control/dilution water is to be filter (~1 µm) **(Must)**; with optional further filtration (0.22 µm)	☐	☐	☐

Parameter	Specification	Met Specifics? Y	N	NA
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)**	☐	☐	☐
	Nominal concentrations of the solutions are corrected for the volume of nutrient stock are adopted and reported as the test concentrations **(Must)**	☐	☐	☐
# Replicates/Conc.	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 *Lemna* plant are randomly assigned/transferred to each test vessel **(Must)**	☐	☐	☐
	Care is to be taken to not contaminate the *Lemna* 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 strenghts and in the control for static option; additional measurements at beginning and end of each renewal period for static-renewal option	☐	☐	☐
	Agent concentration should not exceed 0.1mL/L	☐	☐	☐
Biological Endpoints	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 numer 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 *Lemna* 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)**	☐	☐	☐

Parameter	Specification	Met Specifics? Y	N	NA
Calculation of ICp	Calculation of ICp by entering conc. as logarithms **(Must)**	☐	☐	☐
	Initial plot of raw data (increase in frond number or dry weight) against the logarithm of concentration	☐	☐	☐
	Regression analysis for calculation of ICps & 95% confidence limits **(Must)**	☐	☐	☐
	All requirements for regression analysis outlined in Section 4.5.2 of 2nd ed. EPS 1/RM/37 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)**	☐	☐	☐
	Hormesis data entered directly for regression (i.e., no trimming of data points)	☐	☐	☐
	ICPIN analyses used only if regression fails to provide meaningful ICps	☐	☐	☐
	Regression analysis attempted on each of the two growth endpoints independently (i.e., in a given test) before analyst defaults to ICPIN **(Must)**	☐	☐	☐
Stimulatory Effects	A stimulatory effect (increased response at all concentrations or at high concentrations) must be reported for all concentrations in which significant stimulation was observed; % stimulation calculated using formula in Section 4.5.4 of 2nd ed. EPS 1/RM/37**(Must)**	☐	☐	☐
Observations & Measurements				
Temperature	Measured daily in representative solutions (high, medium, low, and controls) as a minimum **(Must)**	☐	☐	☐
pH	For both static and static-renewal tests; measured at start of test, before the plants are added and at test end, in representative solutions **(Must)**	☐	☐	☐
	For static-renewal, pH also measured before & after each test solution renewal (in both fresh & "to be discarded" solutions) in representatives solutions **(Must)**	☐	☐	☐
Light Fluence Rate	Measured at least once during the test, at points the same distance from the light as the *Lemna* fronds and a several locations in the test area **(Must)**	☐	☐	☐
Fronds	# of fronds in each test vessel counted & recorded at test start & end **(Must)**	☐	☐	☐
	The count include every frond and every visible protruding bud **(Must)**	☐	☐	☐
	Total dry weight of fronds in each test vessel at test end **(Must)**	☐	☐	☐
Dry Weight	Colonies (including the roots) are collected, blotted dry, and dried immediately at 100 °C for 6 h or at 60 °C for 24 h	... ☐	... ☐	... ☐
	Upon removal from oven, boats moved immediately to dessicator **(Must)**	☐	☐	☐
	Thereafter, the boats are individually and randomly removed from the dessicator and weighed on a balance the measures consistently to 10 µg	☐	☐	☐
	Rapid weighing and standard timing among weigh boats is necessary	☐	☐	☐
Test Organisms				
Species	*Lemna minor* Linnaeus **(Must)**	☐	☐	☐
	Landolt clones 8434 and 7730 are recommended	☐	☐	☐
Source	Culture collections, government or private labs or commercial suppliers	☐	☐	☐
	Species identification to be confirmed and documented by taxonomist **(Must)**	☐	☐	☐
	All organisms used in a test are to be from the same strain **(Must)**	☐	☐	☐
	The culture (to obtain organisms for test) is to be axenic **(Must)**	☐	☐	☐
Age	Inocula from the culture are 7-10 days old & consist of young, rapidly-growing colonies without visible lesions before used to set up a given test **(Must)**	☐	☐	☐
Health Criteria	The # of fronds in the vessel(s) set up for monitoring culture health increased to ≥ 8-times the original frond number (i.e., ≥24 fronds) by the end of 7 days in the medium to be used in the test (i.e., APHA, SIS, or modified Steinberg) before the culture is used as source organisms for a test **(Must)**	☐	☐	☐
	Lemna plants from the vessels set up for monitoring culture healthare not used in a test **(Must)**	☐	☐	☐
	Organisms are to appear healthy **(Must)**	☐	☐	☐

Parameter	Specification	Met Specifics? Y	N	NA
Culture/Holding Conditions				
Facilities	*Lemna* 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 *Lemna minor* (see Appendix F in 2nd ed. EPS 1/RM/37.	☐	☐	☐
Apparatus	Vessels and accessories in contact with the *Lemna* 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 *Lemna* 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 *Lemna* culture made to ensure the availability of at least one sterile culture in case of contamination.	☐	☐	☐
	Lemna 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 *Lemna* 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/(m^2·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., *Lemna* 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 *Lemna* 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)**	☐	☐	☐

Parameter	Specification	Met Specifics? Y	N	NA
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 *Lemna minor* 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 ± 2°C) using regular ice or frozen gel packs	☐	☐	☐
	Upon collection, if sample > 7 °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± 2°C **(Must)**	☐	☐	☐
Sample Aliquots	Each sample or subsample in a collection container be agitated thoroughly just before pouring **(Must)**	☐	☐	☐

Parameter	Specification	Met Specifics? Y	N	NA
2nd Edition EPS 1/RM/37	Has the laboratory incorporated all of the changes in the 2nd edition of EPS 1/RM37 into their SOPs?	...	...	...
Test Report				
Sample/subsample	Brief description of sample type if and as provided to the lab **(Must)**	...	...	...
	Information on labelling or coding, for each sample/subsample **(Must)**	...	...	...
	Date of sample/subsample collection; date and time sample(s)/subsample(s) received at test facility **(Must)**	...	...	...
	Dates or days during test when individual samples/subsamples used **(Must)**	...	...	...
	For wastewater/receiving water, temperature upon receipt at lab **(Must)**	...	...	...
	pH of sample(s) or subsample(s) just before its preparation and use **(Must)**	...	...	...
	Date of elutriate generation and procedure for preparation **(Must)**	...	...	...
Test Organisms	Species, clone ID code (if known), and origin of culture **(Must)**	...	...	...
	Age of test culture used to provide inocula of organisms at test start **(Must)**	...	...	...
	Indication as to whether test culture is axenic **(Must)**	...	...	...
	Growth medium used for culturing *Lemna minor* **(Must)**	...	...	...
	Test medium in which *Lemna* were acclimated for 18-24h before test **(Must)**	...	...	...
	Data showing increase in frond nb in vessels to monitor culture health **(Must)**			
	Any unusual appearance/treatment of test culture, prior to use in test **(Must)**	...	...	...
Test Facilities	Name and address of test laboratory **(Must)**	...	...	...
	Name of person(s) performing the test **(Must)**	...	...	...
	Brief description of test vessels (size, shape, type of material) **(Must)**	...	...	...
Control/Dilution Water	Type of test medium used as control and dilution water **(Must)**	...	...	...
	Type and source of water used to prepare test medium **(Must)**	...	...	...
	Type/quantity of any chemical(s) used to prepare control/dilution water **(Must)**	...	...	...
Test Method	Citation of biological test method used **(Must)**	...	...	...
	Indication as to whether test is performed with or without renewal of test solutions and, if static-renewal test, frequency of renewals **(Must)**	...	...	...
	Design and description if specialized procedure **(Must)**	...	...	...
	Frequency and type of all observations/measures made during test **(Must)**	...	...	...
	Programs and methods used for calculating statistical endpoints **(Must)**	...	...	...
Test Conditions	Design and description if any deviation from or exclusion of any of the procedures and conditions specified in test method document **(Must)**	...	...	...
	Number, conc., volume, and depth of solutions in test vessels **(Must)**	...	...	...
	Number of fronds per plant and number of plants per test vessel at test start, and number of replicates **(Must)**	...	...	...
	Procedure, rate, and duration if any pre-aeration of samples or test solutions before starting the test **(Must)**	...	...	...
	Description of the procedure for sample filtration **(Must)**	...	...	...
	Type and quantity of chemicals added to test sample before starting the test (ie: nutrient spiking) **(Must)**	...	...	...
	Description of any sample or test solutions receiving pH adjustment, and/or hardness adjustment **(Must)**	...	...	...
	All required measurements of temperature, pH and light fluence rate in test solutions made during the test **(Must)**	...	...	...
	Dates when test was started and ended **(Must)**			
	Statement indicating whether reference toxicity test was performed under the same experimental conditions as those used with the test samples; and description of any deviation(s) from or exclusion(s) of any of the procedures and conditions specified for the reference toxicity test.	...	...	...
Test Results	Number of fronds and frond appearance in each test vessel as noted during each observation period over the 7-day exposure **(Must)**	...	...	...
	For each treatment including control(s): mean ± SD for the increase in frond number, as determined at test end **(Must)**	...	...	...
	For each treatment including control(s): mean ± SD for dry weight of *Lemna* fronds determined at test end **(Must)**	...	...	...

Parameter	Specification	Met Specifics? Y	N	NA
Test Results. . . . . (continued)	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)**.	...	...	...
	Any outliers and the justification for their removal **(Must)**.	...	...	...
	Results/duration of any toxicity tests with reference toxicants started within 14 days of the test period, together with the geometric mean value (± SD) for the same reference toxicant(s), test species, clone, and test medium as derived at the test facility in previous tests **(Must)**.	...	...	...
	Any findings of significant growth stimulation, expressed as % stimulation, at any concentration(s) **(Must)**.	...	...	...
	Anything unusual about the test, any problems encountered, 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 8.2 of the 2nd edition of EPS 1/RM/37 method must be kept on file for 5 years? **(Must)**.	...	...	...
	For details of this information, see the 2nd edition of EPS 1/RM/37, Section 8.2.			