TEST SPECIFIC CHECKLIST

Growth Inhibition Test Using a Freshwater Alga

Revised: Jan 2014

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

Parameter	Specification	Met Specifics? Y	N	NA
2nd edition EPS 1/RM/25	Has the laboratory incorporated all of the changes in the 2nd edition of EPS 1/RM/25 into their SOPs?	☐	☐	☐
Sample Preparation				
Homogenization	Sample shaken vigorously to ensure homogeneity and to resuspend particulates	☐	☐	☐
Filtering	Subsample sufficient to complete the test (e.g., 5-10 mL); filtered through preconditioned 0.45 µm pore diameter membrane **(Must)**	☐	☐	☐
pH Adjustment	pH measured just before sample is used to prepare test solutions **(Must)**	☐	☐	☐
	No adjustment if pH of test solution is within 6.5 - 8.5; a second (pH adjusted) test might be required if pH is beyond this range	☐	☐	☐
T° Measurement	T° measured in sample on arrival at lab **(Must)**	☐	☐	☐
Test Conditions				
Test Facility	Temperature and lighting can be controlled and monitored continuously	☐	☐	☐
	Environmental chamber meets recommended specifications for test type, temperature and light quality/intensity **(Must)**	☐	☐	☐
	Test chamber well ventilated, free of dust and vapors, and protected from external perturbations	☐	☐	☐
	Test conditions uniform throughout environmental chamber and identical to those in culturing facility	☐	☐	☐
Test Equipment	Instruments for routine measurements of chemical, physical and biological variables maintained properly and calibrated regularly **(Must)**	☐	☐	☐
	No equipment made of copper, zinc, brass, galvanized metal, lead or natural rubber allowed **(Must)**	☐	☐	☐
	Equipment not previously used in tests are pre-rinsed in dilution water and tested for cytotoxicity prior to use	☐	☐	☐
Test Type	Static **(Must)**	☐	☐	☐
Test Duration	72h **(Must)**	☐	☐	☐
Test T°	24 ± 2°C **(Must)**	☐	☐	☐
Light Quality	Overhead "cool white" fluorescent **(Must)**	☐	☐	☐
Light Intensity	4000 ± 400 lux at surface; quantal flux ~56 ± 6 µmol/(m^2 • s) **(Must)**	☐	☐	☐
Photoperiod	Continuous light **(Must)**	☐	☐	☐
Aeration	None**(Must)**	☐	☐	☐
Vessel Size & Type	Sterile, disposable, rigid, polystyrene, 96-well microplates **(Must)**; covered recommend untreated	☐	☐	☐
	If electronic particle reader or manual enumeration is used, U-shaped microplates are required **(Must)**	☐	☐	☐
	If photometric method used, flat-bottomed microplates are required **(Must)**	☐	☐	☐
	For chemical testing, glass microplates are used **(Must)**; however lab may use polystyrene, if they can demonstrate that the test chemical does not sorb to polystyrene more than glass in side-by-side comparisons	☐	☐	☐
	If volatility expected, test conc. isolated from one another by using separate plates or polyester seals **(Must)**	☐	☐	☐
Test Volume	≥ 3 mL for each sample conc.; more required if chemical analysis of test concentrations is desired**(Must)**	☐	☐	☐
	Final volume in each well is 220 µL **(Must)**; with 200 µL of sample, 10 µL of enrichment medium and 10 µL of algal inoculum	☐	☐	☐
Renewal of Solution	None	☐	☐	☐
Dilution/Control Water	Reagent water (Millipore Super Q™ water or equivalent), receiving water, groundwater, surface water or reconstituted water;	☐	☐	☐
	All field-collected control/dilution water is filtered through 0.45 µm filter before use to reduce algal contamination **(Must)**	☐	☐	☐
	Same water used for preparing control and test solutions **(Must)**	☐	☐	☐
	If upstream water is used as control/dilution water, a separate set of replicate control solutions is to be prepared using reagent water **(Must)**	☐	☐	☐

Parameter	Specification	Met Specifics? Y	N	NA
Enrichment Medium . .	Stored in dark at 4˚C in a closed inert container **(Must)**	☐	☐	☐
	Can be stored up to 6 months	☐	☐	☐
	If a metal substance or metal mixture is being tested, the final amount of $Na_2EDTA \cdot 2H_2O$ is reduced by 25% for a final concentration of 46.9 µg/L in test medium **(Must)**	☐	☐	☐
Algal Inoculum	Inoculum consists of *P. subcapitata* cells harvested from a liquid stock algal culture that is 3-7 d old and in logarithmic growth phase as demonstrated from growth curves **(Must)**	☐	☐	☐
	Prepared ≤ 2-3 h before incubation of microplate **(Must)**	☐	☐	☐
	Harvested cells centrifuged at 2000g for 15 min, supernatant discarded and cells resuspended in 5 to 10 mL of bicarbonate solution (may be prepared by diluting stock solution #5); determine cell concentration and dilute such that initial cell concentration is 10 000 cells/mL**(Must)**	☐	☐	☐
	Algal inoculum final solution is 220 000 ± 22 000 cells/mL	☐	☐	☐
	Algal cells used for testing are not obtained from the first stock culture derived from a solid-phase (agar slant) starter culture	☐	☐	☐
Vessel Identification . .	Microplate labeled to identify test sample, conc., date and time of test	☐	☐	☐
	Test solutions are dispensed to wells in a predetermined pattern	☐	☐	☐
# Test Conc.	≥7 plus control to calculate ICp **(Must)**; ≥10 are recommended	☐	☐	☐
	For tests with wastewater and receiving water, actual concentrations are lower due to dilution with nutrient spike and algal inoculum; actual (calculated) concentration must be used in endpoint calculations and reporting **(Must)**	☐	☐	☐
# Replicates/Conc. . . .	4 (for 2 controls) or 5 (for 1 control) per test conc. set up.; ≥3 per test conc. are enumerated; 10 for standard control(s) and any additional dilution water (if used); 2 of the 10 standard control wells are used to measure pH and the remaining 8 for cell enumeration **(Must)**	☐	☐	☐
# Algal Cells/Well	2200 cells per well (initial cell density of 10 000 ± 1000 cells/mL)**(Must)** . . .	☐	☐	☐
Dispensing Solutions	Multi-channel pipette used to dispense 220 µL of reagent water to 36 peripheral wells (these wells excluded from test due to "edge-effect")	☐	☐	☐
	Dispense 200 µL of lowest test conc. (highest dilution) first and highest conc. last to appropriate wells; dispense 200 µL of reagent water to D2 to D11 wells; add 20 µL of mixture containing equal volumes of algal inoculum and enrichment medium to each well of microplate (except peripheral wells) . . .	☐	☐	☐
	An additional row (e.g., row E) containing control/dilution water is used when control/dilution water is not reagent water **(Must)**	☐	☐	☐
Microplate Incubation	Microplates placed in plastic bags and sealed	☐	☐	☐
	Microplates placed in an incubator or environmental chamber **(Must)**	☐	☐	☐
	Microplates distributed randomly throughout the incubator	☐	☐	☐
Vessel Cleaning	Reusable glassware is cleaned and treated to remove trace metals and organics **(Must)**	☐	☐	☐
	Reusable equipment made of any material other than glass also be washed by the recommended method if it can withstand the treatment **(Must)**	☐	☐	☐
Substance Testing . . .	Containers sealed and labeled with chemical name, supplier, date received and grade or purity **(Must)**	☐	☐	☐
	If solubilizing agent used, additional controls (containing highest conc. of the solubilizing agent) be run **(Must)**	☐	☐	☐
	Stock solutions are not filtered **(Must)**; reagent water may be filtered prior to preparation of chemical stock solutions	☐	☐	☐
Biological Endpoints . .	Growth inhibition of algae; based on algal cell yield (subtract initial cell concentration (~10 000 cells/mL) from final cell concentration)	☐	☐	☐

Parameter	Specification	Met Specifics? Y	N	NA
Statistical Endpoints . .	Mean (± SD) cell yield and corresponding CV for each test concentration and control are calculated **(Must)**	☐	☐	☐
	Cell yield in the standard control wells of each sample microplate (ie: D2 to D5 and D8 to D11) be compared statistically using trend analysis *(Mann-Kendall test*) to check that there is no gradient of effect **(Must)**	☐	☐	☐
	Any trend indicates volatile contaminants; test is to be repeated using one microplate per test conc. or using a polyester film which seals the individuals wells **(Must)**	☐	☐	☐
	Strongly recommended that the average cell yield in the standard control wells on each microplate be compared with that obtained for the standard control wells in another test (eg: reference toxicant) using identical conditions and procedures. Test to be repeated if outside ± 2 SD, using one microplate per test conc.	☐	☐	☐
	If microplate includes both a standard reagent control and sample control, a statistical comparison for significant differences of means performed **(Must)**	☐	☐	☐
	For multi-concentration test an ICp based on algal cell yield (with 95% confidence limits) is calculated **(Must)**	☐	☐	☐
	ICp results always be reported with the exposure duration (72 h) and expressed in % v/v for wastewater samples (corrected for vol. of enrichment media and algal inoculum) and in µg/L or mg/L for chemicals **(Must)**	☐	☐	☐
Calculation of ICp	Calculation of ICp by entering conc. as logarithms **(Must)**	☐	☐	☐
	Initial plot of raw data (cell yield) against the log. conc.	☐	☐	☐
	Regression analysis used for calculation of ICp & 95% confidence limits **(Must)**	☐	☐	☐
	All requirements for regression analysis outlined in Section 4.6.2 of 2nd ed. EPS 1/RM/25 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 . . .	☐	☐	☐
	For hormesis data analyzed by ICPIN, control responses are entered for those concentrations demonstrating hormesis **(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 4.6.3 of 2nd ed. EPS 1/RM/25**(Must)**	☐	☐	☐
Observations & Measurements				
pH	Measured in one standard control well per microplate at the start (t = 0 h) and end (t = 72 h) of the test (eg: median wells D6 and D7), using a microprobe or pH measurement paper. The pH at t = 72 h made before the algal cells are resuspended. Algal counts not made for these 2 wells	☐	☐	☐
	Test to be repeated if the pH difference between these 2 readings (t = 72h and t = 0h) differ by more than 1.5 pH units	☐	☐	☐
T°	Constant and monitored continuously in incubator throughout the test **(Must)**	☐	☐	☐
Visual Observations . .	Record any presence of condensation on lid or in bag and describe location of condensation at test end	☐	☐	☐
	Visually examine plate (at test end) for algal/bacterial growth and record observations	☐	☐	☐
Enumeration	Enumerate the cells in the remaining 8 standard control wells (D2 to D5 and D8 to D11) and in at least 3 wells containing each test conc., and also, if it applies, in each of the sample control wells	☐	☐	☐

Parameter	Specification	Met Specifics? Y	N	NA
Enumeration (continued)	If enumeration results from 3 replicates of test conc. are inconsistent (i.e., high variation), counts are completed of additional replicates **(Must)**	☐	☐	☐
	Less than 7 concentrations are enumerated only if :(i) cell counts of lower test conc. show that a large effect (e.g., > IC50) has been reached, then counts at higher test conc.are not required; (ii) cell counts show that there is no effect, then only 6 concentrations are required for enumeration and the highest test concentration must be enumerated **(Must)**	☐	☐	☐
Manual Enumeration	Algal cells resuspended in each well prior to subsampling or counting **(Must)**	☐	☐	☐
	Algal cells counted using a hemocytometer	☐	☐	☐
	Manual enumeration is performed on the same day the test is terminated; microplates are not stored at 4°C **(Must)**	☐	☐	☐
Electronic Enumeration	Electronic particle counter calibrated according to SOP **(Must)**	☐	☐	☐
	Operative aperture diameter of 70 µm recommended	☐	☐	☐
	170 µL from each well withdrawn and diluted with isotonic diluent	☐	☐	☐
	Counted 1-3 times, ideally immediately after isotonic solution added- (alternatively cap and store in dark at 4˚C for up to 24h after adding isotonic solution, then resuspend and count)	☐	☐	☐
Photometry	Absorbance and fluorescence measurements may also be used as a surrogate after it is established that there is a consistent, quantifiable, and reliable relationship with cell yield **(Must)**	☐	☐	☐
	Additionally, absorbance or fluorescence measurements may only be performed with sample if the test solutions containing algal inocula in microplates have been centrifuged and if the algal cells are then resuspended in a clear solution before deriving the endpoints **(Must)**	☐	☐	☐
	For ≥ 3 wells of test solutions (high, medium & low cell densities), concurrent counts made using electronic particle counter or hemocytometer **(Must)**	☐	☐	☐
	Results for these 3 direct counts compared to the estimates of cell density obtained by the photometry method for the same wells **(Must)**	☐	☐	☐
	Direct counts of cell density within the expected variation (± 2 SD) for the respective points on the standard calibration curve (absorbance vs cell conc.); if not, algal counts for each wells determined only by hemocytometer or electronic particle counter	☐	☐	☐
	Before measuring absorbance (wavelength 430 nm), algal cells in each well resuspended in reagent water **(Must)**	☐	☐	☐
Test Organism				
Species	*Pseudokirchneriella subcapitata*	☐	☐	☐
	Strains ATCC 22662, UTEX 1648, or UTCC37 recommended	☐	☐	☐
	Identified to species by microscopic examination; confirmed by an algal taxonomist **(Must)**	☐	☐	☐
Source	Commercial biological supply house	☐	☐	☐
Age	Algal culture between 3 and 7 days old and in exponential growth phase (culture very green and cell conc. ~ 2 x 10^6 - 3 x 10^6 cells/mL)	☐	☐	☐
Health Criteria	Alga uncontaminated with other species of algae or microorganisms **(Must)**	☐	☐	☐
	Alga in an exponential growth phase **(Must)**	☐	☐	☐
	Culture health and performance evaluated by periodically measuring rate of growth and relative sensitivity to reference toxicant **(Must)**	☐	☐	☐
	An algal growth curve, starting with an inoculum from the algal stock culture, be determined over an 8 to 10 d period using an Erlenmeyer flask **(Must)**	☐	☐	☐
	An algal growth curve is to be performed ≥2 X/year **(Must)**; recommend ≥4	☐	☐	☐
	If algal testing is performed throughout the year, preparation of growth curves should be separated by 5-6 months; if algal testing is seasonal (i.e., during summer months only), growth curves should be conducted at the beginning and end of the testing period for that year	☐	☐	☐

Parameter	Specification	Met Specifics? Y	N	NA
Culture/Holding Conditions				
Starter Culture	Agar slant, liquid culture, or frozen in an ampule as a dried pellet	☐	☐	☐
	Stored in the dark at 4 ˚C and remain viable for at least 6 months	☐	☐	☐
	Every 12 months a new culture is purchased **(Must)**	☐	☐	☐
	Aseptically transferred to, and resuspended in, a defined growth medium to maintain a stock culture as a source for toxicity tests **(Must)**	☐	☐	☐
	A new algal culture for toxicity testing is set up from the "starter" culture every 2 months **(Must)**	☐	☐	☐
Stock Culture . . . . . (Liquid or Solid)	Routine microscopic examination of stock algal culture to evaluate culture health (cell morphology, colour, clumping, and contamination)	☐	☐	☐
Liquid Growth Medium for Stock Culture	Volume-to-flasks ratio of 20% (eg: 25 mL medium in a 125 mL flask; 50 mL medium in a 250 mL flask)	☐	☐	☐
	Aseptically transfer an inoculum of starter culture using a disposable sterile pipette or a group of cells using a sterile loop to liquid growth medium	☐	☐	☐
	Incubate at 24 ± 2˚C; continuous light ("cool white" fluorescent; 4000 lux at surface); continuous shaker at 100 rpm or shaken manually 2 x daily	☐	☐	☐
	Culture renewed weekly to ensure regular supply of exponentially growing algal cells by aseptically transferring 1.0 mL of stock algal culture (3 to 7 days post inoculation) to clean growth medium	☐	☐	☐
	Purity verified at each transfer by microscopic examination and by transferring an inoculum of algal stock culture to a petri-dish containing solid bacterial nutrient medium and incubating at 24˚C for 48h (to reveal presence of contaminating bacteria undetectable by microscope) **(Must)**	☐	☐	☐
Solid Growth Medium for Stock Culture	Algal cells aseptically transferred from liquid culture to sterile solid growth medium using streak-plate procedures	☐	☐	☐
	Plates incubated upside down under conditions which match culturing conditions (i.e., 24 ± 2°C under continuous "cool white" fluorescent light with an intensity of 4000 lux; no agitation necessary) until colonies are visible (~ 2 weeks)	☐	☐	☐
	Stored at 4˚C in dark, cells remain viable for up to 3 months	☐	☐	☐
Aeration	No aeration for any liquid algal cultures since axenic cultures	☐	☐	☐

Parameter	Specification	Met Specifics? Y	N	NA
QA/QC				
Test Validity Criteria . .	Test is valid if:			
	• Homogeneity demonstrated for the standard control wells, among the measurements or photometric estimates of cell yield (C.V.≤ 20%) **(Must)**	☐	☐	☐
	• The C.V. is ≥10% but <20% in standard controls; application of trend analyses (*Mann-Kendall test*) to estimates of cell yield in the standard control wells indicate that no trend or gradient in algal cell conc. is present in the control treatment; and **(Must)**	☐	☐	☐
	• Number of algal cells measured or estimated (if photometry used) for the standard controls have increased by a factor of more than 16 in 72h **(Must)**	☐	☐	☐
Reference Toxicant . .	One or more of the following 3 chemicals: copper sulphate, zinc sulphate or phenol	☐	☐	☐
	Start within 14 d of the toxicity test period and under the same experimental conditions as those used with the test sample(s) **(Must)**	☐	☐	☐
	The same batch of organisms be used for tests on both the reference toxicant and the sample	☐	☐	☐
	Reagent water routinely used in algal toxicity test to be used as control/dilution water in reference toxicant tests	☐	☐	☐
Warning Chart	Prepared and updated for each reference toxicant used **(Must)**	☐	☐	☐
	Results within acceptable warning limits (± 2 SD on log scale)	☐	☐	☐
	Log concentrations used for mean & SD calculations and plotting **(Must)** . .	☐	☐	☐
Quality Control Microplate	A quality control microplate (220 µL of reagent water in peripheral wells and 200 µL of reagent water, 10 µL of algal inoculum and 10 µL of enrichment medium in the other wells) incubated to provide a standard for appraising algal growth under the test conditions and also to monitor the pH inside the wells	☐	☐	☐
Sample Handling				
Containers	Non-toxic materials for sample and transport containers, new containers or thoroughly cleaned used containers **(Must)**	☐	☐	☐
	Container be rinsed with the sample to be collected, and then filled to the brim and sealed	☐	☐	☐
Volume	1L	☐	☐	☐
Labeling	Include at least sample type, source, date and time of collection and name of sample collectors	☐	☐	☐
	Chain of custody during sample collection, transport, and storage recorded .	☐	☐	☐
Holding Time	Test to be initiated within 3 days after sampling (or following preparation of elutriate) **(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)**	☐	☐	☐
	Samples or portions of samples to be stored for subsequent use be held in sealed containers without air headspace, in the dark at 4 ± 2°C **(Must)**	☐	☐	☐

Parameter	Specification	Met Specifics? Y	N	NA
Test Report				
Sample Data	Brief description of sample type if and as provided to the lab **(Must)**	☐	☐	☐
	Information on labelling or coding of sample **(Must)**	☐	☐	☐
	Date of sample collection; date and time of sample received at lab **(Must)**	☐	☐	☐
	For effluent or leachate, T° of sample upon receipt at lab **(Must)**	☐	☐	☐
	pH of sample just before its preparation and use in toxicity test **(Must)**	☐	☐	☐
	For elutriate, dates for sample generation and use **(Must)**	☐	☐	☐
Test Organism	Species, strain number, and origin of culture **(Must)**	☐	☐	☐
	Age of known-age culture used to provide inocula of test organisms, at the start of the test **(Must)**	☐	☐	☐
	Any unusual appearance or treatment of known-age culture, before its use in the test **(Must)**	☐	☐	☐
Test Facilities	Name and address of test laboratory **(Must)**	☐	☐	☐
	Name of person(s) performing the test **(Must)**	☐	☐	☐
Control/Dilution Water	Type and source of water used as control and dilution water **(Must)**	☐	☐	☐
	Type/quantity of any chemical(s) added to control or dilution water **(Must)**	☐	☐	☐
Test Method	Citation of biological test method used **(Must)**	☐	☐	☐
	Design and description if specialized procedure **(Must)**	☐	☐	☐
	Program(s) 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)**	☐	☐	☐
	Mean test temperature **(Must)**	☐	☐	☐
	Number and concentration of test solutions **(Must)**	☐	☐	☐
	Number of replicate test wells per treatment (including controls) **(Must)**	☐	☐	☐
	Initial cell density of the algal inoculum **(Must)**	☐	☐	☐
	Statement and description (ie: procedure, rate, and duration) if any aeration of sample or test solutions before starting the test **(Must)**	☐	☐	☐
	Description of procedure for sample filtration **(Must)**	☐	☐	☐
	Brief description of any sample or test solutions receiving pH adjustment, and/or hardness adjustment, including procedure(s) **(Must)**	☐	☐	☐
	pH of sample before any dilution, at the start of the test **(Must)**	☐	☐	☐
	pH of the two median control wells at start and end of test **(Must)**	☐	☐	☐
	Dates when test was started and ended; duration of test **(Must)**	☐	☐	☐
Test Results	Cell concentration in each replicate (including controls) at test end **(Must)**	☐	☐	☐
	If absorbance is used, cell concentration (direct count) in the three wells containing high/medium/low test concentrations, and their corresponding values estimated using the absorbance method **(Must)**	☐	☐	☐
	Mean cell (± SD) cell yield at 72h for each treatment (including controls), with corresponding CV (CV = 100 X standard deviation/mean) **(Must)**	☐	☐	☐
	ICp (with its 95% confidence limits) using concentrations corrected for the volume of algal inoculum and enrichment media; details regarding any weighting techniques applied to the data; and indication of quantitative method used **(Must)**	☐	☐	☐
	Any outliers and the justification for their removal **(Must)**	☐	☐	☐
	Details regarding any statistical transformation of data required **(Must)**	☐	☐	☐
	ICp and 95% confidence limits for any toxicity tests with the reference toxicant(s) performed within one month of the test, together with the geometric mean value (± 2 SD) for the same reference toxicant(s) as derived at the test facility in previous tests **(Must)**	☐	☐	☐
	Any findings of growth 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 are signed or initialed, and dated by the laboratory personnel conducting the tests **(Must)**	☐	☐	☐

Parameter	Specification	Met Specifics? Y	N	NA
Info Kept On-File	Do lab SOPs indicate that the information on Section 8.2 of the 2nd edition of EPS 1/RM/25 method must be kept on file for 5 years? **(Must)** . . . . . For details of this information, see the 2nd edition of EPS 1/RM/25, Section 8.2.	☐	☐	☐