This checklist is a summary of the requirements and recommendations in the Environment and Climate Change Canada supporting guidance document. 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 guidance document, the guidance document is taken as the definitive source.

Y= Yes, meets requirements; N= No, does not meet requirements; NA= not applicable
DO = dissolved oxygen; temp = temperature; conc = concentration(s); h = hour(s); min – minutes; psi = pounds per square inch (of force); pH i = pH as measured on composite 100% sample at 15°C before any aeration of the test solutions

TEST SPECIFIC CHECKLIST

Procedure for pH Stabilization During the Testing of Acute Lethality of Wastewater Effluent to Rainbow Trout

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
General							
Purpose	pH stabilization techniques are add-on procedures used in conjunction with EPS 1/RM/13 on samples of wastewater effluent **(must)**	☐	☐	☐	☐	☐	☐
Conditions for Use	All three of the following conditions are met before any pH stabilization procedures are used **(must)**:	☐	☐	☐	☐	☐	☐
	1) Conc of total ammonia (mg/L) is measured on wastewater effluent sample and used in the calculation of un-ionized ammonia at the initial pH (pH i) of the effluent at 15°C **(must)**	☐	☐	☐	☐	☐	☐
	2) Wastewater effluent sample previously collected from the same source failed (i.e., > 50% mortality) the rainbow trout acute lethality test (EPS 1/RM/13) **(must)**	☐	☐	☐	☐	☐	☐
	3) Un-ionized ammonia conc in 100% wastewater is <1.25 mg/L at 15°C, or total ammonia conc is < maximum total ammonia conc (y) in mg/L determined using the following formula at the initial pH of the wastewater effluent sample at 15°C: $y = 1.25 \times (10^{(9.564136638-pH)} + 1)$ **(must)**	☐	☐	☐	☐	☐	☐
pH Stabilization Methods	One of three techniques for pH stabilization is used to control the pH of the sample at the level measured at test initiation (pH i): (1) CO_2 Injection, (2) Recycling, or (3) pH Controller **(must)**	☐	☐	☐	☐	☐	☐
Total Ammonia	Measured (in mg/L) on all wastewater effluent samples submitted for toxicity testing using EPS 1/RM/13 **(must)**	☐	☐	☐	☐	☐	☐
Un-ionized Ammonia	Given that "total ammonia" = $NH_3 + NH_4^+$, un-ionized ammonia is calculated using the following formula **(must)**: un-ionized ammonia = (total ammonia) x $[1/(1 + 10^{pK - pH})]$ where: - pK = 9.56 at 15°C - pH is the initial pH (pH i) of the wastewater effluent at 15°C - total ammonia is in mg/L as measured for Condition #1, described above	☐	☐	☐	☐	☐	☐
Sample Preparation	All solutions are prepared before aeration is started **(must)**	☐	☐	☐	☐	☐	☐
	Stabilization of pH starts when pre-aeration is initiated **(must)**	☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Procedure for pH Stabilization During the Testing of Acute Lethality of Wastewater Effluent to Rainbow Trout

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Pre-aeration (before exposure of fish, as per EPS	Upon preparation, all test solutions and controls are pre-aerated for 30 min at a rate of 6.5 ± 1 mL/min·L **(must)**	☐	☐	☐	☐	☐	☐
	Second period of pre-aeration if DO (measured after initial 30 min pre-aeration) in highest test conc (normally 100% effluent) is < 70% or >100%; pre-aeration of all solutions including control(s) is continued at 6.5 ± 1 mL/min·L until DO is 70 - 100% or 90 additional min, whichever is shorter **(must)**	☐	☐	☐	☐	☐	☐
	Fish are randomly placed in test solutions and test initiated immediately after pre-aeration regardless of whether 70 - 100% aeration achieved **(must)**	☐	☐	☐	☐	☐	☐
Air Delivery	Clean air stones are used for delivery of CO_2 mix for CO_2 Injection technique and for delivery of laboratory air in Recycling and pH Controller techniques **(must)**	☐	☐	☐	☐	☐	☐
	Glass pipette is used for delivery of CO_2 gas in pH Controller technique (highly recommended)	☐	☐	☐	☐	☐	☐
Test Conditions							
Aeration	Oil-free compressed laboratory air at a controlled rate of 6.5 ± 1 mL/min·L throughout test period **(must)**	☐	☐	☐	☐	☐	☐
Vessel Size & Type	Glass aquaria or non-toxic containers; glass aquaria recommended for Recycling technique	☐	☐	☐	☐	☐	☐
pH	pH of each effluent conc (i.e., 100, 50, 25...) is maintained at the pH value measured at test initiation (before any aeration is started) in each individual exposure conc and the control **(must)**	☐	☐	☐	☐	☐	☐
Results	LC50 is not calculated if there is a non-dose related response that may be due to a gradient of pH values observed during testing across concs; 100% wastewater effluent sample are still acceptable if other validity criteria are met	☐	☐	☐	☐	☐	☐
Observations & Measurements							
pH, Total Ammonia, Hardness	pH, total ammonia, and hardness are measured in each full strength effluent sample after sub-samples (aliquots of a sample divided between two or more containers) have been combined, thoroughly mixed, and adjusted to 15 ± 1°C (and in each test solution for multi-conc tests) **(must)**	☐	☐	☐	☐	☐	☐
Un-ionized Ammonia	Total ammonia is measured to at least two decimal places **(must)**	☐	☐	☐	☐	☐	☐
	Un-ionized ammonia conc is calculated using total ammonia measurement at 15 °C and pH i of the sample before any aeration of test solutions **(must)**	☐	☐	☐	☐	☐	☐
	pH stabilization technique is not used if this conc of un-ionized ammonia is ≥1.25 mg/L **(must)**	☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Procedure for pH Stabilization During the Testing of Acute Lethality of Wastewater Effluent to Rainbow Trout

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Alkalinity	For CO_2 Injection technique, alkalinity is measured 100% wastewater only, after sub-samples have been combined, thoroughly mixed and adjusted to 15 ± 1°C **(must)**	☐	☐	☐	☐	☐	☐
Total Residual Chlorine	Total residual chlorine is measured in each effluent sample received	☐	☐	☐	☐	☐	☐
	Total residual chlorine is measured if fish display stressed or atypical behaviour **(must)**	☐	☐	☐	☐	☐	☐
	If total residual chlorine >0.1 mg/L, pH stabilization is not used	☐	☐	☐	☐	☐	☐
Validity Criteria	A test is considered invalid if any of the following occur **(must)**:	☐	☐	☐	☐	☐	☐
	1) The average pH in pH stabilized 100% wastewater effluent test solution shifts more than ± 0.2 units from pH i **(must)**	☐	☐	☐	☐	☐	☐
	2) The instantaneous pH in the pH stabilized 100% wastewater effluent test solution is greater than ± 0.3 units from pH i **(must)**	☐	☐	☐	☐	☐	☐
	3) If >10% of the fish (combined data if replicates are used) in the pH stabilized control die or exhibit atypical or stressed behaviour **(must)**	☐	☐	☐	☐	☐	☐
	For a multi-conc test, the calculation of the LC50 does not include any exposure conc where the pH validity criteria were not met **(must)**	☐	☐	☐	☐	☐	☐
CO_2 Injection Technique							
pH Control	Upward drift of pH is controlled by aerating wastewater test solutions (including control) using a mixture of 15% CO_2, 21% oxygen (O_2) and 64% nitrogen (N_2)(referred to as CO_2 mix) blended with a source of lab air **(must)**	☐	☐	☐	☐	☐	☐
Setup for Delivery of CO_2 Mixture	Apparatus for CO_2 stabilization is set up as described in Section 2.2 and Figures 1 to 5 in EPS 1/RM/50; i.e., CO_2 is delivered to test vessels from a compressed gas cylinder containing CO_2 mix, via gas cylinder regulator, flexible Tygon® air tubing, through a 4-way gang valve, through a flow meter, and combined with the normal lab airflow (delivered via Tygon® tubing through another flow meter) using "Y" plastic connector, and into the test solution via an air stone	☐	☐	☐	☐	☐	☐
	Cylinder containing CO_2 mix is securely attached near exposure vessels	☐	☐	☐	☐	☐	☐
	For a multi-conc test (i.e., 5 test concs, plus control), 6 flow meters with a 0 to 137 mL/min flow rate, and 6 flow meters with a 0 to 300 mL/min flow rate are used **(must)**	☐	☐	☐	☐	☐	☐
	All flow meters have adjustable valves	☐	☐	☐	☐	☐	☐
	Each conc has 2 flow meters, 1 for the CO_2 mix and 1 for lab air	☐	☐	☐	☐	☐	☐
	Oil or grease is not used on any regulator or cylinder fittings **(must)**	☐	☐	☐	☐	☐	☐
% CO_2 for pH Stabilization	Initial % CO_2 mixture required to stabilize pH is based on the measured pH i (of each test conc for a multi-conc test) and alkalinity of 100% test solution **(must)**	☐	☐	☐	☐	☐	☐
	CO_2 Calibration Table (Table 1 in EPS 1/RM/50) is used to estimate the % CO_2 that is applied for a given pH i and alkalinity, to provide pH control	☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Procedure for pH Stabilization During the Testing of Acute Lethality of Wastewater Effluent to Rainbow Trout

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
	% CO_2 is adjusted by 0.5% increments if there is an upward or downward trend in pH after initiation of aeration with the CO_2 mixture (pH measured within the first 30 min of aeration); CO_2 increased if there is an upward trend in pH, and decreased if there is a downward trend	☐	☐	☐	☐	☐	☐
Flow Rates	Total aeration rate (CO_2 and lab air) is 6.5 ± 1 mL/min · L throughout test in all exposures and control **(must)**	☐	☐	☐	☐	☐	☐
	Adjusted using adjustable valves on flow meters, based on test solution volume and require final % CO_2	☐	☐	☐	☐	☐	☐
	Flow rates for CO_2 and lab air determined using following equations: ***(1)*** combined flow to test vessel (mL/min) = 6.5 mL/min·L x test volume (L) ***(2)*** flow rate of CO_2 mix = $\frac{\text{required \% } CO_2}{\% CO_2 \text{ in mix (15\%)}}$ x combined flow to vessel ***(1)*** ***(3)*** flow of lab air = combined flow to vessel ***(1)*** - flow rate of CO_2 mix ***(2)***	☐	☐	☐	☐	☐	☐
pH Measurements	Frequent pH measurements and adjustments to flow of CO_2 mix are made during first three hours of the test to stabilize pH **(must)**	☐	☐	☐	☐	☐	☐
	pH is measured and recorded immediately before any aeration (pH i), at t = 0 h (test start, when fish are introduced), and at t = 0.5, 1, 2, 3, 24, 48, 72, and 96 h in all exposure concs and control **(must)**	☐	☐	☐	☐	☐	☐
	pH is also measured and recorded with any adjustment to CO_2 flow and a subsequent pH reading taken within 30 min after the adjustment **(must)**	☐	☐	☐	☐	☐	☐
	Final pH is recorded if there is 100% mortality in a test conc before test end **(must)**	☐	☐	☐	☐	☐	☐
Operating Check	Air line tubing is inspected at least once daily to ensure continual delivery of CO_2 mixture and lab air to all test solutions **(must)**	☐	☐	☐	☐	☐	☐
Recycling Technique							
pH Control	Upward drift of pH is controlled by recycling CO_2 in a closed system; i.e., test vessels sealed with lids, and air, containing CO_2, re-circulated in headspace, preventing loss of CO_2 and maintaining pH **(must)**	☐	☐	☐	☐	☐	☐
	Recycle technique may accentuate decline in DO for samples with high BOD	☐	☐	☐	☐	☐	☐
Setup for Recycling Technique	Recycling apparatus is set up as described in Section 2.3.1 and Figures 6 and 7 of EPS 1/RM/50; i.e., closed system with specially fabricated “Recycle” lid; aeration pump that generates 6.5 ± 1 mL/min · L; catch flask to prevent condensate from entering flow meter; flow meter; Tygon® tubing; air stone; and siphoning tube for removing samples for observation and physicochemical measurements	☐	☐	☐	☐	☐	☐
	Test containers filled to very top with sample to reduce headspace	☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Procedure for pH Stabilization During the Testing of Acute Lethality of Wastewater Effluent to Rainbow Trout

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Setup for Recycling Technique (cont)	Recycle lid is secured and tightly sealed to the top of the test container by fastening all O-rings and elastics	☐	☐	☐	☐	☐	☐
	pH stabilization begins in tanks sealed with Recycling lid, during pre-aeration when pump is started and flow meter adjusted	☐	☐	☐	☐	☐	☐
	After pre-aeration, fish are added and system is re-sealed	☐	☐	☐	☐	☐	☐
Flow Rates	Aeration rate is 6.5 ± 1 mL/min · L throughout test in all exposures and control **(must)**	☐	☐	☐	☐	☐	☐
pH Measurements	pH is measured and recorded immediately before any aeration (pH i), at t = 0 h (test start, when fish are introduced), and at t = 24, 48, 72, and 96 h in all exposure concs and control **(must)**; pH measurements at t = 0.5, 1, and 2 h are also recommended	☐	☐	☐	☐	☐	☐
	pH is also measured and recorded any time the test container is opened **(must)**	☐	☐	☐	☐	☐	☐
	Final pH is recorded if there is 100% mortality in a test conc before test end **(must)**	☐	☐	☐	☐	☐	☐
	Care is taken when using the siphoning tube for sampling to avoid loss of sample **(must)**	☐	☐	☐	☐	☐	☐
	Dead fish are removed every 24 h, quickly to prevent pH drift **(must)**	☐	☐	☐	☐	☐	☐
Operating Check	Visual checks are made at least once daily to ensure air lines, pumps, and flow meters are working properly **(must)**	☐	☐	☐	☐	☐	☐
pH Controller Technique							
pH Control	Upward drift of pH is controlled by aerating wastewater test solutions (including control) using pure CO_2 (or a mixture of 15% CO_2, 21% O_2, and 64% N_2) with separate lines for lab air addition; CO_2 addition is regulated by a controller that is triggered by a drift in pH above a programmed set point and shut off when pH returns to the acceptable limit **(must)**	☐	☐	☐	☐	☐	☐
Setup for pH Controller Technique	Apparatus for Controller Technique is set up as described in Section 2.4.1 and Figures 8 to 13 of EPS 1/RM/50; i.e., CO_2 is delivered to test vessels from a compressed gas cylinder containing CO_2, via gas cylinder regulators, and individual pressure regulators with needle valve assemblies, connected to the gauge assembly (manifold); solenoids, one for each exposure conc, are used to control the flow of CO_2; and pH controllers, one for each exposure conc, to monitor and regulate CO_2 delivery through backflow valves and glass pipettes	☐	☐	☐	☐	☐	☐
	Oil or grease is not used on any regulator or cylinder fittings **(must)**	☐	☐	☐	☐	☐	☐
	All solenoids are turned off before the valve on the CO_2 cylinder is opened **(must)**	☐	☐	☐	☐	☐	☐
	Valve on CO_2 cylinder is opened and pressure adjusted to 40 psi	☐	☐	☐	☐	☐	☐
	Working pressure on solenoid is adjusted to 20 psi (i.e., solenoid regulator gauge reads 20 psi)	☐	☐	☐	☐	☐	☐
	Connections are tested for leaks	☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Procedure for pH Stabilization During the Testing of Acute Lethality of Wastewater Effluent to Rainbow Trout

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Setup for pH Controller Technique (cont)	pH Controller is calibrated daily using certified pH standards **(must)**	☐	☐	☐	☐	☐	☐
	Sensitivity of pH Controller is set before test initiation (± 0.2 pH units) **(must)**	☐	☐	☐	☐	☐	☐
	CO_2 tubing is removed from the exposure solution during calibration **(must)**	☐	☐	☐	☐	☐	☐
	Meter calibration is completed rapidly to prevent pH drift **(must)**	☐	☐	☐	☐	☐	☐
	Instructions for calibration and maintenance provided by manufacturer are reviewed before test initiation	☐	☐	☐	☐	☐	☐
	One pH probe and controller is used for each test solution for test duration	☐	☐	☐	☐	☐	☐
	Probe is secured 3-5 cm below the surface of the test solution	☐	☐	☐	☐	☐	☐
	CO_2 delivery pipette is directly beneath the pH probe (for accurate pH control)	☐	☐	☐	☐	☐	☐
	Back-flow is prevented using spring-loaded (stainless steel) back-flow check valves	☐	☐	☐	☐	☐	☐
	Durable pH probes are used to reduce risk of electrode-filling solution (e.g., KCl) leaks	☐	☐	☐	☐	☐	☐
	Frequent pH measurements and adjustment of CO_2 flow (i.e., to ensure stabilization of pH) are carried out during first few hours of the test	☐	☐	☐	☐	☐	☐
	pH values on the controllers are closely monitored to ensure proper operation of the solenoids **(must)**; controller cycles on and off to control the flow of CO_2; if solenoid remains open, CO_2 flow is gradually increased until required pH value is reached and the solenoid closes	☐	☐	☐	☐	☐	☐
	pH stabilization begins during pre-aeration when CO_2 cylinder is opened	☐	☐	☐	☐	☐	☐
Flow Rates	Aeration rate for delivery of laboratory air (using oil-free compressed air) through air stone is 6.5 ± 1 mL/min · L throughout test in all exposures and control **(must)**; note that addition of CO_2 will slightly increase aeration rate when pH Controller cycles on	☐	☐	☐	☐	☐	☐
pH Measurements	pH is measured and recorded immediately before any aeration (pH i), at t = 0 h (test start, when fish are introduced), and at t = 24, 48, 72, and 96 h in all exposure concs and control **(must)**	☐	☐	☐	☐	☐	☐
	pH is also measured and recorded any time the CO_2 flow is manually adjusted and a subsequent pH reading taken within 30 min after the adjustment **(must)**	☐	☐	☐	☐	☐	☐
	Final pH is recorded if there is 100% mortality in a test conc before test end **(must)**	☐	☐	☐	☐	☐	☐
Operating Check	Visual checks are made at least once daily to ensure pH Controllers and air lines are working properly **(must)**	☐	☐	☐	☐	☐	☐

TEST SPECIFIC CHECKLIST

Procedure for pH Stabilization During the Testing of Acute Lethality of Wastewater Effluent to Rainbow Trout

Parameter	Specification	Document Review			Implementation		
		Y	N	NA	Y	N	NA
Test Report							
General	In addition to reporting requirements outlined in EPS 1/RM/13, the following information is reported when conducting a pH stabilized test with wastewater effluent **(must)**	☐	☐	☐	☐	☐	☐
	Type of pH stabilization technique used (pH Controller, Recycling, or CO_2 Injection) **(must)**	☐	☐	☐	☐	☐	☐
	pH i, total ammonia, and hardness; all measured in the 100% wastewater effluent sample, after all effluent to be used in testing has been composited, thoroughly mixed, and temp of the sample adjusted to 15 ± 1°C **(must)**	☐	☐	☐	☐	☐	☐
	Calculated un-ionized ammonia conc, based on the measurement of total ammonia, a temp of 15°C and the pH i of the 100% wastewater effluent sample **(must)**	☐	☐	☐	☐	☐	☐
	Total residual chlorine in the wastewater effluent sample, if fish display stressed or atypical behaviour at test initiation **(must)**	☐	☐	☐	☐	☐	☐
	For multi-conc tests, pH of the diluted effluent concs at the start of testing (before any aeration is started at 15°C) in each individual exposure conc **(must)**	☐	☐	☐	☐	☐	☐
	Average pH based on all readings taken during test **(must)**	☐	☐	☐	☐	☐	☐
For CO_2 Injection Technique	Percentage of CO_2 gas mix used during test **(must)**	☐	☐	☐	☐	☐	☐
	Alkalinity in the 100% wastewater effluent sample **(must)**	☐	☐	☐	☐	☐	☐
	pH readings taken at t = 0 h (test start, when fish are introduced) and at t = 0.5, 1, 2, 3, 24, 48, 72, and 96 h in the control and all exposure concs **(must)**	☐	☐	☐	☐	☐	☐
	Any additional pH readings taken during testing, or after adjustment of CO_2 gas mix **(must)**	☐	☐	☐	☐	☐	☐
For Recycling Technique	pH readings taken at t = 0 (test start, when fish are introduced) and at 24, 48, 72, and 96 h in the control and all exposure concs **(must)**	☐	☐	☐	☐	☐	☐
	Additional pH readings (if taken) at t = 0.5, 1 and 2 h **(must)**	☐	☐	☐	☐	☐	☐
	Any additional pH readings taken during testing, or after removal of Recycling lid **(must)**	☐	☐	☐	☐	☐	☐
For pH Controller Technique	Percentage of CO_2 gas mix or CO_2 used during test **(must)**	☐	☐	☐	☐	☐	☐
	pH readings taken at t = 0 (test start, when fish are introduced) and at 24, 48, 72, and 96 h in the control and all exposure concs **(must)**	☐	☐	☐	☐	☐	☐
	Any additional pH readings taken during testing, or after adjustment of CO_2 or CO_2 gas mix **(must)**	☐	☐	☐	☐	☐	☐

Notes: