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

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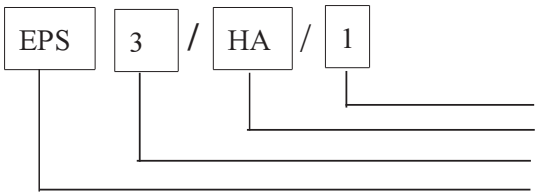

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Procedure for pH Stabilization During the Testing of Acute Lethality of Wastewater Effluent to Rainbow Trout

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Environmental Science and Technology Centre
Science and Technology Branch
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Abstract

This document provides detailed techniques, conditions, and guidance for the pH stabilization of wastewater effluent samples. The procedure described herein must be used in conjunction with the explicit instructions given in the reference method EPS 1/RM/13 "Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout" (Environment Canada, 2000). This procedure is not stand-alone; it is an add-on to the rainbow trout method for acute lethality.

In many wastewater effluent samples, the carbon dioxide (CO_2) content may be elevated as a result of high biological activity. Aeration of these samples may cause the pH to rise because of a loss of CO_2, and this change in pH can alter the toxicity of the ammonia present in the wastewater effluent sample. The purpose of pH stabilization is to replace the CO_2 lost due to aeration in order to maintain the pH throughout the test at the same levels found in the initial samples.

In order to use this add-on procedure, the wastewater effluent sample must meet three conditions: (i) total ammonia must be measured on all wastewater effluent samples submitted for testing with EPS 1/RM/13, (ii) the wastewater effluent must have failed an acute lethality test using rainbow trout (EPS 1/RM/13) on a previously collected sample, and (iii) pH stabilization techniques may only be used when the un-ionized ammonia concentration present in the 100% wastewater effluent sample does not equal or exceed 1.25 mg/L at 15°C or when the total ammonia concentration does not equal or exceed the maximum total ammonia concentration (y) in mg/L determined using the following formula and the initial pH of the wastewater effluent sample at 15°C:

$$y = 1.25 \times (10^{(9.564136638-pH)} + 1)$$

This procedure document describes three pH stabilization techniques that can be used as an add-on to EPS 1/RM/13: (i) CO_2 injection; (ii) Recycling; and (iii) pH Controller.

This pH stabilization procedure applies to both single-concentration tests and multi-concentration tests to determine the median lethal concentration (LC50). Instructions are included on the apparatus setup, observations and measurements to be made, and maintaining pH control throughout the test. Validity criteria for this add-on procedure are outlined, and these must be met in addition to those outlined in EPS 1/RM/13.

Résumé

Le présent document expose dans le détail des techniques, des conditions et des conseils permettant la stabilisation du pH d'échantillons d'effluent d'eau usée. Les modes opératoires décrits doivent être utilisés conjointement avec les consignes explicites données dans la méthode de référence SPE 1/RM/13 intitulée Méthode d'essai biologique : méthode de référence pour la détermination de la létalité aiguë d'effluents chez la truite arc-en-ciel *(Environnement Canada, 2000). Cette procédure n'est pas autonome ; elle est complémentaire à la méthode de la détermination de la létalité aiguë chez la truite arc-en-ciel.*

Dans beaucoup d'échantillons d'effluent d'eau usée, la teneur en dioxyde de carbone (CO_2) risque d'être élevée en raison d'une forte activité biologique. L'aération de ces échantillons peut provoquer l'augmentation du pH, celle-ci causée par la perte de CO_2, ce qui peut modifier la toxicité de l'ammoniac présent. La stabilisation du pH a pour but de remplacer le CO_2 perdu à cause de l'aération pour maintenir le pH durant l'essai aux valeurs où il se trouvait initialement dans les échantillons.

*Pour qu'on lui applique cette procédure complémentaire, l'échantillon doit satisfaire à trois conditions : (i) il faut doser l'ammoniac total de tous les échantillons d'effluent d'eau usée soumis à un essai par la méthode SPE 1/RM/13 ; (ii) un échantillon antérieur de l'effluent doit avoir échoué à un essai de létalité aiguë pour la truite arc-en-ciel (SPE 1/RM/13) ; (iii) les techniques de stabilisation du pH ne peuvent être utilisées que lorsque la concentration d'ammoniac non ionisé présent dans l'échantillon d'effluent d'eau usée non dilué est inférieure à 1,25 mg/L à 15 °C ou lorsque la concentration d'ammoniac total est inférieure à la concentration maximale d'ammoniac total (*y*) en mg/L déterminée à l'aide de la formule suivante et du pH initial de l'échantillon à 15 °C :*

$$y = 1{,}25 \times (10^{(9{,}564\,136\,638 - pH)} + 1)$$

Dans le présent document, on décrit trois techniques de stabilisation du pH utilisables en complément de la méthode SPE 1/RM/13 : (i) l'injection de CO_2 ; (ii) le recyclage ; (iii) la technique du pH mètre régulateur.

Cette procédure de stabilisation du pH s'applique aux essais à concentration unique comme aux essais à concentrations multiples visant à déterminer la concentration létale médiane (CL50). Elle comprend des instructions sur le montage de l'appareillage, les observations et mesures à effectuer et la régulation du pH durant l'essai. On expose brièvement les critères de validité de la procédure auxquels il faut satisfaire en plus de ceux qui sont exposés dans la méthode SPE 1/RM/13.

Foreword

The three techniques for stabilizing the pH of a wastewater effluent during an acute lethality test function as ***add-on procedures*** *to the reference method, EPS 1/RM/13; the technique chosen must be used in conjunction with this reference method for measuring and assessing the toxic effect(s) of wastewater effluent on rainbow trout. It may only be used when the test sample has met the three conditions outlined within this document; these conditions pertain to the measurement of total ammonia on all wastewater effluent samples submitted for toxicity testing, the failure of the reference method on a previously collected sample of wastewater effluent, and the amount of un-ionized ammonia in the sample of wastewater effluent to be tested.*

This ***add-on procedure*** *outlines explicit sets of instructions and conditions to be used with EPS 1/RM/13 and are applied only to wastewater effluent samples as defined in this document.*

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Terminology

The words defined in this section are italicized when first used in the body of the report according to the definition. All definitions are given in the context of the procedures in this report, and might not be appropriate in another context.

Grammatical Terms

Can is used to mean "is (are) able to."

May is used to mean "is (are) allowed to."

Must is used to express an absolute requirement.

Should is used to state that the specified condition or procedure is recommended and ought to be met if possible.

Technical Terms

Acute means happening within a short period of time (≤96-h for the rainbow trout acute lethality test).

Alkalinity means the acid-neutralizing capacity of water, reported as mg/L as calcium carbonate ($CaCO_3$) (see also APHA *et al.,* 2005).

Ammonia means total ammonia [$NH_3 + NH_4$, as nitrogen (N)], un-ionized ammonia (NH_3, as N) and ionized ammonia (NH_4^+, as N). The percentage of un-ionized ammonia (NH_3) in total ammonia is determined by pH and temperature. The following formulae are used to calculate the fraction of un-ionized (NH_3) and ionized (NH_4^+) ammonia. Since $NH_3 = 1/(1 + 10^{pK - pH})$ and $NH_4^+ = 1/(1 + 10^{pH - pK})$, and total ammonia = $NH_3 + NH_4^+$, the concentration of un-ionized ammonia (assuming a pK of 9.56 at 15 ºC) is calculated as: un-ionized ammonia = (total ammonia) × $[1/(1 + 10^{pK - pH})]$ (USEPA, 1999).

BOD means biological oxygen demand and refers to the amount of oxygen consumed when organic matter in a volume of water is biodegraded (see also APHA *et al.,* 2005).

Buffering capacity is the ability of water to maintain a stable pH which is controlled by the amount of carbonate ions (alkalinity) present in water.

Control means, in this test method, a treatment that duplicates all the conditions and factors that might affect the results, except the specific condition that is being studied. In an aquatic toxicity test, the control must duplicate all conditions of the exposure treatment(s), but must contain no test material. The control is used to determine the absence of toxicity due to basic test conditions (e.g., temperature, quality of dilution water, health of test organisms, or effects due to their handling).

Control/dilution water means water that is used for diluting the sample of effluent, and for the control test.

Dead fish are fish in which all visible signs of movement or other activity have ceased (see also Section 4.5 of EPS 1/RM/13).

Effluent is any liquid waste (e.g., industrial, municipal) discharged to the aquatic environment. See definition of *wastewater effluent* for the meaning of a specific category of effluent.

Hardness means the concentration of cations in water that will react with a sodium soap to precipitate an insoluble residue. In this method, hardness means a measure of the concentration of calcium (Ca^{++}) and magnesium (Mg^{++}) ions in water, expressed as mg/L calcium carbonate ($CaCO_3$) (see also APHA *et al.,* 2005).

LC50 (median lethal concentration) means the concentration of effluent in water that is estimated to be lethal to 50% of the test organisms with a 96-hour exposure period. The LC50 and its 95% confidence limits are derived by statistical analysis of percent mortalities in several test concentrations, after a fixed period of exposure.

Lethal means causing death by direct action.

pH is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions on a scale from 0 to 14, with 7 representing neutrality, numbers less then 7 signifying increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.

pH i (initial pH) refers to the pH as measured on composite 100% sample at 15 ± 1°C before any aeration of the test solution at the lab.

pH stabilized test means the EPS 1/RM/13 test method with a pH stabilization technique applied on a wastewater effluent sample.

Reference method means a specific biological test method for performing a toxicity test, i.e., a toxicity test method with an explicit set of instructions and conditions which are described precisely in a written document. Unlike other multi-purpose (generic) biological test methods published by Environment Canada, the use of a *reference method* is frequently restricted to testing requirements associated with specific regulations; testing to assess whether there has been a violation of the General Provisions of the Canadian Fisheries Act.

Static means toxicity tests in which the test solutions are not renewed during the test.

Sublethal means deleterious to fish, but below the level that directly causes death of fish within the 96-hour test period.

Toxicity means the inherent potential or capacity of a substance to cause deleterious effect(s) on fish. The effect(s) may be lethal or sublethal.

Wastewater means a mixture of liquid wastes primarily composed of domestic sewage that can also include other liquid wastes from industrial, commercial and institutional sources.

Wastewater effluent means untreated or treated wastewater that is released from the outfall(s) of a wastewater system, excluding combined sewer overflows from the wastewater system.

Wastewater system means any works for the collection or treatment and release of wastewater or any part of such works.

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Introduction

In 1990, Environment Canada published a biological test method for conducting *acute* lethality tests with rainbow trout: *Reference Method* (RM) for Determining Acute Lethality of *Effluent* to Rainbow Trout (EPS 1/RM/13) (EC, 2000). The method (revised in 2007) was developed specifically for determining the acute lethality of effluent, and has been used across Canada by the federal, provincial, and territorial governments in the monitoring and control of industrial and *wastewater effluent*.

The test is conducted at 15 ± 1°C for 96 hours under *static* conditions (i.e., no renewal of test solution). Tests *can* be conducted using the full-strength (100%) wastewater only, or as a multiple concentrations (e.g., 100%, 50% etc.) to determine the *LC50* (the concentration of wastewater sample that is estimated to be *lethal* to 50% of the animals exposed to that concentration within a defined period of exposure). Under most existing provincial and federal regulations, a sample is considered to "fail" the acute lethality test if >50% rainbow trout mortality is observed in the full-strength wastewater.

Aeration of both the *control* and test solutions at a rate of 6.5 ± 1 mL/min · L is a requirement of this test method. This aeration rate is sufficient to maintain the dissolved oxygen concentration in the control solution within 70% to 100% of the oxygen saturation value. The aeration rate is kept to a minimum, however, because excessive aeration of the wastewater can increase the rate of *pH* change and the removal of volatile compounds (ESG, 2002).

Aeration of wastewaters during acute lethality testing *may* cause the pH to rise from the equilibration of carbon dioxide (CO_2) partial pressure in the wastewater with that in the atmosphere. The loss of CO_2 due to aeration causes a shift in the carbonate buffering system of an effluent, and this leads to the rise in pH.

In many wastewater effluent samples, the CO_2 content may be artificially elevated as a result of high biological activity, or from wastewater acidification prior to discharge (Mount and Mount, 1992). Any change in wastewater pH during an acute lethality test may affect mortality if the *toxicity* of the substance responsible is pH-dependent.

Ammonia, which could be of concern in wastewater effluent, would be one such example of a pH-dependent toxicant. Ammonia toxicity is attributable to the free or un-ionized (NH_3-N) form as opposed to the ionized species. The relative concentration of un-ionized ammonia increases with increases in pH and water temperature. Depending on the initial pH of the full-strength wastewater and the magnitude of the upwards pH drift during testing, concentrations of un-ionized ammonia that were below lethal levels at test initiation, could increase sufficiently during testing to cause rainbow trout mortality by test completion.

To address the potential for residual ammonia toxicity in a wastewater effluent due to pH drift, Environment Canada has standardized three pH stabilization techniques for the control of pH during rainbow trout acute lethality testing. These pH stabilization techniques are add-on procedures to the EPS 1/RM/13 test method, however, a *pH stabilized test* can only be performed if three conditions have been met as outlined in Sections 1.1, 1.2 and 1.3.

Additional supporting background information on the use of these pH stabilization techniques is provided in "Supplementary Background and Guidance for Investigating Acute Lethality of Wastewater Effluent to Rainbow Trout" (EC, 2008).

1.1 Condition #1 – Total Ammonia Measurement

Total ammonia (in mg/L) *must* be measured on all wastewater effluent samples submitted for toxicity testing using EPS 1/RM/13. This measurement will be used to determine if pH stabilization is appropriate for subsequent samples. This concentration of total ammonia is used in the calculation of un-ionized ammonia at the initial pH (*pH i*) [1] of the effluent at 15°C (refer to Condition #3, Section 1.3).

1.2 Condition #2 – Failure of Acute Lethality Reference Method EPS 1/RM/13

The techniques described herein for the pH stabilization of wastewater effluent must only be used when it has been shown that a previously collected sample of wastewater effluent from the same source failed the rainbow trout acute lethality test (EPS 1/RM/13; i.e., > 50% mortality)[2].

1.3 Condition #3- Maximum Ammonia Concentration

The procedures described herein may only be used when the un-ionized ammonia concentration present in the 100% wastewater effluent sample does not equal or exceed 1.25 mg/L at 15°C or when the total ammonia concentration does not equal or exceed the maximum total ammonia concentration (y) in mg/L determined using the following formula and the initial pH of the wastewater effluent sample at 15°C:

$$y = 1.25 \times (10^{(9.564136638-pH)}+1)$$

These maximum values for ammonia are set to pre-screen those wastewaters that would result in rainbow trout mortality regardless of the pH drift observed during the acute lethality test. In other words, pH stabilization techniques are not appropriate if the ammonia concentration is already sufficiently high to cause rainbow trout mortality at the start of the acute lethality test. If this maximum un-ionized ammonia value is exceeded, it clearly identifies that a wastewater effluent is not of a quality where the pH drift phenomenon would be a consideration (i.e., ammonia is already at an acutely lethal concentration prior to testing). For additional information and supporting rationale please refer to Environment Canada (2008).

Given that "total ammonia" = $NH_3 + NH_4^+$, the un-ionized ammonia concentration in mg/L must be calculated using the following formula (USEPA, 1999):

$$\text{Un-ionized ammonia} = (\text{total ammonia}) \times [1/(1 + 10^{pK - pH})]$$

where:
pK is 9.56 at 15 ºC;
pH is the initial pH of the wastewater effluent at 15 ºC; and
total ammonia is in mg/L as measured for Condition #1, Section 1.1.

1.4 Overview of pH Stabilization Techniques

Three techniques can be used for pH stabilization during rainbow trout acute lethality testing of wastewater effluent samples when the conditions for use of pH stabilization are met:

1) CO_2 Injection,
2) Recycling, and
3) pH Controller.

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[1] The initial pH of the wastewater effluent at 15°C is already a required measurement in EPS 1/RM/13.

[2] If the sample was not acutely lethal using the standard reference method, there is no need to use an add-on pH stabilization procedure. If the sample was acutely lethal, the ammonia measurement (from Condition #1, Section 1.1) and the pH during the failed test can be used to indicate if un-ionized ammonia was likely responsible for trout mortality.

Regardless of the technique selected, application of a pH stabilization procedure with an acute lethality test using rainbow trout will require hands-on training prior to testing an actual wastewater effluent sample. Some experimentation will also likely be required with each individual sample, since the specific water chemistry will vary among (or even within) *wastewater systems*.

In addition to specific test requirements for pH stabilization techniques, all method requirements and procedures for EPS 1/RM/13 must be followed during the conduct of the tests. The rationale behind each technique is to replace the CO_2 lost during test aeration in order to maintain the pH at initial pH of the sample (*pH i*). The techniques are not intended to add more CO_2 than is already present in a wastewater effluent. A detailed description of each pH stabilization technique is provided in Section 2.

Procedure Options for Conducting pH Stabilization of Wastewater Effluents

This section provides details for conducting each pH stabilization technique with wastewater effluent:

1) CO_2 Injection,
2) Recycling, and
3) pH Controller.

In the pH stabilization test, the pH of the sample is controlled at the level measured at test initiation (pH i) using one of the three techniques set out below. The pH stabilization procedure does not supercede the existing acute lethality test method using rainbow trout (EC, 2000), but describes "add-on" techniques. All tests must meet the requirements and procedures outlined in EPS 1/RM/13. However, additional monitoring (Section 2.1) and reporting (Section 3) requirements are mandatory with these pH stabilization techniques.

2.1 General Requirements

The pH stabilization procedures apply when the tests from EPS 1/RM/13 [Section 5 – single concentration test; Section 6 – multi-concentration (LC50) test] are performed. In either case, the highest concentration tested is the 100% wastewater effluent.

Prior to testing for regulatory purposes, some preliminary investigations may be required with each wastewater sample, since the specific effluent chemistry will vary among (or even within) facilities. For example, wastewater samples or *control/dilution water* with low *alkalinity* or low *hardness* may be susceptible to significant shifts in pH due to minimal *buffering capacity*, and therefore, require less CO_2. The data generated during the development of the CO_2 Injection and pH Controller techniques indicated that both could be successfully applied to wastewater effluent samples without any significant declines in pH. Difficulties encountered in control/dilution waters with low buffering capacity can be avoided by ensuring each test solution and the control is injected with only enough CO_2 to maintain a stable pH. This ensures that a control solution with low buffering capacity will likely receive less CO_2 than a wastewater test solution with a higher buffering capacity. This approach reduces the chances of control mortality that could result if excess CO_2 were to be added, while still meeting the objective of pH stabilization, which is to maintain the initial pH of a sample.

For the CO_2 Injection or pH Controller techniques, tests *should* be conducted in either glass aquaria or in non-toxic containers (e.g., polyethylene, polypropylene containers). When using the Recycling technique, glass aquaria are recommended due to the need for specially adapted lids required to control the headspace above the test solution.

All solutions must be aerated with oil-free compressed laboratory air (lab air) throughout the test, at a controlled rate of 6.5 ± 1 mL/min · L. All solutions for tests must be prepared before aeration is started. Upon preparation of the test solutions, all solutions must be aerated for 30 minutes at 6.5 ± 1 mL/min · L. Stabilization of pH must start when aeration is initiated. After 30 minutes aeration, the concentration of dissolved oxygen must be measured in at least the highest test concentration (normally 100% effluent). If (and only if) oxygen in the highest test concentration is <70% or >100% of air saturation, then aeration (i.e., before exposure of fish) of all solutions including the control(s) must be continued at 6.5 ± 1 mL/min · L. This period of aeration must be restricted to the lesser of 90 additional minutes and attaining 70% saturation in the highest test concentration (or 100% saturation if super-saturation is evident). Immediately thereafter, fish must be placed

randomly in each test solution and the test must be initiated, regardless of whether 70 to 100% saturation was achieved in all test solutions.

Environment Canada (2000) requires that compressed air be bubbled through a clean air stone. For the CO_2 Injection technique, air stones must be used in the delivery of the CO_2 mix. For the Recycling and pH Controller techniques, air stones must be used in the delivery of laboratory air. For the pH Controller technique, a glass pipette is highly recommended for use in the delivery of the CO_2 gas. The use of a glass pipette in the delivery of CO_2 gas in the pH Controller technique provides better control of the amount of CO_2 gas that is delivered to the sample when the controller is activated.

Test results may be confounded or difficult to interpret in cases when there is a difference in pH (> 0.2 pH units) between the 100%wastewater effluent sample and laboratory dilution water used to prepare exposure solutions for a multiple concentration test. The pH of each effluent concentration (i.e., 100, 50, 25, 12.5, 6.25%) must be maintained at the pH value measured at test initiation (before any aeration is started) in each individual exposure concentration and the control. However, there may be a gradient of pH values observed during testing that could result in a non-dose related response. In this case (i.e., when mortality is observed in diluted effluent concentrations, but not in the 100% concentration), an LC50 should not be calculated. Results for the 100% wastewater effluent sample will still be considered acceptable, provided that all other validity criteria are met (see Section 2.1.3).

2.1.1 Observations and Measurements

In addition to the observations and measurements described in EPS 1/RM/13 (e.g., temperature, dissolved oxygen, colour, turbidity, odour, and floating or settling solids), the laboratory must measure the pH, total ammonia, and hardness in each wastewater effluent sample. Total ammonia must be measured to at least two decimal places. Alkalinity must be measured if the CO_2 Injection technique is to be used. Measurements must be taken only after the contents of all containers have been thoroughly mixed and the temperature of the sample has been adjusted to 15 ± 1 °C. These parameters must be measured in the full strength sample after sub-samples (e.g., aliquots of a sample divided between two or more containers) have been combined. When a multiple concentration (LC50) test is conducted, pH, ammonia and hardness must be measured in each test solution; if the CO_2 Injection technique is used, alkalinity must be measured in the 100% wastewater effluent sample.

Before any aeration of the test solutions, the un-ionized ammonia concentration must be calculated using the measurement of total ammonia, a temperature of 15 °C, and the initial pH (pH i) of the sample (pH i = pH as measured on composite 100% sample at 15°C before any aeration of the test solutions). A pH stabilization technique must not be used if the concentration of un-ionized ammonia in a wastewater sample equals or exceeds 1.25 mg/L.

The pH must be measured and recorded at the beginning of the test (when fish are added to the wastewater effluent and control) and at each time interval required by each procedure, in all concentrations and the control (Sections 2.2.4, 2.3.2, and 2.4.3). Additional monitoring of pH during the first 8 hours of testing may be needed when using the pH stabilization procedure. For the remainder of the test, pH must be measured at each 24-h interval (at minimum) to track changes in pH and to ensure that the pH is maintained within test validity criteria (Section 2.1.3). More frequent monitoring of pH (e.g., twice daily) may be needed if the wastewater effluent sample has a low buffering capacity (low alkalinity), which may result in rapid changes in pH.

2.1.2 Chlorinated Wastewater Samples

Laboratories should measure total residual chlorine (TRC) concentrations in each wastewater effluent sample received (i.e., at the same time ammonia measurements are made).

Total residual chlorine must be measured in the sample if fish display stressed or atypical behaviour at test initiation. If chlorine is present in the sample (TRC >0.1 mg/L), pH stabilization procedures should not be used, since trout mortality, stress or atypical behaviour will occur, regardless of pH drift. Testing laboratories should contact the wastewater facility to review their wastewater treatment operations (i.e., determine if the wastewater is chlorinated and then dechlorinated prior to discharge) and obtain historical data (i.e., determine typical total chlorine concentrations in the final discharge) because pH stabilization would not be necessary if the wastewater effluent samples contained a lethal level of total chlorine. Additional information on procedures to remove chlorine from wastewater effluent samples for investigative purposes is provided in Environment Canada (2008).

2.1.3 Test Validity Criteria

A test is considered invalid if any of the following occur:

1) the average pH in the pH stabilized 100% wastewater effluent test solution shifts more than ± 0.2 units from pH i;
2) the instantaneous pH in the pH stabilized 100% wastewater effluent test solution is greater than ± 0.3 units from pH i; or
3) if >10% of the fish (combined data if replicates are used) in the pH stabilized control die or exhibit atypical or stressed behaviour.

In the case of a multiple concentration test, an LC50 calculation must not include any exposure concentration where the pH criteria were not met.[3]

[3] For example, if the concentration series used for an LC50 test is 100, 50, 25, 12.5 and 6.25% (plus a control) and the average pH in the 12.5% concentration is not maintained within ± 0.2 pH units from the pH i in the 12.5% concentration, then the 12.5% mortality results must not be included in the LC50 calculation.

2.2 pH Stabilization Using the CO_2 Injection Technique

In the CO_2 Injection pH stabilization technique, the upward drift of pH is controlled by aerating the 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.

In addition to the standard equipment and facilities required to conduct EPS 1/RM/13, the following materials and equipment are required to use this pH stabilization technique:

- certified compressed cylinder containing a mixture of 15% CO_2, 21% O_2, and 64% N_2 with a CGA 590 outlet connector from a certified compressed gas supplier (e.g., Praxair, Air Liquide, BOC Gases)
- dual stage CP brass CGA 590 compressed gas regulator (e.g., Concoa, Fisherbrand, Restek, Cole-Parmer, VWR)
- six 150-mm flow meters with adjustable valves (approximate flow rate range 0 to 137 mL/min) (e.g., Cole Parmer, Gilmont, Scienceware) [4]
- six 150-mm flow meters with adjustable valve (approximate flow rate range 0 to 300 mL/min) (e.g., Cole Parmer, Gilmont, Scienceware) [4]
- plastic male pipe adapters 1/8 × 3/16" (0.32 × 0.48 cm) or equivalent to connect flow meters to plastic tubing
- flexible plastic air tubing
- Tygon® tubing (R-3606) or equivalent
- tapered “Y” shaped plastic air tubing connectors (e.g., VWR, Fisherbrand)

[4] Additional flow meters will be required if more than the minimum number of concentrations is used (0, 6.25, 12.5, 25, 50 and 100%).

- assorted plastic tubing connectors
- two-way and four-way manifold valves (e.g., Cole Parmer)

Diagrams and photos showing a CO_2 injection technique test setup are provided in Figures 1 to 5.

2.2.1 Apparatus for Delivering CO_2 Mixture

The CO_2 is delivered to a test vessel from a compressed gas cylinder containing 15% CO_2, 21% O_2, and 64 % N_2 mix via a gas cylinder regulator, flexible Tygon® air tubing through a 4-way gang valve, through a flow meter, combined with the normal lab airflow using a "Y" plastic connector and into the test solutions via an air stone. The normal lab air is delivered via flexible Tygon® R-3603 air tubing through a flow meter and combined with the CO_2 mix flow using the above-mentioned "Y" connector (Figures 1 and 3).

The cylinder containing the CO_2 mix should be securely attached near the exposure vessels by chaining to a wall or other stable structure. Never use oil or grease on regulator or cylinder fittings, as this could contaminate the pure gas mix, or create a fire hazard.

2.2.2 Estimating Percent CO_2 Mixture Required to Stabilize pH

The initial percent CO_2 mixture required to stabilize pH is estimated by measuring the initial pH (pH i) and alkalinity of the 100% test solution. In a multiple concentration (LC50) test, in order to determine the correct percent CO_2 mix at the start of the test: (i) initial pH (pH i) must be measured in each concentration, and (ii) alkalinity must be measured in the 100% wastewater effluent. Alkalinity in the remaining dilutions may be estimated, based on the known alkalinity values of the control/dilution water and the 100% wastewater effluent.

Once the initial pH and alkalinity have been determined, the CO_2 calibration table (Table 1) is used to estimate the percent CO_2 that is applied for a given initial pH and alkalinity to provide pH control. For example, a test solution with an alkalinity of 300 mg/L, as calcium carbonate ($CaCO_3$), with an initial pH of 7.1 will require a final flow of 5% CO_2 to maintain the initial pH. The CO_2 calibration table is for estimation and guidance purposes only.

Some adjustment to the percent CO_2 will be required if there is an upward or downward trend in pH after initiation of aeration with the CO_2 mixture. For example, the percent CO_2 mixture should be increased if there is an upward trend in the pH, while the percent CO_2 mixture should be lowered (decreased) if there is a downward trend in the pH.

The percent of CO_2 mixture required to stabilize pH depends on the chemical characteristics (i.e., alkalinity/buffering capacity) of the test solution. For example, wastewater effluent samples with a low buffering capacity require less CO_2 to control pH drift compared to those that have a greater buffering capacity.

2.2.3 Setting Flow Rates for CO_2 Mixture and Laboratory Air

In a multiple concentration (LC50) test setup (5 test concentrations, plus control), six flow meters with a 0 to 137 mL/min flow rate, and six flow meters with a 0 to 300 mL/min flow rate are required. All flow meters have adjustable valves. Each test concentration and control has one of each type of flow meter: one to control the flow of CO_2 mix, and the other to control the flow of lab air.

After the required percent CO_2 has been determined (Table 1), the flow rate of the CO_2 mix and lab air can be adjusted to achieve the required percent CO_2 using the adjustable valves on the flow meters. The test solution volume and required final percent CO_2 determines which flow meter is used to control the two flows of CO_2 mix and lab air.

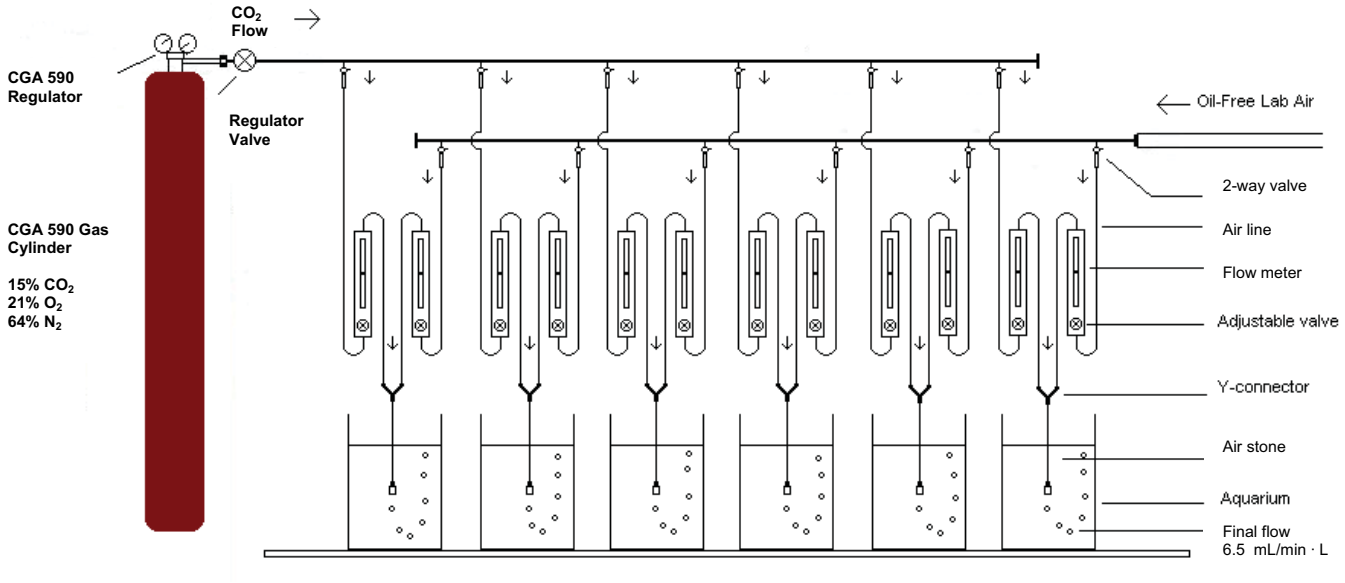

Figure 1 Schematic Diagram of a Six-concentration Test Using the CO_2 Injection Technique
Each concentration has two flow meters. One flow meter is for the CO_2 mix and the other is for the lab air flow. One flow meter has a maximum flow rate of 300 mL/min and the other has a maximum flow rate of 137 mL/min. This allows for a range of 0.5% to 15% CO_2 for sample volumes between 20 and 40 L. The final percent CO_2 will determine which flow meter controls the CO_2 and lab air flows. Refer to the CO_2 Injection Tecnhique section for guidance on determining the final percent CO_2. The final flow to the aquaria must be 6.5 mL/min · L (i.e., for a 20 L sample volume, the final flow from the two flow meters must add up to 130 mL/min).

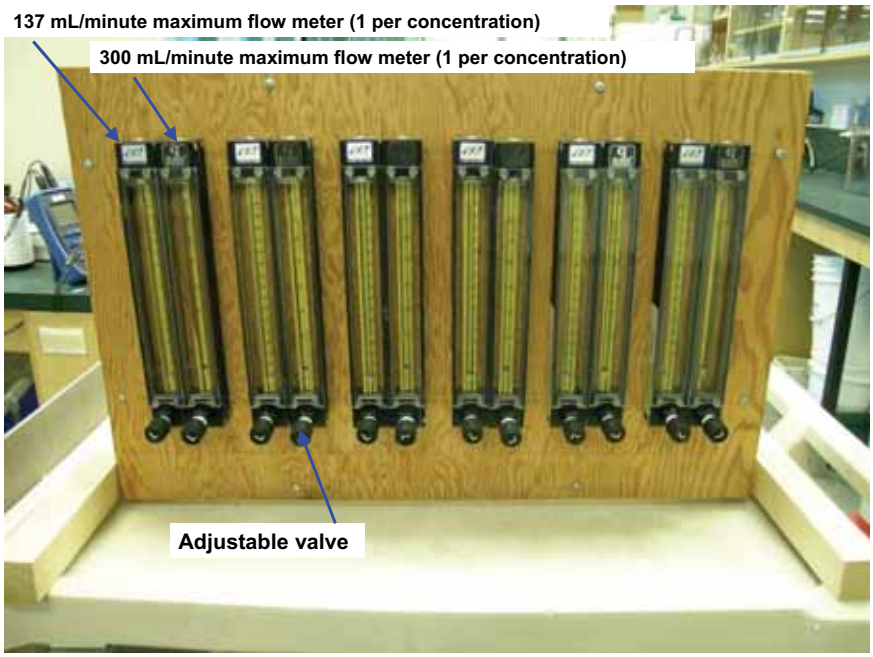

Figure 2 Front View of CO_2 Injection Technique- Control Board with Flow Meters
Each concentration has two flow meters with 137 mL/min and 300 mL/min maximum flow rates. The required percent CO_2 determines which flow meter controls the flow of CO_2 mix, and which flow meter controls the laboratory air.

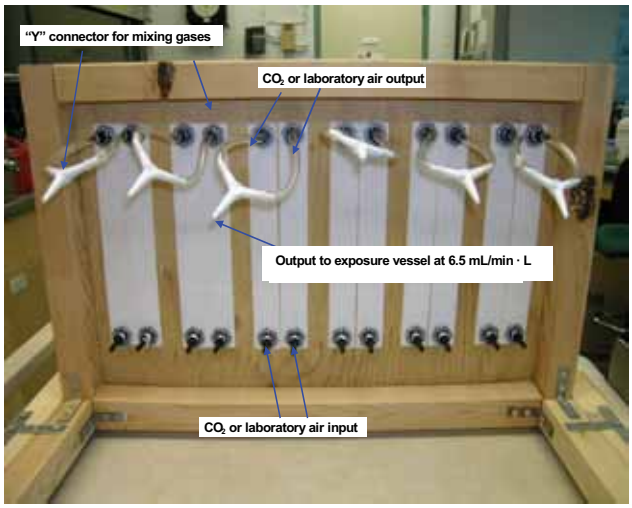

Figure 3 Rear View of CO_2 Injection Technique-Control Board with Connections

Figure 4 Glass Aquaria Used for CO_2 Injection Technique

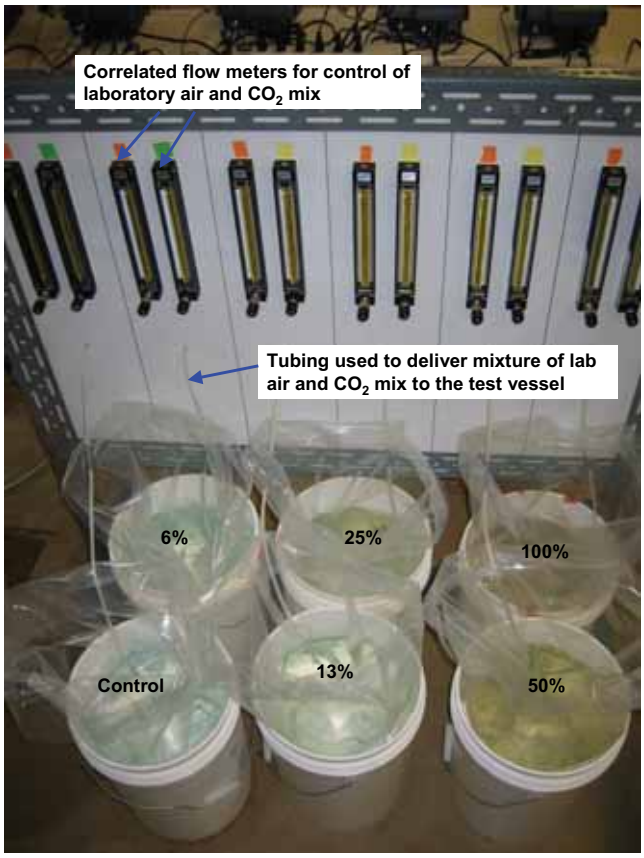

Figure 5 Plastic Pails Used for CO_2 Injection Technique

Table 1 CO_2 Calibration Table for Estimating the Percent CO_2 Mixture Required to Maintain pH Control Based on Test Solution pH and Alkalinity[a]

Percent CO_2 (%)	Alkalinity (mg/L as $CaCO_3$)					
	100	200	300	400	500	600
	pH					
0.5	7.68	7.91	8.05	8.15	8.22	8.29
1	7.11	7.31	7.52	7.73	7.93	8.14
2	6.97	7.17	7.38	7.58	7.78	7.98
3	6.89	7.10	7.31	7.52	7.73	7.94
4	6.80	6.92	7.15	7.38	7.61	7.84
5	6.76	6.89	7.10	7.27	7.44	7.61
6	6.68	6.85	7.03	7.20	7.37	7.55
7	6.56	6.81	7.01	7.15	7.30	7.48
8	6.54	6.78	6.94	7.09	7.25	7.41
9	6.48	6.66	6.84	7.02	7.20	7.38
10	6.39	6.56	6.73	6.91	7.08	7.26
15	6.23	6.41	6.60	6.78	6.96	7.14

[a] All percent CO_2 calibration numbers are based on linear regressions of actual results tested using 20 L volumes of synthetic water prepared as described by the USEPA (2002); the actual percent CO_2 required to produce a pH for a given alkalinity may be different.

The following equations are used to determine flow rates for the CO_2 mixture and lab air: [5]

(1) **Combined flow to the test vessel** (mL/min) =

6.5 mL/min·L × test volume (L)

(2) **Flow rate of CO_2 mix** =

$$\frac{\text{Required \% } CO_2 \text{ (from Table 1)}}{\text{\% } CO_2 \text{ in the mix (i.e., 15\%)}} \times \text{Combined flow to the test vessel } \textit{(1)}$$

(3) **Flow of lab air** =

Combined flow to the test vessel ***(1)*** − Flow rate of CO_2 mix ***(2)***

The CO_2 mix flow may be adjusted to maintain pH control anytime during the test.

[5] For example, to achieve a final 10% CO_2 mix flow for a 20 L exposure volume, the flow on the 15% CO_2, 21% O_2, and 64% N_2 gas is set to 86.7 mL/min and the lab air flow is set to 43.3 mL/min for a combined total of 130 mL/min (or 6.5 ± 1 mL/min · L). In this case, the 0 to 137 mL/min flow meter is used to control the CO_2 gas flow and the 0 to 300 mL/min flow meter is used to control the lab air flow. Because the 0 to 137 mL/min flow meters generally allow for finer adjustments, using this flow meter with the CO_2 mix in this example affords more precise control of pH. In another example, to achieve a final 10% CO_2 mix flow for a 40 L exposure volume, the flow on the 15% CO_2, 21% O_2, and 64% N_2 gas is set to 173.3 mL/min and the lab air flow is set to 86.7 mL/min for a combined total of 260 mL/min (or 6.5 ± 1 mL/min · L). In this case, the 0 to 137 mL/min flow meter is used to control the lab air flow and the 0 to 300 mL/min flow meter is used to control the CO_2 gas flow.

2.2.4 Controlling pH Drift

Stabilization of pH commences immediately upon initiation of aeration at 6.5 ± 1 mL/min · L (see Section 2.1). Total aeration rates (CO_2 and lab air) must be 6.5 ± 1 mL/min · L throughout the test in all exposure concentrations, including the control (as per EPS 1/RM/13). Each test vessel is aerated through an air stone with lab air and CO_2 gas mix at a combined rate of 6.5 ± 1 mL/min · L at a percent CO_2 that maintains the average pH (for all effluent concentrations, excluding the control) within ± 0.2 pH units and the instantaneous pH within ± 0.3 pH units of pH i. [6]

Frequent pH measurements and appropriate adjustments to the flow of the CO_2 mix to stabilize pH must be conducted particularly during the first three hours of the test. Most adjustments to the flow of CO_2 mix occur within the first few hours from the start of aeration. Fewer adjustments (one to two times daily) will likely be required in the days after test initiation.

The pH must be 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 the control and all exposure concentrations. This will provide data to show that the pH has been maintained throughout the entire duration of the test. The pH must also be measured and recorded whenever there is an adjustment to the CO_2 flow. A subsequent pH reading must be taken 30 minutes or sooner after an adjustment, to ensure the pH is being maintained. The final pH is recorded if there is 100% rainbow trout mortality in a test concentration before the end of the 96-h test period.

If the test solution pH begins to decrease within the first 30 minutes of aeration, the percent CO_2 should be decreased in 0.5% increments until the pH is maintained within ± 0.2 pH units of the initial pH. If the test solution pH begins to increase in the first 30 minutes of aeration, increase the percent CO_2 in 0.5% increments until the pH is maintained within ± 0.2 pH units of the initial pH. Continue to make adjustments in 0.5% CO_2 increments until the pH is maintained within ± 0.2 pH units of the initial pH. The percent CO_2 delivered to each test vessel must be recorded.

In cases where the control/dilution water has a lower buffering capacity than the wastewater effluent test solution, it is unlikely that the amount of CO_2 needed to maintain pH in a sample would maintain a pH within ± 0.2 pH units of the laboratory control/dilution water initial pH. Therefore, in the single-concentration pH stabilization test, it is not required to add the same amount of CO_2 to both the 100% wastewater effluent sample and the control, since the purpose of this pH stabilization procedure is to control the pH drift of the effluent sample by replacing CO_2 that has been lost from the original test solution due to aeration during a rainbow trout acute lethality test.

Air line tubing must be inspected at least once a day to ensure continual delivery of CO_2 mixture and laboratory air to all test solutions.

2.3 pH Stabilization Using the Recycling Technique

The Recycling pH stabilization technique controls upwards pH drift by recycling CO_2 in a closed system (Elliott *et al.*, 2003). A lid is placed securely on the test vessel and air, which contains CO_2, is re-circulated in the contained headspace with an air pump, thereby preventing loss of CO_2 to the atmosphere and maintaining the pH.

In addition to the standard equipment and facilities required to conduct EPS 1/RM/13, the following materials and equipment are required to use this pH stabilization technique:

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[6] For example, a test volume of 20 L is aerated at a rate of 130 mL/min. Although the contribution of each flow (normal laboratory air and CO_2 mix) to the final flow will vary depending on the pH control requirements of each individual test solution, the total flow rate must be 6.5 ± 1 mL/min · L.

- Tygon® Tubing (siphon tubes and air lines)
- aquarium aeration pump that generates 6.5 ± 1 mL/min · L and conforms with the lid (e.g., Elite 799 & 800, 115-volt aquarium aeration pump)
- 10 mL disposable pipettes
- recycle lids [with neoprene seals, O-rings, and elastics attached; lids are fabricated to fit test vessels used; can be fabricated and purchased from a plastic fabricator, e.g., Allwest Plastic Fabricators in Edmonton (www.allwestplastic.com)]
- 250-mL Erlenmeyer catch flask used to prevent condensate from entering flow meters
- test vessels (aquaria or other containers such as pails)

Diagrams and photos showing a Recycle technique test setup are provided in Figures 6 and 7.

2.3.1 Recycling Technique Setup

To reduce headspace, the test container is filled to the very top with sample, without immersing the pump. The Recycle lid is then placed loosely on top of the test container. The air line tubing (#1) runs from the aeration pump to the catch flask as shown in Figure 6, and air line tubing (#2) then runs from the catch flask to the flow meter. The air line tubing (#3) proceeds from the flow meter outlet to a connector in the top of the Recycle lid. An air line (# 4) is attached to the bottom of the same connector and an air stone is attached to the end of this air line which is immersed in the sample. A siphoning tube is also attached to the Recycle lid for removing an aliquot of sample at each 24-h observation for physicochemical measurements. This is accomplished by attaching Tygon® tubing (#5) to a connector in the top of the Recycle lid and Tygon® tubing (#6) to the bottom of the same connector. A 10-mL pipette is attached to the end of this Tygon® tubing (#6) and immersed in the sample. After an aliquot has been removed, ensure that the end of the siphoning tube is stored above the sample level to avoid loss of sample. A tubing clamp can be used to seal the end of the siphoning tube as a precaution. Extra care must also be taken when collecting sub-samples for monitoring water quality parameters (e.g., pH, temperature), as a vacuum can form and result in a significant loss of sample from the test vessel.

The Recycle lid is secured and sealed to the top of the test container by fastening all O-rings and elastics around the plastic knob catches on the test vessel. The Recycling technique will only be successful in stabilizing pH if a tight seal is obtained.

Highly coloured, opaque, or foamy test solutions in the sealed test vessel used for the Recycling technique could make test observations difficult when inspecting fish for stress and mortality. Checks and removal of *dead fish* must be completed as quickly as possible to prevent pH drift.

For wastewater effluent samples with a high *BOD*, a decline in dissolved oxygen could be observed during an acute lethality test with rainbow trout when the pH is not stabilized. This dissolved oxygen decline could be accentuated if the Recycle technique were to be used to stabilize pH.

2.3.2 Controlling pH Drift

Stabilization of pH commences immediately upon initiation of aeration for 30 minutes at 6.5 ± 1 mL/min · L, before fish are added (see Section 2.1). To begin aeration, the pump is started and the flow meter setting is adjusted. All aeration rates must be 6.5 ± 1 mL/min · L throughout the test in all exposure concentrations, including the control (as per EPS 1/RM/13).

After the required aeration has been completed (i.e., minimum 30 minutes), the elastics from the sampling port on the Recycling lids for all concentrations are removed. The sampling port is opened, and fish are added in a quick and random manner. The sampling port is resealed before proceeding with the test.

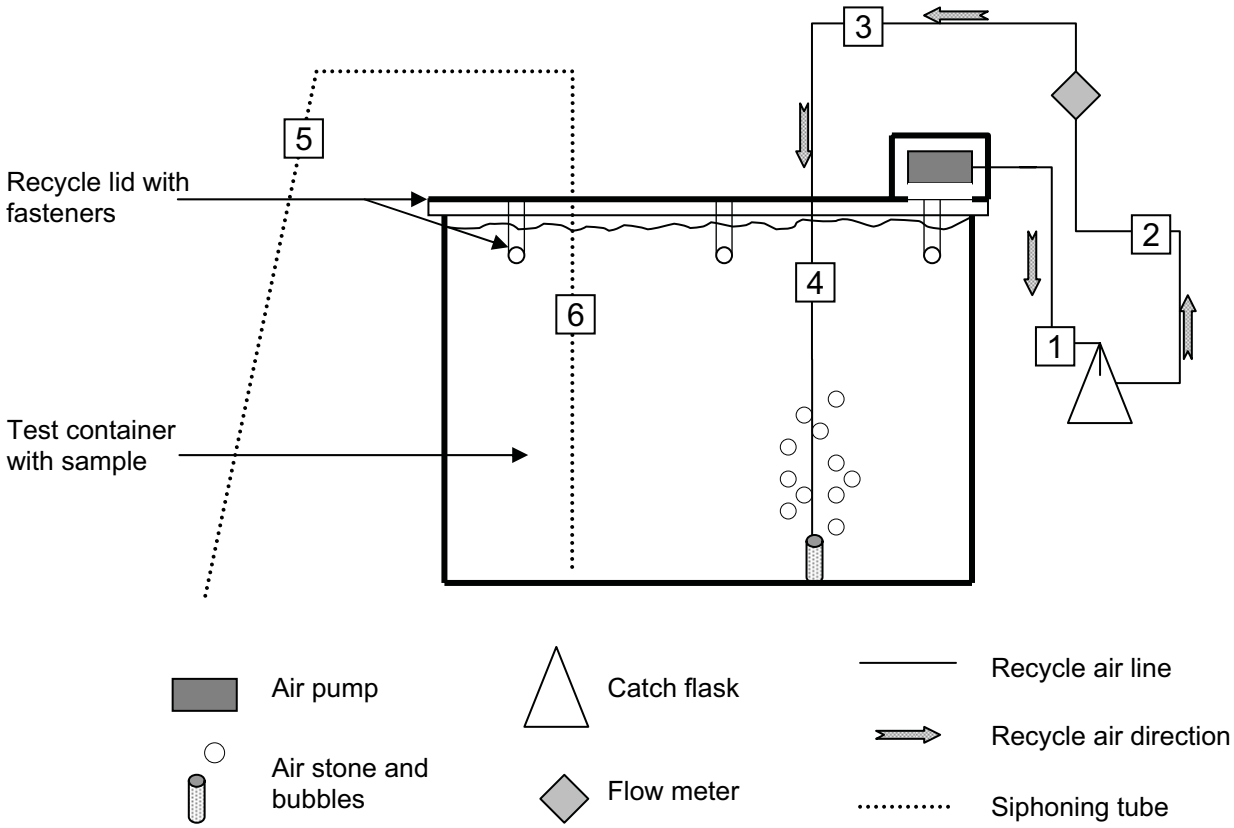

Figure 6 Schematic Diagram of a Recycling Technique Test Unit

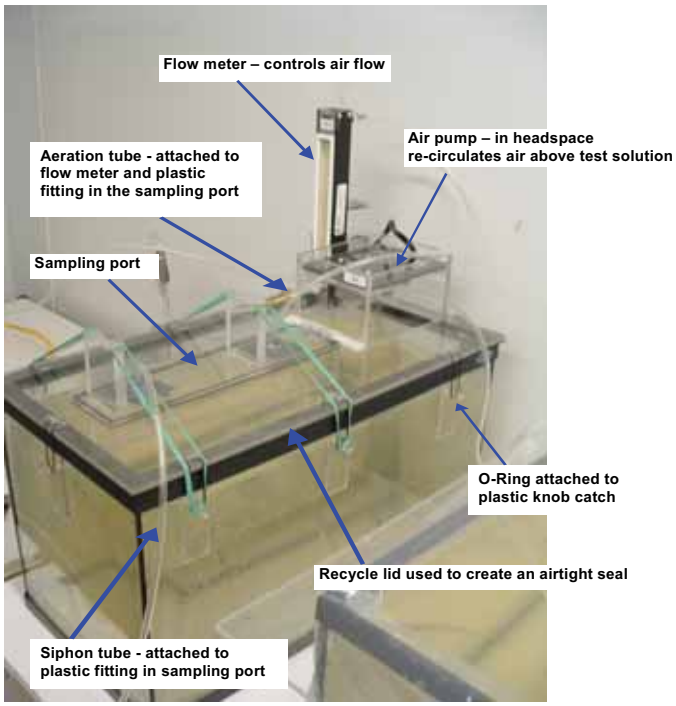

Figure 7 Glass Aquaria with Sampling Port, Air Pump, and Recycle Lid for Recycling Technique

The pH must be measured and recorded immediately before any aeration (pH i), at t = 0 h (test start, when fish are introduced) and then at 24, 48, 72, and 96 h in the control and all exposure concentrations. This will provide data to show that the pH has been maintained throughout the entire duration of the test. It is also recommended that pH be measured at t = 0.5, 1 and 2 h to ensure that the lid has been properly sealed. The pH must also be taken and recorded whenever the test container is opened (i.e., to remove dead fish). The final pH is recorded if there is 100% rainbow trout mortality in a test concentration before the end of the 96-h test period.

Any dead fish observed at each 24-h interval must be removed as quickly as possible. To remove dead fish from the test vessel, the elastics from the sampling port on the Recycle lid are removed and the dead fish are taken out. The elastics are then replaced to seal the test vessel. It is important that any opening of the test vessels be completed quickly, as a loss of CO_2 during this step may result in pH increases.

Visual checks must be made at least once per day to ensure that the air lines, pumps, and flow meters are working properly.

2.4 pH Stabilization Using the pH Controller Technique

The pH stabilization controller technique uses pure CO_2 (or a gas mix of 15% CO_2, 21% O_2 and 64% N_2) with separate lines for lab air addition. If pH drifts above a predetermined and programmed set point, the controller is activated and CO_2 is added to reduce pH. Once

pH returns to the acceptable limit, the injection of CO_2 is automatically shut off.

In addition to the standard equipment and facilities required to conduct EPS 1/RM/13, the following materials and equipment are required to use this pH stabilization technique:

- solenoids (one for each exposure concentration) to control the flow of CO_2
- CO_2 pressure regulator and needle valve assembly
- certified compressed cylinder containing 100% CO_2 from a certified compressed gas supplier (e.g., Praxair) [note: gas mixture (15% CO_2, 21% O_2, and 64% N_2) from CO_2 Injection technique can also be used for pH Controller stabilization technique]
- pH controller [e.g., American Marine Inc. (cat.# CRT4) or equivalent; available from Fish Farm Supply, Elmira, Ontario]
- glass pipettes (1 mL)
- backflow valves (e.g., available from Hagen®)
- various fittings [1/2" (1.25 cm) black pipe (natural gas fittings) for LC50 setup; 1/2" × 90 deg; 1/2" 'T'; 1/2" × 2.5" nipples; 1/8" (0.32 cm) inner diameter Tygon® tubing to connect pipettes; 1/2" × 6" (15.2 cm) nipples]

A glass pipette is highly recommended for use in the delivery of the CO_2 gas because it provides better control of the amount of CO_2 gas that is delivered to the sample when the controller is activated. Diagrams and photos showing a pH Controller technique test setup are provided in Figures 8 to 13.

2.4.1 Regulator/Solenoid Assembly

Individual pressure regulators are connected to the gauge assembly (manifold) (Figures 8 and 9). The CO_2 gas regulator is connected to a CO_2 cylinder. Never use oil or grease on regulator or cylinder fittings, as this could contaminate the pure gas mix, or create a fire hazard.

The manifold is connected to the regulator on the CO_2 cylinder using high-pressure polypropylene tubing (1/4" [0.64cm] outer diameter). All needle valves on the solenoids

must be turned to the off position. The locking hex cap from the regulator on the solenoid assembly is removed to expose the adjustment screw (hex wrench or Allen key may be required) and the screw is turned counterclockwise until there is no more resistance.

The valve on the CO_2 cylinder is opened and pressure is adjusted to approximately 40 psi. The working pressure on the solenoid is adjusted (using the hex wrench or Allen key) to approximately 20 psi. Connections should be tested for leaks using a dilute dish detergent (any bubble formation suggests there may be leaks and the system should be rechecked and sealed as required).

An appropriate length of silicone airline (1/4" outer diameter) is connected to the needle valve and attached to the pipette/back-flow preventer (Figure 13).

2.4.2 pH Controller

The pH Controller (see Figure 12) must be calibrated daily using certified pH standards. The tolerance of the pH Controller (i.e., the sensitivity of pH control) must be set before test initiation (± 0.2 pH units). The CO_2 tubing must be removed from the exposure solution during calibration. Meter calibration must be completed rapidly to prevent pH drift from occurring. Instructions for calibration and maintenance should be provided by the manufacturer and reviewed before test initiation.

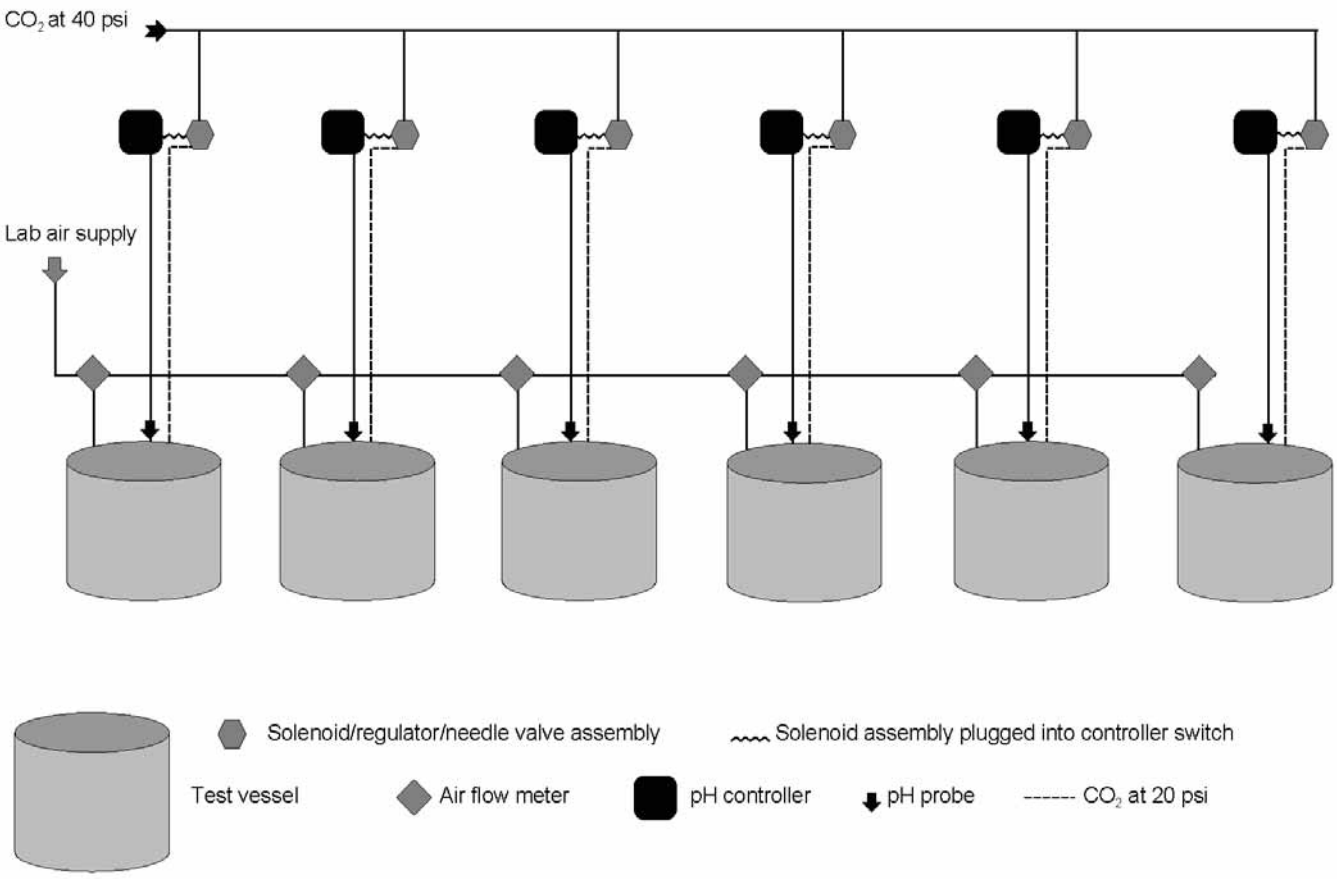

Figure 8 Schematic Diagram of a Six-concentration Test Using pH Controller Technique

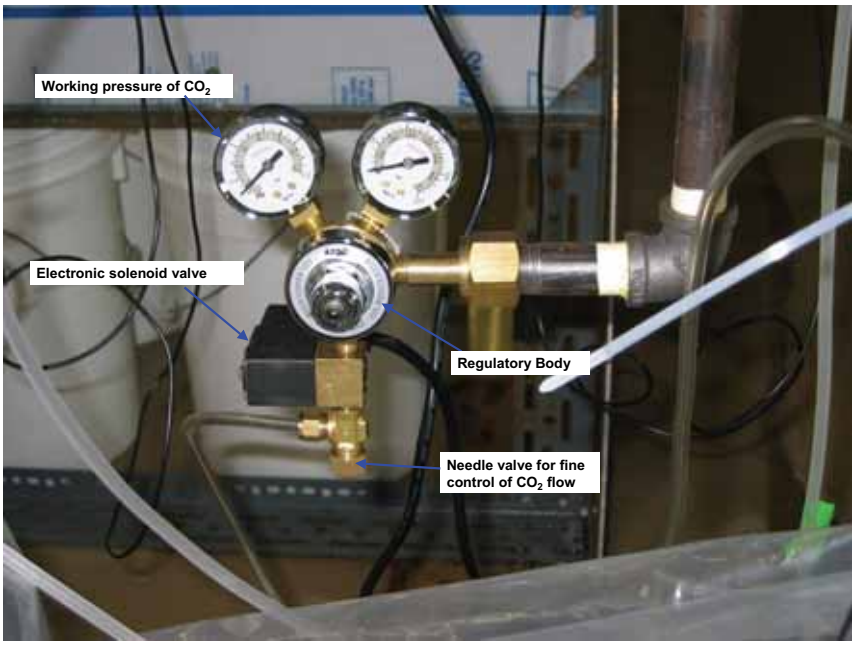

Figure 9 **Solenoid with Regulator and Needle Valve Assembly for pH Controller Technique**

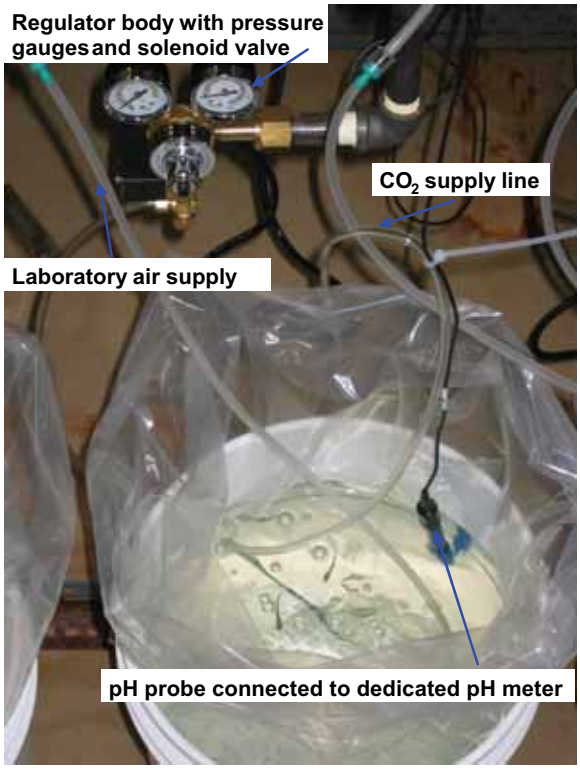

Figure 10 **Test Setup for pH Controller Technique**

Figure 11 Overview of Setup for LC50 Test Using pH Controller Technique

Figure 12 Example of pH Controller Unit

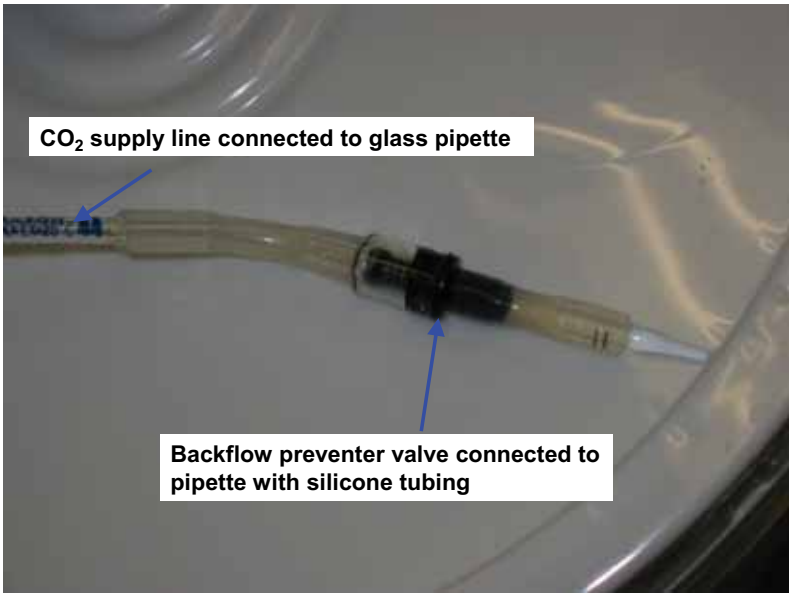

Figure 13 Supply Line for CO_2 Showing Backflow Preventer Valve

The pH probe from one controller is placed into a single test solution for the duration of the test (the probe can be temporarily removed for calibration). The probe should be secured 3–5 cm below the surface of the test solution. The CO_2 delivery pipette should be directly beneath the pH probe, and tied to the probe conductor. This is important for accurate pH control. Back siphoning into the CO_2 line could occur, but this can be prevented by using a spring-loaded (stainless steel) back-flow check valve (Figure 13). Durable pH probes should be used to reduce the risk of leaks of potassium chloride (KCl) solution from the probe into the exposure solution.

2.4.3 Controlling pH Drift

Stabilization of pH commences upon initiation of aeration for 30 minutes at 6.5 ± 1 mL/min · L, before fish are added (see Section 2.1). When aeration is started, the main valve on the CO_2 cylinder is opened to approximately 40 psi. Pressure readings at the solenoid regulator gauge should be approximately 20 psi.

Aeration rates of laboratory air must be at 6.5 ± 1 mL/min · L throughout the test in all exposure concentrations, including the control (as per EPS 1/RM/13). Each test vessel is aerated through an air stone with lab air at 6.5 ± 1 mL/min · L. The addition of CO_2 will slightly increase the aeration rate each time the pH controller cycles on or off, in order to maintain the mean pH in the 100% test solution within ± 0.2 pH units and the instantaneous pH within ± 0.3 pH units of the initial pH. The increase in aeration rate is considered insignificant, since it only occurs periodically to control upwards pH drift and should still be within the allowable limits.

Taking frequent pH measurements and making appropriate adjustments to the flow of CO_2 is critical to stabilizing pH, particularly during the first few hours of the test. The pH value on the controllers must be closely monitored to ensure proper operation of the solenoid. It is important for the controller to cycle on and off to control the flow of CO_2. If cycling does not occur within two to five minutes of operation, and the solenoid remains open (powered), then CO_2 flow should be gradually increased using the needle valve until the required pH value is reached and the solenoid closes.

The pH must be measured and recorded immediately before any aeration (pH i), at t = 0 h (test start, when fish are introduced) and then at 24, 48, 72 and 96 h in the control and all exposure concentrations. This will provide data to show that the pH has been maintained throughout the duration of the test. The pH must also be measured and recorded whenever there is a manual adjustment to the CO_2 flow. A subsequent pH reading must be taken 30 minutes or sooner after an adjustment, to ensure the pH is being maintained. The final pH is recorded if there is 100% rainbow trout mortality in a test concentration before the end of the 96-h test period.

Visual checks must be made once per day to ensure that the pH Controllers and air lines are working properly.

Reporting Requirements

In addition to the reporting requirements outlined in the Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout, EPS 1/RM/13, the following supplementary information must be reported when conducting a pH stabilized test with wastewater effluent. Specific reporting requirements are as follows:

- type of pH stabilization technique used (pH Controller, Recycling or CO_2 Injection);
- for CO_2 Injection technique, percentage of CO_2 gas mix used during testing;
- for pH Controller technique, percentage of CO_2 gas mix or CO_2 used during testing;
- measured concentrations of the following parameters in the 100% wastewater effluent sample, after all effluent to be used in testing has been composited, thoroughly mixed and temperature of the sample has been adjusted to 15 ± 1 °C — pH i (pH i = pH as measured on composite 100% sample at 15°C before any aeration of the test solutions), total ammonia, and hardness;
- for the CO_2 Injection technique, alkalinity in the 100% wastewater effluent sample;
- calculated un-ionized ammonia concentration, based on the measurement of total ammonia, a temperature of 15 °C and the initial pH (pH i) of the 100% wastewater effluent sample;
- total residual chlorine in the wastewater effluent sample, if fish display stressed or atypical behaviour at test initiation;
- for multiple concentration (LC50) tests, pH of the diluted effluent concentrations at the start of testing (before any aeration is started at 15 °C) in each individual exposure concentration;
- for the CO_2 Injection technique, 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 concentrations;
- for the pH Controller and Recycling techniques, pH readings taken at t = 0 h (test start, when fish are introduced) and at 24, 48, 72, and 96 h in the control and all exposure concentrations;
- for the Recycling technique, report additional pH readings (if taken) at t = 0.5, 1, and 2 h;
- any additional pH readings taken during testing, or after adjustment of CO_2 or CO_2 gas mix, or removal of Recycling lid; and
- average pH based on all readings taken during testing.

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