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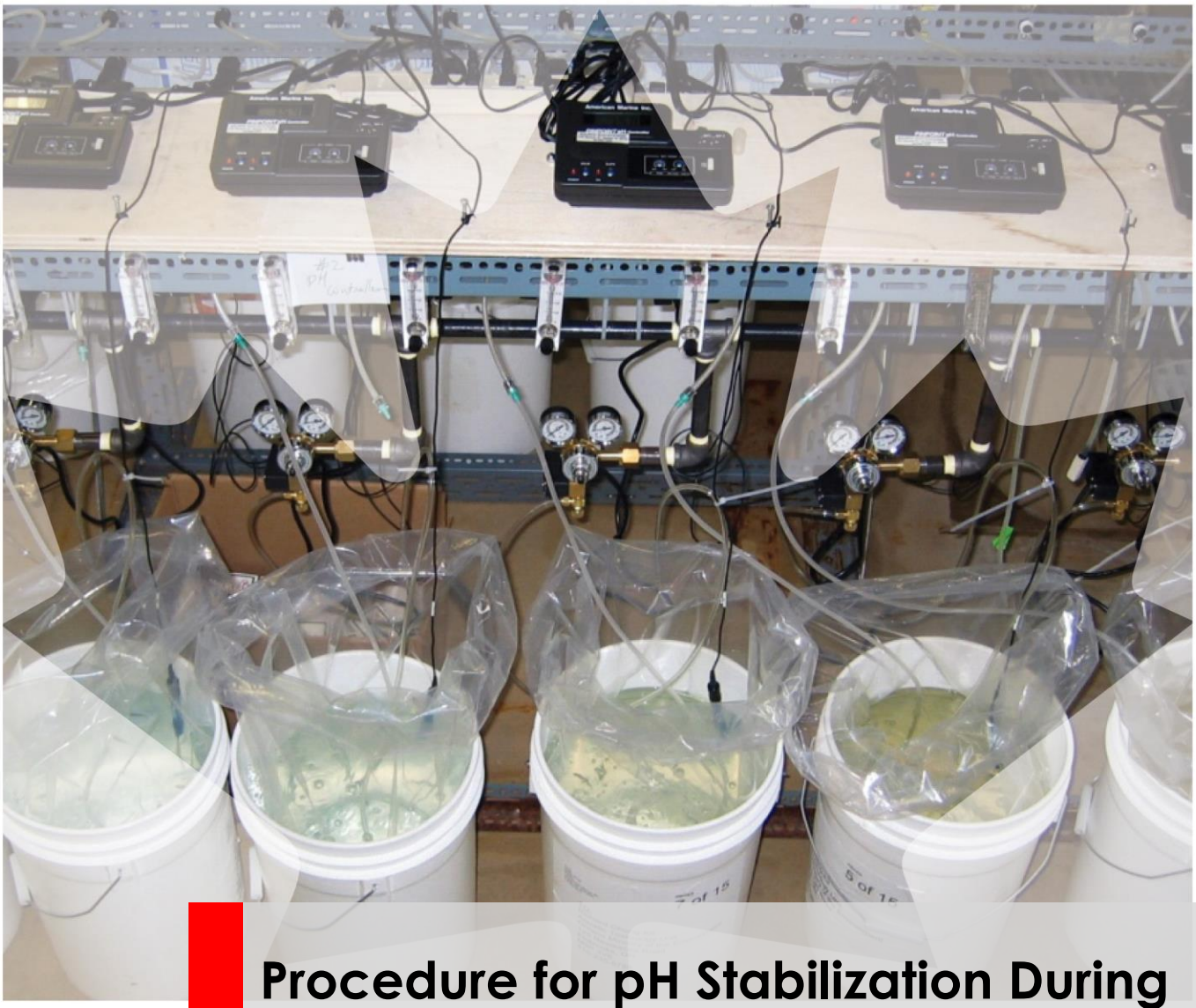

Procedure for pH Stabilization During the Testing of Acute Lethality of Pulp and Paper Effluent to Rainbow Trout

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Photo on cover: pH stabilized toxicity test © Environment and Climate Change Canada

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Environment and Climate Change Canada
Ottawa, Ontario

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Review Notice

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Abstract

This document provides detailed techniques, conditions, and guidance for the pH stabilization of pulp and paper 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”. This procedure is not stand-alone; it is an add-on procedure to Section 5 (Single Concentration test) of the EPS 1/RM/13 test method.

Aeration of pulp and paper effluent samples may cause the pH to rise because of a loss of CO_2, and this change in pH can alter the toxicity of ammonia if present in the effluent sample. To address the potential for residual ammonia toxicity in a pulp and paper effluent sample due to pH drift, Environment and Climate Change Canada has developed the use of the pH Controller technique for the control of pH during the single concentration rainbow trout acute lethality test.

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 sample. In order to use this add-on procedure, the effluent sample must meet four conditions: (i) total ammonia (in mg/L) must be measured on all pulp and paper effluent samples submitted for toxicity testing using EPS 1/RM/13; (ii) the pH stabilization technique may only be used when the un-ionized ammonia concentration present in the 100% 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 effluent sample at 15°C: $y = 1.25 \times (10^{(9.564-pH)}+1)$; (iii) the technique described herein for the pH stabilization of pulp and paper effluent is only acceptable for use with the 100% full strength sample (i.e., the Single Concentration test in Section 5 of EPS 1/RM/13); and (iv) any pH-stabilized test must be run concurrently with a standard rainbow trout test conducted according to EPS 1/RM/13 (i.e., where the pH is not controlled).

This procedure document describes the pH Controller technique as the add-on to EPS 1/RM/13, and includes instructions on the apparatus setup, observations and measurements to be made, and on 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.

Foreword

The pH Controller technique for stabilizing the pH of a pulp and paper effluent sample during an acute lethality test functions as an add-on procedure to Section 5 (Single Concentration test) of the reference method, EPS 1/RM/13, and must be used in conjunction with this reference method for measuring and assessing the toxic effect(s) of pulp and paper effluent on rainbow trout. It may only be used when the test sample has met the four conditions outlined within this document; these conditions pertain to the measurement of total ammonia on all effluent samples submitted for toxicity testing, the amount of un-ionized ammonia in the sample to be tested, the use of the method with the 100% concentration only, and the conduct of parallel tests with and without pH stabilization.

The add-on procedure described herein outlines an explicit set of instructions and conditions to be used with Section 5 (Single Concentration Test) of EPS 1/RM/13, and is applied only to pulp and paper effluent.

Words defined in the Terminology section of this document are italicized when first used in the body of the report according to the definition. Italics are also used to emphasize these and other words throughout the report.

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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 15ºC) is calculated as: un-ionized ammonia = (total ammonia) × $[1/(1 + 10^{pK - pH})]$ (USEPA, 1999).

Buffering capacity is the ability of water to maintain a stable pH; it 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.

Effluent is any liquid waste (e.g., industrial, municipal) discharged to the aquatic environment.

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 than 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 a composite 100% sample at 15 ± 1°C before any aeration of the test solution in the lab.

pH-stabilized test means the EPS 1/RM/13 test method with a pH stabilization technique applied on an 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 multipurpose (generic) biological test methods published by Environment and Climate Change 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.

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

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Introduction

In 2000, Environment and Climate Change Canada published the second edition of 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 or as amended from time to time). The method (last revised in 2016) 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.

In 1992, the requirement to conduct acute lethality testing with rainbow trout (along with *Daphnia magna*, EPS 1/RM/14) was included in the *Pulp and Paper Effluent Regulations* (PPER). The PPER introduced enforceable effluent quality standards for all mills based on standards achievable using secondary effluent treatment, including a requirement that effluents not be acutely *lethal* to rainbow trout (EC, 2014). Tests *can* be conducted using the full-strength (100%) pulp and paper effluent only, or with multiple concentrations (e.g., 100%, 50%etc.) to determine the *LC50*. A sample is considered to "fail" the acute lethality test if >50% rainbow trout mortality is observed in the full-strength sample.

In addition to the required regulatory rainbow trout test following EPS 1/RM/13, pulp and paper facilities began to use *pH* stabilization methods developed by Environment and Climate Change Canada for use with municipal wastewater effluents (EPS 1/RM/50; EC 2008a). The pH stabilization methods have been used on an ad hoc basis for investigative testing by the pulp and paper industry for several years, especially since the publication of EPS 1/RM/50 in 2008. With the ongoing use of municipal wastewater pH stabilization methods, additional research was undertaken by a Canadian industrial research association, FPInnovations, to provide the necessary method research and inter-laboratory validation of a pH stabilization technique specifically for use with pulp and paper effluents (FPI, 2013, 2014, 2016).

The EPS 1/RM/13 test is conducted at $15 \pm 1^{o}C$ for 96 hours under *static* conditions (i.e., no renewal of test solution). 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 sample aeration of the effluent can increase the rate of *pH* change and the removal of volatile compounds (ESG, 2002).

In some cases, aeration of pulp and paper effluents during acute lethality testing can cause the pH to rise from the equilibration of carbon dioxide (CO_2) partial pressure in the effluent 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 some effluent samples, the CO_2 content may be artificially elevated as a result of high biological activity. Any change in pulp and paper effluent pH during an acute lethality test may affect mortality if there are pH-sensitive substances in the contaminant mixture.

Ammonia, which could be of concern in pulp and paper 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 pulp and paper effluent 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.

In pulp and paper biological treatment systems, bacteria use nitrogen (ammonia) and phosphorus (orthophosphate) to metabolize organic chemicals. Ammonia is the primary form of nitrogen used by bacteria for biological oxidation. However, organic nitrogen must first be converted into ammonia before it can be used as a nutrient. Bacteria require a certain level of nitrogen and phosphorus to effectively metabolize the organics released from pulping operations and final effluents can become toxic if the bacteria lack the proper amount of nitrogen (and phosphorus) to break down complex (and toxic) organic substances (FPAC, 2008). As a result, mills typically need to add nitrogen and phosphorus to their treatment systems for the biological degradation of organics; however, nitrogen addition can result in some residual ammonia in the final effluent (Kovacs et al., 2004). Therefore, proper nutrient addition rates and procedures are critical to ensure the existence of a healthy bacterial population and effective breakdown of organic contaminants, while also keeping residual nutrient concentrations in the final effluent to a minimum (FPAC, 2008). In addition, increases in ammonia concentrations could also occur as facilities undertake water conservation efforts resulting in more concentrated effluents.

To address the potential for residual ammonia toxicity in a pulp and paper effluent due to pH drift, Environment and Climate Change Canada developed this standardized pH stabilization technique (i.e., the pH Controller technique) to control the pH during rainbow trout acute lethality testing. The pH stabilization technique is an add-on procedure to Section 5 (single concentration test) of the EPS 1/RM/13 test method. All test requirements of EPS 1/RM/13 must be met when this add-on procedure is used. Additional supporting guidance on the use of pH stabilization is provided in "Supplementary Guidance for Investigating Acute Lethality of Pulp and Paper Mill Effluents due to Ammonia" (ECCC, 2018).

A *pH-stabilized test* can only be performed if four conditions have been met, as outlined in Sections 1.1, 1.2, 1.3, and 1.4.

1.1 Condition #1 – Total Ammonia Measurement in 100% Sample

Total ammonia (in mg/L) *must* be measured on all samples of pulp and paper effluent submitted for toxicity testing using EPS 1/RM/13. This measurement in the 100% (full strength) sample will be used to determine if pH stabilization is appropriate. This concentration of total ammonia (taken from the 100% sample after receipt at the testing laboratory and after adjustment to 15°C) is used in the calculation of un-ionized ammonia at the initial pH (*pH i*) of the effluent at 15°C (refer to Condition #2, Section 1.2).

1.2 Condition #2 – Maximum Ammonia Concentration

The procedure described herein may only be used when the un-ionized ammonia concentration present in the 100% 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 effluent sample at 15°C:

$$y = 1.25 \times (10^{(9.564-pH)}+1)$$

These maximum values for ammonia are set to pre-screen those effluents that would result in rainbow trout mortality regardless of the pH drift observed during the acute lethality test. In other words, the pH stabilization technique would not be 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 an effluent is not of a quality where pH stabilization would be worthwhile (i.e., ammonia is already at an acutely

lethal concentration prior to testing). For additional information and a supporting rationale, please refer to Environment Canada (2008b). 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):

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

where:

pK is 9.56 at 15ºC;

pH is the initial pH of the effluent at 15ºC; and

total ammonia is in mg/L as measured for Condition #1, Section 1.1.

1.3 Condition #3 – pH Stabilization Technique Using Single Concentration Tests

The pH Controller technique described herein for the pH stabilization of pulp and paper effluent is only allowed for use with the 100% full strength sample in conjunction with the Single Concentration test in Section 5 of EPS 1/RM/13. The single concentration test application was validated during an inter-laboratory test program (FPI, 2016).

1.4 Condition #4 – Parallel Testing with Reference Method EPS 1/RM/13

Any pH-stabilized test must be run concurrently according to the Environment and Climate Change Canada Reference Method, EPS 1/RM/13 (i.e., where the pH is not controlled). In the test without pH stabilization, the sample is tested according to EPS 1/RM/13, either the single or multi-concentration test.[1] In the pH-stabilized sample, the pH is controlled at the level measured at test initiation (pH i) using the pH Controller technique and the single concentration test (i.e., 100% effluent).

Parallel testing is required on all samples to consistently demonstrate the presence of ammonia toxicity and pH drift, and to confirm that other pH-sensitive toxicants are not present at acutely lethal concentrations. This is necessary for all samples since the quality of the discharge, concentration of ammonia, the initial sample pH and the rate of pH drift can vary over time.

Tests with and without pH stabilization must be initiated on the same day. The same type of exposure vessel, exposure volume, and batch of fish must be used when conducting parallel tests with and without pH stabilization.

1.5 Overview of the pH Stabilization Technique

Only the pH Controller technique described herein can be used for pH stabilization during rainbow trout acute lethality testing of pulp and paper effluent samples.[2] The pH stabilization procedure does not supersede the existing Environment Canada test method using rainbow trout (EPS 1/RM/13), but is rather an add-on technique.

Application of a pH stabilization procedure with an acute lethality test using rainbow trout will

[1] Single concentration and multiple concentration tests can be used when following EPS 1/RM/13.

[2] An inter-laboratory (round robin) study was conducted in 2014 and 2015 by the industrial research organization FPInnovations to evaluate the effectiveness of the pH controller technique using final effluents from Bleached Chemi-ThermoMechanical Pulp (BCTMP), Kraft, Thermo-Mechanical Pulp (TMP) and Recycled pulp operations. Five contract laboratories participated in the round robin study. The primary objective was to determine if acutely lethal pulp and paper effluents (created to be toxic by the addition of acutely lethal concentrations of ammonia) could be rendered non-acutely lethal using the pH Controller technique described in EPS 1/RM/50 (EC 2008a). Results from the round robin study demonstrated the pH Controller technique could be effectively used (i.e., 28 out of 30 toxic effluents were rendered non-toxic) to prevent pH drift and reduce or eliminate acute lethality in pulp and paper effluents (FPI, 2016).

require training. Some experimentation may be necessary with samples from an individual facility, since the specific water chemistry will vary among (or even within) different types of pulp and paper effluent samples.

In addition to specific test requirements for the pH stabilization technique, all method requirements and procedures for EPS 1/RM/13 must be followed while the tests are being conducted. The rationale behind the technique is to replace the CO_2 lost during test aeration in order to maintain the effluent pH at the initial pH of the sample (*pH i*). The technique is not intended to add more CO_2 than is already present in a pulp and paper effluent, nor is it intended to reduce toxicity resulting from other contaminants.

Procedure for Conducting pH Stabilization of Pulp and Paper Effluents

This section provides details for conducting pH stabilization using the pH Controller technique with pulp and paper effluent. In the pH stabilization test, the pH of the sample is controlled at the level measured at test initiation (pH i) using the technique described below. The pH stabilization procedure does not supersede any regulatory requirements or the existing acute lethality test method using rainbow trout, 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 this pH stabilization technique.

2.1 General Requirements

The pH stabilization procedure can only be used when the single concentration test (Section 5) from EPS 1/RM/13 is performed. The data generated during the development of the pH Controller technique (EC, 2008a), as well as during the pulp and paper inter-laboratory study (FPI, 2016), indicated this method can be successfully applied to effluent samples.[3] However, prior to testing for regulatory purposes, some preliminary investigations may be required, since the specific pulp and paper effluent chemistry will vary among (or even within) facilities. For example, during the inter-laboratory study with effluents from various pulping processes, difficulties were encountered with Thermo-Mechanical Pulp (TMP) mill effluents where initial pH was low (~ 7.0) and pH drift was limited. Similarly, 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. Alternatively, samples with high alkalinity may require the addition of excess CO_2.[4]

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 an effluent 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.

All solutions must be aerated with oil-free compressed laboratory 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. Because the aeration of test solutions will significantly impact the rate of pH drift, which in turn influences the proportion of ammonia present in the un-ionized (acutely lethal) form, rigorous attention is needed for both airflow regulation and for tracking of pH during the test. Additional details are provided in Sections 2.1.1 and 2.1.2.

[3] Elphick et al., (2005) also demonstrated that aeration with low concentrations of carbon dioxide could be used to control pH drift and resulting toxicity due to un-ionized ammonia in municipal wastewater effluent samples.

[4] Kovacs et al. (2004) reported CO_2 concentrations >125 mg/L would be sufficient to cause rainbow trout mortality.

Stabilization of pH must start when aeration is initiated. After 30 minutes of aeration, the concentration of dissolved oxygen must be measured in the 100% test concentration. 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.

EPS 1/RM/13 requires that compressed air be bubbled through a clean air stone. 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.

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 and total ammonia in each effluent sample. The alkalinity of each effluent sample should also be measured. Un-ionized ammonia must be calculated based on the formula provided in Section 1.2. The detection limit for total ammonia should be 0.05 mg/L. Precision and accuracy for the total ammonia measurement should be ± 20%.

Measurements must be taken only after the contents of all containers have been thoroughly combined and 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.

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 an effluent 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 effluent and control) in the 100% sample and the control. 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 least to track changes in effluent pH and to ensure that the pH is maintained within the test validity criteria (Section 2.1.4). More frequent monitoring of pH (e.g., twice daily) may be needed if the initial sample pH is low (e.g., ~7) or if the effluent sample has a low buffering capacity (low alkalinity; e.g., 10 mg/L as $CaCO_3$), which may result in rapid changes in pH and/or necessitate the addition of excess CO_2 to stabilize and maintain the pH.

In order to allow for a full comparison of results, the frequency of measurements (i.e., pH measurements at each 24-h interval) must be conducted in both the unstabilized (EPS 1/RM/13) and pH-stabilized tests (100% concentrations and controls). Furthermore, if monitoring is increased in the pH-stabilized sample (e.g., twice daily pH measurements; or more frequent monitoring during the first 8 hours of the test), the same measurements should be made at the same frequency in the unstabilized test.

2.1.2 Aeration Requirements

The aeration rate can influence the rate of pH drift in the stabilized and unstabilized (EPS 1/RM/13) tests, and consequently the potential rainbow trout mortality to occur due to increases in the concentration of un-ionized ammonia. Control over the aeration rate during the toxicity tests could be the difference between an acutely lethal or non-lethal effluent (FPI, 2016). However, the impact of variability in the aeration rate would be detected during the daily measurement of pH in the stabilized and unstabilized test solutions (see Section 2.1.1).

In most cases, airflow meters will be pre-calibrated by the supplier or manufacturer. Airflow meters used in the pH-stabilized and unstabilized tests must be verified according to industry accepted techniques and practices (i.e., positive-displacement) for air delivery rate by the laboratory. Airflow meters must be visually inspected prior to use and daily while in use. If for any reason aeration rates are suspected to be outside of the required range in any test vessel, the aeration rates must be immediately verified and adjusted as needed.[5]

2.1.3 Ammonia Measurement Requirements

Total ammonia (in mg/L) must be measured on all pulp and paper effluent samples submitted for toxicity testing. The samples for total ammonia measurement must be taken from the 100% effluent sample after receipt at the testing laboratory and after adjustment to 15°C.

In biological treatment systems, nutrients (including ammonia) released from anaerobic breakdown of settled sludge/solids is referred to as benthic feedback (FPAC, 2008). If this occurs, there is a potential for ammonia to be released during a toxicity test, resulting in a gradual increase in total ammonia during the 96-h exposure. This increase can also occur during transportation or holding of the sample prior to testing. Nutrients can also be consumed by bacteria (resulting in a net decrease in ammonia in pulp and paper effluent samples submitted for toxicity testing). For these reasons, total ammonia (in the 100% sample) must also be measured at test completion (96 h), and/or at any time during the test when >50% mortality is observed. Furthermore, in order to allow for a full comparison of results, the measurement for total ammonia must be conducted simultaneously in both the unstabilized (EPS 1/RM/13) and pH-stabilized tests (100% test concentration). In other words, if 60% mortality or greater is observed at any time in either test, a sample for total ammonia measurement must be collected and analyzed from both the pH-stabilized and unstabilized samples (and include calculations of un-ionized ammonia).

Consistent sample collection, storage, preservation, and analytical techniques must be used for the samples collected and analyzed for total ammonia. A variety of standardized and proven analytical methods are available for analysis of total ammonia in effluent samples (e.g., titration, ammonia-selective electrode, colorimetric methods). The analytical method selected for conducting ammonia measurements should take into consideration the predicted/expected concentration of total ammonia in each sample as well as the existence of potential interferences. However, not all methods may be appropriate for use with pulp and paper effluent samples. More specifically, there are two factors that influence the selection of the most appropriate method: ammonia concentration and the presence of interferences (APHA et al., 2005). Depending on the analytical method chosen, interferences can include a variety of chemicals (e.g., chlorine, amines, urea, and others) as well as colour. In the latter case, a study by FPInnovations showed that laboratories using a colorimetric technique for ammonia analysis of pulp and paper effluent samples resulted in an under-estimation of total ammonia concentrations, but this could be overcome by

[5] During the inter-laboratory study, variations in daily aeration rates were observed, and for this reason aeration rates must be closely monitored during the 96-h exposures.

using an ammonia-selective electrode (FPInnovations, 2014). Interferences, including those from coloured or turbid samples, could also be reduced by sample dilution or pre-treatment using distillation (USEPA, 1993).[6]

Storage and preservation conditions must also be carefully considered when collecting samples for total ammonia analysis. According to APHA et al. (2005), the most reliable results are obtained on fresh samples that can be refrigerated (4°C) un-acidified as long as analyses are conducted within 24 h of collection. Sample can also be preserved (for up to 28 days) by acidification to pH 2 using H_2SO_4 (sample must be neutralized immediately prior to analysis), or by freezing (un-acidified to -20°C). However, APHA et al., (2005) also provide cautionary guidance that could be relevant to certain types of pulp and paper effluents in that "although acidification is suitable for certain types of samples, it produces interferences when exchangeable ammonium is present in unfiltered solids".

2.1.4 Test Validity Criteria

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

i) the average pH in the pH-stabilized 100% effluent test solution shifts more than ± 0.2 units from pH i;

ii) the instantaneous pH in the pH-stabilized 100% effluent test solution is greater than ± 0.3 units from pH i; or

iii) if >10% of the fish (combined data if replicates are used) in the pH-stabilized control die or exhibit atypical or stressed behaviour.

2.2 pH Stabilization Using the pH Controller Technique

The pH 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 laboratory 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 that the gas mixture (15% CO_2, 21% O_2, and 64% N_2) can also be used for the 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; examples as follows: [1/2" (1.25 cm) black pipe (natural gas fittings); ½" × 90 deg; ½" 'T'; ½" × 2.5" nipples; 1/8" (0.32 cm) inner diameter Tygon® tubing to connect pipettes; ½" × 6" (15.2 cm) nipples]

[6] The pulp and paper pH stabilization inter-laboratory study was not designed to validate various methods for ammonia analysis. However, variability in total ammonia results was observed among the participating labs. Reasons for the variability was not known or evaluated as each laboratory used a variety of sample handling methods, analytical procedures, storage conditions, etc. Furthermore, ammonia can be either produced or consumed by bacteria present in pulp and paper effluent samples, thereby also contributing to variability in ammonia results. These factors were not evaluated in the round robin study (FPI, 2016).

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 1 to 6.

2.2.1 Regulator/Solenoid Assembly

Individual pressure regulators are connected to the gauge assembly (manifold) (Figure 3). 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 can be turned to the off position (or the controllers and solenoids powered off). 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 liquid leak detector (e.g., 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 6).

2.2.2 pH Controller

The pH Controller (see Figure 5) must be calibrated at the start of the test and verified daily thereafter using certified pH standards. If the pH Controller and associated probe is used to document and record pH in the pH-stabilized test, the pH readings should be verified using the meter/probe used for the unstabilized test.[7] This ensures the pH readings from the stabilized test can be compared to the unstabilized test (e.g., both tests will have the same pH at test start). The tolerance of the pH Controller (i.e., the sensitivity of pH control) must be set before test initiation (typically ± 0.1 pH units, maximum ± 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.

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 6). Durable pH probes should be used to reduce the risk of the electrode-filling solution (e.g., potassium chloride) leaking from the probe into the exposure solution. In the event a probe leaks, the sample is compromised and the test must be discontinued.

2.2.3 Controlling pH Drift

Stabilization of effluent pH commences upon initiation of aeration for 30 minutes at 6.5 ± 1 mL/min · L, before fish are added (see Section 2.1.2). When aeration is started, the main valve on the CO_2 cylinder is opened to

[7] Some laboratories have observed declining performance (with age) of probes supplied with the pH Controller. (G. Schroeder, Pacific and Yukon Laboratory for Environmental Testing; personal communication, 2017).

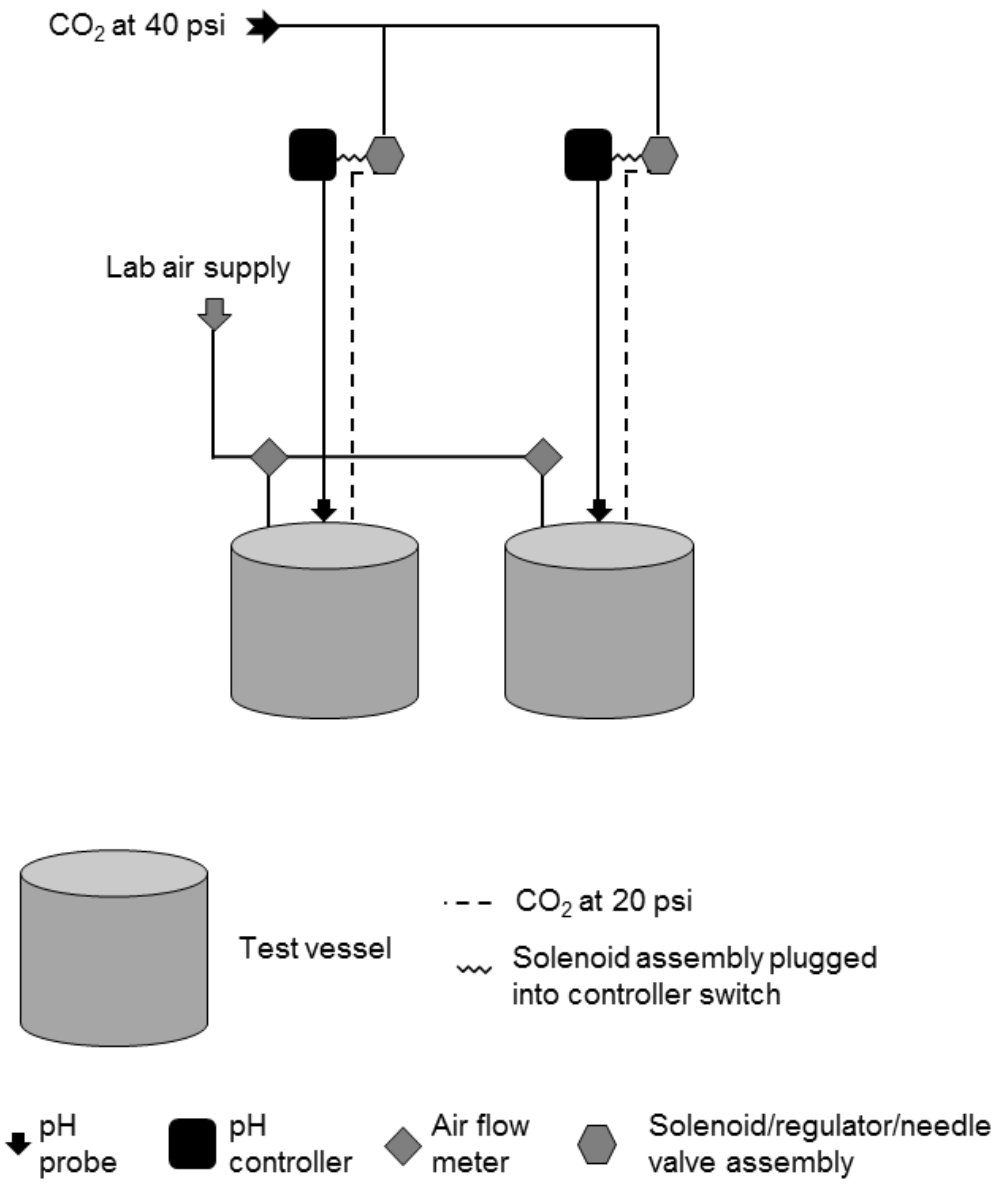

Figure 1 **Schematic diagram of a single concentration test using the pH Controller technique**

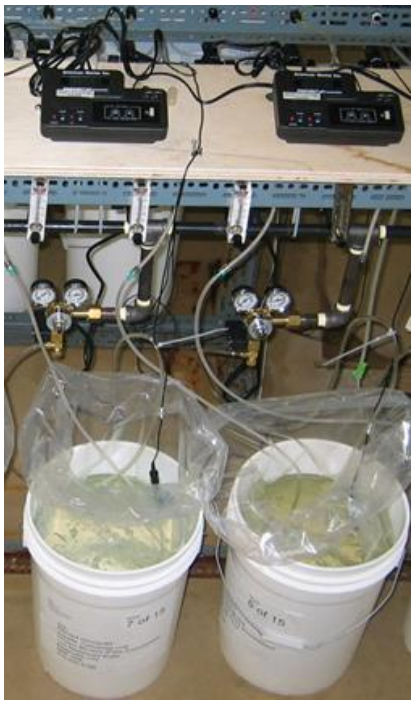

Figure 2 **Overview of setup for single concentration test using the pH Controller technique**

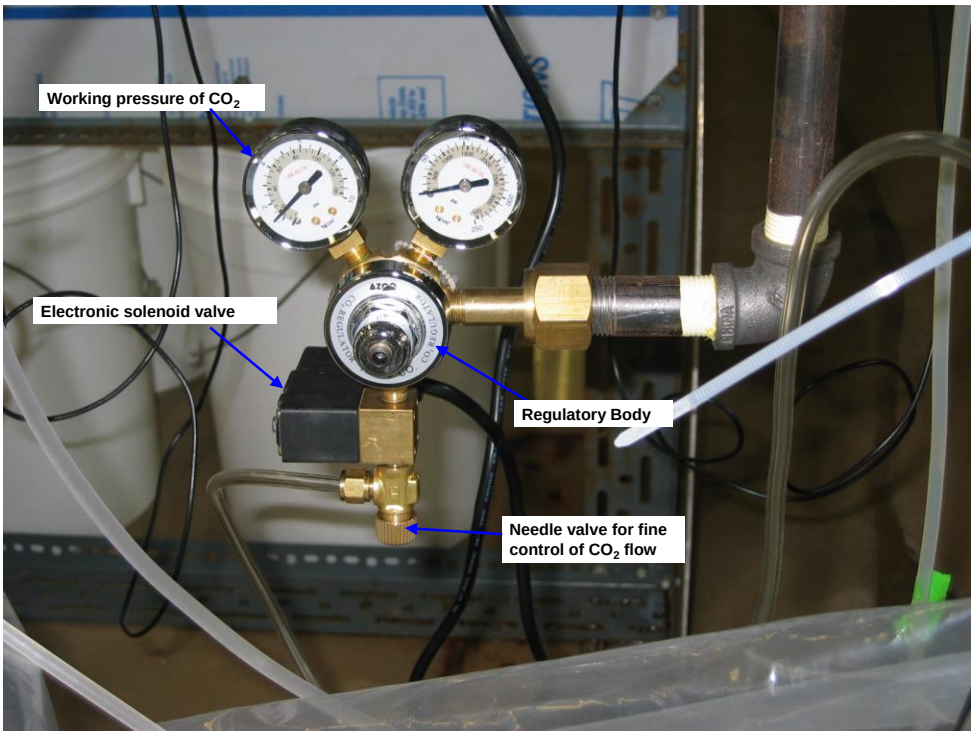

Figure 3 **Solenoid with regulator and needle valve assembly for the pH Controller technique**

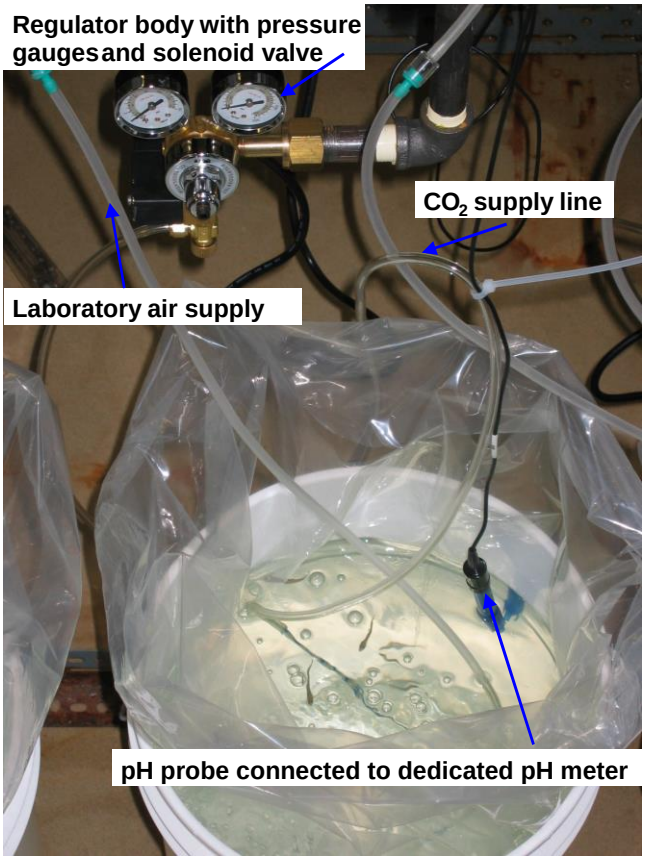

Figure 4 Exposure setup for the pH Controller technique

Figure 5 Example of pH Controller unit

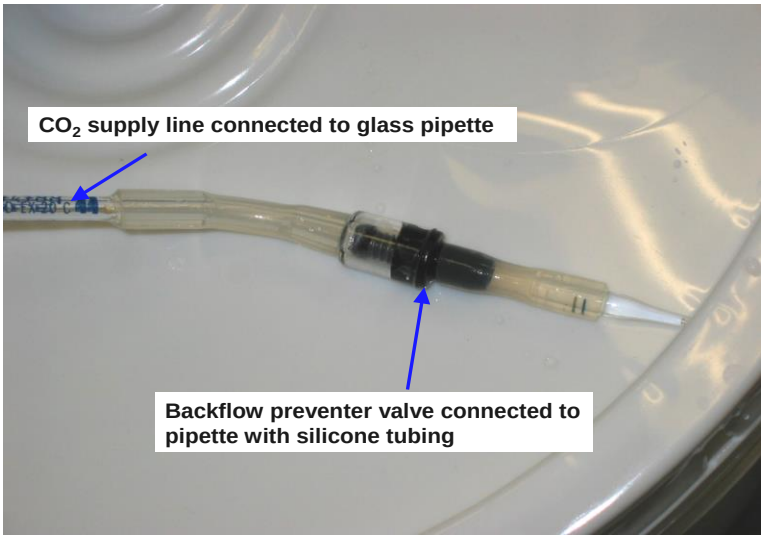

Figure 6 Supply line for CO_2 showing backflow preventer valve

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 laboratory 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 for 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 (minimum) 24, 48, 72, and 96 h in the control and in 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 or if there is a change to the set point on a pH controller. A subsequent pH reading must be taken 30 minutes or sooner after an adjustment, to ensure the pH is being maintained.

In order to allow for a full comparison of results, the frequency of measurements (i.e., pH measurements at each 24-h interval) must be conducted in both the unstabilized (EPS 1/RM/13) and pH-stabilized tests (100% samples and controls). Furthermore, if monitoring is increased in the pH-stabilized sample (e.g., twice daily pH measurements or more frequent monitoring during the first 8 hours of the test), the same measurements should be made at the same frequency in the unstabilized (EPS 1/RM/13) test.

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 pulp and paper effluent. Specific reporting requirements are as follows:

- percentage of CO_2 gas mix or CO_2 used during testing;
- measured concentrations of the following parameters in the 100% effluent sample, after all effluent to be used in testing has been composited and 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 (if measured) alkalinity;
- confirmation that airflow meters were verified; visually inspected prior to use and daily when in use; if aeration rates suspected to be outside range, confirmation that aeration rates were verified and adjusted;
- 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% effluent sample;
- pH measurements taken at a minimum of t = 0 h (test start, when fish are introduced) and at 24, 48, 72, and 96 h in the control and 100% concentration;
- any additional pH measurements and the time taken;
- total ammonia (in the 100% sample) measured at test completion (96 h), and/or at any time during the test when >50% mortality is observed;
- calculated un-ionized ammonia concentrations corresponding to all measurements of both pH and total ammonia;
- coinciding pH and total ammonia measurements in the parallel EPS 1/RM/13 test;
- for total ammonia measurements, description of sample collection, storage and preservation techniques, analysis method, and detection limit (with precision and accuracy to be held on file); and
- average pH based on all readings in the 100% effluent measured during testing.

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