Environmental Protection Series

Biological Test Method:

Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout

Reference Method EPS 1/RM/13
July 1990

PRINTED IN CANADA

Canada

Environment Canada | Environnement Canada

Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout

Environmental Protection
Conservation and Protection
Environment Canada

Reference Method EPS 1/RM/13
July 1990

Canadian Cataloguing in Publication Data

Main entry under title:

Biological test method. Reference method for determining acute lethality of effluents to rainbow trout

(Reference method ; EPS 1/RM/13)
Text in English and French with French text on inverted pages.
Title on added t.p.: Méthode d'essai biologique, méthode de référence pour la détermination de la létalité aiguë chez la truite arc-en-ciel.
Includes bibliographical references.
ISBN 0-662-57746-9
DSS cat. no. En49-24/1-13

1. Rainbow trout -- Mortality -- Testing -- Standards -- Canada. 2. Rainbow trout -- Microbiology -- Standards -- Canada. 3. Effluent quality -- Testing -- Standards -- Canada. I. Canada. Environmental Protection Directorate. II. Canada. Environment Canada. III. Series: Report (Canada. Environment Canada) ; EPS 1/RM/13. IV. Title. V. Title: Reference method for determining acute lethality of effluents to rainbow trout.

QL638.S2 1990 597.'55 C90-098700-6E

Catalogue No. En 49-24/1-13
ISBN 0-662-57746-9
BEAUREGARD PRINTERS LIMITED

Readers' Comments

Comments regarding the content of this report should be addressed to:

Gary Sergy
Technology Development Branch
Environment Canada
No. 210, 4999 - 98 Avenue
Edmonton, Alberta
T6B 2X3

Richard Scroggins
Industrial Programs Branch
Environment Canada
Ottawa, Ontario
K1A 0H3

Abstract

*Explicit standard or reference methods for measuring the acute lethal toxicity of effluents to rainbow trout (*Oncorhynchus mykiss*) are provided in this report. Specific instructions for performing and reporting acute lethality tests with samples of effluent are given, and the guidance provided in the generic methodology report "Acute Lethality Test Using Rainbow Trout" is built upon (Environment Canada, 1990a).*

Methods are given for: 1). a single-concentration test, with full-strength effluent unless otherwise specified; 2). a multi-concentration test to determine the median lethal concentration (LC_{50}); and 3). a test with a reference toxicant. Instructions are included on holding trout in the laboratory, facilities and water supply, handling and storage of samples, preparation of solutions, test conditions, observations to be made, endpoints with methods of calculation, and the use of reference toxicants.

Table of Contents

Abstract v
Terminology ix
Acknowledgements xii

Section 1
Introduction 1

Section 2
Organisms and Holding 2
2.1 Species and Source 2
2.2 Holding and Acclimation 2
2.3 Water 3
2.4 Physicochemical Conditions 3

Section 3
Facilities 4

Section 4
General Procedure for Determining Acute Lethality of Effluent 5
4.1 Sample Labelling, Transport, and Storage 5
4.2 Test Conditions 5
4.3 Preparing Test Solutions 6
4.4 Beginning the Test 6
4.5 Observations and Measurements 7

Section 5
Procedure for Testing a Single Concentration of Effluent 8

Section 6
Procedure for Determining an LC_{50} for Effluent 9

Section 7
Procedure for Tests With a Reference Toxicant 10

Section 8
Reporting Requirements 11
8.1 Data to be Reported 11
8.1.1 Effluent 11
8.1.2 Test Facilities and Conditions 11

8.1.3 Results 12
8.2 Data to be Held on File 12
8.2.1 Effluent 12
8.2.2 Test Facilities and Conditions 12
8.2.3 Results 13

References **15**

Appendix
List of Members of the Inter-Governmental Aquatic Toxicity Group and Conservation & Protection Regional and Headquarters' Office Addresses **17**

Terminology

The following definitions are given in the context of this report, and additional definitions in the detailed companion document (Environment Canada, 1990a) apply here.

Acclimation means to become physiologically adapted to a particular level of one or more environmental variables such as temperature. The term usually refers to controlled laboratory conditions.

Acute means happening within a short period of time, usually taken as ≤ 96-h for fish.

Alevin is a recently-hatched, non-feeding fish with an evident yolk sac (for nutritive requirements). Often referred to as yolk-sac fry.

Conductivity is a numerical expression of the ability of an aqueous solution to carry electric current. This depends on concentrations of ions in solution, their valence and mobility, and on temperature. Conductivity is reported as millisiemens/metre, an SI unit (Système international d'unités), or as micromhos/cm (1 mS/m = 10 μmhos/cm).

Control is a treatment in an investigation that duplicates all the factors that might affect results, except the specific condition being studied. In toxicity tests, the control must duplicate all conditions in the exposure treatment(s), but must contain no test material. The control is used to check for toxicity due to basic conditions such as quality of dilution water, or health and handling of organisms.

Control/dilution water is the water used for diluting the sample of effluent, or for the control test, or both.

Dechlorinated water is a chlorinated water (usually municipal drinking water) that has been treated to remove chlorine.

Dilution water is that which is used to dilute a test material, to prepare different concentrations for the toxicity test.

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

Fingerling is a young (underyearling), actively feeding fish.

Flow-through describes tests in which solutions in test vessels are renewed continuously by the constant inflow of a fresh solution, or by a frequent intermittent inflow.

Fork length is the length of a fish, as measured from the tip of the nose to the fork of the tail.

Hardness is used to mean Total Hardness, the sum of calcium and magnesium concentrations, both expressed as calcium carbonate in milligrams per litre (mg/L).

LC_{50} (median lethal concentration) is the concentration of material (in this case effluent) in water that is estimated to be lethal to 50% of test organisms after a defined period of exposure (e.g., 96-h LC_{50}).

Lethal means causing death by direct action. Death of fish is defined here as the cessation of all visible signs of movement or other activity.

Lux is a unit of illumination based on units per square metre. One lux = 0.0929 foot-candles and one foot-candle = 10.76 lux.

Overt means obviously discernible under the test conditions employed.

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.

Photoperiod is the duration of illumination and darkness within a 24-h day.

Pre-treatment means, in this report, treatment of a sample or dilution thereof, prior to exposure of fish.

Reference toxicant is a standard chemical used to assess the sensitivity of organisms and the validity of measurements of effluent toxicity.

Salinity is the total amount of solid material, in grams, dissolved in 1 kg of seawater. It is determined after all carbonates have been converted to oxides, all bromide and iodide have been replaced by chloride, and all organic matter has been oxidized. Salinity can also be measured directly using a salinity/conductivity meter or other means (see APHA *et al.*, 1989). It is usually reported in parts per thousand (‰).

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

Static replacement describes toxicity tests in which solutions are renewed (replaced) periodically during the test, usually every 24 h. Synonymous terms are "renewal", "batch replacement", and "semi- static".

Sublethal means detrimental to an organism, but below the level which directly causes death within the test period.

Swim-up fry is a young, post-alevin fish which has commenced active feeding.

Toxicity means the inherent potential or capacity of a material to cause adverse effects in living organisms.

Acknowledgements

This report was shaped by the objectives, needs, and comments of members of a subcommittee from Environmental Protection (EP): N. Bermingham (EP, Montreal); C. Blaise (EP, Longueuil); G. Coulombe (EP, Montreal); K. Doe (EP, Dartmouth); G. Elliott (EP, Edmonton); D. MacGregor (EP, Ottawa); J. MacLatchy (EP, Ottawa); W. Parker (EP, Dartmouth); D. Robinson (Fisheries & Oceans Canada, Ottawa); T. Ruthman (EP, Ottawa); R. Scroggins (EP, Ottawa); G. Sergy (EP, Edmonton); and R. Watts (EP, North Vancouver). Special thanks to provincial members of the Inter-Governmental Aquatic Toxicity Group (G. Joubert, ministère de l'Environnement du Québec; G. Westlake, Ontario Ministry of Environment; W. Young, Manitoba Environment and Public Safety; K. Lauten, Saskatchewan Environment and Public Safety; J. Somers, Alberta Environment; S. Horvath and G. van Aggelen, B.C. Ministry of Environment) who provided valuable input and comment. The contributions of the following external reviewers are acknowledged with sincere thanks: D. Vaughan (EP, Dartmouth); S. Wade (EP, Dartmouth); A. Beckett (EP, Edmonton); F. Zaal (EP, Edmonton); S. Yee (EP, North Vancouver); D. Moul (EP, North Vancouver); V. Zitko (Fisheries & Oceans Canada, St. Andrews); V. Cairns (Fisheries & Oceans Canada, Burlington); S. Samis (Fisheries & Oceans Canada, Vancouver); R. Martin (Newfoundland Dept. Environ. & Lands, St. John's); J. Lee (Ontario Ministry of Environment, Rexdale); J. Moores (Alberta Environment, Vegreville); P. Martel (Pulp & Paper Research Inst. of Canada, Pointe Claire); Y. Bois (Technitrol · Eco Inc., Pointe Claire); C.R. Cook (E.B. Eddy Forest Products Ltd., Espanola); G. Craig (Beak Consultants Ltd., Brampton); and D. Bradley, D. Monteith, and J. Pickard (B.C. Research Corp., Vancouver). The laboratory testing support of Environment Canada (see Appendix) is also acknowledged.

Messrs. R. Scroggins and G. Sergy (EP, Environment Canada) acted as Scientific Authorities and provided technical input and guidance. The document was prepared by Don McLeay (McLeay Associates Ltd., West Vancouver, B.C.) and John Sprague (J.B. Sprague Associates Ltd., Guelph, Ontario).

Section 1

Introduction

The procedures for an acute lethality test with rainbow trout, specified by the Canadian government in pollution control regulations for various categories of industry, are given in this report. Rainbow trout have been used for two decades in Canada for testing effluents under a series of regulations and guidelines (EPS, 1971; 1973; 1974; 1977a-c; 1984). An existing standard procedure (EPS, 1980) provided the foundation for the present method, which should be used in conjunction with a more extensive report which gives supporting rationale and additional details (Environment Canada, 1990a).

Many components of procedures in this report are similar to Canadian provincial methods (McGuinness, 1982; Rocchini *et al.*, 1982; Craig *et al.*, 1983; OME, 1989), methods used in the United States (ASTM, 1980; U.S. EPA, 1985a; U.S. EPA, 1985b; APHA *et al.*, 1989), or international techniques (BHSC, 1982; UKWRC, 1983; OECD, 1984). The contribution of the above-mentioned methods to all parts of the present report is acknowledged, and they are recommended as sources of supporting information. Procedures stipulated in this report should, however, be taken as the definitive ones for regulatory purposes.

The organism to be tested is the rainbow trout *Oncorhynchus mykiss* (formerly *Salmo gairdneri*), native to western North America but now inhabiting waters of all Canadian provinces and widely introduced around the world. It thrives in cool, fresh water, runs to sea on both Atlantic and Pacific coasts, and is commonly reared in hatcheries and commercial aquaculture. It has also become the world's standard cool-water fish for freshwater toxicity tests, with a toxicological data bank of appreciable magnitude.

Three basic procedures are described. One uses a single concentration of effluent (full strength unless otherwise specified) and a control, as would be suitable for a pass/fail test. A second procedure estimates the median lethal concentration (LC_{50}) (i.e., it determines the degree of toxicity using several concentrations of effluent including full strength). A third procedure is a multi-concentration test with a reference toxicant, to assess the sensitivity of the test fish to a standard toxicant and the precision of the data produced by the laboratory.

This test is to be used with effluents containing fresh water or having a salinity of $\leq$ 10 ‰, defined as conductivity $\leq$ 1400 mS/m at a temperature of 15 °C. Saline (> 10 ‰) effluents discharging into fresh water should also be tested with rainbow trout acclimated to fresh water. Saline effluents discharging directly to seawater should be tested with a species authorized by the regional Environment Canada laboratory (see Appendix) and acclimated to seawater of similar salinity to that of the effluent.

Section 2

Organisms and Holding

2.1 Species and Source

Test fish are to be rainbow trout (*Oncorhynchus mykiss*), either swim-up fry that have been actively feeding for at least two weeks, or fingerlings. Their average wet weight should be between 0.3 and 5 g, and the largest fish should not be more than twice the length of the smallest in the same test.

Fish may be acquired as eyed eggs, fry, or fingerlings, all from the same hatchery and stock and free of known diseases. Procurement and shipment of fish should be approved by regional representatives of the Federal (Fisheries and Oceans Canada) - Provincial Transplant Committee, in provinces where this committee acts to control movements of fish stocks. Advice on sources of trout can be obtained from regional offices of Environment Canada (see Appendix).

2.2 Holding and Acclimation

Fish should be reared using tanks and other facilities made of nontoxic materials such as stainless steel, porcelain, fiberglass-reinforced polyester, acrylic, polyethylene, or polypropylene. Eggs and alevins may be incubated in vertical-flow hatchery trays or flowing water troughs (Leitritz and Lewis, 1976). Fry and fingerlings may be reared and acclimated in troughs or tanks with flowing water, located away from physical disturbances and preferably separate from the test tanks.

Rearing may be done outdoors, but two and preferably three or more weeks of acclimation to the lighting and temperature to be used in tests (see Section 2.4) are required. This may be done indoors or outdoors using lids with photoperiod-controlled lights. See Environment Canada (1990a) for additional details on holding and acclimating fish for use in toxicity tests.

Tanks should be kept clean, with siphoning of excess food and faeces as frequently as necessary. Tanks with central, double standpipes are partially self-cleaning and are recommended. Tanks should be disinfected and rinsed thoroughly with water used for holding/acclimating fish before introducing a new batch of fish. Disinfectants such as those containing chlorinated or iodophore compounds or n-alkyldimethyl-benzylammonium chloride should be used.

Unless specified otherwise by the feed manufacturer, feeding should be once or more per day with a recognized (standard) commercial pelleted fish food, at a daily ration approximating 1 to 5% of wet body weight, depending on temperature and fish size (Environment Canada, 1990a). Pellet size and type should be chosen in consideration of fish size and age, water temperature, and the manufacturer's recommendations. The duration and method of food storage should also follow the manufacturer's recommendation.

Dead and moribund fish should be removed immediately after daily inspection.

Mortalities in the stock tank(s) from which test fish are to be taken should be less than 2% during the seven days before a test. If mortality is 2 to 10%, acclimation should be extended for at least seven days until a mortality of ≤ 2% is achieved in a seven-day period. Mortalities > 10% per week make the group of fish unacceptable for future use if deaths are caused by disease or aquatic contaminants. If deaths result from other factors (e.g., high initial mortalities during transition from alevins to swim-up fry or following fish transfer), the fish may be used for future toxicity tests provided that mortalities in the stock tank(s) from which fish are to be taken decline to < 2% during the seven days immediately preceding a test. Chemical treatment of diseased fish should be avoided, and if treated they must not be used in toxicity tests for at least four weeks. The test with a reference toxicant (see Section 7) gives some indication of suitability of the fish for toxicity tests.

2.3 *Water*

Water for holding and acclimating fish can be uncontaminated ground- , surface, or dechlorinated municipal water. The water should consistently support good survival, health, and growth of trout. Chemical quality of the laboratory supply should be measured as often as necessary to document variation. This should include at least hardness, pH, conductivity, dissolved oxygen, and residual chlorine (if municipal water is used), and if possible, alkalinity, suspended solids, total organic carbon, total dissolved gases, ammonia, nitrite, metals, and total organophosphorus pesticides. Any supersaturation with gases should be remedied (see Environment Canada, 1990a).

If municipal water is used, it must be free of any harmful concentration of chlorine upon fish exposure. The target value for total residual chlorine in holding tanks, and for that in control/dilution water within test tanks, is ≤ 0.002 mg/L (see Environment Canada, 1990a).

Flow of fresh (new) water through tanks used for holding and acclimating fish must be ≥ 1.0 L/min for every kilogram of fish being held (1.4 L/g fish·d^{-1} or 0.69 g fish· d/L). In addition, a tank must contain at any given time, ≥ 1.0 L/10 g of fish. Under unusual circumstances, cleaning and recirculation of water may be permissible (Environment Canada, 1990a).

2.4 *Physicochemical Conditions*

Lighting should be by overhead full-spectrum fluorescent lights, with intensity at the water surface ≤ 500 lux. For at least two weeks before a test, photoperiod must be constant at 16 ± 1 h of light and 8 ± 1 h of darkness, preferably with a 15- to 30-min. transition period.

Holding temperature may be 4 to 18 °C, but fish must be acclimated for ≥ 2 weeks, and preferably ≥ 3 weeks, at 15 ± 2 °C before use in a test. Change between temperature levels may proceed at ≤ 3 °C/d. Dissolved oxygen within tanks should be 80 to 100% air saturation. Supplementary aeration using filtered, oil-free compressed air, should be provided if necessary. The pH of water should be within the range 6.0 to 8.5. Temperature, oxygen, pH, flow, and fish mortalities should be monitored for each holding or acclimation tank, preferably daily; weekly or more frequent monitoring of levels of ammonia, nitrite, and total residual chlorine (if municipal water source) in holding or acclimation tanks is recommended.

Section 3

Facilities

Tests must be performed in a facility isolated from general laboratory disturbances, either a separate room or a section walled or curtained off. Dust and fumes should be minimized. Control of test temperature ($15 \pm 1°C$) may be achieved by thermostatically-controlled air conditioning or by immersing test vessels in regulated water baths.

Test vessels and all other material and equipment that may contact the test solutions or control/dilution water must not contain leachable substances, nor should they sorb toxicants from the test solution. Test vessels must be glass or Plexiglas™, acrylic, polypropylene, polyethylene, or have polyethylene liners. If liners are used, they must be discarded at the end of the test. It is recommended that test vessels be covered (Environment Canada, 1990a). All containers for a test should be identical, and the minimum water depth must be 15 cm. Equipment must be thoroughly cleaned and rinsed in accordance with good laboratory procedures.

The control/dilution water should be the type described in Section 2.3, and it should preferably be identical to that used for holding and/or acclimating the fish.

Section 4

General Procedure For Determining Acute Lethality of Effluent

4.1 Sample Labelling, Transport, and Storage

Sample-volume requirements depend on fish size and numbers per test solution, loading-density requirements, test concentrations, and the use of replicates. For single-concentration tests, sample volumes of 25 to 50 L or more are normally required. For tests to determine an LC_{50}, sample volumes of 50 to 100 L or more are normally required.

Containers for storage and transportation of samples must be of nontoxic material (e.g., polyethylene or polypropylene carboys or pails). The containers must be new or thoroughly cleaned and dried, and should be rinsed with clean water, then with the sample to be collected. They should be filled with sample to exclude air and then be sealed. Labelling must include at least sample type, source, date and time of collection, and name of sampler(s).

Samples should be kept from freezing. During transport, samples should be kept dark, and at a temperature of 1 to 8 °C if more than two days are spent in transit. Upon arrival at the laboratory, samples may be adjusted immediately or overnight to 15 °C, then testing commenced. If stored at the test facility, samples must be kept dark and cool (8 °C or less, preferably 4 ± 2 °C).

Testing of samples should commence as soon as possible after collection. The test should begin within three days and must commence no later than five days after termination of sampling. Samples must be agitated thoroughly just before pouring aliquots to prepare solutions. Sub-samples (a sample divided between two or more containers) must be combined.

4.2 Test Conditions

This is a 96-h static test, i.e., there is no replacement of solutions. Loading of fish into each test vessel should not exceed a density of 0.5 g/L $\cdot d^{-1}$ over four days (i.e., $\geq$ 2 L/g $\cdot 4d^{-1}$; $\geq$ 0.5 L/g $\cdot d^{-1}$). Fish are not fed during the test, nor during the 24-h period immediately preceding it. The test is not valid if > 10% of control fish die or exhibit atypical/stressed behaviour (Environment Canada, 1990a).

The test must be conducted at 15 ± 1 °C. All solutions are aerated throughout the test. Lighting must be the same as that defined for acclimation (see Section 2.4). Photoperiod (a light : dark cycle of 16 ± 1 h : 8 ± 1 h) must coincide with the timing which prevailed during acclimation.

The test must be conducted without adjustment of sample or solution pH. However, if it is desired to understand the extent to which extremes in solution or sample pH (e.g., outside the range 5.5 to 8.5) may contribute to acute lethality, a parallel (pH-adjusted) test may be used. If both pH-adjusted and non-adjusted tests are run, definitive results should be those derived from the non-adjusted test. Rationale and

procedural details regarding pH adjustment are provided in Environment Canada (1990a). Adjustment of pH is also one of a number of "Toxicity Identification Evaluation" techniques for characterizing the cause of sample toxicity (Mount and Anderson-Carnahan, 1988).

4.3 Preparing Test Solutions

Adjustment of the effluent sample and control/dilution water to 15 ± 1° C must be done if temperature is outside that range.

For a given test, the same water is to be used for preparing the control(s) and all test concentrations less than 100%. This is almost always the same water as used for acclimation. If temperature of this water is adjusted upwards, supersaturation with gases must be avoided. The water must have an oxygen content within the range of 90 to 100% air-saturation, achieved if necessary by vigorous aeration with oil-free compressed air passed through air stones or glass diffusers.

Test vessels should be rinsed with control/dilution water just before use, although that is not necessary if disposable polyethylene liners are used. Test solutions must be made up and well mixed with a glass rod, Teflon™ stir bar, or other non-reactive device, just before their use. All test vessels, measurement devices, stirring equipment and fish-transfer pails must be thoroughly cleaned and rinsed in accordance with standard operational procedures.

Upon preparation of the test solutions, each should be aerated for a period of 30 minutes at a rate ≤ 7.5 mL/min $\cdot L^{-1}$. Thereafter, 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 pre-aeration (i.e., before exposure of fish) of all solutions including the control(s) must be continued at ≤ 7.5 mL/min $\cdot$ L $^{-1}$. This period of pre-aeration must be restricted to the lesser of 90 minutes and attaining 70% saturation in the highest test concentration (or 100% saturation if supersaturation is evident). Immediately thereafter, fish must be placed in each test solution and the test initiated, regardless of whether 70 to 100% saturation was achieved in all test solutions. Aeration of test solutions should be provided by bubbling compressed air through a clean silica-glass air diffuser or disposable glass pipette (Environment Canada, 1990a). Bubble size should be in the range of 1 to 3 mm.

4.4 Beginning the Test

Each test vessel must be clearly coded or labelled as to concentration, date, and time of start. Vessels should be positioned for easy observation of fish, and the concentrations should preferably be positioned at random. Aeration of each test solution at a rate no greater than 7.5 mL/min $\cdot L^{-1}$ should be continued throughout the test, using one of the air diffusers specified in Section 4.3.

If one or more test solutions are highly coloured, opaque or foamy, baskets made of nontoxic, nonabrasive material (e.g., nylon, polyethylene, polypropylene) may be used to permit inspections of fish during the test. If used, a basket should be placed in each test vessel including the control(s). Baskets should be big enough to permit fish movement throughout the test vessel. Each basket must be thoroughly cleaned and

rinsed with control/dilution water before being used.

Equal numbers of fish are to be introduced into each test solution including the control(s). At least ten fish should be used per treatment. They may be divided between two or more vessels at the same concentration to meet the required limit on loading. The order of adding fish to vessels should be randomized beforehand. Individual fish are to be used only once, and handling methods should minimize stress (Environment Canada, 1990a).

4.5 Observations and Measurements

Colour, turbidity, odour, and floating or settling solids in the sample should be noted at the start of the test. The appearance of test solutions should also be noted, and any obvious changes during the test should be recorded.

Measurements of dissolved oxygen and pH must be made in each test solution including the control(s), at the start and end of the test as a minimum. Final measurements should be done after biological observations are complete. Conductivity of each test solution should be measured at the start of the test as a minimum. Temperature should be measured in representative solutions, daily.

Each vessel should be inspected at least at 24, 48, 72, and 96 h, and dead fish recorded and removed. Fish are considered to be dead when they fail to show evidence of opercular or other activity, and do not respond to subsequent gentle prodding. Overt sublethal toxic effects should also be recorded (see Environment Canada, 1990a). For highly-coloured, opaque, or foamy test solutions, fish may be inspected using a dip net (cleaned and rinsed before use) or by raising them to the surface within a suitable basket (Section 4.4). Mean (± SD) fork lengths and wet weights of control fish should be determined at the end of the test.

Section 5

Procedure for Testing a Single Concentration of Effluent

All conditions, procedures, and facilities specified in Sections 1, 2, 3, 4, 7 and 8 apply to the procedure for testing a single concentration of effluent.

This procedure uses one concentration of effluent, 100% unless otherwise specified, plus a control. Replicate solutions may be tested but are not required. At least ten fish should be exposed to each solution of effluent and control. The test is invalidated if >10% of the control fish exhibit atypical/stressed behaviour and/or mortality.

The end point for this test is percentage mortality at 96 h. Mortality of 50% is commonly used, and is associated with greater precision of measurement than percentages such as 20% or 80% which are closer to the extremes of the distribution of effects. For example, the 1990 proposed Pulp and Paper Effluent Regulations of Environment Canada (1990b) define an effluent as failing this test if the effluent at 100 % concentration kills 50 % or more of the fish.

Section 6

Procedure for Determining an LC_{50} for Effluent

All conditions, procedures, and facilities specified in Sections 1, 2, 3, 4, 7 and 8 apply to this procedure.

At least five concentrations of effluent plus a control (dilution water only) must be used in tests to estimate an LC_{50}. The highest concentration must be full-strength effluent, and each successive concentration must have at least 50% of the strength of the next higher one. A geometric (logarithmic) series is beneficial (e.g., percentage concentrations such as 100, 50, 25, 12.5, 6.3). Concentrations may be based on other proportions or on standard dilution series (see Appendix D of Environment Canada, 1990a).

Replicates of each concentration may be used but are not required. If replicates are used, their data are combined for calculating the LC_{50}. The precision of the estimate of LC_{50} increases with the number of organisms used, but not necessarily its accuracy.

The 96-h LC_{50} and its 95% confidence limits should be calculated and the method of calculation reported. Computer programs for calculating LC_{50} and confidence limits are available (Environment Canada, 1990a) and should be used. A recommended program is available for copying onto a user-supplied disk through courtesy of C.E. Stephan (Stephan, 1977), from Environment Canada (address in Appendix). A check of the computer-derived LC_{50} should be made by examining a plot on logarithmic-probability scales, of percent mortalities at 96 h for the various effluent concentrations (see Environment Canada, 1990a).

The test is invalidated if >10% of the control fish (combined data if replicates are used) exhibit atypical/stressed behaviour and/or mortality.

Section 7

Procedure for Tests With a Reference Toxicant

A reference toxicant must be used to assess the relative sensitivity of test fish and the precision of data produced by the laboratory. The selected chemical(s) must be tested at least once during each calendar month when an effluent is tested, and upon acclimation of a new batch of fish. The procedures and conditions to be followed are identical to those in Section 4 and as described in Environment Canada (1990a, c), except that a reference chemical is measured out and tested, instead of an effluent. The control/dilution water used routinely in effluent tests should also be used for the reference toxicant.

Reagent-grade phenol and/or zinc sulphate ($ZnSO_4 \cdot 7H_2O$) are recommended for use as reference toxicants. The 96-h LC_{50} should be determined for the reference toxicant(s) used and expressed as mg/L based on phenol or zinc (Zn^{++}) (see Environment Canada, 1990a). Stock solutions of phenol must be made up on the day of use, and those for zinc should be prepared fresh on the day of use or stored in the dark at pH 3 to 4.

Concentrations of reference toxicant in all stock solutions should be measured chemically by appropriate methods (APHA *et al.*, 1989). Upon preparation of the test solutions, aliquots are to be taken from at least the control, low, middle, and high concentrations, and analyzed directly or stored for future analysis should the LC_{50} be atypical (outside warning limits). If stored, sample aliquots must be held in the dark at 4 ± 2 °C. Both zinc and phenol aliquots should be preserved (APHA *et al.*, 1989) before storage. Stored aliquots requiring chemical measurement should be analyzed promptly upon completion of the toxicity test. It is desirable, but not required, to measure concentrations in the same solutions at the end of the test after completing biological observations. Calculations of LC_{50} should be based on measured concentrations if they are appreciably (i.e., $\geq 20\%$) different from nominal ones.

A warning chart (U.S. EPA, 1985a; Environment Canada, 1990c) or similar record must be prepared for each reference toxicant used, and continually updated. Successive LC_{50}s are plotted to determine whether they are within ± 2 SD of the geometric mean (or arithmetic mean, in those instances where data are not normally distributed) of previous LC_{50}s. The geometric mean with its upper and lower warning limits (± 2 SD, still calculated on a logarithmic basis) is recalculated with each successive LC_{50} until the statistics stabilize (U.S. EPA, 1985a; Environment Canada, 1990c). If a particular LC_{50} falls outside the warning limits, sensitivity of the fish and validity of recent effluent tests are both suspect. A check of all acclimation and test conditions is required under these circumstances. Depending on the findings, further acclimation and re-evaluation of the fish with one or more reference toxicants should be undertaken, or a new population of fish should be procured and acclimated for use in toxicity tests.

Section 8

Reporting Requirements

The following is a summary of reporting and record-keeping requirements associated with this reference method. Further details or explanation can be found within previous sections of this method.

Unless otherwise specified by Environment Canada, all items listed in Section 8.1 must be reported to Environment Canada for each completed toxicity test. The information is to be provided in accordance with pertinent regulations, and in a manner and format specified by Environment Canada* (i.e., manual or electronic; transmission mode; form and content).

Information additional to that in Section 8.1, such as that required by or distinctive to a regulation, or that which is necessary to clarify reporting and data assessment, may also be specified by Environment Canada.

Unless otherwise specified by Environment Canada, those items listed under Section 8.2 must be recorded and held on file for a period of five years. This information is to be provided as and when requested by Environment Canada. It will be required on a less frequent basis such as during an audit or investigation.

8.1 Data to be Reported

8.1.1 Effluent

- name and location of operation generating the effluent;
- date and time of sampling;
- type of sample (e.g. "whole effluent from plant", "final mill effluent", "discharge from emergency spill lagoon", "leachate");
- brief description of sampling point;
- sampling method (e.g., "grab", "batch", "24-h composite with sub-samples at 1-h intervals");
- person collecting sample; and
- indication if sample frozen or partially frozen in transit.

8.1.2 Test Facilities and Conditions

- test type and protocol (e.g., "single-concentration test", protocol specified in Reference Method "EPS 1/RM/13";
- indication of any differences from method given in this report;
- name and city of testing laboratory;

*Contact an office listed in the Appendix for details.

- species of test organism;
- date and time for start of definitive test;
- persons performing the test and verifying the results;
- the pH, temperature, dissolved oxygen, and conductivity of unadjusted, undiluted effluent, just before preparing test solutions;
- indication of aeration of test solutions (rate, time) before introduction of fish; method and rate of aeration throughout the test;
- concentrations and volumes tested, including controls, and indication of replication;
- measurements of dissolved oxygen, pH, temperature, and conductivity determined for each test solution (including control(s)) during the test; and
- number of fish per vessel; mean (± SD) wet weight and loading density (g/L) of fish in test solutions.

8.1.3 Results

- number of mortalities of fish in each test solution (including control(s)) at 96 h; number of control fish showing atypical/stressed behaviour;
- estimate of 96-h LC_{50} and 95% confidence limits in multi-concentration tests, by computer calculation; indication of statistical method (e.g., log-probit, moving average) on which result is based; and
- most recent 96-h LC_{50} (with 95% confidence limits) for reference toxicant(s); chemical(s), date test initiated; historic geometric mean LC_{50} and warning limits (± 2 SD).

*8.2 Data to be Held on File**

8.2.1 Effluent

- detailed description of sampling point, source and type of effluent;
- type of container(s) and label or code used;
- volume and/or weight of sample;
- transport and storage conditions (times, temperatures);
- appearance and other properties (observations on colour, turbidity, odour, floating or settleable material);
- colour change, precipitation, flocculation, release of volatiles or other changes when making up test solution(s); and
- procedures and results for any chemical analyses (e.g., suspended solids content, hardness).

8.2.2 Test Facilities and Conditions

- address of testing laboratory;
- description of rearing/acclimation and test facilities including general layout of each and means of isolation;
- normal holding and acclimating conditions (containers, location, lighting, temperatures including maximum rate of change, aeration, volumes and flows of water, method of water renewal, numbers

---

* To be stored for a five-year period at the test facility and/or the offices of the discharger. Some of this information may be comon to a series of tests, and recorded and held on file as a general report.

and densities of fish, handling methods, food type, ration and frequency of feeding, disease incidence and treatment if any, weekly percent mortality);

- source of test fish;
- brief history of test-specific conditions and procedures for holding and acclimating fish (e.g., times, water source, and characteristics such as temperature, pH and dissolved oxygen content, food type and ration, disease incidence and treatment, weekly percent mortality) if different from usual practice;
- description of source(s) of water used for rearing and acclimating fish and as control/dilution water;
- pre-treatment of acclimation/control/dilution water, if any (e.g., adjustment of temperature, aeration rate and duration, quantity of any chemical added);
- quality (mean and range values) of acclimation and control/dilution water as measured for source water and within holding tank(s); to include hardness, pH, conductivity, dissolved oxygen content, and total residual chlorine (if municipal water); preferably also total dissolved gases, alkalinity, solids, organic carbon, colour, mineral ions, metals, ammonia, nitrite, and organophosphorus pesticides;
- systems to regulate light and temperature;
- light source, photoperiod, and past measures of intensity at rearing/acclimation tanks and at surface of test vessels;
- description of test vessels (size, shape, and material), covers and baskets (if used for inspecting fish), and routine cleaning procedures for each;
- conditions and apparatus for aeration of test solutions;
- fork length and wet weight of fish used in test (means and standard deviations, sample size);
- appearance of solutions; any changes during test;
- test concentrations of reference toxicant(s), both nominal and measured; indication of data set used to estimate LC_{50}; and
- any measurements of water quality in test solutions not included in data reported (Section 8.1.2).

8.2.3 Results

- any observations of mortalities of fish not included in data reported (e.g., at 24, 48, and 72 h) (see Section 8.1.3);
- observations of fish behaviour and appearance recorded for each test solution during the test; and
- confirmation that graph verified any computer-derived LC_{50}(s).

References

APHA *et al.*, "Toxicity Test Methods for Aquatic Organisms", p. 8-1 – 8-143 in: *Standard Methods for the Examination of Water and Wastewater*, 17th ed. American Public Health Association, American Water Works Association, and Water Pollution Control Federation, Washington, D.C., Part 8000, (1989).

ASTM, "Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians", Amer. Soc. Testing and Materials, Philadelphia, PA, Report E729-80, 25 p. (1980).

BHSC, "Methods for the Determination of Ecotoxicity", Approved Code of Practice, Notification of New Substances Regulations 1982, British Health and Safety Commission, London, U.K., Report COP 8, 13 p. (1982).

Craig, G.R., K. Flood, J. Lee, and M. Thomson, "Protocol to Determine the Acute Lethality of Liquid Effluents to Fish", Ontario Ministry of Environment, Rexdale, Ontario, 9 p. (1983).

Environment Canada, "Acute Lethality Test Using Rainbow Trout", Conservation and Protection, Ottawa, Ontario, Report EPS 1/RM/9 (1990a).

Environment Canada, 1990 proposed "Pulp and Paper Regulations", (1990b).

Environment Canada, "Guidance Document for Control of Toxicity Test Precision Using Reference Toxicants", Conservation and Protection, Ottawa, Ontario, Report EPS 1/RM/12 (1990c).

EPS, "Pulp and Paper Effluent Regulations", Environ. Prot. Serv., Environment Canada, Regulations, Codes and Protocols, Ottawa, Ontario, Report EPS 1-WP-72-1 (1971).

EPS, "Guidelines on the Use and Acceptability of Oil Spill Dispersants", Environ. Prot. Serv., Environment Canada, Report EPS 1-EE-73-1, Ottawa, Ontario (August, 1973).

EPS, "Petroleum Refinery Effluent Regulations and Guidelines", Environ. Prot. Serv., Environment Canada, Regulations, Codes and Protocols, Ottawa, Ontario, Report EPS 1-WP-74-1 (1974).

EPS, "Metal Mining Liquid Effluent Regulations and Guidelines", Environ. Prot. Serv., Environment Canada, Regulations, Codes and Protocols, Ottawa, Ontario, Report EPS 1-WP-77-1, (1977a).

EPS, "Meat and Poultry Products Plant Liquid Effluent Regulations and Guidelines", Environ. Prot. Serv., Environment Canada, Regulations, Codes and Protocols, Ottawa, Ontario, Report EPS 1-WP-77-2 (1977b).

EPS, "Potato Processing Plant Liquid Effluent Regulations and Guidelines", Environ. Prot. Serv., Environment Canada, Regulations, Codes and Protocols, Ottawa, Ontario, Report EPS 1-WP-77-4 (1977c).

EPS, "Standard Procedure for Testing the Acute Lethality of Liquid Effluents", Environ. Prot. Serv., Environment Canada, Regulations, Codes and Protocols, Ottawa, Ontario, Report EPS 1-WP-80-1 (1980).

EPS, "Guidelines on the Use and Acceptability of Oil Spill Dispersants", Environ. Prot. Serv., Environment Canada, Regulations, Codes and Protocols, Ottawa, Ontario, Report EPS 1-EP-84-1 (1984).

Leitritz, E. and R.C. Lewis, "Trout and Salmon Culture (Hatchery Methods)", University of California, CA, Fish Bulletin No. 164, 197 p. (1976).

McGuinness, E.J., "Procedures Manual, Aquatic Bioassay Service Projects", Alberta Environmental Centre, Vegreville, Alberta, Report AECV-82M1, 56 p. (1982).

Mount, D.I. and L. Anderson-Carnahan, "Methods for Aquatic Toxicity Identification Evaluations, Phase I. Toxicity Characterization Procedures", U.S. Environmental Protection Agency, Duluth, MN, Report EPA-600/3-88/034 (1988).

OECD, "Guideline for Testing of Chemicals - Fish, Acute Toxicity Test", Organization for Economic Cooperation and Development, Paris, France, Document No. 203, 12 p. (1984).

OME, "Protocol for Single Concentration Acute Lethality Tests on Liquid Effluent Samples Using Rainbow Trout", Ontario Ministry of Environment, Rexdale, Ontario, 10 p. (1989).

Rocchini, R.J., M.J.R. Clark, A.J. Jordan, S. Horvath, D.J. McLeay, J.A. Servizi, A. Sholund, H.J. Singleton, R.G. Watts and R.H. Young, "Provincial Guidelines and Laboratory Procedures for Measuring Acute Lethal Toxicity of Liquid Effluents to Fish", B.C. Ministry of Environment, Victoria, B.C., 18 p. (1982).

Stephan, C.E., "Methods for Calculating an LC_{50}", p. 65-84 in: *Aquatic Toxicology and Hazard Evaluation,* F.L. Mayer and J.L. Hamelink (eds.), Amer. Soc. Testing and Materials, Philadelphia, PA, ASTM STP 634 (1977).

UKWRC, "Acute Toxicity to Fish. Determination of the 96-hour LC_{50} of Test Substances to the Rainbow Trout Under Flow-through Conditions", Water Research Centre, Great Britain, Medmenham, U.K., Standard Operating Procedure, No. EP 120 02, 19 p. (1983).

U.S. EPA, "Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms", W.H. Peltier and C.I. Weber (eds.), U.S. Environmental Protection Agency, Report EPA/600/4-85/013, Cincinnati, OH (1985a).

U.S. EPA, "Acute Toxicity Test for Freshwater Fish; Standard Evaluation Procedure", U.S. Environmental Protection Agency, Hazard Evaluation Division, Washington, DC, Report EPA-540/9-85-006, 12 p. (1985b).

Appendix

List of Members of the Inter-Governmental Aquatic Toxicity Group and Conservation & Protection Regional and Headquarters' Office Addresses

Members of the Inter-Governmental Aquatic Toxicity Group*

Federal**

P. Wells (Chairperson)
EP, Dartmouth, Nova Scotia

B. Moores
St. John's, Newfoundland

K. Doe
Dartmouth, Nova Scotia

W. Parker
Dartmouth, Nova Scotia

N. Bermingham
Longueuil, Québec

C. Blaise
Longueuil, Québec

G. Elliott
Edmonton, Alberta

R. Watts
North Vancouver, British Columbia

K. Day
National Water Research Institute
Burlington, Ontario

B. Dutka
National Water Research Institute
Burlington, Ontario

C. Kriz
Federal Programs Branch
Ottawa, Ontario

D. MacGregor
Commercial Chemicals Branch
Ottawa, Ontario

P. MacQuarrie
Commercial Chemicals Branch
Ottawa, Ontario

R. Scroggins
Industrial Programs Branch
Ottawa, Ontario

G. Sergy
Technology Development Branch
Edmonton, Alberta

P. Farrington
Water Quality Branch
Ottawa, Ontario

Provincial

C. Bastien
Ministère de l'Environnement du Québec
Sainte Foy, Québec

G. Westlake
Ontario Ministry of Environment
Rexdale, Ontario

W. Young
Manitoba Environment and Public Safety
Winnipeg, Manitoba

K. Lauten
Saskatchewan Environment and Public Safety
Regina, Saskatchewan

J. Somers
Alberta Environment
Vegreville, Alberta

S. Horvath
B.C. Ministry of Environment
Vancouver, British Columbia

G. van Aggelen
B.C. Ministry of Environment
North Vancouver, British Columbia

---

* IGATG membership as of July 1990
** Conservation and Protection, Environment Canada

Conservation & Protection, Regional and Headquarters' Office Addresses

Headquarters
351 St. Joseph Boulevard
Place Vincent Massey
Hull, Québec
K1A 0H3

Atlantic Region
15th Floor, Queen Square
45 Alderney Drive
Dartmouth, Nova Scotia
B2Y 2N6

Québec Region
1141 Route de L'Eglise
P.O. Box 10100
Sainte Foy, Quebec
G1V 4H5

Ontario Region
25 St. Clair Ave. East, 6th Floor
Toronto, Ontario
M4T 1M2

Western and Northern Region
Room 210, Twin Atria # 2
4999 -98 Avenue
Edmonton, Alberta
T6B 2X3

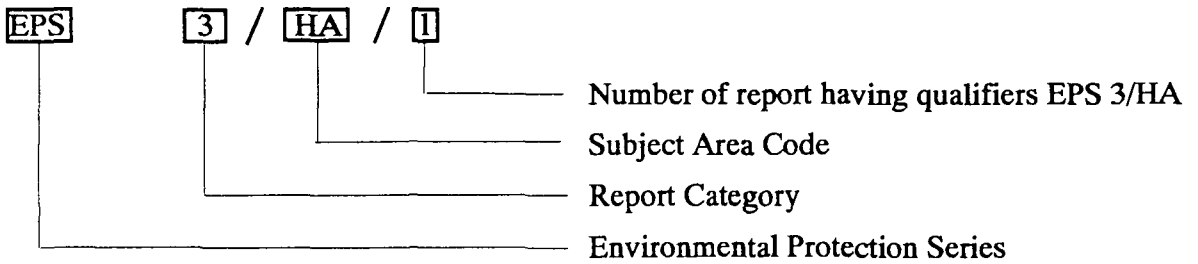
Pacific and Yukon Region*
224 West Esplanade Street
North Vancouver, British Columbia
V7M 3H7

---

* A BASIC computer program for calculating LC50s is avaible for copying onto a formatted 13-cm IBM-compatible floppy disk supplied by the user, by contacting the Aquatic Toxicity Laboratory at this address.

ENVIRONMENTAL PROTECTION SERIES

Sample Number:



Categories

1 Regulations/Guidelines/Codes of Practice
2 Problem Assessments, and Control Options
3 Research and Technology Development
4 Literature Reviews
5 Surveys
6 Social, Economic and Environmental Impact Assessments
7 Surveillance
8 Policy Proposals & Statements
9 Manuals

Subject Areas

AG Agriculture
AN Anaerobic Technology
AP Airborne Pollutants
AT Aquatic Toxicity
CC Commercial Chemicals
CE Consumers and the Environment
CI Chemical Industries
FA Federal Activities
FP Food Processing
HA Hazardous Wastes
IC Inorganic Chemicals
MA Marine Pollution
MM Mining and Ore Processing
NR Northern and Rural Regions
PF Paper and Fibres
PG Power Generation
PN Petroleum and Natural Gas
RA Refrigeration and Air Conditioning
RM Reference Methods
SF Surface Finishing
SP Oil and Chemical Spills
SRM Standard Reference Methods
TS Transportation Systems
TX Textiles
UP Urban Pollution
WP Wood Protection/Preservation

New subject areas and codes are introduced as they become necessary. A list of EPS reports may be obtained from Environmental Protection Publications, Conservation and Protection, Environment Canada, Ottawa, Ontario, Canada, K1A 0H3