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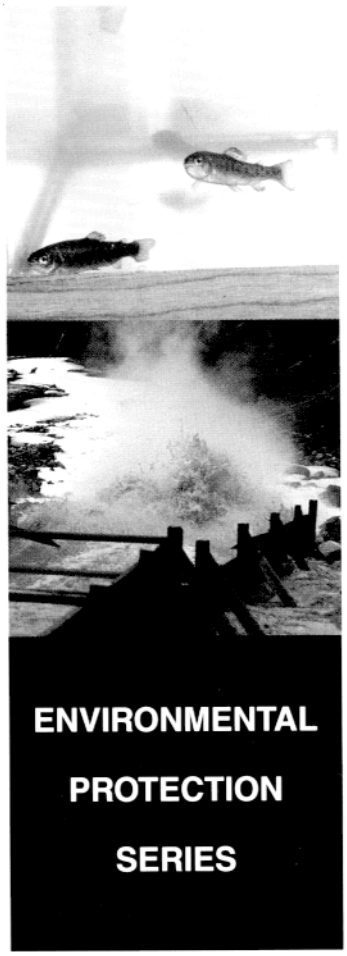

Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout

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Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout

Method Development and Applications Section
Environmental Technology Centre
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Review Notice

This report has been reviewed by the staff of the Environmental Technology Advancement Directorate, Environment Canada, and approved for publication. Mention of trade names or commercial products does not constitute endorsement by Environment Canada. Other products of similar value are available.

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 acute lethality tests with samples of effluent are given. The guidance provided in the generic methodology report "Acute Lethality Test Using Rainbow Trout" (EC, 1990a, including its May 1996 Amendments) is built upon herein.*

The present report represents the second edition of Reference Method EPS 1/RM/13, published in July 1990 and amended in May 1996 (EC, 1990b, including its May 1996 Amendments). It supersedes that earlier version, and is to be applied as Environment Canada's current reference method *for determining the acute lethality of effluents to rainbow trout.*

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 (LC50); 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.

Foreword

This is one of a series of **reference methods** *for measuring and assessing the toxic effect(s) on single species of aquatic or terrestrial organisms, caused by their exposure to samples of test materials or substances under controlled and defined laboratory conditions.*

A **reference method** *is defined herein as a specific biological test method for performing a toxicity test, i.e., a toxicity test method with an explicit set of instructions and conditions which are described precisely in a written document. Unlike other multi-purpose (generic) biological test methods published by Environment Canada, the use of a* **reference method** *is frequently restricted to testing requirements associated with specific regulations.*

Reference methods *are those that have been developed and published by Environment Canada (EC), and are favoured:*

- *for regulatory use in the environmental toxicity laboratories of federal and provincial agencies;*
- *for regulatory testing which is contracted out by Environment Canada or requested from outside agencies or industry;*
- *for incorporation in federal, provincial, or municipal environmental regulations or permits, as a regulatory monitoring requirement; and*
- *as a foundation for the provision of very explicit instructions.*

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Terminology

The following definitions are given in the context of the procedures in this report. Additional definitions in the detailed companion document (EC, 1990a, including its May 1996 Amendments) apply here.

Grammatical Terms

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

Technical Terms

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). It is often referred to as yolk-sac fry.

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

Control is a treatment in an investigation or study 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 measurable 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 is the water used for diluting the sample of effluent, and for the control test.

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

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, measured from the tip of the nose to the fork of the tail.

Hardness is the concentration of cations in water that will react with a sodium soap to precipitate an insoluble residue. In general, hardness is a measure of the concentration of calcium and magnesium ions in water, expressed as mg/L calcium carbonate.

LC50 (median lethal concentration) is the concentration of material (in this case, effluent) in water that is estimated to be lethal to 50% of the test organisms. The LC50 and its 95% confidence limits are usually derived by statistical analysis of percent mortalities in several test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 96-h LC50).

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 then 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.

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

Reference method refers to a specific biological test method for performing a toxicity test, i.e., a

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

Reference toxicant is a standard chemical used to measure the sensitivity of the test organisms to establish confidence in the toxicity data obtained for a test material. In most instances, a toxicity test with a reference toxicant is performed to assess the sensitivity of the organisms at the time the test material is evaluated, and the precision of results obtained by the laboratory for that chemical.

Salinity is the total amount of solid substance, in grams, dissolved in 1 kg of water. 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.*, 1998). It is usually reported in grams per kilogram or parts per thousand (‰).

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

Static replacement describes toxicity tests in which test 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 the fish, 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 is the inherent potential or capacity of a material to cause adverse effect(s) on fish or other living organisms. The effect(s) could be lethal or sublethal.

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The following persons contributed to the May 1996 Amendments to the first edition of this *reference method* (EC, 1990b), which are now incorporated (with changes, as deemed appropriate) in this second edition: C. Bastien (ministère de l'Environnement du Québec, Sainte-Foy); B. Bayer (Manitoba Environment, Winnipeg); K. Doe (Environmental Conservation Service [ECS], Moncton); G. Elliott (EPS, Edmonton); P. Jackman (ECS, St. John's); J. Miller (EPS, Ottawa); G. van Aggelen (ECS, North Vancouver); G. Westlake (Ontario Ministry of Environment, Rexdale); and R. Watts (ECS, North Vancouver).

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Introduction

This report replaces the first edition of Environment Canada's *reference method* EPS 1/RM/13 (EC, 1990b), which was published in July 1990 and amended in May 1996. Testing instructions, guidance, and wording in this (second) edition of EPS 1/RM/13 remain the same as in the amended first edition except for the changes or additions included here, which were considered by Environment Canada to be necessary or prudent improvements.

The procedures for an acute lethality test with rainbow trout, as specified by Canadian governments involved in pollution monitoring and control of industrial or municipal effluents, are given in this report. Rainbow trout have been used for three decades in Canada for testing effluents under a series of regulations and guidelines (EPS, 1971, 1973, 1974, 1977a–c, 1980, 1984; Government of Canada, 1992). This *reference method* should be used in conjunction with a more extensive report which gives supporting rationale and additional details (EC, 1990a, including its 1996 Amendments).

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; USEPA, 1985a, 1985b; APHA *et al.*, 1998), 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 *reference method* should, however, be taken as the definitive ones for regulatory purposes.

Rainbow trout *(Oncorhynchus mykiss*; formerly *Salmo gairdneri*) are to be used as the test organisms when performing this *reference method.* This species is native to western North America. It now inhabits waters of all Canadian provinces and has been widely introduced around the world. It thrives in cool, fresh water, runs to the sea on both Atlantic and Pacific coasts, and is commonly reared in hatcheries and commercial aquaculture operations. 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 (LC50) (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 for that chemical.

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 (>10‰) effluents discharging directly to estuarine or marine receiving waters 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.

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 must be between 0.3 and 2.5 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. Each group of fish should be obtained from a certified "disease-free" hatchery with an ongoing health monitoring and certification program. 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, fibreglass-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 in a location separate from the test tanks.

Following the transport of fish to the rearing/acclimation facilities of the testing laboratory, they must be held under conditions specified in Section 2.4 for a minimum period of two weeks. This acclimation period must immediately precede their use in a test. Fish acclimation 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 (EC, 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 or 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 monitored and recorded daily, and as a minimum must be monitored and recorded five days per week. The cumulative rate of fish mortality during the 7-day period preceding the day that the toxicity test is started must be less than 2%. If the cumulative fish mortality rate during this period is 2 to 10%, acclimation must be extended for at least an additional seven days and until a cumulative 7-day mortality rate of <2% is achieved for the 7-day period preceding the day that the toxicity test is started. A cumulative mortality rate of >10% per week, during any 7-day period, makes 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 the day that the test is started.

Chemical treatment of diseased fish should be avoided. If the use of chemically treated fish cannot be avoided, a minimum two-week period must follow their treatment before they are used in tests. The test with a reference toxicant (see Section 7) gives some indication of the suitability of the fish for use in toxicity tests.

2.3 Water

Water for holding and acclimating fish can be uncontaminated groundwater, surface water, or dechlorinated municipal drinking water. The water supply should consistently support good survival, health, and growth of trout. Chemical quality of the laboratory's water 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 drinking water is used), and as appropriate, alkalinity, suspended solids, total organic carbon, total dissolved gases, ammonia, nitrite, metals, and total organophosphorus pesticides. Any supersaturation with gases should be remedied (see EC, 1990a).

If dechlorinated municipal drinking water is used, it must be free of any harmful concentration of chlorine or chlorinated compounds 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 EC, 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 or 0.69 g fish · d/L). In addition, a tank must contain at any given time, ≥1.0 L/10 g fish. Water flow rates to each tank should be measured and recorded at regular (e.g., daily or, as a minimum, weekly) intervals. Additionally, ten or more fish should be removed randomly from each holding/acclimation tank at regular (e.g., weekly) intervals to determine individual wet weights and assure that these requirements are met. The mean wet weight of individual fish should be determined and recorded, for each of these samples. These measurements should also be used as a guide when determining the volume of effluent required for a test, and to ensure that the maximum loading density of 0.5 g fish/L

solution in each test vessel during the toxicity test (see Section 4.2) will not be exceeded.

2.4 Physicochemical Conditions

Lighting should be full spectrum, with 100 to 500 lux intensity at the water surface. 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-minute 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 to the tanks should be provided if necessary, using filtered, oil-free compressed air. The pH of water should be within the range of 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 and nitrite in holding or acclimation tanks is recommended. Weekly or more frequent monitoring of water in these tanks for total residual chlorine is also recommended if dechlorinated municipal drinking water is used as the water source.

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 ± 1°C) may be achieved by thermostatically controlled air conditioning or by immersing test vessels in regulated water baths.

Test vessels and all other 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 high quality (nontoxic) polyethylene liners. If liners are used, they must be discarded at the end of the test. It is recommended that test vessels be covered if necessary to prevent fish from escaping (EC, 1990a). All containers used for a test must 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.

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 LC50, sample volumes of 50 to 100 L or more are normally required.

Containers for storage and transportation of samples must be made 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 sealed (e.g., using a snap-on lid if the sample container is a pail). Labelling must include at least sample type, source, date and time of collection, and name of sampler(s).

Samples must be kept from freezing. During transport, samples should be kept in the dark, and at a temperature of 1 to 8° C if more than two days are spent in transit. Upon the receipt of sample(s) at the laboratory, the temperature of the effluent in each sample container should be measured and recorded. That portion of each sample to be used in the toxicity test must be adjusted to 15 ± 1°C before the toxicity test can be started.

To enable the toxicity test to be started on the day that the sample is received in the laboratory, temperature adjustment of the effluent sample(s) may be done quickly (see Section 4.3). Alternatively, the laboratory may choose to store the sample in the dark at 4 ± 2°C for a brief period (e.g., over the weekend, if the sample arrived on a Friday afternoon). Using this option, the sample must be stored in full, sealed containers which are held in the dark within a refrigerated facility. A third option is to hold the sample overnight within a facility adjusted to the test temperature (i.e., 15 ± 1°C), in which instance the test must be started the next day. If a sample is warmed or cooled at 15 ± 1°C overnight, it must be kept in one or more full, sealed containers during that time.

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. The contents of each sample container must be agitated thoroughly just before pouring aliquots to prepare solutions. Sub-samples (i.e., aliquots of 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 must not exceed a density of 0.5 g/L; adherence to this requirement is based on the mean wet weight of control fish at the end of the test (Section 4.5). Fish must not be fed during the test, nor during the 16-h period immediately preceding it. The test is not

valid if >10% control fish die or exhibit atypical/stressed behaviour (EC, 1990a).

The test must be conducted at 15 ± 1°C. All solutions must be aerated throughout the test, at a controlled rate of 6.5 ± 1 mL/min · L. 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 of 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 (USEPA, 1991).

4.3 Preparing Test Solutions

Adjustment of the effluent sample and control/dilution water to 15 ± 1°C must be done if the temperature is outside that range. The sample may be cooled using a cold-water bath or immersion cooler made of nontoxic material, or warmed using a hot-water bath. Samples or test solutions must not be heated by immersion heaters.

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 the 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 clean air stones. Air stones acceptable for use are: (i) Aqua Fizzz, 2.5 cm length × 1.5 cm diameter, cylindrical (one use only); or (ii) AS1 silica glass, 3.8 cm length × 1.3 cm width, rectangular (re-usable after proper cleaning; see EC 1990a and associated May 2007 amendments for details).

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

The depth of solution in each test vessel must be at least 15 cm. Upon preparation of the test solutions, each must be aerated for a period of 30 minutes at 6.5 ± 1 mL/min · L. 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 6.5 ± 1 mL/min · L. This period of pre-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 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 must be provided by bubbling compressed air through clean

air stones (see page 8 for details). Aeration of each test solution at a rate of 6.5 ± 1 mL/min · L must be continued throughout the test.

4.4 Beginning the Test

One or more control solutions must be prepared and included as part of each test conducted on each sample. The multiple use of a control solution and its fish for more than one toxicity test and/or more than one effluent sample is unacceptable.

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. If a multi-concentration test is being performed (Section 6), the concentrations should be positioned at random.

Healthy fish which have been acclimated for a minimum of two weeks to the temperature and lighting conditions used in the test (see Sections 2.1, 2.2, 2.3, and 2.4) must be taken randomly from the acclimation tank(s) for use in the test. Handling and transfer procedures should minimize stress (EC, 1990a).

At least ten fish must be introduced into each test concentration including each control solution. They may be divided between two or more vessels at the same concentration to meet the required limit on loading (see Section 4.2).

Besides positioning the test vessels at random within the testing facility, the order of adding fish to each test solution should also be randomized. One or two fish should be introduced sequentially to each test solution, including the control solution(s), until 10 fish have been placed in each.

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 allow fish to move throughout the test vessel. Each basket must be thoroughly cleaned and rinsed with control/dilution water before being used.

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, pH, and temperature 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 must be measured at the start of the test as a minimum.

The frequent and routine observation of fish in each test vessel is required to obtain information regarding their time to death as well as any overt sublethal responses evident, and to remove dead fish which could otherwise foul the test solution. Whenever possible, fish in each test vessel should be inspected at least at 24, 48, 72,

and 96 h; more frequent observations are recommended during the initial day of the test. During each observation period, all dead fish must be 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 EC, 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).

The mean fork length of control fish must be determined and recorded at the end of the test. The measurements of fork length, for each control fish, should be used to assess if the size of the test fish met with the recommendation that "*the largest fish should not be more than twice the length of the smallest in the same test*" (Section 2.1).

The mean wet weight of individual control fish must be determined and recorded at the end of the test. This measurement must be used to confirm that the acceptable range of weights for test fish, specified in Section 2.1 (i.e., "*their average wet weight must be between 0.3 and 2.5 g*"), was met. It must also be used to confirm that the requirement for fish-loading density in test solutions specified in Section 4.2 (i.e., "*the loading of fish in each test vessel must not exceed a density of 0.5 g/L"*) was met.

All surviving fish (including the controls) used in the test must be disposed of in a humane manner at the end of the test. Overdosing the fish with an anaesthetic such as tricaine methanesulphonate is recommended.

Procedure for a Single-concentration Test to Determine the Mortality Rate at 96 Hours

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. The use of replicate solutions (e.g., three replicates of the 100% concentration and three replicate control solutions, using 10 fish in each replicate solution) is recommended for this test, to provide greater confidence in the test results and their interpretation. At least ten fish must be exposed to each solution of effluent and control. The test is invalidated if >10% of the control fish (combined data, if replicates are used) exhibit atypical/stressed behaviour and/or mortality.

The endpoint for this test is percentage mortality at 96 h. Mortality of 50% is commonly used. For example, the 1992 Federal Pulp and Paper Effluent Regulations promulgated under the Federal Fisheries Act (Government of Canada, 1992) define an effluent as failing this test if the effluent at 100% concentration kills more than 50% of the fish.

Procedure for a Multi-concentration Test to Determine the 96-h LC50

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 LC50. At least ten fish must be exposed to each test concentration, including the undiluted (100%) concentration and the control. The highest concentration must be full-strength (100%) 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., percent concentrations such as 100, 50, 25, 12.5, 6.3). Concentrations may be based on other proportions or on standard dilution series (see EC, 1990a; Appendix D).

Since this LC50 test must include full-strength (100%) effluent as the highest concentration, the single-concentration endpoint of percent mortality in 100% effluent at 96 h (see Section 5) can also be determined from the results of this test.

Replicates of each concentration may be used but are not required. If replicates are used, their data are combined for calculating the LC50. The precision of the estimate of LC50 increases with the number of fish exposed to each test concentration, although the accuracy of this estimate is not necessarily improved.

The 96-h LC50 and its 95 % confidence limits should be calculated and the method of calculation reported. Computer programs for calculating LC50 and confidence limits are available (EC, 1990a) and should be used. A recommended program is available from Environment Canada (address in Appendix) for copying onto a user-supplied diskette, through the courtesy of C.E. Stephan (Stephan, 1977). A check of the computer-derived LC50 should be made by examining a plot on logarithmic-probability scales, of percent mortalities at 96 h for the various effluent concentrations (see EC, 1990a).

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

Procedure for Tests with a Reference Toxicant

A reference toxicant must be used to assess the relative sensitivity of the population of fish used in the toxicity test, and the precision and reliability of data produced by the laboratory personnel for that reference toxicant under standardized test conditions. The selected reference 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. Fish used in the reference toxicity test conducted in conjunction with a test for determining the acute lethality of effluent must be from the same group held at the laboratory and used in the effluent test. 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 must also be used for the reference test.

Reagent-grade phenol and/or zinc sulphate ($ZnSO_4 \cdot 7H_2O$) are recommended for use as reference toxicants. The 96-h LC50 should be determined for the reference toxicant(s) used and expressed as mg/L based on phenol or zinc (Zn^{++}) (see EC, 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 using appropriate methods (APHA *et al.,*1998). 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 LC50 be atypical (i.e., 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.,*1998) 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 LC50 should be based on measured concentrations if they are appreciably (i.e., ≥20%) different from nominal ones.

Once sufficient data are available (EC, 1990c), a warning chart which plots values for LC50 must be prepared, and continually updated, for each reference toxicant used. The warning chart should plot logarithm of concentration on the vertical axis against date of the test or test number on the horizontal axis. Each new LC50 for the reference toxicant should be compared with the established warning limits of the chart; the LC50 is acceptable if it falls within the warning limits. All calculations of mean and standard deviation must be made on the basis of log(LC50). The mean of log(LC50), together with its upper and lower warning limits (± 2 SD) as calculated by using the available values of log(LC50), are recalculated with each successive LC50 until the statistics stabilize (USEPA, 1985a; EC, 1990c).

The warning chart may be constructed by simply plotting mean and ± 2 SD as the logarithms, or if desired, by converting them to arithmetic values and plotting LC50 and ± 2 SD on a logarithmic scale of concentration. If a particular LC50 fell outside the warning limits, the sensitivity of the test fish and the performance and precision of the test would be 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 subsequent toxicity tests with effluent and reference toxicant(s).

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 toxicity test that is initiated. 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 information that 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"); and
- person collecting the sample.

8.1.2 Test Facilities and Conditions

- test type and method; e.g., "single-concentration test method as specified in the second (December 2000) edition of EPS 1/RM/13";
- indication of any deviation from one or more "must" requirements delineated in Sections 2 to 7 of this report, including details as to the deviation;
- name and city of testing laboratory;
- percent mortality of fish in stock tank(s) from which test fish are taken, as recorded daily (or, as a minimum, for five of the seven days spanning a weekly period) for the seven-day period immediately preceding the test;
- species of test organism;
- date and time for start of definitive test;

* Contact an office listed in the Appendix for details.

- person(s) performing the test and verifying the results;

- the pH, temperature, dissolved oxygen, and conductivity of unadjusted, undiluted effluent, just before preparing test solutions;

- confirmation that no adjustment of sample or solution pH occurred; indication of procedure used for any pH adjustment if both pH-adjusted and non-adjusted tests were run (see Section 4.2);

- indication of aeration of test solutions (rate, time) before introduction of fish; rate of aeration throughout the test;

- concentrations and volumes tested, including controls, and indication of any replication;

- measurements of dissolved oxygen, pH, and temperature determined for each test solution including control(s) at the beginning and end of the test, as well as conductivity of each test solution at the beginning of the test;

- number of fish added to each test vessel;

- mean fork length of control fish at the end of the test, together with the range of the values measured;

- mean wet weight of individual control fish at the end of the test; and

- estimated loading density (g/L) of fish in test solutions.

8.1.3 Results

- number of mortalities of fish in each test solution including the control(s), at 96 h; number of control fish showing atypical/ stressed behaviour;

- mean mortality rate in solutions of effluent and control water, if a single-concentration or multi-concentration test is performed using replicate solutions; mean number of control fish showing atypical/stressed behaviour if replicate control solutions;

- estimate of 96-h LC50 and 95% confidence limits in multi-concentration tests, if statistically achievable; indication of statistical method (e.g., log-probit, moving average) on which result is based; and

- most recent 96-h LC50 (with 95% confidence limits) for reference toxicity test(s) performed with the group of fish used in the effluent test; reference chemical(s); date test initiated; historic geometric mean LC50 and warning limits (± 2 SD).

*8.2 Data to be Held on File**

8.2.1 Effluent

- all information (e.g., code, sample description, date/time of sampling) affixed to label(s) on sample container(s);

- volume of sample;

- transport and storage conditions (times; temperature on receipt at laboratory and during storage; indication if sample frozen or partially frozen on arrival);

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

- 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 performed on the effluent, if available (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, procedure for water renewal, numbers and densities of fish, handling procedures, food type, ration and frequency of feeding, disease incidence and treatment if any, weekly cumulative 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 cumulative percent mortality) if different from usual practice;

- description of source(s) of water used for rearing and acclimating fish and as control/dilution water;

- pretreatment of acclimation and 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 dechlorinated municipal drinking 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;

- procedures used to randomize the introduction of fish to test vessels;

- procedure and apparatus for aeration of test solutions;

- all measurements of fork length and wet weight of individual fish used in test (together with the mean value and range of values for each sample);

- depth of test solutions; appearance of solutions, including any changes evident during test;

- test concentrations of reference toxicant(s), both nominal and measured; indication of data set used to estimate LC50; 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

- any manual plot(s) of data used to verify a computer-derived LC50.

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Appendix

Members of the Inter-Governmental Environmental Toxicity Group and Addresses of Environment Canada's Headquarters and Regional Offices

Members of the Inter-Governmental Environmental Toxicity Group (as of December, 2000):

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J. Bruno
Pacific Environmental Science Centre
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C. Buday
Pacific Environmental Science Centre
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* A computer program for calculating LC50s is available for copying onto an IBM-compatible diskette supplied by the user, by contacting the Environmental Toxicology Section at this address.