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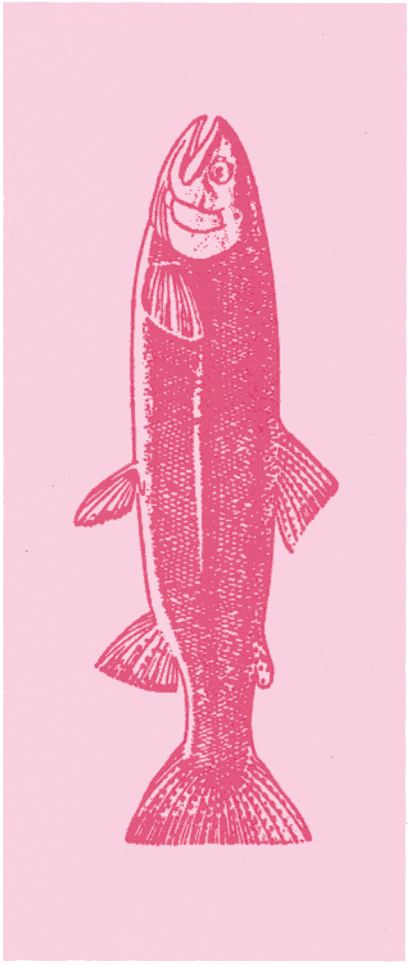

Biological Test Method:

Acute Lethality Test Using Rainbow Trout

Report EPS 1/RM/9 July 1990 (with May 1996 and May 2007 amendments)

Canada

Environment Canada

Environnement Canada

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Acute Lethality Test Using Rainbow Trout

Method Development and Applications Section
Environmental Technology Centre
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Abstract

Methods recommended by Environment Canada for performing acute lethality tests with rainbow trout (Oncorhynchus mykiss, *formerly named* Salmo gairdneri), *are described in this report.*

General or universal conditions and procedures are outlined for undertaking an acute lethality test using a variety of test materials. Additional conditions and procedures are stipulated which are specific for assessing samples of chemicals, effluents, elutriates, leachates, or receiving waters. Included are instructions on holding and acclimating test organisms, sample handling and storage, test facility requirements, procedures for preparing test solutions and test initiation, specified test conditions, appropriate observations and measurements, endpoints, methods of calculation, and the use of reference toxicants.

Résumé

Le présent document expose les méthodes recommandées par Environnement Canada pour l'exécution d'essais de létalité aiguë sur la truite arc-en-ciel (Oncorhynchus mykiss, *auparavant* Salmo gairdneri).

Il présente les conditions et méthodes générales ou universelles permettant de réaliser des essais de létalité aiguë sur un large éventail de substances à expérimenter. Il précise d'autres conditions et méthodes propres à l'évaluation d'échantillons de produits chimiques, d'effluents, d'élutriats, de lixiviats ou de milieux récepteurs. Le lecteur y trouvera des instructions pour la détention et l'acclimatation des organismes soumis à l'essai, la manipulation et le stockage des échantillons, les installations d'essai requises, les méthodes de préparation des solutions d'essai et de mise en route des essai, les conditions prescrites pour les essais, les observations et mesures appropriées, les résultats des essais, les méthodes de calcul et l'utilisation de produits toxiques de référence.

Foreword

*This reference method is one of a series of **recommended methods** for measuring and assessing the aquatic biological effects of toxic substances. Recommended methods are those which have been evaluated by the Environmental Protection Service (EPS), and are recommended:*

- *for use in Environment Canada and provincial aquatic toxicity laboratories;*
- *for testing which is contracted out by Environment Canada or requested from outside agencies or industry;*
- *in lieu of more specific instructions, such as are contained in regulations; and*
- *as a foundation for the provision of very explicit instructions as may be required in a legal protocol or standard reference method.*

The different types of tests included in this series were selected on the basis of their acceptability for the needs of environmental protection and conservation programs in Environment Canada. These documents are intended to provide guidance and to facilitate the use of consistent, appropriate, and comprehensive procedures for obtaining data on toxic effects of samples of chemicals, effluents, elutriates, leachates, and receiving water.

Mention of trade names in this document does not constitute endorsement by Environment Canada; other products with similar value are available.

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Glossary

°C . . . . . degree(s) Celsius
$CaCO_3$ . . . . . calcium carbonate
$CaSO_4$ . . . . . calcium sulphate
d . . . . . day
DO . . . . . dissolved oxygen (concentration)
g . . . . . gram
h . . . . . hour
HCl . . . . . hydrochloric acid
H_2O . . . . . water
KCl . . . . . potassium chloride
L . . . . . litre
LC50 . . . . . median lethal concentration
LT50 . . . . . time to 50% mortality
mg . . . . . milligram
min. . . . . minute
mL . . . . . millilitre
$MgSO_4$ . . . . . magnesium sulphate
N . . . . . normal
$NaHCO_3$ . . . . . sodium bicarbonate
NaOH . . . . . sodium hydroxide
OD . . . . . outside diameter
OECD . . . . . Organization for Economic Cooperation and Development
SD . . . . . standard deviation
SI . . . . . Système international d'unités
TIE . . . . . Toxicity Identification Evaluation
™ . . . . . trade mark
µ . . . . . micro
> . . . . . greater than
< . . . . . less than
≥ . . . . . greater than or equal to
≤ . . . . . less than or equal to

Terminology

Note: All definitions are given in the context of the procedures in this report, and may not be appropriate in another context.

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".

General 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.

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

Compliance means in accordance with governmental permitting or regulatory requirements.

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

Dispersant is a chemical substance which reduces the surface tension between water and a hydrophobic substance (e.g., oil), thereby facilitating the dispersal of the hydrophobic material throughout the water as an emulsion.

Emulsifier is a chemical substance that aids the fine mixing (in the form of small droplets) within water, of an otherwise hydrophobic substance.

Eyed egg is an encapsulated embryo that has reached a stage of development where its eyes are clearly evident to the casual observer.

Fingerling is a young (underyearling), actively feeding fish.
Flocculation is the formation of a light, loose precipitate (i.e., floc) from a solution.

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, and is expressed as mg/L calcium carbonate or equivalent.

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.

Monitoring is the routine (e.g., daily, weekly, monthly, quarterly) checking of quality or collection and reporting of information. In the context of this report, it means either the periodic (routine) checking and measurement of certain biological or water-quality variables, or the collection and testing of samples of effluent, elutriate, leachate, or receiving water for toxicity.

Percentage (%) is a concentration expressed in parts per hundred parts. One percent represents one unit or part of material (e.g., effluent, elutriate, leachate, or receiving water) diluted with water to a total of 100 parts. Concentrations can be prepared on a volume-to-volume or weight-to-weight basis, and are expressed as the percentage of test material in the final solution.

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.

Precipitation is the formation of a solid (i.e., precipitate) from a solution.

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

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 replace 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 expressed in parts per thousand (‰).

Surfactant is a surface-active chemical substance (e.g., detergent) which, when added to a non-aqueous liquid, decreases its surface tension and facilitates dispersion of materials in water.

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

Turbidity is the extent to which the clarity of water has been reduced by the presence of suspended or other matter that causes light to be scattered and absorbed rather than transmitted in straight lines through the sample. It is generally expressed in terms of Nephelometric Turbidity Units.

Terms for Test Materials

Chemical is, in this report, any element, compound, formulation, or mixture of a chemical substance that may enter the aquatic environment through spillage, application, or discharge. Examples of chemicals which are applied to the environment are insecticides, herbicides, fungicides, sea lamprey larvicides, and agents for treating oil spills.

Control is a treatment in an investigation or study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific condition that is being studied. In an aquatic toxicity test, the control must duplicate all the 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., quality of the control/dilution water, health or handling of test organisms).

Control/dilution water is the water used for diluting the test material, or for the control test, or both.

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

Deionized water is water that has been passed through resin columns to remove ions from solution and thereby purify it.

Dilution water is the water used to dilute a test material in order to prepare different concentrations for the various toxicity test treatments.

Distilled water is water that has been passed through a distillation apparatus of borosilicate glass or other material, to remove impurities.

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

Elutriate is an aqueous solution obtained after adding water to a solid waste (e.g., tailings, drilling mud, dredge spoil), shaking the mixture, then centrifuging or filtering it or decanting the supernatant.

Leachate is water or wastewater that has percolated through a column of soil or solid waste within the environment.

Receiving water is surface water (e.g., stream, river, or lake) that has received a discharged water, or else is about to receive such a waste (e.g., it is just upstream from the discharge point). Further descriptors must be provided to indicate which meaning is intended.

Reconstituted water is de-ionized or glass-distilled water to which reagent-grade chemicals have been added. The resultant synthetic fresh water is free from contaminants and has the desired pH and hardness characteristics.

Reference toxicant is a standard chemical used to measure the sensitivity of the test fish in order 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.

Stock solution is a concentrated aqueous solution of the material to be tested. Measured volumes of a stock solution are added to dilution water in order to prepare the required strengths of test solutions.

Upstream water is surface water (e.g., in a stream, river, or lake) that is not influenced by the test material, by virtue of being removed from it in a direction against the current or sufficiently far across the current.

Wastewater is a general term which includes effluents, leachates, and elutriates.

Toxicity Terms

Acute toxicity is a discernible adverse effect (lethal or sublethal) induced in the test organisms within a short period of exposure to a test material, usually ≤ 4 days for fish.

Endpoint means the variables (i.e., time, reaction of the organism, etc.) that indicate the termination of a test, and also means the measurement(s) or value(s) derived, that characterize the results of the test (LC50, LT50, etc.).

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.

LC50 is the medial lethal concentration (i.e., the concentration of material 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 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 as the cessation of all visible signs of movement or other activity.

LT50 is the time (period of exposure) estimated to cause 50% mortality in a group of fish held in a particular test solution.

Overt means obviously discernible under the test conditions employed.

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 24h. 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.

Toxicity is the inherent potential or capacity of a material to cause adverse effects on fish.

Toxicity Identification Evaluation describes a systematic sample pre-treatment (e.g., pH adjustment, filtration, aeration) followed by tests for acute toxicity. This evaluation is used to identify the causative agent(s) which are primarily responsible for acute lethality in a complex mixture.

Toxicity test is a determination of the effect of a material on a group of selected organisms under defined conditions. As aquatic toxicity test usually measures the proportions of organisms affected by their exposure to specific concentrations of chemical, effluent, elutriate, leachate, or receiving water.

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Introduction

1.1 Background

No single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection. Delivery of the preventative and remedial measures necessary to manage the environment requires the effective use of a selected battery of well-defined aquatic toxicity tests. Sergy (1987), in consultation with the Inter-Governmental Aquatic Toxicity Group (IGATG; members listed in Appendix A), proposed a set of tests which would be broadly acceptable, and measure different types of toxic effects in different organisms. The acute lethality tests using rainbow trout was one of several "core" aquatic toxicity tests which was selected to be standardized sufficiently to help meet Environment Canada's testing requirements.

Universal test procedures generically applicable to any acute lethality test with rainbow trout performed under controlled laboratory conditions are described in this report. Also presented are specific sets of test conditions and procedures, required or recommended when using the acute lethality test for evaluating different types of materials (namely samples of chemicals, effluents, elutriates, leachates, or receiving water) (Figure 1). Those specific procedures and conditions of relevance to the conduct of the test and its standardization are delineated and, as appropriate, discussed in an explanatory note. In developing these procedures, an attempt was made to balance scientific, practical, and financial considerations, and to ensure that the results will be accurate and precise enough for the majority of situations in which they will be applied.

The authors assume that the user has a certain degree of familiarity with aquatic toxicity tests. Explicit instructions on every detail such as may be required in a specific regulatory protocol are not provided, although this report is intended to serve as a guideline document useful for this and other applications.

1.2 Species Distribution and Historical Use in Tests

Rainbow trout *(Oncorhynchus mykiss;* formerly *Salmo gairdneri*)* are native to western North America, mostly west of the Rocky Mountains, although this fish species (which includes steelhead and Kamloops trout) now frequents waters of all Canadian provinces as a result of intentional or unintentional releases. It thrives in most cool, fresh water bodies (lakes, streams, and rivers). Additionally, there are subspecies of rainbow trout (i.e., steelhead) on both coasts that run to sea and return to streams for spawning (Scott and Crossman, 1973). The species has been introduced around the world with considerable success and now is probably the most widespread of the salmonids. In Canada and elsewhere, it is widely reared in hatcheries for stocking natural waters to support sports fishing, and is among the most common species used in commercial aquaculture.

The rainbow trout has also become the world's standard cool-water fish for freshwater pollution studies and research in aquatic toxicology. Culturing of rainbow trout is well established and many hatcheries will provide eggs or young fish appropriate for toxicity test purposes. A

* North American taxonomists recently renamed this species.

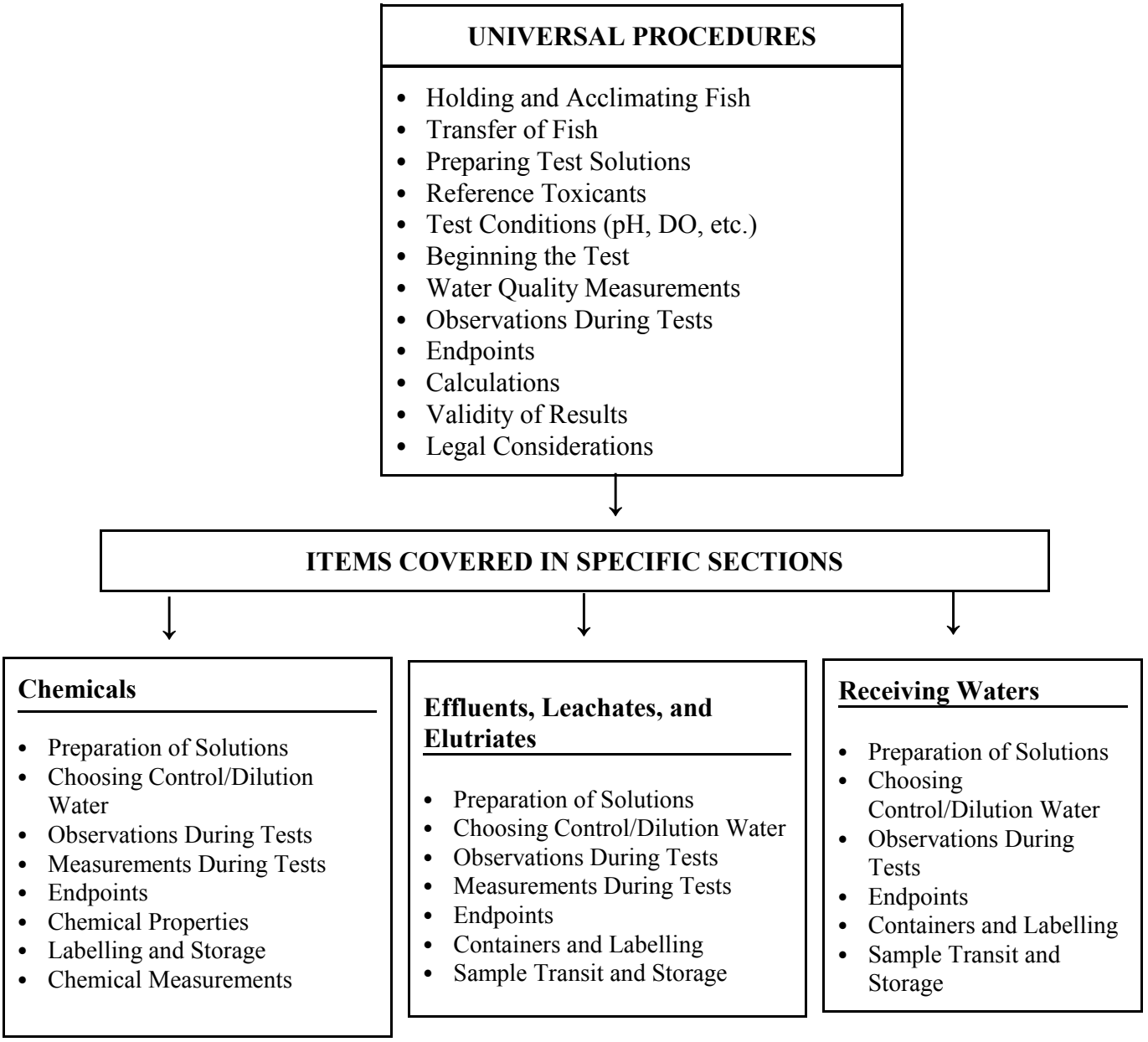

Figure 1 Diagram of Approach Taken in Delineating Test Conditions and Procedures Appropriate to Various Types of Materials

toxicological data bank of appreciable magnitude has been assembled for this species. The routine and extensive use of this species in Environment Canada laboratories (Appendix A) and other laboratories across Canada has facilitated studies such as: toxicity comparisons for different effluents and chemicals, comparisons over time for a given industry or location, and evaluation of toxic components in a complex waste. The background of previous toxicity data for this fish species, its proven sensitivity to aquatic contaminants, its commercial value, and its widespread availability make the rainbow trout a logical choice for standard toxicity tests using cool, fresh water.

For two decades, rainbow trout have been used extensively in Canada for evaluating the acute (short-term) lethal effects toward salmonid fish (and, by inference, other sensitive aquatic organisms) associated with exposure or potential exposure to chemicals or effluents (Sprague, 1969; Pessah and Cornwall, 1980; Wells and Moyse, 1981; Dafoe *et al.*, 1984). A series of Canadian regulations and guidelines for undertaking acute lethality tests with rainbow trout and specific types of industrial effluents was promulgated by Environment Canada during the 1970s (EPS, 1971; 1974; 1977a–c). A standard procedure for testing aqueous effluents using rainbow trout was prepared in 1980 (EPS, 1980). Environment Canada guidelines on use of oil spill dispersants have also specified procedures using rainbow trout (EPS, 1973; 1984). Some provinces developed guidelines and laboratory procedures for measuring acute lethal toxicity of liquid effluents to rainbow trout (Rocchini *et al.*, 1982; McGuinness, 1982; Craig *et al.,* 1983; OME, 1989).

The test procedures detailed in these and other governmental documents differ in endpoints, and some do not address important issues such as pH adjustment, variations in test methodology associated with differing test objectives, acceptable criteria for selecting and preparing control/dilution water, or how to deal with samples which contain appreciable solids or floating material. Most existing methodology reports on acute lethality tests using rainbow trout give procedures for performing tests with effluents or chemicals, but provide no guidance for testing elutriates, leachates, or receiving water. Additionally, the rationale for selecting certain specific conditions or approaches is often omitted. A review of procedural variables and approaches given in existing methodology reports is provided in Appendix B.

The issues previously discussed have been considered in the development of this methodology report. It has been designed for use with freshwater-acclimated fish (rainbow trout), test solutions that are essentially fresh water (i.e., salinity $\leq$ 10‰) or saline but destined for discharge to fresh water, and fresh water as the dilution and control water. Its application may be varied but includes instances where the impact or potential impact of materials on the freshwater environment is under investigation. Other tests, using other species acclimated to seawater, may be used to assess the impact or potential impact of materials in estuarine or marine environments, or to evaluate test solutions having a salinity > 10‰ which are destined for estuarine/marine discharge.

Test Organisms

2.1 Test Species

Rainbow trout *(Oncorhynchus mykiss)* are to be used as the test species.

2.2 Life Stage and Size

Either swim-up fry or fingerling life stages may be used. The average wet weight of test fish should be between 0.3[a] and 2.5g. The length of the largest fish should not be more than twice that of the smallest in the same test. Mean fork lengths and wet weights should be measured routinely for a representative sample of fish (e.g., weekly measurements of ≥ 10 fish taken from the holding tank or measurements of controls at the end of the test), to ensure adequate loading rates and uniformity of size in tests.

2.3 Source

Fish may be acquired as eggs, fry, or fingerlings. All fish used in a test should be derived from the same population and source. These must be free of known diseases (Roberts and Shepherd, 1986) and from hatchery stock. Procurement and shipment of fish should be approved by regional representatives of the Federal (Department of Fisheries and Oceans; DFO)–Provincial Transplant Committee in provinces where this committee acts to control movements of fish stocks. Advice regarding representatives of the Transplant Committee, and sources or rainbow trout suitable for conducting aquatic toxicity tests, can be obtained by contacting regional Environmental Protection offices (Appendix A).

2.4 Holding and Acclimation

A summary checklist of recommended conditions for holding and acclimating rainbow trout is provided in Table 1.

2.4.1 Facilities

Eggs and alevins may be incubated in vertical-flow hatchery trays or flowing water troughs made of nontoxic materials such as: stainless steel, porcelain, fibreglass-reinforced polyester, acrylic, polyethylene, or polypropylene. Procedures for the handling and incubation of eggs and alevins should be according to standard hatchery practice (Leitritz and Lewis, 1976).

Fry and fingerling life stages may be reared and acclimated in troughs or tanks receiving flowing water. These must also be made of nontoxic materials (as previously listed). Troughs and tanks used for this purpose should be located away from any physical disturbances and preferably in a location separate from the test tanks. Holding (rearing) troughs or tanks may be outdoors or indoors; tanks for acclimating fish to laboratory lighting and other test conditions should be indoors or, if outdoors, covered with lids fitted with photoperiod-controlled lights.

2.4.2 Lighting

Depending on test requirements and intent, lighting during acclimation may be natural or as

[a] Very young fry with mean weight <0.3 g might in some instances (e.g., for research purposes) be suitable for use in toxicity tests provided that they have been actively feeding for a minimum of two weeks and have been acclimated for that period of time to the lighting and temperature conditions specified for the test.

Table 1 Checklist of Recommended Conditions and Procedures for Holding and Acclimating Rainbow Trout

Source of fish	–	hatchery stock free of known diseases; approved by Federal (DFO) – Provincial Transplant Committee in provinces where this committee acts
Water	–	uncontaminated ground, surface, or dechlorinated municipal water
	–	holding volume and flow, 1.0 L/10 g of fish and 1.4 L/g fish per day, respectively
Temperature	–	holding temperature within the range 4 to 18° C; acclimation temperature achieved at rate $\leq$3° C/d and held at 15 ± 2° C for $\geq$2 weeks
Oxygen/aeration	–	dissolved oxygen 80 to 100% saturation, maintained by aeration with filtered, oil-free air if necessary
pH	–	within the range 6.0 to 8.5
Water quality	–	temperature, dissolved oxygen, pH, and flow to each holding or acclimation tank to be monitored, preferably daily
Lighting	–	full-spectrum fluorescent, 100–500 lux at surface, 16 ± 1 h light:8 ± 1 h dark, preferably gradual transition
Feeding	–	at least once a day with standard commercial pelleted food; feeding rate, 1 to 5% of wet body wt/d (depending on fish size and water temperature and manufacturer's recommendations); ration type, pellet size, feeding frequency, and storage conditions as recommended by manufacturer
Cleaning	–	siphoning of debris daily, or as required; transfer to clean, disinfected tanks as necessary
Disease	–	mortalities monitored daily and moribund fish removed; mortality rate for group to be used in tests, <2% during seven days preceding test; if treated for disease, not to be used within two weeks thereafter

provided by overhead full-spectrum[b] fluorescent fixtures. If photoperiod control is required, the photoperiod should normally be a constant sequence of 16 ± 1 hours of light and 8 ± 1 hours of darkness. Light intensity at the water surface should be 100 to 500 lux. A 15- to 30-minute transition period between light and dark is recommended if artificial lighting is provided.[c] Fish should be acclimated to lighting conditions (including photoperiod and intensity) consistent with those used in the test, for a period of at least two weeks and preferably three or more weeks prior to testing.

2.4.3 Water

Sources of water for holding and acclimating fish can be "uncontaminated" supplies of groundwater, surface water, or dechlorinated municipal drinking water. The water supply should previously have been demonstrated to consistently and reliably support good survival, health, and growth of rainbow trout. Monitoring and assessment of variables such as residual chlorine, fluoride, pH, hardness, alkalinity, total organic carbon, conductivity, suspended solids, dissolved oxygen, total dissolved gases, temperature, ammonia nitrogen, nitrate, metals, and total organophosphorus pesticides, should be performed as frequently as necessary to document water quality.

If municipal drinking water is to be used for culturing fish and as control/dilution water, effective dechlorination must rid the water to which fish are exposed of any harmful concentration of chlorine. The target value for total residual chlorine in water within stock tanks and control/dilution water in test vessels is
$\leq$ 0.002 mg/L (CCREM, 1987). Vigorous aeration of the water supply (prior to pumping it to holding/acclimation tanks) can be applied to strip out volatile chlorine gas. The use of activated carbon (bone charcoal) filters and subsequent ultraviolet radiation (Armstrong and Scott, 1974) is recommended for removing residual chloramine and other chlorinated organic compounds.

If reconstituted water is to be used as dilution and control water (see Section 5.3), fish must be acclimated to this or a water of similar hardness for at least five days immediately prior to testing.[d] Acclimation could be to the reconstituted water, to a natural water with hardness within 20% of the reconstituted water, or to a natural water adjusted to such a hardness with deionized water (if too hard) or with the appropriate quantity and ratio (e.g., ASTM, 1980) of reagent-grade salts (if too soft).

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[b] Fluorescent or other tubes with a full-spectrum wavelength lamp, supplemented if desired with natural outdoor illumination, should be used to simulate the visible range of natural light. However, it should be noted that full-spectrum lights do not emit the intensity of ultraviolet (UV-B) radiation approaching that of natural illumination, and that the toxicity of certain effluents and chemicals can be altered markedly by photolysis reactions caused by UV-B radiation. For certain tests (e.g., photoactivation or photodegradation of toxic materials due to ultraviolet radiation), special lights (e.g., high-pressure mercury arc lamps) with differing spectral qualities may be used. ASTM (1995) provides useful guidance in this regard. Studies wishing to determine the influence of lighting conditions on toxicity could conduct concurrent side-by-side comparisons with replicate solutions held under differing (e.g., full-spectrum versus mercury arc) lighting conditions.

[c] A "dawn/dusk" transition period is recommended since abrupt changes in intensity startle and stress fish. Automated dimmer control systems are available for dimming and brightening the intensity of fluorescent lights, although they are costly. Alternatively, a secondary incandescent light source, regulated by time clock and automated rheostat, may be used to provide the transition period.

[d] Without such acclimation, the benefit of a standardized dilution water might be lost. For example, it takes several days for fish to readjust their tolerance to heavy metals when moved to a water of different mineral content (Lloyd, 1965).

A constant flow of water through the holding and acclimation tanks is necessary. To prevent a buildup of metabolic wastes, at least one litre per minute of fresh (new) water should flow into the tank for every kilogram of fish being held (equals 1.4 L/g fish · d or 0.69 g fish · d/L)[e]. Additionally, to prevent overcrowding, a tank should contain at any given moment at least one litre of water for every 10 grams of fish held (Sprague, 1973). Unusual circumstances such as acclimation of fish to reconstituted water may require the filtration and recirculation of water, or its periodic renewal in static systems. In such cases, ammonia and nitrite should be measured frequently to check that they do not reach harmful levels. Target values, recommended for the protection of freshwater aquatic life, are ≤ 0.02 mg/L of un-ionized ammonia (OME, 1984) and ≤ 0.06 mg/L of nitrite (CCREM, 1987).

Water entering holding and acclimation tanks must not be supersaturated with gases. In situations where gas supersaturation within the water supply is a valid concern, total gas pressure within water supplies should frequently be checked (Bouck, 1982). Remedial measures (e.g., use of aeration columns or vigorous aeration in an open reservoir) must be taken if dissolved gases exceed 100% saturation. Water, temperature, dissolved oxygen, pH, and flow should be monitored for each holding or acclimation tank, preferably daily. Weekly or more frequent monitoring levels of ammonia, nitrite, and total residual chlorine (if municipal water source is used) in holding or acclimation tanks is recommended.

[e] If necessary (e.g., fish are being acclimated to reconstituted water, receiving water or some other water source that is restricted in amount), water-volume requirements for fish acclimation may be decreased substantially by recirculating the flow to the fish tank through a filter suitable for removing metabolic wastes. If a recirculation system is used, ammonia and nitrite concentrations in the acclimation tank should be monitored and kept below levels harmful to fish health.

2.4.4 Temperature

The water temperature for holding populations of fish for subsequent test purposes may be outside the acceptable limits for the test provided that it is compatible with good fish health (i.e., 4 to 18° C). When preparing a batch of fish for the acclimation period, water temperature may be changed at a rate not exceeding 3° C/d, until the acclimation temperature of 15 ± 2° C is achieved. Fish are to be acclimated to 15 ± 2° C for a minimum of two weeks, and preferably ≥ three weeks, prior to initiating the toxicity test.

2.4.5 Dissolved Oxygen

The dissolved oxygen (DO) content of the water within the holding and acclimation tanks should be 80 to 100% air saturation. Supplementary aeration to the tanks using filtered, oil-free compressed air, should be provided if necessary to maintain this level of DO.

2.4.6 pH

The pH of the water used for holding and acclimating fish should be within the range of 6.0 to 8.5. Water with pH values between 7.5 and 8.0 is desirable (Klontz *et al.*, 1979).

2.4.7 Feeding

Fish should be fed a recognized standard commercial pelleted fish food suitable for rainbow trout. Depending on water temperature and fish size, feeding should be one or more times daily, normally with a daily ration approximating 1 to 5% of wet body weight (Appendix C). The pellet size and type, feed ration and frequency, and method and maximum duration for storing food, should be chosen in consideration of fish size and age, water temperature and the manufacturer's recommendation.

2.4.8 Cleaning of Tanks

Troughs and tanks used for holding and acclimating fish should be kept clean.

Siphoning of excess food and faeces should be conducted once a day or as frequently as necessary to eliminate the buildup of excess food or faecal material. Tank designs that provide partial self-cleaning (e.g., those with central, double standpipes) are recommended as they reduce maintenance requirements.

To minimize the occurrence of disease, tanks should be disinfected prior to introducing a new batch of fish. Suitable disinfectants include those containing chlorinated or iodophore compounds or n-alkyldimethyl-benzylammonium chloride (e.g., Comet™, Ovidine™, Argentyne™, Roccal™). As disinfectants are toxic to fish, tanks should be rinsed throughly with water used for holding/acclimating fish, following their use.

2.4.9 Fish Morbidity, Mortality, and Treatment

Fish should be inspected daily for signs of disease[f], and a record kept of their appearance and behaviour. Dead and moribund individuals should be removed immediately.

Mortality in the stock tank(s) from which test fish are to be taken should be monitored and recorded daily or, as a minimum, must be monitored and recorded at least five days per week (e.g., Monday to Friday). The rate of mortality must be less than 2% during the seven days preceding a test. If mortality is between 2 and 10%, the acclimation period should be extended for at least another seven days (i.e., <2% mortality in seven days is realized). If mortalities exceed 10% per week during the acclimation period, the group of fish is unacceptable for future use if death is caused by disease or aquatic contaminants.[g] If death results from other causes (e.g., high initial mortalities during transition from alevins to swimup fry or following fish transfer), the fish may be used for future toxicity tests provided that mortalities in the stock tank(s) from which test fish are to be taken decline to <2% during the seven days immediately preceding the test.

Treatment of fish with chemicals for disease prevention or control should be avoided if possible. It is strongly recommended that fish stocks showing signs of disease be discarded rather than treated. If the use of chemically treated fish cannot be avoided, a minimum two-week period should follow their treatment before they are used in tests. Records of any disease and chemical treatment of fish intended for use should be obtained from hatchery suppliers, and similar records kept throughout the holding and acclimation periods at the test facility.

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[f] Symptoms of unhealthy fish include loss of appetite, abnormal distribution in the tank, lethargy, erratic or atypical swimming behaviour, darkened colouration, pale gills, eroded or frayed fins, and external lesions. The *Handbook of Trout and Salmon Diseases, 2nd ed.* (Roberts and Shepherd, 1986) is a useful guide for the preliminary identification and diagnosis of diseases of salmonid fish.

[g] Based upon mortality criteria specified by the Organization for Economic Cooperation and Development (OECD, 1984).

Test System

3.1 Facilities

The test is to be conducted in a facility isolated from general laboratory disturbances. If a separate room is unavailable, the test area should be surrounded with an opaque curtain (e.g., black plastic) to minimize stress to fish during testing. Dust and fumes should be minimized.

A test facility is required that will maintain the temperature of all test solutions within the range specified (15 ± 1° C). This may be achieved using various types of equipment such as a thermostat-controlled air conditioning unit or a series of temperature-controlled water baths in which test vessels are immersed.

3.2 Lighting

Lighting conditions to which test fish are subjected should be the same as those defined in Section 2.4.2. The photoperiod is to be timed to coincide with that to which the fish have been acclimated.

3.3 Test Vessels

Vessels for testing chemicals should be glass[h] (jars or aquaria, depending on size and numbers of fish per container). Vessels for testing samples of effluents, elutriates, leachates, or receiving waters may be glass, Plexiglas™, acrylic, polypropylene, polyethylene, or polyethylene-lined. If disposable vessel liners are employed, they need not be rinsed with control/dilution water but must not be reused.

The minimum water depth in any test vessel should be 15 cm. For a given test, water depth, and container type, size, and shape should be identical for each test solution.

3.4 Control/Dilution Water

Depending on the test material and intent (see Sections 5 to 7), the control/dilution water may be: "uncontaminated" sources of ground or surface water (river or lake); dechlorinated municipal water[i] (see Section 2.4.3); reconstituted fresh water of a desired pH and hardness (e.g., simulating that of the receiving water); or a sample of receiving water collected upstream of the influence of the contaminant source, or adjacent to it, but removed from it. Conditions for collection , transport, and storage of samples of receiving water should be as described in Section 6.1.

Control/dilution water is to be adjusted to the test temperature prior to use. Supersaturation of this water with excess gases must be prevented (see Section 2.4.3).

Before it is used, the control/dilution water should have a dissolved oxygen content that is 90 to 100% of the air-saturation value. As necessary, the required volume of control/dilution water should be aerated vigorously (oil-free compressed air passed through air stones) immediately prior to use, and its dissolved oxygen content checked to confirm that 90 to 100% saturation has been achieved.

[h] Glass containers are inert and easily cleaned, and permit the unimpeded observation of test fish. Adsorption to non-glass containers (e.g., polyethylene, polypropylene, stainless steel) is markedly different for certain chemicals.

[i] The addition of thiosulphate or other chemicals to dilution water in order to remove residual chlorine is not recommended. Such chemical(s) could alter sample toxicity.

Universal Test Procedures

Procedures described in this section apply to all the tests of particular chemicals and wastewaters described in Sections 5, 6, and 7. All aspects of the test system described in the preceding section must be incorporated into these universal test procedures.

A summary checklist of recommended conditions and procedures for the acute lethal toxicity test using rainbow trout is given in Table 2. This checklist includes universal procedures as well as those recommended for testing specific types of test materials.

4.1 Preparing Test Solutions

All test vessels, measurement devices, stirring equipment, and fish-transfer pails must be thoroughly cleaned and rinsed in accordance with standard operational procedures. Each test vessel should be rinsed with control/dilution water just prior to use.*

The test concentrations and numbers of test solutions to be prepared will depend on the purpose of the test. For tests intended to estimate a 96-h LC50, at least five test concentrations plus a control solution (100% dilution water) are to be prepared[j]. An appropriate geometric dilution series may be used, in which each successive concentration is about 50% of the previous one (e.g., 100, 50, 25, 12.5, 6.3). Test concentrations may be selected from other appropriate logarithmic dilution series (see Appendix D).

When receiving water is used as dilution and control water, a second control solution should be prepared using the laboratory water to which fish have been acclimated for two or more weeks. Upstream water cannot be used if it is clearly toxic according to the criteria of the test for which it was intended.[k] In such cases, the laboratory water to which fish have been acclimated should be used as the control water and for all dilutions.

For a given test, the same dilution water is to be used for preparing the control and all test concentrations. 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 non-toxic material.

4.2 Beginning the Test

Each test vessel placed within the test facility must be clearly coded or labelled to identify the test substance and concentration, date and time of starting the test. The vessels should be positioned for easy observation of fish behaviour and mortalities. Preferably, the test solutions should be placed in random order (Sprague, 1973). It is recommended that, if necessary, test vessels be covered by clean, nontoxic screens or glass to prevent fish from escaping. The latter material should be used if concern exists with respect to contaminants entering the test solution

* Rinsing is not necessary if disposable polyethylene liners are used.

[j] A preliminary or range-finding test may be conducted before starting the definitive test. A range-finder normally covers a broader concentration range, and is frequently terminated in 24 h or less. For each definitive LC50, one or more control solutions must be prepared and included as part of the test.

[k] A comparison of fish appearance, behaviour, and survival in this control water versus the receiving-water control will distinguish any toxic responses that may be attributable to contaminants within the upstream water.

Table 2 Checklist of Recommended Test Conditions and Procedures

Universal		
Test type	–	static, 96-h duration*
Control/dilution water	–	ground, surface, or dechlorinated municipal water; "upstream" receiving water to assess toxic effect at a specific location,** reconstituted water if requiring a high degree of standardization; dissolved oxygen (DO) content 90 to 100% saturation at time of use
Fish	–	swim-up fry or fingerlings, mean weight 0.3 to 2.5 g; normally a minimum of 10/test solution; fish-loading density ≤0.5 g/L
Solution depth	–	≥15 cm
Temperature	–	15 ± 1° C
Oxygen/aeration	–	upon preparation, pre-aerate each test solution for 30 min. at 6.5 ± 1 mL/min · L if required or necessary (see Sections 5.2, 6.2, and 7.2); thereafter, and only if necessary, pre-aerate each test solution at 6.5 ± 1 mL/min · L for the lesser of an additional period not exceeding 90 min or achieving ≥70% saturation in the highest test concentration; aerate solutions at this rate throughout the test
pH	–	no adjustment if pH of test solution within the range 5.5 to 8.5***; a second (pH-adjusted) test may be required or appropriate if sample/solution pH beyond this range
Lighting	–	full-spectrum fluorescent, 100–500 lux at surface, normally 16 ± 1 h light:8 ± 1 h dark, preferably gradual transition
Feeding	–	do not feed for 16 h before start of test, nor during test
Observations	–	fish death, appearance, and behaviour; at least 24, 48, 72, and 96 h
Measurements	–	temperature, pH, and DO; at least at start and end (preferably daily); conductivity at least at start
Endpoints	–	as specified and/or depending on test objectives and test material; may be 96-h LC50 (requiring 95% confidence limits) or single-concentration test (% mortality at 96 h or earlier; LT50)
Reference toxicant	–	phenol and/or zinc (as zinc sulphate); conduct static 96-h LC50 upon acclimation and at least monthly thereafter
Test validity	–	invalid if >10% of control fish die or exhibit atypical/stressed behaviour

Chemicals

Solvents	–	to be used only in special circumstances
Concentration	–	recommended to be measured at beginning and end of exposure, in high, medium, and low strengths and in the control(s); if concentrations decline ≥20%, re-evaluate by flow-through or static replacement test
Control/dilution water	–	as specified and/or depending on intent; reconstituted if a high degree of standardization is required; receiving water if concerned with local toxic impact; otherwise, laboratory water

Effluents and Leachates

Transport and storage	–	transport at ambient temperature (>1° C, <30° C) or at 1 to 8° C if transit time >2 d; sample should not freeze during transit; store in the dark at 1 to 8° C (preferably 4 ± 2° C); the test should begin within three days and must start within five days after sampling
Control/dilution water	–	as specified and/or depending on intent; laboratory water or "upstream" receiving water for monitoring and compliance
High solids or floatables	–	may choose to recirculate test solutions

Elutriates

Transport and storage	–	extract within seven days of sample receipt; store in the dark at 1 to 8° C (preferably 4 ± 2° C); test within ten days of sample receipt
Control/dilution water	–	as specified and/or depending on intent; reconstituted water if a high degree of standardization is required

Receiving Water

Transport and storage	–	as for effluents and leachates
Control/dilution water	–	as specified and/or depending on intent; if studying local impact use "upstream" receiving water as control/dilution water

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* special situations (e.g., volatile or unstable chemicals in solution) may require the use of flow-through or static replacement tests, or a modified test duration

** if receiving water is used as the dilution and control water, an additional control is required using the uncontaminated laboratory water supply to which fish were previously acclimated

*** if pH is outside this range, results may reflect toxicity due to biologically adverse pH

from other sources, or the loss of volatiles from solution. Temperature, dissolved oxygen, and pH levels in the vessels should be checked and adjusted, if required/permitted, to acceptable levels prior to the introduction of fish. The conductivity of each prepared test solution should also be measured and recorded at this time.

A minimum of ten fish per test solution is recommended although circumstances may justify fewer[l]. Fish are to be introduced into each test solution and control water in equal numbers. These may be divided between two or more vessels to accommodate the required fish-loading density (≤0.5 g/L)[m]. The order of adding fish to test vessels should be randomized beforehand. Individual fish are to be used only once as test or control organisms.

Fish in the acclimation tank must not be fed for at least 16 h prior to testing, nor during the test. To minimize stress, transfer of fish from the acclimation tank to test vessels should be done as quickly as possible. Any fish dropped or injured during transfer are to be discarded. Dip nets should be rinsed (dilution water) between transfers if contact is made with a test solution. Water within fish-transfer pails should be aerated if necessary to maintain dissolved oxygen levels at 80 to 100% of air saturation during the period required for introduction of fish to test vessels.

4.3 Test Conditions

The test is to be static* (no replacement of solutions during test).

Test temperature should be within the range of 15 ± 1° C.

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[l] Reduction of numbers of fish per test solution from ten to seven results in a minimal loss of precision of the LC50 (Douglas *et al*., 1986). Such an approach may be necessary and allowable in cases where LC50s are being determined, and available fish are insufficient to provide 10/solution.

In instances where sample volume is insufficient to provide an acceptable fish-loading density (≤0.5 g/L) using 10 fish per solution, it might also be allowable to use fewer fish per test solution. This will result in an accurate but less precise answer, whereas exceeding the acceptable loading density may result in an inaccurate result.

[m] The total wet weight of fish in any test solution (including the control) must not be greater than 0.5 g/L. A lower density of fish loading could be used routinely when feasible, to reduce the buildup of metabolic wastes and the depletion of toxicant(s) from the water by the fish. A favourable density of 0.125 g/L has been suggested (i.e., Sprague, 1973).

A high rate of fish-loading can reduce the apparent toxicity of certain samples. Maximum loadings of 0.4 g/L (Davis and Mason, 1973) and 0.5 g/L (Craig and Beggs, 1979) have been recommended for four-day tests because higher densities resulted in longer survival or higher LC50s, for fish exposed to effluent or chemicals.

The static tests recommended here may indicate less toxicity than would a flow-through test. Bleached kraft pulp mill effluent may reveal only half of its acute toxicity in a static test, compared to a flow-through test (Walden *et al.,* 1975). Very toxic pulp mill effluents may show four times as much toxicity in flow-through tests, although there may be little difference for mildly toxic effluents (Loch and MacLeod, 1974).

The loading rate recommended in this report, therefore, is considered to be an acceptable maximum. As is the case if static tests are employed, it should be recognized that the use of this maximum loading could influence apparent toxicity. Both are compromises that acknowledge the economy of shipping smaller samples of effluent to testing laboratories. Because of day-to-day variability of industrial effluents, it would usually be more useful to expend available resources in testing small samples more frequently, than to conduct definitive but infrequent tests with large samples. Still, the possibility should be recognized that greater toxicity could become apparent in tests that used better-than-minimum conditions.

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* Special situations (e.g., volatile or unstable chemicals in solution) may required the use of flow-through or static replacement tests, or a modified test duration.

The depth of solution in each test vessel must be at least 15 cm. Fish-loading density in each test vessel must not exceed 0.5 g/L.

Test solutions (including controls) are to be aerated at a rate no greater than 6.5 mL/min · L.

Fish are not to be fed during the test.

The test is rendered invalid if mortality in the control water exceeds 10%, or if more than 10% of the fish in the control water display atypical swimming or other behaviour such as twitching, skittering at the surface, or loss of equilibrium (see Appendix E).

4.3.1 Dissolved Oxygen and Aeration

Depending on the test material, pre-aeration of each test solution (including the controls) under defined conditions just before the addition of test fish might or might not be recommended (see Sections 5.2, 6.2, and 7.2).

For those instances where pre-aeration is recommended (see Sections 5.2, 6.2, and 7.2), each solution including the control(s) should be aerated gently for a period of 30 minutes at a rate of 6.5 ± 1 mL/min · L. Immediately thereafter, the dissolved oxygen content of each test solution should be measured. If (and only if) the measured value in one or more solutions is <70% or >100% of air saturation, the pre-aeration of all test solutions should be continued at the same rate (i.e., 6.5 ± 1mL/min · L) for an additional period not to exceed 90 minutes[n]. This additional period of pre-aeration should 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 introduced to each test solution and the test initiated, regardless of whether 70 to 100% saturation was achieved in all test solutions.

At the start of the test, the aeration of test (and control) solutions should be commenced or continued at a rate of 6.5 ± 1 mL/min · L. This aeration should be maintained throughout the test period. Any aeration (or pre-aeration) of test solutions should be provided by bubbling compressed air, previously filtered so as to be free of oil, 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)[***]. The aeration rate should be verified and monitored at least daily using a suitable gas flow meter.

If using the prescribed aeration rate, the dissolved oxygen levels to which fish are exposed are or become depressed below 60% saturation (OECD, 1984; EPA, 1985a) and the intent of the test is to distinguish the degree to which oxygen depletion may contribute to fish deaths, a second test may be conducted with the sample (or a portion thereof) using a higher aeration rate sufficient to maintain dissolved oxygen values ≥70% saturation. Alternatively, the second test may be conducted using compressed oxygen gas bubbled at a controlled (6.5 ±1 mL/min · L) rate into each test solution, provided that supersaturation does not occur.

4.3.2 pH

Toxicity tests should normally be carried out without adjustment of pH. In instances where the chemical, wastewater, or receiving-water

[n] Aeration may strip volatile chemicals from solution or may increase their rate of oxidation and degradation to other substances. However, aeration of test solutions prior to fish exposure may be necessary because of the oxygen demand of the test material (e.g., oxygen depleted in the sample during storage). Aeration also assists in re-mixing the test solution.

[**] The Aqua Fizzz (also known as the Elite Aqua Fizzz) air stones are available from numerous local suppliers and from Rolf C. Hagen Inc. (780-467-3302). For a complete description, go to http://www.hagen.com/ and search for product A-962 or A-960. The silica glass air stones are available from Dynamic Aqua Supply, Surrey, BC (604-543-7504); Fish Farm Supply, Elmira, ON (519-669-1096); and Valox Ltd., Fredericton, NB (506-458-5430).

[***] Acceptable cleaning procedures for the AS1 silica glass air stones include: (i) an overnight soak in 33% concentrated nitric acid, followed by a rinse with tap water for about 1 h (or until overlying water is not acidic), five rinses with distilled or control water, and finally a two hour soak in distilled or control water; air stones may then be stored dry; (ii) a rinse with hot tap water, followed by a 24 h or overnight soak in 500 ppm (µL/L) hydrogen peroxide, a rinse with tap water, three rinses with dechlorinated water over the period of a workday, and finally a flush with dechlorinated water from the air stone backwards through an air line; air stones may then be stored in dechlorinated water.

sample causes the pH of any test solution to be outside the range 5.5 to 8.5, and it is desired to assess toxic chemicals rather than the lethal or modifying effects of pH, then the pH of the test solutions or sample should be adjusted before adding the fish, or a second (pH-adjusted) test should be conducted concurrently[o]. For this (second) test, the initial pH of the sample, or of each test solution[p] may (depending upon the test objectives) be neutralized (adjusted to pH 7.0) or adjusted to within ± 0.5 pH units of that of the dilution water, prior to fish exposure. Another acceptable approach for this second test is to adjust each test solution (including the control) to pH 5.5 to 6.0 (if test sample has/causes pH <5.5) or to pH 8.0 to 8.5 (if sample has/causes pH >8.5). Solutions of hydrochloric acid (HCl) or sodium hydroxide (NaOH) at strengths ≤ 1 *N* should normally be used for all pH adjustments.

Some situations (e.g., effluent samples with highly buffered pH) may require higher strengths of acid or base.

Abernethy and Westlake (1989) provide useful guidelines for adjusting pH. Test solutions or aliquots of samples receiving pH-adjustment should be allowed to equilibriate after each incremental addition of acid or base. The amount of time required for equilibration will depend on the buffering capacity of the solution/sample. For effluent samples, a period of 30 to 60 minutes is recommended for pH adjustment (Abernethy and Westlake, 1989). Once the test is initiated, the pH of each test solution is monitored (Section 4.4) but not adjusted.

If the purpose of the toxicity test is to better understand the nature of the toxicants in an effluent, elutriate, leachate, or receiving-water sample, pH adjustment is frequently used as one of a number of treatment techniques (e.g., oxidation, filtration, air stripping, addition of chelating agent) for characterizing sample toxicity. Mount and Anderson-Carnahan (1988) list pH adjustment as one of nine "Toxicity Identification Evaluation" (TIE) techniques which, when performed with an acutely toxic aqueous sample, provide the investigator with a useful method for assessing the physical/chemical nature of the toxicant(s) and their susceptibility to detoxification.

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[o] The main reason for not adjusting sample/solution pH is that pH may have a strong influence on the toxicity of a chemical, or substances in a wastewater. For the (generally) low concentrations of waste found in receiving water after dilution, any changes from the natural pH, with concomitant modification of toxicity, should be accepted as part of the pollution "package". That leads to the rational that the pH should not be adjusted.

Some chemicals and wastewaters will, however, cause lethal levels of pH in high concentrations of test solution. That is especially true in monitoring or compliance tests with full-strength effluent. It seems unlikely that an investigator would be primarily interested in ascertaining whether extreme pH in full-strength effluent was lethal to fish, since such a pH would be unrepresentative of what would prevail after even moderate dilution in receiving water. If pH *per se* were of primary interest, a toxicity test would not seem necessary, since the lethality of extreme pH is well-documented and any danger could be much more economically assessed by a simple chemical measurement. The investigator would usually wish to know if toxic substances were present in a wastewater, and determining that requires that masking by lethal action of pH be eliminated. That rationale leads to the use of pH-adjusted samples or test solutions, where appropriate. The rationale is exactly parallel to standardizing the temperature and dissolved oxygen in the toxicity tests, even if the wastewater itself were 90°C or had low (e.g., <2 mg/L) dissolved oxygen, either of which would rapidly be lethal to fish in itself.

[p] Tests with chemicals or samples of effluent, leachate, or elutriate requiring pH adjustment usually require the separate adjustment of each test solution (including the control). Those with samples of receiving water normally adjust an aliquot of the undiluted sample, prior to preparing the test concentrations.

4.4 Test Observations and Measurements

Unless indicated otherwise, the fish in each test vessel should be observed at least at 24, 48, 72, and 96 hours after commencement of the test. Any fish mortalities, abnormal appearance, or behaviour observed should be recorded.

At each observation, numbers of dead fish in each test vessel should be recorded and these fish removed. Fish are considered dead when they fail to show evidence of opercular or other activity, and do not respond to subsequent gentle prodding. Fish should also be examined for overt sublethal toxic effects (e.g., increased respiratory "coughing" rates, erratic swimming behaviour, surfacing, discolouration, loss of equilibrium). Any differences from control fish should be noted. An example of terms suitable for recording changes in fish behaviour and appearance is given in Appendix E.

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 and preferably at the start of each 24-h period of exposure. 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. Daily measurement of the conductivity of each test solution might be desirable, as changes during the test are indicative of chemical alterations.

Mean fork length and wet weight of control fish must be determined at the end of the test.

4.5 Test Endpoints and Calculations

In multi-concentration tests, record the percentages of fish killed in ≤96 h for each test solution of the wastewater or chemical.

Calculate the 96-h LC50 and its 95% confidence limits, and report the method used for those calculations.

Various computer programs for calculating LC50 and confidence limits are available and may be used. Stephan (1977) developed an LC50 program which uses probit, moving average, and binomial methods, and adapted it for the IBM-compatible personal computer. This BASIC program is recommended, and is available for copying onto a user-supplied floppy disk through courtesy of C.E. Stephan (USEPA, Duluth, MN), from Environment Canada (see Appendix A). An efficient micro-computer program for probit analysis is also available from Hubert (1987), and other satisfactory computer and manual methods (APHA *et al.,* 1989; USEPA, 1985a) may be used. Programs using the Trimmed Spearman-Kärber method (Hamilton *et al.*, 1977) are available for personal computers but are not recommended here because divergent results may be obtained by operators who are unfamiliar with the implications of trimming off ends of the dose-response data.

The recommended program of C.E. Stephan provides estimates of LC50 and confidence lifts by each of its three methods, if there are at least two partial mortalities in the set of data. For smooth or regular data, the three results will likely be similar, and values from the probit analysis should be taken as the preferred ones and reported. The binomial estimate may differ somewhat from the others. If the results do not include two partial mortalities, the probit and moving average methods do not function, and the binomial method can be used to provide a best estimate of the LC50 with conservative (wide) confidence limits.

A check of any computer-derived LC50 should be made by examining a plot on logarithmic-probability scales of percent

mortalities at 96 hours for the various test concentrations[q] (Figure 2) (APHA *et al.,* 1989). Any major disparity between the estimated LC50 derived from this plot and the computer-derived LC50 must be resolved.

For single-concentration test, the endpoints are dependent upon the objective of the test. Appropriate endpoints may include:

a) determination of percent mortality upon exposure of fish to the undiluted sample for 96 h; b) percent mortalities at various times for toxicity comparisons; or c) measurement of times to death for individual fish in each solution.

If successive measurements are made (items b or c), the median time to death (LT50) may be estimated if desired, by plotting in similar fashion to Figure 2 except that the horizontal axis is the logarithm of time instead of concentration. The 95% confidence limits may be estimated and compared by carrying the graphic analysis a stage further (Litchfield, 1949). It should be recognized that neither an LT50 nor percentage survival at short exposures is a dependable method of judging ultimate toxicity; therefore, comparisons based on those endpoints give only semi-quantitative guidance.

4.6 Reference Toxicant

The routine use of reference toxicant(s) is necessary to assess, under standardized test conditions, the relative sensitivity of the population of test fish and the precision and reliability of data produced by the laboratory (Environment Canada, 1990). Fish sensitivity to the reference toxicant(s) should be evaluated upon acclimation of a new batch of fish for possible use and at least once each month that the population of acclimated fish is used in toxicity tests.

Criteria used in recommending appropriate reference toxicants for this test may include:

- chemical readily available in pure form;
- stable (long) shelf life of chemical;
- highly soluble in water;
- stable in aqueous solution;

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[q] Figure 2 was based on concentrations of 1.8, 3.2, 5.6, 10, and 18 mg/L, with mortalities of 0, 2, 4, 9, and 10 fish, out of 10 per concentration. The eye-fitted line estimated the LC50 as 5.6 mg/L.

Computer programs gave very similar estimates to the graphic one, for the regular data of Figure 2. The LC50s (and 95% confidence limits) were as follows:

Probit analysis of Hubert (1987):		5.56	(4.28–7.21)
Stephan (1977):	probit analysis	5.58	(4.24–7.37)
	moving average	5.58	(4.24–7.33)
	binomial	6.22	(1.8–10)
Spearman-Kärber method:			
(Hamilton *et al.*, 1977)	0% trim	5.64	(4.38–7.26)
	10% trim	5.73	(4.34–7.58)
	20% trim	5.95	(4.34–9.80)

The binomial method did not estimate confidence limits, but selected two concentrations from the test as outer limits of range within which the true confidence limits would lie.

In fitting a line such as that in Figure 2, relatively more significance should be assigned to points that are near 50% mortality. If successive concentrations yield a series of 0% mortalities, only one such value should be used in fitting the line (i.e., the one that is "closest to the middle" of the distribution of data). Similarly, only the first of a series of successive 100% values should be used. The same principle applies to computer programs; only one successive 0% or 100% should be entered; additional ones may distort the estimate of LC50. Logarithmic-probability paper ("log-probit", as in Figure 2) may be purchased in, or ordered through good technical bookstores.

If it is desired to estimate LT50, a graph such as Figure 2 can be plotted using logarithm of time as the horizontal axis. Individual times to death of fish could be used but they are seldom available since tests are not inspected continuously. The cumulative percent mortality at successive inspections is quite satisfactory for plotting, and an eye-fitted line leads to estimates of confidence limits following the steps in Litchfield (1949).

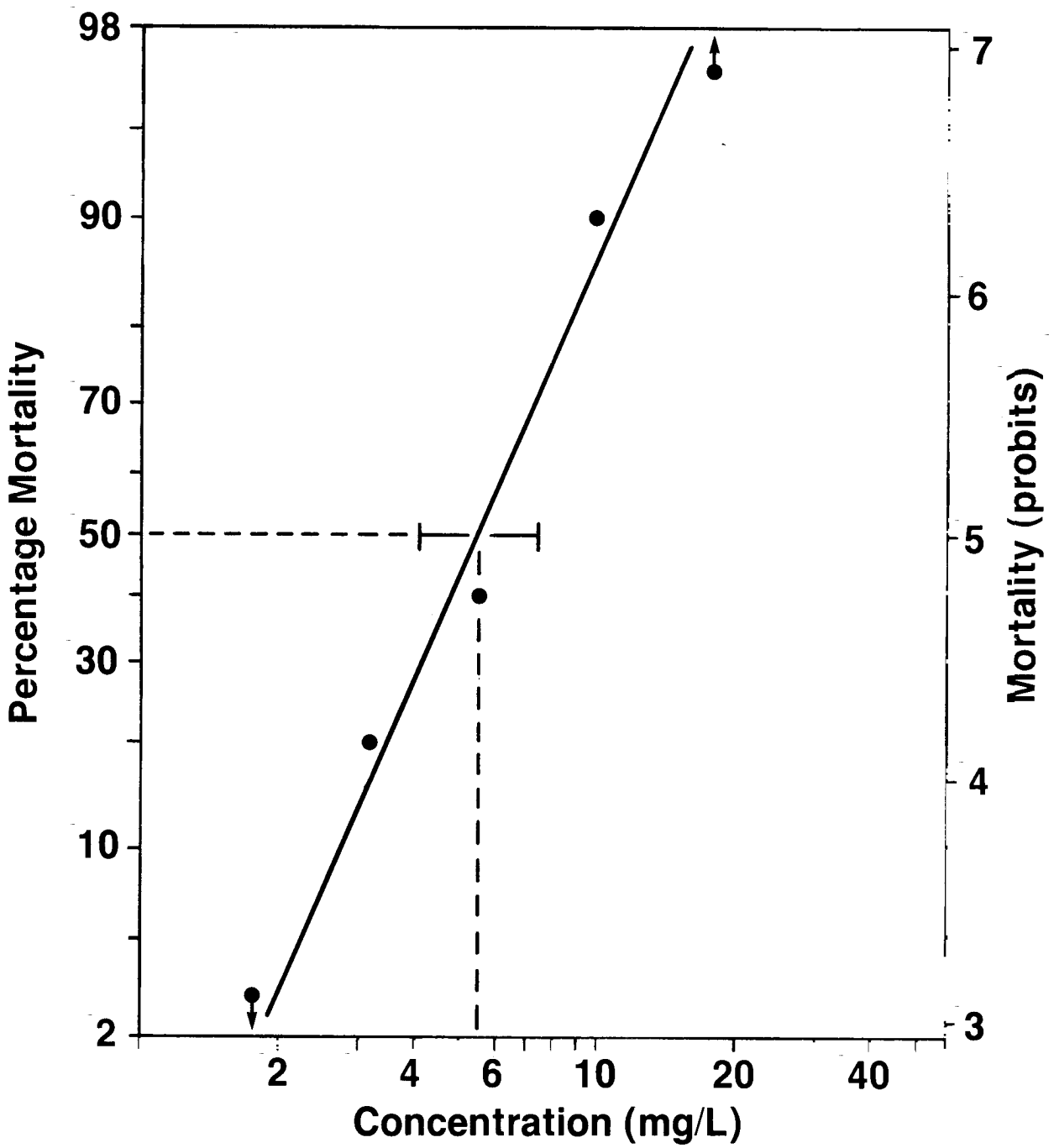

Figure 2 Estimating a Median Lethal Concentration by Plotting Mortalities on Logarithmic-probability Paper. In this hypothetical example, there were ten fish tested at each of five concentrations. The line was fitted by eye. The concentration expected to be lethal to 50% of the fish may be read by following across from 50% (broken line) to the intersection with the fitted line, then down to the horizontal axis for an estimated LC50 (5.6 mg/L).

- minimal hazard posed to user;
- easily analyzed with precision;
- good dose-response curve for test organism;
- known influence of pH on toxicity to test organism; and
- known influence of water hardness on toxicity to test organism.

Reagent-grade phenol and/or zinc (prepared using zinc sulphate) are recommended for use as the reference toxicants for this test. Fish sensitivity should be evaluated by static tests to measure the 96-h LC50 for one or both of these chemicals, using the dilution water used routinely by the laboratory.* Test conditions (including diluent-water type and quality) and procedures for undertaking reference toxicant tests are to be consistent and as described in this document.[r]

A warning chart should be prepared and updated for each reference toxicant used. The warning chart should plot logarithm of concentration on the vertical axis against date of the test 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 (± SD) as calculated by using the available values of log(LC50), are recalculated with each successive LC50 until the statistics stabilize (USEPA, 1985a; Environment Canada, 1990). 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 falls outside the warning limits, the sensitivity of the fish and the test system are suspect. Since this may occur 5% of the time due to chance alone, an outlying LC50 does not necessarily mean that the sensitivity of the population of fish or the precision of the toxicity data produced by the test laboratory are in question. Rather, it provides a warning that this may be the case. A check of all holding and test conditions is required at this time. Depending on the findings, it may be necessary to commence the acclimation of a new population of fish or provide further acclimation and evaluation (with reference toxicants) of the existing population before its use in toxicity tests.

Stock solutions of phenol should be made up on the day of use. Zinc sulphate (usually $ZnSO_4 \cdot 7H_2O$, molecular weight 4.3982 times that of zinc) should be used for preparing stock solutions of zinc. Stock solutions of zinc should be acidic (pH 3 to 4). Acidic zinc solutions may be used when prepared, or stored in the dark at 4 ± 2° C for several weeks until used. Concentration of zinc should be expressed as mg Zn^{++}/L.

Concentrations of reference toxicant in all stock solutions should be measured

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* Reconstituted water may be used if a greater degree of standardization is desired.

[r] Since the pH, hardness, and other characteristics of the dilution water can markedly influence the toxicity of the test material, use of a standard reconstituted water provides results that may be compared in a meaningful way with results from other laboratories.

Soft reconstituted water is recommended for this purpose. This water is prepared by adding the following quantities of reagent-grade salts to carbon-filtered, de-ionized water or glass-distilled water (ASTM, 1980):

salt	mg/L
$NaHCO_3$	48
$CaSO_4 \cdot 2H_2O$	30
$MgSO_4$	30
KCl	2

The reconstituted water should be aged several days (USEPA, 1985b) and intensely aerated before use.

chemically by appropriate methods (e.g., APHA *et al.,* 1989). Upon preparation of the test solutions, aliquots should 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 (outside warning limits). If stored, sample aliquots must be held in the dark at 4 ± 2° C. Both zinc and phenol solutions 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 to measure concentrations in the same solutions at the end of the test, after completing biological observations. Calculations of LC50 should be based on the average measured concentrations if they are appreciably (i.e., ≥20%) different from nominal ones and if the accuracy of the chemical analyses is reliable.

4.7 Legal Considerations

Complete and detailed specifications for acute lethality tests undertaken for legal purposes are beyond the scope of this document. It is most important that care be taken to ensure that samples collected and tested with a view to prosecution will be admissible in court. For this purpose, legal samples must be: representative of the substance being sampled; uncontaminated by foreign substances; identifiable as to date, time, and location of origin; clearly documented as to the chain of continuity; and analyzed as soon as possible after collection. Persons responsible for conducting the test and reporting the findings must maintain continuity of evidence for court proceedings (McCaffrey, 1979), and ensure the integrity of the test results.

Specific Procedures for Testing Chemicals

This section gives particular instructions for testing chemicals, in addition to the procedures listed in Section 4.

5.1 Properties, Labelling, and Storage of Sample

Information should be obtained on the properties of the chemical to be tested, including water solubility, vapour pressure, chemical stability, dissociation constants, and biodegradability. Material safety data sheets should be consulted, if available. Where aqueous solubility is in doubt or problematic, acceptable procedures used previously for preparing aqueous solutions of the chemical should be obtained and reported. Other available information such as structural formula, degree of purity, nature and percentage of significant impurities, presence and amounts of additives, and n-octanol–water partition coefficient should be obtained and recorded.[s]

Chemical containers must be sealed and coded or labelled (e.g., chemical name, supplier, date received) upon receipt. Storage conditions (e.g., temperature, protection from light) are frequently dictated by the nature of the chemical. Standard operating procedures for chemical handling and storage should be followed.

5.2 Preparing Test Solutions

For testing chemicals, a multiple-concentration test is usually performed, to determine the LC50. It may be desirable to have replicates (two to three) of each test concentration, for purposes of evaluating new chemicals. Replicates could be required under regulations for registering a pesticide or similar category of chemical.

Solutions of the chemical may be prepared either by adding pre-weighed (analytical balance) quantities of chemical to each test vessel as required to give the nominal strengths to be tested[t], or by adding measured volumes of a stock solution. Stock solutions should be prepared by dissolving the test chemical in control/dilution water. For chemicals that do not dissolve readily in water, stock solutions may be prepared using the generator column technique (Billington *et al.,* 1988; Shiu *et al.,* 1988) or, less desirably, by ultrasonic dispersion. The investigator should be aware that ultrasonic dispersion can result in variations in the biological availability (and therefore the resulting toxicity) of the test chemical, due to the production of droplets differing in size and uniformity.

Organic solvents, emulsifiers, or dispersants should not be used to increase chemical solubility except in instances where these substances might be formulated with the test chemical for its commercial purposes. If used, an additional control solution should be prepared containing the same concentration of solubilizing agent as that present in the most concentrated solution of the test chemical.

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[s] Knowledge of the properties of the chemical will assist in determining any special precautions and requirements necessary while handling and testing it (e.g., testing in a well-ventilated facility, need for solvent). Information regarding chemical solubility and stability in fresh water will also be useful in interpreting results.

[t] This approach is normally used only for preparing high concentrations or large volumes of test solutions. Otherwise, greater accuracy can be achieved by preparing a stock solution.

Such agents should be used sparingly and should not exceed 0.5 mL/L in any test solution (USEPA, 1985b). If solvents are used, the following are preferred (USEPA, 1985b): dimethyl formamide, triethylene glycol, methanol, acetone, and ethanol.

Upon preparation of each test solution including the control(s), its dissolved oxygen content should be measured. Thereafter, either fish should be introduced and the test initiated (see Section 4.2), or each test solution should be pre-aerated (see Subsection 4.3.1) and then fish added. In most instances, the pre-aeration of test solutions is not necessary nor warranted (see footnote "n"). For those situations where pre-aeration is appropriate (i.e., if, upon preparation, the dissolved oxygen content of one or more test solutions is <70% or >100% of air saturation), the guidance for pre-aeration of solutions given in Subsection 4.3.1 should be followed.

5.3 Control/Dilution Water

Control/dilution water may be reconstituted water, the freshwater source to which the fish are acclimated (natural groundwater, surface water or dechlorinated municipal water), or a particular sample of receiving water if there is special interest in a local situation. The choice of control/dilution water depends upon the intent of the test.

If a high degree of standardization is required (e.g., the measured toxicity of a chemical is to be compared and assessed relative to values derived elsewhere, for this and/or other chemicals), soft reconstituted water (hardness 40 to 48 mg/L as $CaCO_3$, pH 7.2 to 7.5) should be prepared and used for all dilutions and as the control water (see footnote "r") (USEPA, 1985b).

If the toxic effect of a chemical on a particular receiving water is to be appraised, sample(s) of the receiving water could be taken from a place that was isolated from influences of the chemical, and used as the control/dilution water[u, v, w]. Examples of such situations includes appraisals of the toxic effect of chemical spills (real or potential) or intentional chemical applications (e.g., spraying of a pesticide) on a particular water body. The laboratory supply of natural water or dechlorinated water may also be used for this purpose, especially where logistical constraints make the collection and use of receiving water impractical. Natural water or dechlorinated municipal water to which test fish have been acclimated is also appropriate for use in other instances (e.g., preliminary or intra-laboratory assessment of chemical toxicity).

5.4 Test Observations and Measurements

During solution preparation and at each of the prescribed observation periods during the test,

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[u] Contaminants already in the receiving water may add toxicity to that of the chemical or wastewater under investigation. In such instances, uncontaminated dilution water (reconstituted, natural, or dechlorinated municipal) would give a more accurate estimate of the individual toxicity of the spill or spray, but not necessarily of the total impact on the site of interest.

[v] While it would be desirable to acclimate a group of fish to the receiving water before using them in a test with that water used for dilution and control, that is seldom feasible because of the need to transport large volumes of water. If possible and appropriate, tests using receiving water could be carried out near the site of interest, in which case acclimation should last at least five days.

[w] An alternative (compromise) to using receiving water as dilution and control water is to adjust the pH and hardness of the laboratory water supply (or reconstituted water) to that of the receiving water. Depending upon the situation, the adjustment may be to seasonal means, or to values in the receiving water at a particular time.

each test solution should be examined for evidence of chemical presence and change (e.g., solution colour and opacity, precipitation or flocculation of chemical). Any observations should be recorded.

It is desirable and recommended that test solutions be analyzed to determine the concentrations of chemicals to which fish are exposed[x]. In instances where chemicals are to be measured, samples should be taken from the high, medium, and low test concentrations and the control(s) at the beginning and end of the test, as a minimum. These should be preserved, stored, and analyzed according to best proven methodologies available for determining the concentration of the particular chemical in aqueous solution.

If chemical measurements indicate that concentrations declined by more than 20% during the test, the acute lethal toxicity of the chemical should be re-evaluated by a test in which solutions are renewed periodically (static replacement test) or continuously (flow-through test) (OECD, 1984).

Toxicity results for any test in which concentrations are measured should be calculated and expressed in terms of those measured concentrations, unless there is good reason to believe that the chemical measurements are not accurate. In making these calculations, each test solution should be characterized by the geometric average measured concentration to which fish were exposed.

5.5 Test Endpoints and Calculations

The endpoint for tests performed with chemicals will usually be a 96-h LC50. Accepted procedures for calculating the LC50 and its 95% confidence interval are given in Section 4.5.

If a solvent control is used, the test is rendered invalid if mortality in this control (or in the untreated control water) exceeds 10%. The test is also invalid if >10% of the fish in either control exhibit atypical/stressed behaviour (Appendix E).

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[x] Such analyses need not be undertaken in all instances, due to analytical limitations, cost, or previous technical data indicating chemical stability in solution under conditions similar to those in the test.

Chemical analyses are particularly advisable if (USEPA, 1985b): the test solutions are aerated; the test material is volatile, insoluble, or precipitates out of solution; the test chemical is known to sorb to the material(s) from which the test vessels are constructed; or a flow-trough system is used. Some situations (e.g., testing of pesticides for purposes of registration) may require the measurement of chemical concentrations in test solutions.

Specific Procedures for Testing Effluent, Elutriate, and Leachate Samples

This section gives particular instructions for testing samples of effluent, elutriate, and leachate, in addition to the procedures listed in Section 4.

6.1 Sample Labelling, Transport, and Storage

Containers for transportation and storage of samples of effluents, leachates, and elutriates must be made of nontoxic material (e.g., polyethylene or polypropylene containers manufactured for storing drinking water or gasoline). The containers must either be new or throughly cleaned and rinsed with uncontaminated water. They should also be rinsed with the sample to be collected. Containers should be filled to minimize any remaining air space.

Upon collection, each sample container must be filled, sealed, and labelled or coded. Labelling should include at least sample type, source, date and time of collection, and name of sampler(s). Unlabelled or uncoded containers arriving at the laboratory should not be tested. Nor should samples arriving in partially filled containers be routinely tested, since volatile toxicants escape into the air space. However, if it is known that volatility is not a factor, such samples might be tested at the discretion of the investigator.

Testing of effluent and leachate 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 collected for extraction and subsequent testing of the elutriate should be tested within ten days of receipt. Elutriates should be tested within three days of sample preparation or as specified.

It is desirable to refrigerate samples of effluent and leachate upon collection and during their transport. In situations where this is impractical (e.g., shipment of large volumes of sample), effluent and leachate samples may be held at ambient temperature during transport. However, when ambient temperatures are extreme (i.e., >30° C or <1° C) or when transit times greater than two days are anticipated, the temperature of the samples should be controlled (1 to 8° C) in transit.

Samples should not freeze during transport. Upon arrival at the laboratory, effluent and leachate samples may be adjusted immediately or overnight to 15° C, and testing may be commenced. If more prolonged sample storage is needed, sample containers should be stored in darkness at 1 to 8° C and preferably at 4 ± 2° C.

Unless otherwise specified, temperature conditions during transit and storage of elutriates, as well as samples intended for aqueous extraction and subsequent testing of the elutriate, should be as indicated previously.

6.2 Preparing Test Solutions

Samples in the collection chambers must be agitated thoroughly just prior to pouring, to ensure the re-suspension of settleable solids. Sub-samples (i.e., a sample divided between two or more containers) must be mixed together to ensure their homogeneity. If

further sample storage is required, the composited sample (or a portion thereof) should be returned to the sub-sample containers and stored (Section 6.1) until used. If necessary, the temperature of samples or test solutions may be adjusted to the test temperature by heating or chilling in a water bath, or by the use of an immersion cooler made of nontoxic material (e.g., stainless steel). Samples or test solutions must not be heated by immersion heaters, since this could alter chemical constituents and toxicity.

One or more control solutions must be prepared and included as part of each test. Upon preparation and mixing (see Section 4.1), each solution including the control(s) should be aerated gently for period of 30 minutes at a rate of 6.5 ± 1 mL/min · L. Thereafter, guidance provided in Subsection 4.3.1, paragraph 2 should be reviewed and followed before starting the test.

6.3 Control/Dilution Water

Tests conducted with samples of effluent or leachate for monitoring and regulatory compliance purposes should use either the laboratory water supply to which fish have been acclimated for two or more weeks, or a sample of the receiving water, as the control/dilution water. Since results could be quite different for the two sources of water, the objectives of the test must be decided before a choice is made. Shipping difficulties and costs should also be considered, since the use of receiving water as control/dilution water greatly increases the volume of liquid to be shipped.

The use of receiving water as the control/dilution water may be desirable in certain instances where site-specific information is required regarding the potential toxic effect of an effluent, leachate, or elutriate on a particular receiving water[u, v, w]. Conditions for the collection, transport, and storage of such receiving-water samples should be as described in Section 6.1.

If a sample of upstream receiving water is to be used as control/dilution water, a separate control solution should be prepared using the laboratory water supply to which fish have been acclimated for two or more weeks[k]. Fish survival, appearance, and behaviour (Section 4.4) in the laboratory control water should be compared to that shown in the sample of receiving water.

Tests requiring a high degree of standardization may be undertaken using reconstituted water as the dilution and control water[r]. Situations where the use of reconstituted water is appropriate include investigative studies intended to interrelate toxicity data for various effluent, leachate, or elutriate types and sources, derived from a number of test facilities or from a single facility where water quality is variable. In such instances, it is desirable to minimize any modifying influence due to (differing) dilution-water chemistry.

6.4 Test Conditions

Samples of effluent, leachate, or elutriate are normally not filtered or agitated during the test. However, the presence of high concentrations of suspended solids in a sample may be stressful to exposed fish, and can be acutely lethal if present in sufficiently high strengths (e.g., ≥2000 mg/L, Noggle, 1978; McLeay *et al.,* 1987; Servizi *et al.,* 1987). High concentrations of biological solids in certain types of treated effluent may also contribute to sample toxicity from ammonia and/or nitrite production (Servizi and Gordon, 1986). If there is concern about toxicity contribution from elevated concentrations of suspended or settleable solids in samples of effluent, elutriate, or leachate, an additional test may be conducted by maintaining solids in suspension throughout the period of fish exposure. Test vessels with vertical sides and steeply sloped, conical-shaped bottoms (Noggle, 1978;

McLeay *et al.,* 1983) may be used for this purpose. Using this or similar apparatus, test suspensions can be continuously agitated during the test by aeration from the conical bottom or by use of a pump which draws from the bottom and redistributes to the surface. The insertion of a basket into each test vessel will permit their periodic inspection and protection from the recirculating apparatus. A third test, using a portion of the sample treated by filtering or decanting to remove solids, may also be performed using otherwise identical procedures if the intent of the study is to quantify the degree to which sample solids contribute to acute lethal toxicity.

If the sample contains an appreciable quantity of floatable material (e.g., oil or surfactants) and there is concern about the possible contribution of this material to sample toxicity, solutions may be agitated throughout the test to ensure mixing and exposure of fish to soluble constituents. The recirculating conical vessels described previously may be used for this purpose, or alternatively, cylindrical vessels with individual impellers could be used (EPS, 1973; Blackman *et al.,* 1978). Fish must be protected from impellers.

Solutions of certain biotreated effluents (e.g., municipal) containing appreciable quantities of ammonia can increase in toxicity during the test (Clement *et al.,* 1989). This can be attributable to a progressive increase in pH of solutions during the test (associated with a progressive decline in dissolved CO_2 due to aeration), resulting in an increasing amount of toxic un-ionized ammonia in solution (CCREM, 1987). If effluent samples contain an appreciable quantity of ammonia or other constituent whose toxicity is highly pH-dependent, and concern exists about pH drift during testing and its contribution to sample toxicity, a second (concurrent) test may be conducted. This second test could be undertaken using various means (e.g., oxygenating rather than aerating solutions, addition of CO_2 to test solutions or enclosed atmospheres above the solutions, testing solutions in sealed containers with oxygen atmospheres) to reduce or prevent pH drift during the test.

6.5 Test Observations and Measurements

Colour, turbidity, odour, and homogeneity (i.e., presence of floatable material or settleable solids) of the effluent, leachate, or elutriate sample should be observed at the time of preparing test solutions. Precipitation, flocculation, colour change, release of volatiles, or other reactions upon dilution with water should be recorded, as should any changes in appearance of solutions during the test (e.g., foaming, settling, flocculation, increase or decrease in turbidity, colour change).

For tests with highly coloured or opaque solutions, or for samples producing foam in the test vessel, fish should be inspected for appearance, behaviour, and survival (as per Section 4.4) by raising them to the solution's surface at the intervals specified. Housing fish in a suitable basket constructed of nontoxic, nonabrasive material is recommended for this purpose, although dip nets may also be used provided that fish are not injured or unduly stressed during capture. If baskets are used, one should be placed in each test vessel including the control(s). Baskets should be large enough to permit fish movement throughout the test vessel. Each basket must be throughly cleaned and rinsed with control/dilution water before being used.

6.6 Test Endpoints and Calculations

Tests for monitoring and compliance with regulatory requirements should normally include, as a minimum, one or more undiluted portions of the samples and one or more control solutions. Depending on

specified regulatory requirements, tests for regulatory compliance may use a single concentration (100% wastewater unless otherwise specified) or may determine the 96-h LC50 (see Section 4.5).

Tests undertaken for monitoring effluent, leachate, or elutriate toxicity may also be single-concentration tests to measure precent fish mortality at 96 h, tests to determine an LT50 at full strength and/or with sample dilution, or tests to measure the LC50. The endpoint will depend on a number of considerations including the objectives of the monitoring program, compliance requirements, test costs, and past history of fish survival in the undiluted wastewater.

Toxicity tests conducted for other purposes (e.g., determination of in-plant sources of toxicity, treatment effectiveness, effects of process changes on toxicity) may, depending on the study objectives, be single-concentration tests (100% or an appropriate dilution, plus a control), or multiple-concentration tests. Single-concentration tests are often cost-effective for determining the presence or absence of acute lethal toxicity or as a method for screening a large number of samples for relative toxicity. Endpoints for these tests would again depend upon the objectives of the undertaking, but could include arbitrary "pass" or "fail" ratings, percent fish mortality at 96 h or an earlier time period (e.g., 24 h), or times to death for individual fish in each solution. Items discussed in Section 4.5 are relevant here.

Specific Procedures for Testing Receiving-water Samples

Instructions for testing samples of receiving waters, additional to those provided in Section 4, are given here.

7.1 Sample Labelling, Transport, and Storage

Procedures for the labelling, transportation, and storage of samples should be as described in Section 6.1. 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.

7.2 Preparing Test Solutions

Samples in the collection containers should be agitated before pouring to ensure their homogeneity. Compositing if sub-samples should be as described in Section 6.2.

Upon preparation of each test solution including the control(s), its dissolved oxygen content should be measured. Thereafter, either fish should be introduced and the test initiated (see Section 4.2), or each test solution should be pre-aerated (see Subsection 4.3.1) and then fish added. In most instances, the pre-aeration of test solutions is not necessary nor warranted (see footnote "n"). For those situations where pre-aeration is appropriate (i.e., if, upon preparation, the dissolved oxygen content of one or more test solutions is <70% or >100% of air saturation), the guidance for pre-aeration of solutions given in Subsection 4.3.1 should be followed.

7.3 Control/Dilution Water

For receiving-water samples collected in the vicinity of a wastewater discharge, chemical spill or other point-source of possible contamination, "upstream" water may be sampled concurrently and used as control water and diluent for the downstream samples[v, w]. This control/dilution water, should be collected as close as possible to the contaminant source(s) of concern, but upstream of or outside the zone of influence.

If upstream water is used as control/dilution water, a separate control solution should be prepared using the laboratory water supply to which fish have been acclimated for two or more weeks[k]. Fish survival, appearance, and behaviour (Section 4.4) in laboratory control water should be compared to that for fish held under identical conditions in the upstream control water. If mortalities or signs of distress are evident for fish held in this receiving-water sample and if dilutions of downstream water are being prepared for testing (toxicity anticipated), a separate set of dilutions should be prepared at this time using the laboratory water supply to which fish have been acclimated. Investigators anticipating this eventuality should collect sufficient volumes of receiving-water samples to permit these additional dilutions to be prepared.

Logistic constraints, expected toxic effects, or other site-specific practicalities may prevent or rule against the use of upstream water as the control/dilution water. In such cases, the laboratory water supply used for rearing and acclimating fish should be used as a control water for all dilutions. It could be adjusted to partially simulate upstream water[w].

7.4 Test Observations and Measurements

Observations made of sample and solution colour, turbidity, foaming, precipitation, etc.

should be made as described in Section 6.5, both during preparation of test solutions and subsequently during the tests. These are in addition to the preliminary observations on fish described in Section 4.4.

7.5 Test Endpoints and Calculations

Endpoints for tests with samples of receiving water should be consistent with the options and approaches identified in Sections 4.5 and 6.6.

Tests for monitoring and compliance purposes should normally include, as a minimum, one or more undiluted portions of the sample and one or more control solutions. Endpoints for tests with receiving-water samples may be restricted to a determination of percent fish-mortality at 96 h in the undiluted sample, together with time-to-death data where applicable. In instances where toxicity of receiving-water samples is likely and information is desired concerning the degree of dilution necessary to permit short-term survival of fish, a test to determine the 96-h LC50 should be conducted. One or more undiluted (100% sample) concentrations and at least four dilutions should be included in this test, together with one or more control solutions. Assuming that data permit, the LC50 and its confidence limits should be computed.

Reporting Requirements

The test report should describe the materials and methods used, as well as the test results. The reader should be able to establish from the report whether the conditions and procedures rendered the results acceptable for the use intended.

Procedures and conditions that are common to a series of ongoing tests (e.g., routine toxicity tests for monitoring and compliance purposes) and consistent with specifications in this document may be referred to by citation or by attachment of a general report which outlines standard laboratory practice. For the various reporting requirements identified here as bullets in Sections 8.1 to 8.7 inclusive, those that relate to test-specific information must be included in the individual test report. Procedural information that reflects "standard" laboratory practice in the performance of this biological test method may be restricted to the general report.

Each test-specific report must indicate if there has been any deviation from any of the “must” requirements delineated in Sections 2 to 7 of this Biological Test Method, and, if so, provide details as to the deviation. Specific monitoring programs or related test protocols might require selected items (e.g., procedures and results for tests requiring pH adjustment, modified aeration, or oxygenation) in the test report, or might relegate certain procedural-specific information as “data to be held on file”. Details pertinent to the conduct and findings of the test, which are not conveyed by the test report or general reports, should be kept on file by the laboratory so that the appropriate information can be provided if an audit of the test is required.

8.1 Test Material

- sample type, source and description (chemical, effluent, elutriate, leachate or receiving water; sampling location and method; specifics regarding nature, appearance and properties, volume and/or weight);
- information on labelling or coding of the test material;
- details on manner of sample collection, transport and storage (e.g., batch, grab or composite sample, description of container, temperature of sample upon receipt and during storage);
- identification of person(s) collecting and/or providing the sample; and
- dates and times for sample collection, receipt at test facility, and start of definitive test.

8.2 Test Organisms

- species and source;
- description of holding and acclimation conditions (facilities, lighting, water source and quality, water pre-treatment, water exchange rate and method, density of fish in holding and acclimation tanks, temperature during holding and acclimation, acclimation period, food type, ration and frequency of feeding, disease incidence and treatment);
- weekly percentage of mortalities in test population during acclimation; and
- mean fork length and wet weight of control fish at the end of the test, with range and sample size; loading density (g/L) of fish.

8.3 Test Facilities and Apparatus

- name and address of test laboratory;
- name of person(s) performing the test;
- description of systems for regulating light and temperature within the test facility; and
- description of test vessels (size, shape, type of material) and aeration systems and apparatus.

8.4 Control/Dilution Water

- type(s) and source(s) of water used as control and dilution water;
- type and quantity of any chemical(s) added to control or dilution water;
- sampling and storage details if the control/dilution water was "upstream" receiving water;
- water pre-treatment (temperature adjustment, de-gassing, aeration rates, and duration, etc.); and
- measured water quality variables (Section 2.4.3) before and/or at time of commencement of toxicity test.

8.5 Test Method

- brief mention of method used if standard (e.g., as per this report);
- design and description if specialized procedure (e.g., recirculation of test solutions, periodic or continuous replacement of solutions) or modification of standard method;
- procedure used in preparing stock and/or test solutions of chemicals;
- any chemical analysis of test solutions and reference to analytical procedure(s) used;
- use of preliminary or range-finding test; and
- frequency and type of observations made during test.

8.6 Test Conditions

- number, concentration, volume, and depth of test solutions including controls;
- number of organisms per solution and loading density;
- photoperiod, light source, and intensity at surface of test solutions;
- statement concerning aeration (rate, duration, manner of application) of test solutions prior to and during exposure of fish;
- description of any test solutions receiving pH adjustment, including procedure and timing;
- any chemical measurements on test solutions (e.g., chemical concentration, suspended solids content);
- temperature, pH, dissolved oxygen (mg/L and percent saturation) and conductivity as measured/monitored in each test solution; and
- conditions and procedures for measuring the 96-h LC50 of the reference toxicant(s).

8.7 Test Results

- appearance of test solutions and changes noted during test;

- fish behaviour, appearance, number and percentage of mortalities in each test solution (including control) as noted during each observation period; number and percentage of control fish showing atypical/stressed behaviour;

- results for range-finding test (if conducted);

- any 96-h LC50 or LT50 values (including the associated 95% confidence limits) determined, including reference to the statistical method used for their calculation; and

- the 96-h LC50 and 95% confidence limits for the reference toxicant(s) determined within one month of the test using the same group of fish as those from which the test fish were selected, together with the mean value (±2 SD) for the same reference toxicant as derived at the test facility in previous tests.

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Appendix A

Members of the Inter-Governmental Aquatic Toxicity Group and Environment Canada Regional and Headquarters' Office Addresses

Members of the Inter-Governmental Aquatic Toxicity Group (as of July, 1990):

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

Review of Procedural Variations for Undertaking Acute Lethality Tests using Rainbow Trout (as specified in Canadian, Provincial, and International methodology documents)*

1. Type of Test Material

Document	Test Material
EPS 1980	effluent
EPS 1984	oil dispersant
McGuinness 1982	effluent
Rocchini *et al.* 1982	effluent
Craig *et al.* 1983	effluent
USEPA 1985a	effluent
USEPA 1985b	pesticide
OECD 1984	chemical
BHSC 1982	chemical
UKWRC 1983	chemical

2. Type of Test

Document	Test Type
EPS 1980	static, static replacement, or flowthrough
EPS 1984	static
McGuinness 1982	static
Rocchini *et al.* 1982	static, static replacement, or flowthrough
Craig *et al.* 1983	static, static replacement, or flowthrough
USEPA 1985a	static, static replacement, or flowthrough
USEPA 1985b	static or flowthrough
OECD 1984	static, static replacement, or flowthrough
BHSC 1982	static, static replacement, or flowthrough
UKWRC 1983	flowthrough

* Based on methodology documents available to the authors as of August 1988.

3. Acclimation Period for Fish

Document	Duration
EPS 1980	≥3 weeks
EPS 1984	≥3 weeks
McGuinness 1982	NI*
Rocchini *et al.* 1982	≥2 weeks
Craig *et al.* 1983	≥10 days
USEPA 1985a	NI
USEPA 1985b	≥2 weeks
OECD 1984	≥12 days
BHSC 1982	≥12 days
UKWRC 1983	14 days

4. Type of Control/Dilution Water

Document	Recommended Type and Treatment
EPS 1980	dechlorinated (activated carbon, UV)
EPS 1984	dechlorinated (activated carbon, UV)
McGuinness 1982	dechlorinated (activated carbon, UV)
Rocchini *et al.* 1982	receiving water or equivalent
Craig *et al.* 1983	dechlorinated or other
USEPA 1985a	upstream receiving water or other
USEPA 1985b	soft reconstituted water
OECD 1984	dechlorinated, natural or reconstituted (pH 6.0 to 8.5; hardness 50 to 250 mg/L)
BHSC 1982	dechlorinated, natural or reconstituted (pH 6.0 to 8.5; hardness 50 to 250 mg/L)
UKWRC 1983	hard (250 to 280 mg/L) borehole water, pH 6.0 to 8.5

5. pH Adjustment Prior to Test

Document	pH Treatment Specified
EPS 1980	NI
EPS 1984	NI
McGuinness 1982	adjust if pH <6.5 or >8.5
Rocchini *et al.* 1982	NI
Craig *et al.* 1983	NI
USEPA 1985a	test at pH 7.0 and unadjusted, if pH <6.5 or >9.0
USEPA 1985b	NI
OECD 1984	no adjustment; repeat test at pH of dilution water if different
BHSC 1982	no adjustment; repeat test at pH of dilution water if different
UKWRC 1983	adjust before test if necessary

* Not indicated/not addressed

6. Temperature During Test

Document	Acclimation Rate (° C/day)	Test Temperature (° C)
EPS 1980	≤5	15 ± 1
EPS 1984	≤2	15 ± 1
McGuinness 1982	1	15 ± 1
Rocchini *et al.* 1982	NI	15 ± 1
Craig *et al.* 1983	≤5	15 ± 1
USEPA 1985a	≤6	12 ± 2
USEPA 1985b	NI	12
OECD 1984	NI	13 to 17 (± 1)
BHSC 1982	NI	13 to 17 (± 1)
UKWRC 1983	NI	15 ± 12

7. Aeration During Test

Document	Aeration Conditions
EPS 1980	5 to 7.5 mL/min · L
EPS 1984	no aeration if DO ≥70% saturation; otherwise 5 to 7.5 mL/min · L
McGuinness 1982	5 to 7.5 mL/min · L
Rocchini *et al.* 1982	≥7.5 mL/min · L
Craig *et al.* 1983	5 to 7.5 mL/min · L
USEPA 1985a	rate to maintain DO ≥60% saturation
USEPA 1985b	no aeration
OECD 1984	rate to maintain DO ≥60% saturation
BHSC 1982	rate to maintain DO ≥60% saturation
UKWRC 1983	rate to maintain DO ≥60% saturation

8. Lighting Conditions During Test

Document	Photoperiod (L:D)	Intensity (lux)	Type	Dawn/Dusk (min.)
EPS 1980	14h:10h	20 to 30	fluorescent	≥15
EPS 1984	14h:10h	20 to 30	fluorescent	≥15
McGuinness 1982	12h:12h	20 to 30	NI	NI
Rocchini *et al.* 1982	14h:10h	NI	NI	≥15
Craig *et al.* 1983	9h to15h	20 to 30	wide spectrum	NI
USEPA 1985a	NI	NI	NI	NI
USEPA 1985b	16h:8h	NI	NI	15 to 30
OECD 1984	12h to 16h: 12h to 8h	NI	NI	NI
BHSC 1982	12h to 16h	NI	NI	NI
UKWRC 1983	14h:10h	NI	tungsten	30

9. Number of Fish and Number of Replicates per Test solution

Document	No. of Fish	No. of Replicates
EPS 1980	≥5	0
EPS 1984	5	5
McGuinness 1982	5 to 10	0
Rocchini *et al.* 1982	≥10	0
Craig *et al.* 1983	10	0
USEPA 1985a	≥20	0 to 1
USEPA 1985b	≥10	0
OECD 1984	≥10	0
BHSC 1982	≥10	0
UKWRC 1983	≥10	0

10. Weights of Test Fish and Fish-loading Density

Document	Weight Range (g)	g/L · d over four days (static)
EPS 1980	0.5 to 10	≤5
EPS 1984	2 to 10	≤0.25
McGuinness 1982	0.5 to 10	≤5
Rocchini *et al.* 1982	0.2 to 5	≤5
Craig *et al.* 1983	0.5 to 5	≤5
USEPA 1985a	NI (30 to 90 days old)	≤0.8
USEPA 1985b	0.5 to 5	≤0.8
OECD 1984	NI (5 ± 1 cm)	≤1.0
BHSC 1982	NI (6 ± 2 cm)	≤1.0
UKWRC 1983	0.7 to 2 (5 ± 1 cm)	NI

11. Reference Toxicant

Document	Chemical	Test Type
EPS 1980	NI	-
EPS 1984	sodium dodecyl sulphate	LT50
McGuinness 1982	phenol	24-h LC50
Rocchini *et al.* 1982	NI	-
Craig *et al.* 1983	NI	-
USEPA 1985a	sodium dodecyl sulphate, cadmium chloride, sodium pentachlorophenate	LC50
USEPA 1985b	NI	-
OECD 1984	none recommended	-
BHSC 1982	NI	-
UKWRC 1983	NI	-

Appendix C

Daily Feeding Guide for Rainbow Trout* During Holding and Acclimation**

Fish Weight (g)	≤0.4	0.4 to 1.0	1 to 3	3 to 5
Food Size (mm)	0.5	0.7	1.0	1.5
Water Temperature (° C)	Daily Feeding Rate***			
4	2.0	1.4	1.0	0.7
6	3.3	2.3	1.4	1.1
8	4.3	2.7	1.7	1.4
10	4.6	3.2	2.0	1.5
12	4.9	3.3	2.1	1.7
14	5.0	3.4	2.2	1.7
16	5.0	3.5	2.3	1.7

* as provided by EP, Atlantic Region (see Appendix A)

** fish are not to be fed for at least 16 hours prior to testing, nor during the test

*** daily feeding rate (dry feed), expressed as a percentage of the average wet weight of the fish

Appendix D

Logarithmic Series of Concentrations Suitable for Use in Toxicity Tests*

Column (number of concentrations between 100 and 10, or between 10 and 1)**

1	2	3	4	5	6	7
100	100	100	100	100	100	100
32	46	56	63	68	72	75
10	22	32	40	46	52	56
3.2	10	18	25	32	37	42
1.0	4.6	10	16	22	27	32
	2.2	5.6	10	15	19	24
	1.0	3.2	6.3	10	14	18
		1.8	4.0	6.8	10	13
		1.0	2.5	4.6	7.2	10
			1.6	3.2	5.2	7.5
			1.0	2.2	3.7	5.6
				1.5	2.7	4.2
				1.0	1.9	3.2
					1.4	2.4
					1.0	1.8
						1.3
						1.0

* Modified from Rocchini *et al.* (1982).

** A series of five (or more) successive concentrations may be chosen from a column. Mid-points between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed as percentage by volume or weight, mg/L, or µg/L. As necessary, values may be multiplied or divided by any power of 10. Column 1 might be used if there was considerable uncertainty about the degree of toxicity. More widely spaced concentrations (differing by a factor <0.3) should not be used. For effluent testing, there is seldom much gain in precision be selecting concentrations from a column to the right of column 3; the finer gradations of columns 4 to 7 might occasionally be useful for testing chemicals that have an abrupt threshold of effect.

Appendix E

Terms Suitable for Describing Fish Appearance and Behaviour

Term	Definition
INTEGUMENT	**The Epithelial Covering of the Body, Including the Gills**
Shedding	– peeling or loss of portions of the integument
Mucous	– excessive secretions of mucous; especially evident at the gills
Hemorrhaging	– bleeding (e.g., from the gills, anal opening, eyes)
PIGMENTATION	**Colour of Skin due to Deposition or Distribution of Pigment**
Light	– colour lighter than usual for the species (as evident under the test conditions exclusive of the test solution)
Dark	– colour darker than usual for the species (as evident under the test conditions exclusive of the test solution)
Mottled	– colour of individual fish abnormally varied
GENERAL BEHAVIOUR	**Observable Responses of the Test Fish, Individually or in Groups, to their Environment**
Quiescent	– marked by a state of inactivity or abnormally low activity; motionless or nearly so
Hyperexcitable	– reacting to stimuli with substantially greater intensity than control fish
Irritated	– exhibiting more or less continuous hyperactivity
Surfacing	– rising and remaining unusually long at the surface
Sounding	– diving suddenly to the bottom; remaining unusually long at the bottom
Twitching	– sudden jerky movements (muscle spasms) for parts or all of the body
Tetanic	– in a state of tetany, marked by intermittent tonic spasms of the voluntary muscles
Normal	– apparently unaffected by (or not exposed to) the test solution; conforming to the normal appearance and behavioural characteristics of the species under the defined test conditions
SWIMMING	**Progressive Self-propulsion in Water by Coordinated Movement of the Tail, Body, and Fins**
Ceased	– no longer evident
Erratic	– characterized by lack of consistency, regularity, or uniformity; fluctuating; uneven
Gyrating	– revolving around a central point; moving spirally about an axis
Skittering	– skimming hurriedly along the surface with rapid body movements
Inverted	– turned upside down (or approximately so)
On side	– turned 90 degrees laterally, more or less, from the normal body orientation
RESPIRATION	**Physical Exchange of Water at the Gill Surface, Evident by Movement of the Opercula**
Rapid	– faster than normal (obviously exceeding respiratory rate for control)
Slow	– slower than normal (obviously less than respiratory rate for control)
Coughing	– increased (relative to control) rate of coughing (back-flushing of gills, evident by marked flairing of opercula)
Surface	– swimming at surface with mouth open and pumping surface water or air through gills
Irregular	– failing to occur at regular (rhythmic) intervals

* modified from USEPA 1985a