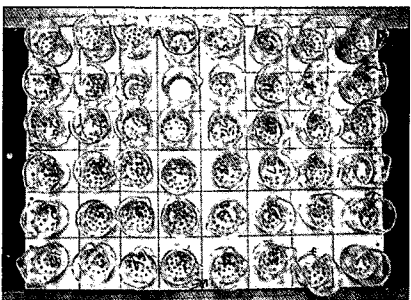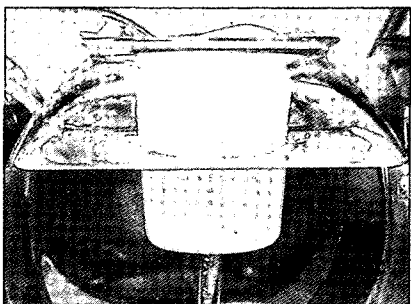# Environmental

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Biological Test Method:
Toxicity Tests Using Early
Life Stages of Salmonid
Fish (Rainbow Trout,
Coho Salmon, or Atlantic
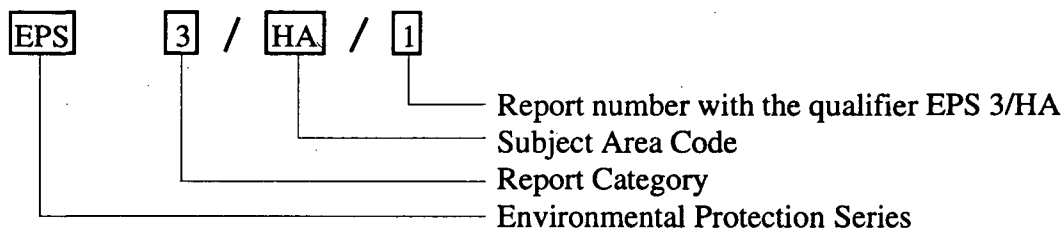Salmon)

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Biological Test Method: Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout, Coho Salmon, or Atlantic Salmon)

Environmental Protection
Conservation and Protection
Environment Canada

Report EPS 1/RM/28
December 1992

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Abstract

Methods recommended by Environment Canada for performing toxicity tests that measure effects on salmonid embryos, alevins, and swim-up fry, are described in this report. Three test methods are described: an embryo test for frequent or routine monitoring; an embryo/alevin test for measuring the effects of toxicants on multiple phases of development; and an embryo/alevin/fry test for more definitive investigations. All three methods start with the onset of embryo development, and measure the development and survival of early life stages. The embryo test ends seven days after fertilization. The embryo/alevin test is terminated during the alevin stage, with no feeding of fish being necessary. The embryo/alevin/fry test ends after 30 days of feeding swim-up fry. Selection of the most appropriate or suitable test method will depend on the objectives of the test and on the physicochemical characteristics of the substance being tested. Because such early life stages are usually a sensitive part of the life cycle of a fish, the tests should be considered as powerful and meaningful assays.

*One or more of the following species must be used in the tests: rainbow trout (*Oncorhynchus mykiss*), coho salmon (*Oncorhynchus kisutch*), or Atlantic salmon (*Salmo salar*). When selecting species, the following factors should be considered: the geographical location of the testing facility and/or study area; seasonal availability of the test organisms; relevance to ecological conditions; and the potential for differences in sensitivity to contaminants.*

Procedures are given for spawning broodstock, handling gametes, and fertilizing eggs before starting the test, and for incubation of embryos and feeding of swim-up fry during the tests. General or universal conditions and procedures are outlined for testing a variety of substances for their effects on the early life stages of salmonids. Additional conditions and procedures, which are specific for testing sample(s) of chemical, effluent, elutriate, leachate, or receiving water, are stipulated. Instructions and requirements are included on apparatus, facilities, handling and storing samples, preparing test solutions and initiating tests, specific test conditions, appropriate observations and measurements, statistical endpoints, methods of calculation, and validation.

Résumé

Le présent rapport décrit les méthodes recommandées par Environnement Canada pour l'exécution d'essais visant à mesurer des effets toxiques chez des embryons, des alevins et des alevins au stade de l'alimentation active de salmonidés. Il renferme trois méthodes d'essai : un essai sur des embryons, destiné aux programmes de surveillance fréquente ou systématique; un essai sur des embryons et des alevins, qui permet de mesurer les effets de substances toxiques sur des sujets à divers stades de leur cycle biologique; et un essai sur des embryons, des alevins et des alevins au stade de l'alimentation active, qui permet d'obtenir des résultats plus concluants. Les trois essais commencent au début du développement des embryons et mesurent le développement et la survie des poissons aux premiers stades de leur cycle biologique. L'essai sur les embryons se termine sept jours après la fertilisation. L'essai sur les embryons et les alevins se termine avant qu'il soit nécessaire de nourrir les alevins. Quant au troisième essai, il se termine 30 jours après que les alevins ont atteint le stade de l'alimentation active. Le choix de la méthode d'essai qui convient le mieux dépend des objectifs de l'essai et des caractéristiques physico-chimiques de la substance à expérimenter. Comme les poissons sont généralement très sensibles aux premiers stades de leur cycle biologique, on devrait considérer ces essais comme puissants et significatifs.

Les essais portent sur une ou plusieurs des espèces suivantes : la truite arc-en-ciel (Oncorhynchus mykiss), le saumon coho (Oncorhynchus kisutch) ou le saumon de l'Atlantique (Salmo salar). L'espèce devrait être choisie en fonction de l'emplacement géographique de l'installation d'essai ou du secteur à l'étude, de la disponibilité saisonnière des organismes destinés à l'essai, des conditions écologiques en cause et de différences éventuelles entre les espèces quant à leur sensibilité aux contaminants.

On donne dans le présent rapport des instructions pour le frai, la manipulation des gamètes et la fertilisation des oeufs avant les essais ainsi que pour l'incubation des embryons et l'alimentation des alevins pendant les essais. On expose des conditions et méthodes générales pour l'évaluation des effets de diverses substances sur des salmonidés aux premiers stades de leur cycle biologique. On précise aussi 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 trouvera des instructions et des exigences concernant l'appareillage, les installations d'essai, la manipulation et le stockage des échantillons, la préparation des solutions d'essai et la mise en route des essais, les conditions prescrites pour les essais, les observations et mesures appropriées, les résultats des essais, les méthodes de calcul et la validation des essais.

Foreword

*This is one of a series of **recommended methods** for measuring and assessing the aquatic biological effects of toxic substances. Recommended methods are those that have been evaluated by Conservation and Protection (C&P), and are favoured:*

- *for use in C&P aquatic toxicity laboratories;*

- *for testing that is contracted out by Environment Canada or requested from outside agencies or industry;*

- *in the absence of more specific instructions, such as are contained in regulations; and*

- *as a foundation for the provision of very explicit instructions as might be required in a regulatory program 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 programs for environmental protection and management carried out by Environment Canada. These reports 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 chemical, effluent, elutriate, leachate, or receiving water.

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

Table of Contents

List of Tables

List of Figures

List of Abbreviations and Chemical Formulae

°C ....degree(s) Celsius

$CaCO_3$ ....calcium carbonate

cm...............centimetre(s)

CV ....coefficient of variation

d..................day(s)

DO..............dissolved oxygen (concentration)

E.................embryo

EA ....embryo/alevin

EAF............embryo/alevin/fry

g..................gram(s)

g/kg ....grams per kilogram (equivalent to ‰)

h..................hour(s)

HCl..............hydrochloric acid

H_2O ....water

ICp..............inhibiting concentration for a (specified) percent effect

L..................litre(s)

LC_{50} ....median lethal concentration

LOEC ....lowest-observed-effect concentration

m ....metre(s)

mg ....milligram(s)

min ....minute(s)

mL...............millilitre(s)

mm ....millimetre(s)

mS ....millisiemen(s)

MSD...........minimum significant difference

N .... Normal

NaOH .... sodium hydroxide

NOEC .... no-observed-effect concentration

P .... probability

SD .... standard deviation

SI .... Système international d'unités

sp. .... species

TEC .... threshold-effect concentration

TIE .... toxicity identification evaluation

TM (™).... Trade Mark

μg .... microgram(s)

μm .... micrometre(s)

> .... greater than

< .... less than

≥ .... greater than or equal to

≤ .... less than or equal to

± .... plus or minus

% .... percentage or percent

‰ .... parts per thousand

~ .... approximately

Terminology

Note: All definitions are given in the context of the procedures in this report, and might 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".

Might is used to mean "could".

General Technical Terms

Acclimation means to become physiologically adjusted 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 salmonid fish with an evident yolk sac (for nutritive requirements). It is often referred to as a yolk-sac fry, and sometimes referred to as a larva.

Broodstock are the adult fish that are undergoing physiological changes to produce either eggs or sperm.

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 solution, their valence and mobility, and the temperature of the solution. Conductivity is normally reported in the SI unit of millisiemens/metre, or as micromhos/centimetre (1 mS/m = 10 μmhos/cm).

Culture, as a noun, is a stock of animals or plants raised under defined and controlled conditions to produce healthy test

organisms. As a verb, it means to carry out this procedure of raising organisms.

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

Egg is an encapsulated, spherical ovum, unfertilized or fertilized, obtained from a sexually mature female fish.

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

Embryo is the undeveloped young fish, before it hatches from the egg.

Exogenous feeding means oral intake and consumption of food available in the water, by free-swimming fry.

Eyed egg is an encapsulated embryo that has reached a stage of development where its pigmented eyes are clearly evident during routine observations.

Flocculation is the formation of a light, loose precipitate (i.e., a floc) from a solution.

Gametes are the eggs or sperm obtained from mature adult fish.

Growth is the increase in size or weight as the result of proliferation of new tissues. In this test, it is limited to increase in dry weight.

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 of calcium carbonate or equivalent.

Impermeable means, in reference to the egg membrane, the extent to which the membrane prevents the passage of molecules (e.g., water, ions, proteins, fats, or toxicants).

Incubation means the rearing of embryos or alevins under defined conditions compatible with normal development.

Larva is a recently hatched fish or other organism that has physical characteristics other than those seen in the adult. For salmonid fish, the term *larva* is synonymous with *alevin*.

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.

Milt is the mixture of sperm and seminal fluid extracted from a sexually mature male fish.

Minimum Significant Difference (MSD) means the difference between groups (in this test with salmonid fish, the difference in average weights or average mortality) that would have to exist before it could be concluded that there was a significant difference between the groups. The MSD is provided by Dunnett's multiple-range test, a standard statistical procedure.

Monitoring means the routine (e.g., daily, weekly, monthly, or 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 substance (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 substance 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 than 7 signifying increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.

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

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

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

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

Spawning is the release of eggs or sperm from mature adult fish, or refers to behaviour related to the readiness of mature adult fish to release gametes.

Stripping means human handling of mature adult fish to extract eggs or sperm from them.

Swim-up fry is a young, post-alevin fish that 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.

Water hardening is the process occurring within the first 2 h after fertilization, when the egg swells due to uptake of water into the perivitelline space, and before the egg membrane becomes relatively impermeable.

Terms for Test Substances

Chemical is, in this report, any element, compound, formulation, or mixture of a chemical substance that might enter the aquatic environment through spillage, application, or discharge. Examples of chemicals that are applied to the environment are insecticides, herbicides, fungicides, sea lamprey larvicides, industrial process additives, 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 substance. The control is used to determine the

absence of measurable toxicity due to basic test conditions (e.g., quality of the dilution water, health of test organisms, or effects due to their handling).

Control/dilution water is the water used for diluting the test substance, 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 purified to remove ions from solution by passing it through resin columns or a reverse osmosis system.

Dilution water is the fresh water used to dilute a test substance 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 substance, to remove impurities.

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

Elutriate is an aqueous solution obtained after adding water to a solid substance (e.g., sediment, tailings, drilling mud, or 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., in a stream, river, or lake) that has received a discharged waste, or else is about to receive such a waste (e.g., it is just "upstream" or up-current from the discharge point). Further descriptive information must be provided to indicate the intended meaning.

Reconstituted water is deionized 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.

Stock solution is a concentrated aqueous solution of the substance to be tested. Measured volumes of a stock solution are added to

dilution water to prepare the required strengths of test solutions.

Substance means a particular kind of material having more or less uniform properties.

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

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

Toxicity Terms

Acute means within a short period in relation to the life span of the organism, usually ≤4 days for fish. An acute toxic effect would be induced and observable within the short period.

Chronic means occurring during a relatively long period, usually a significant portion of the life span of the organism (e.g., 10% or more). A chronic toxic effect might take a significant portion of the life span to become observable, although it could be induced by an acute, a subchronic, or a chronic exposure to a toxic substance.

Chronic toxicity implies long-term effects that are related to changes in such things as metabolism, growth, reproduction, or ability to survive.

Chronic value is the geometric mean of the NOEC and LOEC in tests that have a chronic exposure. Because of the long life span of salmonids, early life-stage tests using salmonid fish normally do not measure chronic toxicity, although the intent of this test is to approximate such a measure. A more appropriate term in the case of the salmonid fish might be *subchronic value*. However, *TEC* (threshold-effect concentration) is the recommended term.

Continuous-flow 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 (same as flow-through).

Endpoint means the variables (i.e., time or reaction of the organisms) that indicate the termination of a test, and also means the

measurement(s) or value(s) that are derived, and which characterize the results of the test (e.g., NOEC or ICp).

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 (same as continuous-flow).

ICp is the inhibiting concentration for a (specified) percent effect. It represents a point estimate of the concentration of test substance that causes a designated percent impairment in a quantitative biological function such as growth of fish. For example, an IC25 could be the concentration estimated to cause a 25% reduction in growth of fish, relative to the control. This term should be used for any toxicological test that measures a quantitative effect or change in rate, such as reproduction, growth, or respiration. (The term EC_{50} or *median effective concentration* is not appropriate in tests of this kind because it is limited to quantal measurements, i.e., number of exposed individuals that show a particular effect.)

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

LC_{50} is the median lethal concentration (i.e., the concentration of substance in water that is estimated to be lethal to 50% of the test organisms). The LC_{50} 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 LC_{50}).

LOEC is the lowest-observed-effect concentration. This is the lowest concentration of a test substance (to which organisms are exposed), that causes adverse effects on the organism, which are detected by the observer and are statistically significant. For example, the LOEC might be the lowest concentration at which growth of fish differed significantly from that in the control. The LOEC is generally reserved for sublethal effects but can also be used for mortality, which might sometimes be the most sensitive effect observed.

NOEC is the no-observed-effect concentration. This is the highest concentration of a test substance to which organisms are exposed, that does not cause any observed and statistically significant adverse effects on the organism. For example, the

NOEC might be the highest tested concentration at which an observed variable such as growth did not differ significantly from growth in the control. The NOEC customarily refers to sublethal effects, and to the most sensitive effect unless otherwise specified.

Replicate is a single test container or aquarium containing a prescribed number of organisms in either one concentration of test solution or in dilution water as a control. In a toxicity test comprising five test concentrations and a control, and using three replicates, 18 aquaria would be used. For each concentration or control, there would be three aquaria or replicates. A replicate is an independent test unit, and therefore, any transfer of organisms or solutions from one replicate to another would invalidate the test.

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

Static renewal describes a toxicity test in which test solutions are renewed (replaced) periodically, usually at the beginning of each 24-h period. Synonymous terms are "batch replacement", "renewed static", "renewal", "static replacement" and "semi-static".

Sublethal means detrimental to the fish, but below the level that directly causes death within the test period.

TEC is the threshold-effect concentration. It is calculated as the geometric mean of NOEC and LOEC. *Chronic value* or *subchronic value* are alternative terms that may be appropriate depending on the duration of exposure in the test. The TEC is equivalent to the MATC (maximum acceptable toxicant concentration) used in other countries.

Toxicity is the inherent potential or capacity of a substance to cause adverse effects on fish or other organisms. The effect could be lethal or sublethal.

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

Toxicity test is a determination of the effect of a substance on a group of selected organisms under defined conditions. An aquatic

toxicity test usually measures either (a) the proportions of organisms affected *(quantal)* or (b) the degree of effect shown *(graded or quantitative)*, after exposure to specific concentrations of chemical, effluent, elutriate, leachate, or receiving water.

Acknowledgements

Introduction

1.1 Background

Aquatic toxicity tests are used within Canada and elsewhere to measure, predict, and control the discharge of substances that could prove harmful to aquatic life. Recognizing that no single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection, the Inter-Governmental Aquatic Toxicity Group (IGATG; members listed in Appendix A) proposed the development and standardization of a set of aquatic toxicity tests that would be broadly acceptable, and would measure different toxic effects using organisms representing different trophic levels and taxonomic groups (Sergy, 1987). A test based on the development, growth, and mortality of early life stages of salmonid fish is one of several "core" aquatic toxicity tests that was selected to be standardized sufficiently for use in Environment Canada's regional laboratories (Appendix B), as well as in provincial and private laboratories, to help meet Environment Canada's testing requirements (e.g., Environment Canada, 1991).

Universal procedures and conditions for conducting early life-stage tests using Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), and/or rainbow trout (*Oncorhynchus mykiss*), are described in this report. Also presented are specific sets of conditions and procedures required or recommended when using the test for evaluating different types of substances (namely, samples of chemical, effluent, elutriate, leachate, or receiving water).

Figure 1 gives a general picture of the topics covered in this report. Some details of methodology are discussed in explanatory footnotes.

The biological test methods presented in this report are based largely on other embryo-larval and early life-stage methods developed in North America and Europe (U.S. EPA, 1985a; Birge *et al.*, 1985; Rexrode and Armitage, 1987; van Aggelen, 1988; Birge and Black, 1990; ASTM, 1991a; Hodson *et al.*, 1991; Paine *et al.*, 1991; Neville, 1992; OECD, 1992a; 1992b). These methods have been developed following a review of specific procedural variations indicated in existing "methodology" documents (Appendix C) and in other related reports and publications. Three test methods are described: an embryo (E) test suitable for frequent or routine monitoring; an embryo/alevin (EA) test for measuring the effects of toxicants on multiple phases of development; and an embryo/alevin/fry (EAF) test for definitive investigations. All three methods start with the onset of embryo development, and measure the development and survival of early life stages. The E test ends seven days after fertilization. The EA test is terminated during the alevin stage, with no feeding of fish being required. The EAF test ends after 30 days of feeding swim-up fry. Any of these methods may be used to evaluate samples of chemical, effluent, elutriate, leachate, or receiving water. Selection of the most appropriate or suitable test method will depend on the objectives of the test and the nature of the substance being tested (see Subsection 4.3.1 and Sections 5.1, 6.1, and 7.1).

UNIVERSAL PROCEDURES

- Obtaining and handling gametes
- Culturing of embryos and alevins
- Preparing test solutions
- Test conditions (temp., DO, etc.)
- Beginning the test
- Water quality measurements
- Reference toxicants for "E" test
- Observations during test
- Endpoints
- Calculations
- Validity of results
- Legal considerations

ITEMS COVERED IN SPECIFIC SECTIONS

Chemicals

- Choosing control/dilution water
- Preparation of solutions
- Observations during tests
- Measurements during tests
- Endpoints
- Chemical properties
- Labelling and storage
- Chemical measurements

Effluents, Elutriates, and Leachates

- Choosing control/dilution water
- Preparation of solutions
- Observations during tests
- Measurements during tests
- Endpoints
- Containers and labelling
- Sample transit and storage

Receiving Waters

- Choosing control/dilution water
- Preparation of solutions
- Observations during test
- Measurements during test
- Endpoints
- Containers and labelling
- Sample transit and storage

Figure 1 **Diagram of Approach Taken in Delineating Test Conditions and Procedures Appropriate for Various Types of Substances**

When formulating these procedures, an attempt was made to balance scientific, practical, and financial considerations, and to ensure that the results would 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. The explicit instructions that might be required in a regulatory test are not provided, although this report is intended to serve as a guidance document for this and other applications.

1.2 Historical Use of Test

Chronic-effect studies with fish have been conducted as either life-cycle (egg-to-egg) or partial life-cycle (egg-to-juvenile) tests, depending on the nature of the studies and the fish species used. For salmonid fish species, complete life-cycle studies are largely impractical because of the time it takes these fish to first reach maturity (2 to 5 years). However, over the past thirty years, results from full and partial life-cycle tests with several fish species and a variety of chemicals indicate that the early developmental stages (i.e., embryo, larval, and early juvenile) can be equally or more sensitive to aquatic contaminants than the adults (Hodson and Blunt, 1981; Woltering, 1984). Based on this experience, a number of procedures have been developed to measure toxic effects on early life stages of salmonid fish (Birge et al., 1985; van Aggelen, 1988; Birge and Black, 1990; Hodson et al., 1991; Paine et al., 1991; Neville, 1992). These procedures are based on the assumption that the

lowest-observed-effect concentrations (LOECs) and no-observed-effect concentrations (NOECs) determined in early life-stage tests, will approximate the chronically safe concentrations for the salmonid species tested.[1]

Early life-stage tests using salmonids for regulatory and research purposes have initiated toxicant exposures at the onset of embryo development, and ended them during the alevin stage, when the fish begin to exhibit swim-up behaviour, or after the fry have been feeding for several weeks (Rexrode and Armitage, 1987; ASTM, 1991a; Hodson et al., 1991; OECD, 1992a; 1992b). Different early life stages can vary in their sensitivity to different toxicants (Mayer et al., 1986; Kristensen, 1990). Therefore, it is preferable to monitor effects of continuous toxicant exposure on several early life stages, and during the transition from one stage to the next, to obtain a good approximation of a chronically safe concentration. Depending on the species and temperature, the duration of the salmonid early life-stage test might be from 50 to 80 days if the assay is ended before the fish exhibit swim-up behaviour (i.e., ~20 days post-hatch), or from 90 to 120 days if the test is ended after fry are fed for 30 days. In any case, such early life-stage tests can be conducted in much less time and at much less expense than full life-cycle tests using salmonid fish.

In situations where frequent, routine testing of toxicity is required, short-term tests of 7 to 28 days have been developed using

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[1] Results of early life-stage toxicity tests are generally useful estimates of the results of comparable life-cycle tests using the same species, but sometimes underestimate chronic toxicity (ASTM, 1991a). Suter et al. (1987) pointed out that fecundity of adults (i.e., the number of viable eggs produced per female surviving to the initiation of reproduction) is usually the most sensitive effect in a full life-cycle test, with larval growth and survival, less sensitive, and about equal in sensitivity to mortality of adults. Birge et al. (1985) showed that, for the substances evaluated, short-term embryo-larval tests with rainbow trout were more sensitive than similar tests using fathead minnows or bluegill sunfish.

embryos and/or alevins (Birge *et al.,* 1985; Birge and Black, 1990; Paine *et al.,* 1991; Neville, 1992; OECD, 1992c). These tests focus on one or more sensitive transitional periods of development (e.g., early embryo development, alevin development and yolk conversion, and feeding and growth of young fry) and have been standardized to use only rainbow trout. These relatively new methods are promising,[2] but in some cases their endpoints might be difficult to define or measure with confidence, or they might require special technical skills to obtain reproducible results. When using these tests, it is advisable to undertake preliminary tests to determine the reproducibility of results, and to compare the sensitivity of results with more conventional early life-stage tests using salmonids (Rexrode and Armitage, 1987; ASTM, 1991a; OECD, 1992b).

The purpose of this report is to provide a "standardized" Canadian methodology for estimating the subchronic or chronic sublethal toxicity of various substances to one or more species of salmonid fish in fresh water, by exposing the early life stages (E, EA, or EAF). The procedures in existing Canadian, American, and international methodology documents vary in duration of exposure, substance examined, test conditions and systems, biological observations and endpoints, statistical design, and criteria for validity (Appendix C). This report gives guidance for evaluating sublethal toxicity of samples of chemical, effluent, leachate, elutriate, or receiving water, and the rationale for selecting certain approaches.

The methods are for use with salmonid fish acclimated to fresh water, with fresh water as the dilution and control water, and with test substances that include wastewaters that are essentially fresh water (i.e., salinity ≤10 g/kg) or are saline but are destined for discharge to fresh water. The application of these methods may be varied but includes instances where the impact or potential impact of substances 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 substances in estuarine or marine environments, or to evaluate wastewaters having a salinity >10 g/kg that are destined for estuarine/marine discharge.

1.3 Salmonid Species Studied and Recommended

In Canada and the United States, the culture and handling of many species of salmonid fish are well established and understood by fisheries scientists, hatchery specialists, fish biologists, and researchers. The performance and sensitivity of several salmonid species have also been examined in a wide variety of toxicity studies in the laboratory. Toxicity to early life stages of salmonid fish has been studied primarily for rainbow trout, brook trout, Pacific salmon, and Atlantic salmon (McKim and Benoit, 1971; Benoit, 1976; Benoit *et al.,* 1976; Davies *et al.,* 1976; Burkhalter and Kaya, 1977; Brenner and Cooper, 1978; Servizi and Martens, 1978; Daye and Garside, 1979; McLeay and Gordon, 1980; Martens *et al.,* 1980; Helder, 1981; Hodson and Blunt, 1981; Birge *et al.,* 1985; NCASI, 1985;

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[2]   Short-term tests with rainbow trout using early embryos (Birge *et al.,* 1985) and late sacfry/early fry (Neville, 1992) have been shown to be more sensitive than similar tests using fathead minnows. The E test given in this report is based on the early embryo test with rainbow trout (Birge *et al.,* 1985; Birge and Black, 1990; Birge, 1992). A 28-day early fry growth/mortality test method using rainbow trout or other fish has been drafted and is under review (OECD, 1992c). This method shows promise as an abbreviated procedure for measuring the toxicity of effluents, chemicals, receiving waters, and other substances.

Peterson *et al.*, 1988; Hodson *et al.*, 1991; Neville, 1992).

Previous studies (Daye and Garside, 1979; McLeay and Gordon, 1980; Peterson *et al.*, 1988; Hodson *et al.*, 1991) have demonstrated that workable methods exist for conducting early life-stage tests using Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), and rainbow trout

(*Oncorhynchus mykiss*). One or more of these three species of salmonid fish is recommended for use in the methods described in this report. These species should provide suitable options for most geographic locations within Canada, study objectives, and availability of gametes. Further information on the biology and distribution of the three species is given in Appendix D.

Test Organisms

2.1 Species and Life Stages

One or more of the following species must be used in the test: Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), or rainbow trout (*Oncorhynchus mykiss*). The selection of species for a study should take into consideration the geographical location of the testing facility and/or study area, seasonal availability of the organisms, relevance to ecological conditions, and the potential for differences in sensitivity to contaminants.

The test is designed to determine effects on the organisms from the onset of embryological development through to a particular stage of development, depending on the test option selected (see Subsection 4.3.1). Because toxicant exposure is initiated immediately after the eggs are fertilized, the test requires that the eggs be fertilized with milt in the laboratory (see Appendix D for recommended procedures). The time taken for embryo/larval development varies with species and with water temperature (see Appendix D). The generalized appearance of salmonid early life stages is shown in Figure 2.

Of the different developmental stages in the early life of salmonids, three major transitions are important in this test. Recently fertilized eggs change to an *embryo*, and develop within an egg membrane that quickly becomes relatively impermeable as the result of "water hardening" after fertilization. Rapid cell division takes place in the developing

embryo, and this phase is used in the shortest test option (the embryo test). A second transition is hatching, and the former embryo becomes an *alevin* or sacfry. This stage is included in the embryo/alevin test. The third transition is from alevin to *swim-up fry*, when the young fish changes from using its yolk as food, to feeding on outside sources. The embryo/alevin/fry test includes this change and 30 days of exposure of the feeding fish. Further details on the stages and the timing of stages with respect to these toxicity tests are given in Subsection 4.3.6.

2.2 Source

Gametes or broodstock should be obtained from a single population and source. The best sources are likely to be government hatcheries, government research stations, and private culture facilities that are known to have disease-free fish. To simplify procedures, it is preferable to obtain gametes, since handling, transport, holding, and stripping of broodstock require additional holding facilities and experienced personnel. If broodstock are obtained, screening of fish for bacterial diseases is recommended before stripping (see Appendix D).

Procurement, shipment, and transfer of gametes or broodstock should be approved, if required, by provincial or regional authorities. Provincial governments might require a permit to import fish or their gametes whether or not the species is native to the area, or movements of fish stocks might be controlled by a Federal - Provincial Introductions and Transplant Committee.

1. Fertilization
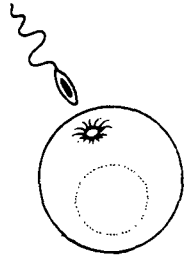
2. Fertilized egg swelling with water uptake
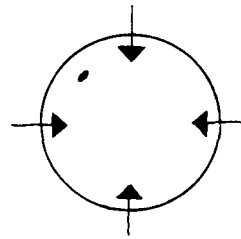
3. Cell division starts
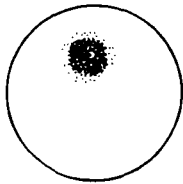
4. Embryo at eyed stage
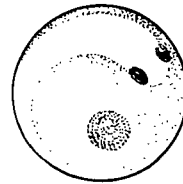
5. Alevin with yolk sac
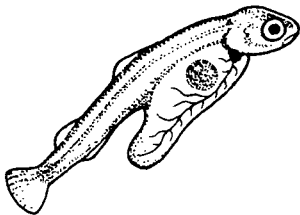
6. Swim-up fry
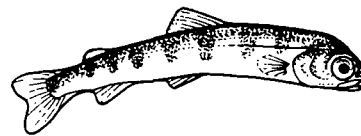
Figure 2 **General Appearance of Salmonid Early Life Stages** (from Sedgewick, 1982)

Advice on contacting the committee or provincial authorities and on sources of fish, can be obtained from the regional Environmental Protection office (Appendix B). In areas where none of the three required species are native, such as in the northern regions of some provinces or the Yukon and Northwest Territories (see species distribution in Appendix D), application for a permit must be made to the previously mentioned committee, the appropriate provincial agency, or the Regional Director General of the Department of Fisheries and Oceans (DFO), depending on procedures in place locally.

2.3 General Biological Characteristics of Test Species

The life cycle and physical characteristics of the test species are summarized in Table 1. This provides some guidance on typical life-cycle characteristics, sizes and/or weights of the life stages of each species. Table 1 can be used as a quick reference to check if control fish are within normal limits, to estimate how many females will be required to supply sufficient eggs for a test, or to plan when gametes might be available from each species.

Table 1   Typical Life Cycle and Physical Characteristics of Test Species [a]

Characteristics	Atlantic Salmon	Coho Salmon	Rainbow Trout
Egg size (mm)	5.5 to 6.8	5.8 to 7.5	3.0 to 5.0
Alevin wet weight (mg)	90 to 110	150 to 375	80 to 175
Swim-up wet weight (g)	0.15 to 0.2	0.3 to 0.7	0.1 to 0.2
Adult wet weight (kg)	3.0 to 6.0	2.7 to 5.4	1.5 to 4.0
Age at maturity (years)	3 to 5	3 to 4	3 to 4
Relative fecundity (no. eggs/kg fish)	1200 to 2000	1500 to 2000	1000 to 1400
Spawning migration [b]	September to November	September to October	March to June

[a]   Values were compiled from Peterson et al., 1977; Beacham et al., 1985; Gordon et al., 1987; March and Walsh, 1987; Peterson and Martin-Robichaud, 1989; Beacham and Murray, 1990; Farmer, 1992; and Peterson, 1992.

[b]   The timing of spawning in local situations, and therefore the availability of gametes, might extend beyond the time of year shown because of manipulation of the life cycle (e.g., photoperiod, temperature, timing of releases) at government or commercial hatcheries.

Section 3

Test System

3.1 Facilities and Materials

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 embryos, alevins, or swim-up fry during testing. Dust and fumes should be minimized within the facilities.

A test facility is required in which the temperature of control/dilution water and all test solutions can be varied from 8 to 15°C, depending on the life stage and species being tested. The temperature must be uniformly maintained within ±1.0°C of each prescribed test temperature (see Subsection 4.3.3). This might require in-line heating and/or cooling of the control/dilution water, a temperature- and photoperiod-controlled wet laboratory, or various types of equipment such as portable water-cooling and/or heating units.

The laboratory must have instruments for measuring the basic water quality variables (temperature, conductivity, dissolved oxygen, pH), and must be prepared to undertake prompt and accurate analysis of

other variables such as hardness, alkalinity, ammonia, and residual chlorine.

Construction materials and any equipment that can contact the test solutions or control/dilution water should not contain any substances that can be leached into the solutions or increase the sorption of test substances. Materials such as borosilicate glass (e.g., Pyrex™), stainless steel, porcelain, or perfluorocarbon plastics (Teflon™), should be used. Other nontoxic plastics, such as polypropylene or polyethylene, may be used, but should not generally be reused after one test, in case the plastic sorbs toxicants that could be released during a subsequent test.

3.2 Lighting

The test should be conducted in the dark until one week after the embryos have hatched.[3] For the remainder of the test, subdued lighting should be used. Light intensity at the water surface should be <220 lux (ASTM, 1991a). Depending on test requirements and intent, lighting might be provided by overhead full-spectrum fluorescent fixtures.[4] The photoperiod should normally be a constant sequence of

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[3]   Minimal incandescent lighting may be used for short periods during observations and maintenance.

[4]   Fluorescent tubes with a full-spectrum wavelength lamp (e.g., Vitalite™, Benelux™) are thought to best simulate natural lighting characteristics. However, fluorescent tubes could introduce bias in studies of compounds that are sensitive to ultraviolet light. In such cases, use of incandescent lighting might be preferable.

16 ± 1 h of light and 8 ± 1 h of darkness. A 15- to 30-minute transition period between light and dark is recommended.[5]

3.3 Test Apparatus

The incubation unit (Figure 3A) for exposing embryos and alevins to a test solution is made from an 800-mL (or larger) plastic beaker or cup having slightly tapered sides (McLeay and Gordon, 1980; Martens *et al.*, 1980; Hodson *et al.*, 1991). The bottom of the beaker is cut off and a replacement floor unit, made out of hard plastic screen, e.g., 16 mesh, is attached. A circular hole is cut in the centre of the plastic screen and a thin cross-section of plastic piping is inset to add strength and provide a secure passage for either the glass tubing or a standpipe drain, depending on which type of incubation unit is being used. At the outer circumference of the plastic screen, a thin cross-section of plastic material is added for support and to help attach the floor unit inside and at the base of the test apparatus.

The incubation unit is suspended in a glass aquarium, of sufficient volume to achieve the fish-loading densities described in Subsection 4.3.2. For a static-renewal test, the aquarium volume would be at least 2 L for every gram of fish biomass expected at the end of the test. For a flow-through test, the aquarium volume would be at least 0.67 L per gram of fish expected at the end of the test (Sprague, 1973).

The incubation unit is suspended in the aquarium by either a Plexiglas™ rack (Figure 3B) or the standpipe drain (Figure 3C). The aquarium should be adapted to accommodate either static-renewal or flow-through conditions, depending on the requirements and objectives of the test.[6]

In tests where aeration is required or desirable[7], the apparatus shown in Figure 3B is recommended. Filtered, oil-free air is bubbled through a disposable, stainless-steel syringe needle located at the end of a plastic air line, and positioned in a glass tube that passes through the centre of the incubation unit. This system, which can be used for either static or flow-through tests, provides a continual current of aerated water past the embryos or alevins.

In tests where aeration is not desired[8], an incubation unit suspended around the standpipe drain is recommended (Figure 3C). The standpipe drain is secured

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[5] 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.

[6] With many types of test substances, static tests with 12- or 24-h renewal of test solutions, when done properly, can be as sensitive and accurate as flow-through tests (Sprague, 1973). Static tests might also be desirable or necessary when the degradation products of the test substance are of concern. High chemical or biological oxygen demand, volatility, or instability of certain substances might necessitate the use of a flow-through test.

[7] Gentle aeration (see Subsection 4.3.4) might be desirable or even necessary during either static-renewal or flow-through tests, to maintain adequate levels of dissolved oxygen when the chemical or biological oxygen demand of the test substance is high.

[8] Aeration might strip volatile chemicals from solution or increase their rate of oxidation and degradation to other substances.

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A (With Aeration / exploded view)

- Glass Tubing or Standpipe
- Embryos or Alevins
- Ring of Plastic Piping
- Plastic Screen
- Support Ring of Plastic

Floor Unit

- Beaker or Cup with Bottom Removed

B (With Aeration)

- Plexiglas™ Support
- Polyethylene Airline Tubing
- Water Level
- Air Bubbles
- Stainless-Steel Syringe Needle
- Plastic Screen Floor Unit
- Standpipe
- Upwelling Current

C (Without Aeration)

- Mesh Insert to Prevent Loss of Embryos or Alevins
- Water Level
- Beaker or Cup with Bottom Removed
- Embryos or Alevins on Plastic Screen Floor Unit
- Solution Flow
- Standpipe Drain

A - Exploded view of incubation unit.

B - Suspended in aquarium in Plexiglas™ rack with aeration through glass tubing secured through the centre of the bottom screen of the incubation unit.

C - Suspended in aquarium on standpipe drain secured through centre of the bottom screen of the incubation unit.

Figure 3   Test Apparatus (from McLeay and Gordon, 1980; Martens *et al.,* 1980; Hodson, 1992)

through the centre of the incubation unit and all flow passes over the embryos and alevins as the test solution drains from the aquarium. This system can only be used for flow-through tests without aeration.

Each aquarium, whether it contains one or more incubation units suspended in it, is one replicate, such that for each test concentration or control, there must be at least three separate aquaria to have true replication that allows calculation of experimental error (see Section 4.1).

Other apparatus for incubating embryos and alevins may also be used, provided that the objectives of the test and criteria for validity are achieved (Section 4.6). However, use of apparatus illustrated in Figure 3 is recommended to provide a greater degree of standardization of conditions during incubation.[9]

3.4 Control/Dilution Water

Depending on the test substance and intent (Sections 5 to 7), the control/dilution water may be: "uncontaminated" ground or surface water from a river or lake; reconstituted water of a desired pH and hardness (e.g., simulating that of the receiving water); a sample of receiving water collected upstream from the source of contamination, or adjacent to the source but removed from it; or dechlorinated municipal water.[10] The water supply should previously have been demonstrated to consistently and reliably support good survival, health, and growth of the test species. Monitoring and assessment of variables such as residual chlorine (if municipal water is used), pH, hardness, alkalinity, total organic carbon, conductivity, suspended solids, dissolved oxygen, total dissolved gases, chemical oxygen demand, temperature, ammonia nitrogen, nitrite, metals, and pesticides, should be performed as frequently as necessary (e.g., monthly or more frequently if deleterious changes in water quality are suspected or found) to document water quality (Table 2). Conditions for the collection, transport, and storage of samples of receiving water, if used as control/dilution water, should be as described in Section 6.1.

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[9] Species-specific modifications to the incubation units might be advantageous. For example, much research has been done on substrates for Atlantic salmon embryos and alevins. Alevins reared on flat surfaces are somewhat stressed and do not use yolk as efficiently as those reared on substrates that provide some lateral support. On flat surfaces, the "innate righting response" of Atlantic salmon alevins results in higher activity levels. Modifications to the plastic screen to provide lateral support, therefore, might be warranted. However, previous experience with both rainbow trout and coho salmon alevins, using the apparatus prescribed in Figure 3 for toxicity tests, did not indicate such a problem with these species.

[10] Dechlorinated water is not recommended for culturing fish and, in particular, not for hatching embryos or rearing of larvae. It is difficult to remove the last traces of residual chlorine and chlorinated organic substances, which might be toxic to the larval fish. If municipal drinking water is to be used for culturing fish and as control/dilution water, effective dechlorination must rid the water of any harmful concentration of chlorine. Vigorous aeration of the water can be applied to strip out part of the volatile chlorine gas followed by use of activated carbon (bone charcoal) filters, and subsequent ultraviolet radiation (Armstrong and Scott, 1974) for removing most of the residual chloramine and other chlorinated organic compounds. Aging the water in aerated holding tanks might also help. The addition of thiosulphate or other chemicals to dilution water to remove residual chlorine is not recommended, as such chemical(s) could alter sample toxicity. The target value for total residual chlorine, recommended for the protection of freshwater aquatic life, is ≤ 0.002 mg/L (CCREM, 1987). Anything greater than 0.002 mg/L might risk interaction of chlorine toxicity with whatever was being tested (Brungs, 1973; NAS/NAE, 1974). In addition to measurements of chlorine, monitoring of egg production and fish survival can provide evidence of satisfactory water.

If surface water is used for culturing fish, it should be filtered. A conventional sand filter or commercial in-line filter would be suitable. Small quantities might be filtered through a fine-mesh net (≤ 60 μm). Ultraviolet sterilization is recommended to reduce the possibility of introducing pathogens into the laboratory and fish-holding system.

The control/dilution water must be adjusted to the required test temperature before use (see Subsection 4.3.3). This water must not be supersaturated with excess gases.[11] Before use, control/dilution water should have dissolved oxygen (DO) that is 90 to 100% of the air-saturation value. If necessary, aerate the control/dilution water vigorously (oil-free compressed air passed through air stones) immediately before use, and confirm that DO of 90 to 100% saturation has been achieved.

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[11] Water entering the aquaria must not be supersaturated with gases. In situations where gas supersaturation within the water supply is a valid concern or the control/dilution water is either actively or passively heated to accommodate a specified test temperature, total gas pressure within water supplies should be checked frequently (Bouck, 1982). Remedial measures must be taken (e.g., use of aeration columns or vigorous aeration in an open reservoir) if dissolved gases exceed 100% saturation. It is not a simple matter to completely remove supersaturation, and frequent checking should be done if the problem is known or suspected to exist.

Table 2 **Recommended Water Quality for Control/Dilution Water** [a]

Variable	Recommended Limits for Exposure
pH	6.5 to 8.5 (7.5 to 8.0 is desirable)
Hardness	15 to 150 mg $CaCO_3$ / L
Alkalinity	20 to 200 mg $CaCO_3$ / L
Aluminum	<5 μg/L (pH ≤6.5)
	<0.1 mg/L (pH >6.5)
Ammonia (un-ionized)	<5 μg/L (preferably not detectable)
Cadmium	<0.3 μg/L (in soft water)
	<0.5 to 0.75 μg/L (in hard water)
Chlorine	<2 μg/L
Copper	<6 μg/L (in soft water)
	<30 μg/L (in hard water)
Dissolved carbon dioxide	0.03 to 15 mg/L
Dissolved oxygen	90 to 100% of saturation
Hydrogen cyanide	<10 μg/L
Hydrogen sulphide	<2 μg/L (preferably not detectable)
Iron	<0.3 mg/L
Lead	<1 μg/L (in soft water)
	<2 μg/L (in hard water)
Mercury	<0.05 μg/L
Nitrite	<60 μg/L (preferably not detectable)
Nitrogen (dissolved gas)	<100 to 103% (max. partial pressure)
	<103% (total gas pressure)
Selenium	<10 μg/L
Total suspended solids	<3 mg/L during incubation
	<25 mg/L during larval and fry stages
	<0.03 mg/L (in soft water)
Zinc	<0.3 mg/L (in hard water)

[a] For salmonid species (from Klontz *et al.*, 1979; CCREM, 1987; and Gordon *et al.*, 1987). Soft water is defined here as ≤60 mg/L total hardness as calcium carbonate ($CaCO_3$). This table is intended as a general guide for water quality. Local water conditions, particularly variations of hardness, alkalinity, and dissolved organic matter can reduce or increase the threshold for metal toxicity. To ensure that the threshold for metal toxicity is not below the limits provided in this table, it is recommended that any available studies of metal toxicity concerning local water quality conditions be consulted.

Other important variables, such as total organic carbon, chemical oxygen demand, and pesticide residues in the control/dilution water, should be monitored and their potential effects on toxicity testing should be evaluated.

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 Section 3 must be incorporated into these universal procedures. The summary checklist of recommended conditions and procedures in Table 3 includes not only universal procedures for each species, but also those for specific types of test substances.

4.1 Preparing Test Solutions

All vessels, measurement devices, stirring equipment, and fish-handling equipment must be thoroughly cleaned and rinsed in accordance with standard operational procedures. Control/dilution water should be used as the final rinse water.

For tests that are intended to estimate the ICp or NOEC/LOEC, at least five concentrations plus a control solution (100% dilution water) are to be prepared. An appropriate geometric series may be used (e.g., 100, 32, 10, 3.2, 1.0; or 100, 46, 22, 10, 4.6, 2.2, 1.0). Concentrations may be selected from other appropriate logarithmic series (see Appendix E). In instances where there is less uncertainty about the range of concentrations likely to be toxic, a geometric series in which each successive concentration is about 50% of the previous one (e.g., 100, 50, 25, 12.5, 6.3) is recommended. There is not usually a great improvement in precision from the use of steps smaller than the 50% dilution factor (i.e., concentrations closer together). Volume requirements for tests will vary

according to the option (E, EA, or EAF) used (see Sections 5.4, 6.1, and 7.1).

Single concentration tests could be used for regulatory purposes (e.g., pass/fail). They would normally use full-strength effluent, elutriate, leachate, or receiving water, or an arbitrary or prescribed concentration of chemical. Use of controls would follow the same rationale as multi-concentration tests. Single concentration tests are not specifically described here, but procedures are evident, and all items apply except for testing a single concentration and a control.

There must be at least three replicates of each concentration including controls for the statistical analysis of results. Replicates are specifically required by Dunnett's test (Gulley *et al.*, 1989). The test must start with an equal number of replicates for each concentration, including controls. If there is accidental loss of a replicate during the test, unbalanced sets of results can be analyzed with less power (Gulley *et al.*, 1989).

The same control/dilution water must be used for preparing the control and all test concentrations. Each test solution should be well mixed using a glass rod, Teflon™ stir bar, or other non-reactive device. Temperatures should be adjusted as required for each life stage of each species (see Subsection 4.3.3). It might be necessary to adjust the pH of the sample of test substance or the solutions (see Subsection 4.3.5), or to provide preliminary aeration of the solutions (Subsection 4.3.4).

Table 3   Checklist of Recommended Test Conditions and Procedures

Universal

Test options	- embryo test (E test) for frequent or routine testing - embryo/alevin test (EA test) for measuring effects on multiple developmental stages - embryo/alevin/swim-up fry test (EAF test) for definitive investigations
Test type	- static-renewal or flow-through; either type for E or EA tests depending on nature of the test substance, EAF test should use flow-through
Test species	- Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), and/or rainbow trout (*O. mykiss*) in the EA or EAF tests; only rainbow trout in the E test
Start of Test	- within 30 minutes of fertilization
End of Test	- for E test, 7 days after fertilization; for EA test, each replicate ends 20 days after ≥90% of its embryos have hatched; for EAF test, a replicate ends after 30 days of feeding its swim-up fry (i.e., 30 days after ≥50% of fry in a replicate show swim-up behaviour)
Control/dilution water	- ground, surface, reconstituted, or if necessary, dechlorinated municipal water; "upstream" water to assess toxic impact at a specific location
Test apparatus	- incubation unit is an 800-mL (or larger) plastic beaker with screened bottom, suspended in a glass aquarium accommodating either static or flow-through conditions
No. organisms, replicates	- initially, ≥40 embryos/replicate for E test and ≥100 embryos/replicate for EA/EAF test; ≥1 incubation unit/aquarium; three replicates per concentration; control plus ≥5 concentrations

Temperature	- *for E test:*
	    Rainbow trout - embryos 12 ± 1.0°C
	- *for EA or EAF tests:*
	    Atlantic salmon - embryos 10 ± 1.0°C, alevins 12 ± 1.0°C, fry 15 ± 1.0°C
	    Coho salmon - embryos 8 ± 1.0°C, alevins 10 ± 1.0°C, fry 12 ± 1.0°C
	    Rainbow trout - embryos 10 ± 1.0°C, alevins 12 ± 1.0°C, fry 15 ± 1.0°C

Oxygen/aeration	- control/dilution water 90 to 100% DO saturation before use; no pre-aeration unless a test solution has DO <60% or >100% upon preparation, in which case aerate all solutions for ≤120 minutes at ≤7.5 mL/min•L before starting test, or renewing or dispensing solution. DO 60 to 100% saturation throughout test, with gentle (≤7.5 mL/min•L) aeration and/or more frequent renewal if required to maintain DO

Table 3   **Checklist of Recommended Test Conditions and Procedures (cont.)**

pH	- no adjustment if pH of test solutions is in range 6.5 to 8.5; a second (pH-adjusted) test might be required or appropriate for pH beyond that range
Lighting	- dark until one week after hatching is completed, then subdued lighting (<220 lux at the water surface) with 16 ± 1 h light : 8 ± 1 h dark, preferably with gradual transition and preferably supplied by full-spectrum fluorescent lights
Feeding	- no feeding in E or EA test; for EAF test, feed fry 4% body weight/day, ≥4 times/day with commercial starter feed, starting in a given replicate when ≥50% of its surviving fish show swim-up behaviour, continuing for 30 days, but no feed in final 24 hours of exposure
Observations	- mortality, deformities, and abnormal behaviour every 24 hours; wet and dry weights of each surviving fish at end of EAF test; optionally, number hatched (EA and EAF tests); and number of swim-up (EAF test) daily in each replicate, weight of subsample from each replicate upon swim-up of ≥50%, and before feeding the fish (EAF test)
Measurements	- temperature at start, at least weekly (preferably daily) in all vessels, and preferably continuously in at least one vessel; DO and pH in representative concentrations, at start and end of 24-h periods in static-renewal, or daily in flow-through tests; optionally, conductivity of each new test solution before dispensing
Endpoints	- number (or percent) of survivors, ICp and/or NOEC/LOEC for mortality (E, EA, and EAF tests), weight (EAF test), and number of deformities/abnormalities (EA and EAF tests); optional for EA and EAF tests are time to ≥90% hatch, percent hatch upon ≥90% hatch of surviving control embryos, and (EAF test) time to ≥50% swim-up fry, percent swim-up upon ≥50% swim-up of surviving control fish
Reference toxicant	- normally for E test only; phenol and/or zinc; determine ICp and/or NOEC/LOEC at the time of the E test or monthly, using conditions and procedures defined for E test
Test validity	- invalid if one of the following occurs: >30% infertility of eggs in any treatment group; >30% of control embryos die or >20% of controls die post-hatch; CV for overall control weight >20% in EA test or >30% in EAF test; instantaneous temperature difference >2°C in ≥2 replicates; time-weighted mean temperature difference >1°C in ≥2 replicates; or usually, if DO in any replicate <60 or >100% saturation

Table 3   Checklist of Recommended Test Conditions and Procedures (cont.)

Chemicals

Solvents — used only in special circumstances; maximum concentration, 0.1 mL/L

Concentration — recommended measurements: weekly in static-renewal tests at beginning and end of 24-h periods in representative high, medium, and low concentrations and control(s); weekly in all replicates of flow-through tests; if concentrations decline >20%, re-test with more frequent renewal

Control/dilution water — as specified and/or depends on intent; reconstituted water if a high degree of standardization required; receiving water for concern with local impact; otherwise, laboratory water

Effluents, Leachates, and Elutriates

Sample requirement — for off-site tests, ≥3 samples collected (effluent, leachate) or prepared (elutriate) weekly; for on-site tests, samples collected daily

Transport and storage — if warm (>7°C), cool to 1 to 7°C with ice or frozen gel packs; transport at 1 to 7°C (preferably 4 ± 2°C) using frozen gel packs as necessary; sample must not freeze during transit; store in the dark at 4 ± 2°C; use in testing should begin as soon as possible after collection, and should start within 72 h of sampling/extraction for off-site tests and within 24 h for on-site tests

Control/dilution water — as specified and/or depends on intent; laboratory water or "upstream" receiving water for monitoring and compliance

High solids — second test with filtered sample is an option, to assess effects of solids in a non-filtered sample

Receiving Water

Sample requirement — as for effluents, leachates, and elutriates

Transport, storage — as for effluents, leachates, and elutriates

Control/dilution water — as specified and/or depends on intent; if studying local impact use "upstream" receiving water as control/dilution water

For site-specific assessments of toxic impact, "upstream" water may be used as control/dilution water. Upstream water cannot be used if it is clearly toxic according to the criteria of the test for which it was intended (see Section 4.6). In such cases, an alternate source of control/dilution water (Section 3.4) should be used.

4.2 Beginning the Test

The test must start within 30 minutes of fertilization of eggs[12] (see Appendix D for fertilization procedure). A minimum of 40 embryos per replicate in the E test, or 100 embryos per replicate in the EA and EAF tests, must be used (see Subsections 4.3.1 and 4.3.6). Uniformity in size of the freshly fertilized eggs is important, as the egg size can affect the alevin and fry size (Beacham *et al.*, 1985). It is recommended that an egg-measuring trough be used (see Von Bayer method in Leitritz and Lewis, 1980), as the freshly fertilized eggs are counted into the test units. Any eggs distinguished visually as under- or oversized should be discarded.

Using 100 embryos per replicate[13], a test with three replicates (including five concentrations and a control), requires 1800 eggs. The eggs should, if possible, be obtained from a batch of eggs stripped from three or more females of similar size. Each concentration, including the control, must

start the test with the same number of replicates (at least three, Section 4.1).

An attempt must be made to achieve "homogeneity of the experimental units" to avoid any differences among vessels that are related to the stripping of gametes. There are two ways to achieve this. They are both valid and are suitable for the same statistical analyses of results (personal communication, Professor J.J. Hubert, Department of Mathematics and Statistics, University of Guelph, Guelph, Ontario). In the first method, embryos from different parents or strippings which have been held separately may be combined (pooled) before exposing embryos to test solutions. In the second method, embryos from a given stripping may be divided evenly among all replicates of all concentrations, then embryos from other strippings are similarly allotted evenly to all incubation units, to make up the full number per replicate. The second method requires more care and effort in culturing and handling. It should, however, reduce the "noise" of the variation between replicates at the same concentration and avoid the chance that exists in the first method, of getting high proportions of unfertilized eggs in a particular replicate, assuming that such stripping-related variation exists.

An attempt to achieve homogeneity must be made with either method by assigning embryos to incubation units in the following

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[12] To maximize test sensitivity and comparability, the start of the test must be standardized to ensure that water hardening occurs during exposure to test solutions. Preferably, gametes would be transported to the laboratory, fertilization would be carried out, and fertilized eggs would be placed into test solutions. In some situations, it might be convenient to transport replicate containers of test solutions to the hatchery or other site where spawning fish are located. The eggs would be fertilized, placed into toxicant solutions for two hours of water hardening, and then transported to the laboratory for distribution into the appropriate test containers. While water-hardened eggs can be transported and handled for several hours without causing undue mortality, there is no period of relative insensitivity to shock in the first few hours during and after water hardening, and great care must be taken when pouring or handling eggs (see Appendix D).

[13] Each replicate concentration must be contained in a single aquarium. However, there may be one or more incubation units suspended in one aquarium.

manner. Immediately after the eggs are fertilized (see Appendix D), embryos should be counted into a series of incubation units, introducing 10 or 20 embryos at a time into each unit in rotation, until the desired total is attained in each unit. Embryos should be counted using an egg measuring trough (see Von Bayer method in Leitritz and Lewis, 1980). Embryos that appear abnormal in any way, or which are noticeably under- or oversized in relation to other eggs, should not be selected for the test. Any embryos possibly damaged or injured during transfer must be discarded; they can be removed by using egg-picking tweezers or a large-bore pipette (7 to 10 mm) with rubber bulb.

Great care must be taken to avoid bumping or dropping individual embryos as they are counted into the incubation units. The embryos must be distributed evenly on the bottom screen of each unit so that they are only one layer thick and are not clumped together or piled on top of one another. The embryos need adequate space to ensure sufficient oxygen exchange and removal of metabolic wastes. This distribution will also facilitate efficient recognition and counting of dead or hatching embryos.

In addition to these procedures, there must be formal random assignment of the groups of the required number of embryos in each incubation unit[14] to particular concentrations and replicate numbers, or vice versa. The individual aquaria must also be in randomized positions in the test facility. Each aquarium must be clearly coded or labelled to identify the substance and concentration being tested, and the date and time of starting. Temperature, dissolved oxygen, and pH levels in the aquaria should

be checked and adjusted, if required/ permitted, to acceptable levels (see Subsections 4.3.3, 4.3.4, 4.3.5) before introducing fish. As a check on concentrations of effluent, it is recommended that conductivity be measured in each new preparation of test solution, before dispensing it to the test vessels.

During approximately the first two weeks that the embryos are exposed to test solutions, or until they reach the "eyed" stage, the embryos are extremely sensitive to any disturbance or mechanical shock (see Appendix D). Therefore, during this time, any routine maintenance procedures (e.g., renewal of test solutions in static-renewal tests) must be done with extra care. Before embryos reach the eyed stage, removal of dead embryos or unfertilized eggs to control fungal infection should be done very carefully (without disturbing any of the surviving embryos) using a large-bore pipette (7 to 10 mm) with rubber bulb.

4.3 Test Conditions and Procedures

4.3.1 Test Methods

One or more of the following three test methods may be used: an embryo (E) test for frequent or routine monitoring; an embryo/alevin (EA) test for measuring toxic effects on multiple developmental stages; or an embryo/alevin/fry (EAF) test for definitive investigations (see Sections 5 to 7). All three methods start with the onset of embryo development, and measure the development and survival of early life stages. Weight attained by the fish is measured in the EAF test. The E test is started with ≥ 40 embryos per replicate and

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[14] Depending on the surface area of the bottom screen in the incubation unit and the size of eggs, it might be necessary to suspend more than one incubation unit in an aquarium to have the required number of embryos distributed only one layer thick for easy identification of dead or hatched embryos. If more than one incubation unit is suspended in an aquarium, the embryos should be distributed equally among them.

ends seven days after fertilization. The EA test starts with ≥100 embryos per replicate and ends 20 days after ≥90% of its surviving embryos have hatched, with no feeding of fish. The EAF test starts with ≥100 embryos per replicate and ends 30 days after ≥50% of the surviving alevins in the replicate have exhibited swim-up behaviour (see Subsection 4.3.6). The EAF test includes feeding of swim-up fry.

Any of these methods may be used to evaluate samples of chemical, effluent, elutriate, leachate, or receiving water, depending on the objectives of the test. The duration of the E test is seven days. The EA test is completed within 50 to 80 days, and the EAF test within 90 to 120 days. Temperature, species of fish, and mode of action of the test substance might influence duration of the EA or EAF tests within these time ranges.[15]

In the E test, only one species (rainbow trout) and one temperature ($12 \pm 1.0°C$) is used. Embryo mortality is the only biological endpoint measured in this test. While the seven-day E test might be convenient for frequent or routine monitoring, its use might not be suitable or appropriate for assessing the toxicity of some substances, depending on their physicochemical characteristics or mode of toxic action. Therefore, before routine use of the E test, an initial comparison of the sensitivity of the E test with the more definitive EAF test is recommended to confirm that the results of the E test will be sufficiently protective for the purpose intended (see Sections 5.1, 6.1, and 7.1).

4.3.2 Test Type and Water Replacement

The test may be run in either a static-renewal or a flow-through mode. With many substances, static tests with 12- or 24-h renewal of solutions, when done properly, can be as sensitive and as accurate as flow-through tests (Sprague, 1973). For some substances having high chemical or biological oxygen demand, volatility, or instability, use of a flow-through test with rapid replacement times for water in the aquaria might be necessary.

The amount of test solution for each replicate should be at least 2 L/g of embryo or other life stage, per day. This can be estimated for the maximum biomass expected during the test, or adjusted periodically through the longer tests (the values in Table 1 are given to assist in calculations). For example, in an EA test using rainbow trout, 100 alevins of medium size (130 mg; as shown in Table 1) would represent 13 g in a replicate vessel. Therefore, at least 26 L of new test solution should be provided every day, in either a static-renewal or flow-through test.

For flow-through tests, a system that continually dispenses and dilutes a stock solution of the test substance (e.g., a metering pump, proportional diluter, or saturator system) is required to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and control/dilution water should be checked

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[15] One effect of exposure to the test substance might be retardation of the rate of development, causing some replicates to hatch or reach swim-up later than the controls or other replicates. Transition benchmarks between the various life stages, therefore, must be applied individually for each replicate, as well as in relation to the controls (see Subsections 4.3.6 and 4.3.7, and Sections 4.4 and 4.5). For any given replicate, test observations, start of feeding, and endpoint of the test should be synchronized with the life-stage transitions in that particular replicate. This could mean that certain replicates with retarded development would cause the duration of EA or EAF tests to be longer than estimated in this report.

daily throughout the test, and should not vary by more that 10%. The flow rate to each replicate should be set at ≥2 L/g·d. In addition, 95% molecular replacement of test solution must take place every 24 hours or less, which requires adjusting the volume in the aquarium so that it is no larger than the volume of inflow during 8 hours (assuming mixing within the aquarium; Sprague, 1973). For the example given having a daily supply of 26 L, the volume of water in an aquarium at any one time would be 26/3 = 8.7 L. Faster replacement times (smaller volume in the aquarium in relation to the flow) are acceptable and might be desirable if volatile toxicants are present.

For the static-renewal tests, an aquarium would normally contain a volume of test solution that equalled the required daily supply of 2 L/g·d, and all or almost all of that solution must be renewed at least every 24 hours. For a 26 L/day requirement of new water, and a daily renewal of 80% of the old water, the volume of water in the aquarium at any one time would be 26 ÷ 0.80 = 32.5 L. More frequent renewal of solutions might be necessary, depending on the nature of the substance being tested.[16]

In static-renewal tests, there are two different procedures for renewing solutions:

1. prepare new solutions in clean aquaria, and gently transfer and resuspend the incubation units containing surviving embryos or alevins in the fresh solutions; or

2. retain the organisms in the same exposure chamber while the solutions are almost completely (≥80%) renewed.

Regardless of the procedure chosen, the renewal of all solutions in static-renewal tests should be done in the latter manner for the E test and during the first two weeks or so in the EA or EAF tests. Old solution should be siphoned out cautiously and new solution added slowly to make up the original volume, because embryos are very sensitive to any disturbance or mechanical shock until they have developed to the eyed stage (see Section 4.2 and Appendix D). Once the embryos have completely developed to the eyed stage, either renewal procedure may be followed.

4.3.3 Temperature

The rate of early development of salmonid fish depends intimately on water temperature (Peterson *et al.*, 1977; Gordon *et al.*, 1987; Peterson and Martin-Robichaud, 1989; Beacham and Murray, 1990), and there can be different temperatures for the optimal development and growth of each life stage and/or species. In the EA and EAF methods, the test temperatures are similar to the optimum temperature for each life stage and species. Temperatures for exposure of embryos must be 10 ± 1.0°C for Atlantic salmon and rainbow trout, and 8 ± 1.0°C for coho salmon. Temperatures for exposure of alevins must be 12 ± 1.0°C for Atlantic salmon and rainbow trout, and 10 ± 1.0°C for coho salmon. Temperatures for exposure of swim-up fry must be 15 ± 1.0°C for Atlantic salmon and rainbow trout, and 12 ± 1.0°C for coho salmon (Table 3).

In the E test, the temperature must be 12 ± 1.0°C for rainbow trout embryos. This temperature, although slightly higher than the optimum for the embryos, is still within the acceptable range for successful

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[16] Test solutions of substances, which are highly volatile or degrade rapidly, will need to be renewed more frequently, perhaps at 12- or even 6-h intervals.

development of trout embryos. At this temperature, development of embryos and toxic action will be modestly accelerated, allowing more definitive endpoints to be reached within the short duration of this test.

In flow-through tests, temperature must be monitored in all replicates at the start of the test and at least weekly (preferably daily) thereafter. In static-renewal tests, the temperature of each newly made solution must be checked before the changeover, and the temperature of all replicates measured and recorded at the end of the first renewal period and at least weekly (preferably daily) thereafter. In addition, temperature should be measured continuously in at least one replicate solution throughout the test. If temperature variation is high in daily/weekly measurements or in concurrent measures of replicates, it could affect the rate or success of early life-stage development, and thereby jeopardize test validity (see Section 4.6).

During the transition from one life stage to the next (see Section 4.3.6), it is preferable to increase the test temperature by 1°C per day, until the desired temperature is reached. However, temperature must not increase by more than 3°C per day during transitional periods.

4.3.4 Dissolved Oxygen and Aeration

The dissolved oxygen content (DO) of the control/dilution water used for preparing test solutions should be 90 to 100% saturation before its use, and, if necessary, it should be

aerated vigorously to achieve this. If (and only if) the measured DO is <60% or >100% of air saturation in one or more test solutions when they have been prepared for use, all solutions should be aerated before the fish are exposed ("pre-aeration"). Oil-free compressed air should be dispensed through a clean silica-glass diffuser[17] or disposable glass pipette, with bubble size 1 to 3 mm. The aeration rate should not exceed 7.5 mL/min.L, and duration of pre-aeration should be the lesser of 120 minutes and attaining 60% saturation in the highest concentration (or 100% saturation, if supersaturation is evident).[18] Any pre-aeration should be discontinued at 120 minutes and the test initiated, whether or not 60 to 100% saturation was achieved in all test solutions.

Dissolved oxygen must be monitored and recorded throughout the test for representative solutions. In static-renewal tests, DO must be measured at the beginning and the end of each renewal interval in at least one replicate of each concentration. In flow-through tests, DO must be measured in each replicate at the start of the test and at least weekly (preferably daily) thereafter in the control and the high, medium, and low concentrations.

Oxygen in the test vessels should not fall below 60% of saturation. If it does, the test becomes invalid as an assessment of the toxic quality, *per se*, of the substance being tested. The test would still be a valid

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[17] A suitable diffuser, measuring 3.8 × 1.3 cm, and fitting 0.5 cm (OD) plastic disposable airline tubing, is available as catalogue item no. AS-1 from Aqua Research Ltd. [P.O. Box 208, North Hatley, Quebec, J0B 2C0, phone: (819) 842-2890].

[18] Aeration might strip volatile chemicals from solution or might increase their rate of oxidation and degradation to other substances. However, aeration of test solutions before fish exposure could be necessary due to the oxygen demand of the test substance (e.g., oxygen depleted in the sample during storage). Aeration also assists in remixing the solution. If it is necessary to aerate any test solution, *all* solutions are to be aerated in an identical manner.

assessment of the total effect of the substance (e.g., effluent) including its deoxygenating influence.[19] Initial measurements will indicate any potential problems with dissolved oxygen, and in such cases, a running check on oxygen concentrations is required. The required use of oxygen-saturated control/dilution water and daily (if semi-static test) or continuous (if flow-through test) renewal of solutions will, in most instances, keep dissolved oxygen above the levels that severely stress the developing salmonids and have a major influence on results.

Depending on the oxygen demand of the test substance (e.g., a particular sample of effluent), gentle aeration of each test solution, including the controls, might be necessary during either static-renewal or flow-through tests, to maintain adequate (i.e., ≥60% saturation) levels of dissolved oxygen. If this is anticipated before test initiation, use of apparatus similar or identical to that illustrated in Figure 3B (Section 3.3) is recommended. If aeration is used, each replicate solution (including the controls) must be aerated at a similar rate not exceeding 7.5 mL/min·L. Alternatively or additionally, more frequent renewal of solutions might be required to maintain DO at ≥60% of saturation.

If the objective for certain tests (e.g., for research) is to include an appraisal of the (high) oxygen demand of the test substance

as part of the measurement of its total effect on salmonid fish, no aeration would be used and the normal daily renewal frequency would be retained.

4.3.5 pH

The pH must be measured in the control solutions and those of high, medium, and low concentrations at the beginning of the test, before embryos are added. The pH should also be measured in representative replicates at the beginning and end of each 24-h period in static-renewal tests, and at least weekly (preferably, daily) in flow-through tests.

Toxicity tests should normally be carried out without adjustment of pH. However, if the sample of test substance causes the pH of any solution to be outside the pH range 6.5 to 8.5, and the toxicity of the test substance rather than the deleterious or modifying effects of pH is being assessed[20], the pH of the solutions or sample should be adjusted, or a second, pH-adjusted test should be conducted concurrently. For this second test, the initial pH of the sample, the stock solution (flow-through tests), or of each fresh solution before renewal (static-renewal tests) may, depending on objectives, be neutralized (adjusted to pH 7.0) or adjusted to within ± 0.5 pH units of that of the control/dilution water, before fish exposure. Another acceptable approach for this second test is to adjust the pH upwards to 6.5 to 7.0 (if sample has/causes pH <6.5), or

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[19] It should be realized that the lower limit of 60% saturation for dissolved oxygen in test solutions is an arbitrary one, and that oxygen levels above that value can also be stressful to the developing fish. Optimal development of salmonid embryos and alevins requires higher (76 to 95%) levels of saturation (Davis, 1975). Any reduction below saturation, in fact, results in some metabolic loading of fish and decreases their performance (Doudoroff and Shumway, 1970). Thus, at oxygen values above the lower limit of 60% saturation for this test, stress from oxygen levels below saturation could interact with any stress from toxicant(s). If this occurs, it will be measured as part of the effect of the sample, be it effluent or other substance. Such interaction has been accepted in this procedure, as part of the effect being measured.

[20] A pH <6.5 might be detrimental in terms of mortality, abnormal behaviour, and poor growth in alevins and older life stages (Gordon *et al.*, 1987).

downwards 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) might require higher strengths of acid or base.

Abernethy and Westlake (1989) provide useful guidelines for adjusting pH. Aliquots of samples or test solutions receiving pH-adjustment should be allowed to equilibrate 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 min is recommended for pH adjustment (Abernethy and Westlake, 1989). Once the test is initiated, the pH of each solution is monitored but not adjusted.

If the purpose of the toxicity test is to gain an understanding of the nature of the toxicants in the test substance, pH adjustment is frequently used as one of a number of techniques (e.g., oxidation, filtration, air stripping, addition of chelating agent) for characterizing and identifying sample toxicity. These "Toxicity Identification Evaluation" (TIE) techniques provide the investigator with useful methods for assessing the physical/chemical nature of the toxicant(s) and their susceptibility to detoxification (U.S. EPA, 1991a; 1991b).

4.3.6 Life-stage Transition

While salmonids go through several developmental phases during their early life stages, there are three major transitions used as benchmarks in the test. The first is the transition from recently fertilized egg to embryo, including the transition from a semi-permeable to a relatively impermeable egg membrane (i.e., water hardening) and the initial period of embryo development (i.e., rapid cell division of the developing embryo). The second is the transition from embryo to alevin (i.e., successful hatching), and the third is from alevin to swim-up fry (i.e., yolk utilization to exogenous feeding). These latter two transition points are used in the EA or EAF test for the purposes of timing changes in test temperature (Subsection 4.3.3) and standardizing the timing of measurements and duration of exposure, to allow for differences in development rate due to the species, temperature, or mode of action of the test substance.

The transition from newly fertilized egg to an embryo in its initial stages of development, before the egg membrane becomes relatively impermeable (until ~2 hours post-fertilization), is a critical period when the developing embryo is highly susceptible to direct exposure to toxic solutions.[21] Therefore, the start of the test (E, EA, and EAF) has been standardized to ensure that this period occurs during exposure to test solutions. To maximize sensitivity and comparability, the test must start within 30 minutes of fertilization.

For the transition stage from embryo to alevin, the start of the alevin stage is defined for a given replicate aquarium, as the time when at least 90% of the surviving embryos in that replicate have hatched. When $\geq 90\%$ of the surviving control embryos have hatched, the percent hatch in all replicates should be recorded. Those replicates in which <90% of the surviving embryos have hatched should be monitored and the time to

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[21] Some toxic substances of low molecular weights (i.e., 400 or less) might diffuse through the membrane after water hardening, and could come in direct contact with the developing embryo.

≥90% hatch recorded for each.[22] The alevins should remain in a given incubation unit for further observation. The duration of this observation period is to be another 20 days after ≥90% of the surviving embryos in that replicate have hatched. It is at this point that the exposure of the replicate is ended in an EA test, and all mortality and abnormalities in the replicate must be recorded. In the EAF test, observation of the alevins continues through the swim-up fry stage.

The start of the swim-up fry stage in a given replicate is defined as the time when ≥50% of the surviving fish in that replicate are exhibiting swim-up behaviour[23]. When ≥50% of the surviving control fish are exhibiting swim-up behaviour, the percent

swim-up in all replicates should be recorded. For replicates in which <50% of the surviving fish have exhibited swimming behaviour, monitoring should continue to determine the time to ≥50% swim-up.[24] When ≥50% of the surviving fish in a given replicate are exhibiting swim-up behaviour in the EAF test, ≥30 fish randomly selected from all surviving fish in that replicate should be released from the incubation unit(s) into the aquarium[25], and feeding of fry should be initiated (see Subsection 4.3.7). Feeding is continued in that replicate for 30 days after ≥50% of the surviving fish in the replicate have exhibited swim-up behaviour. After 30 days of feeding, fish are not fed for 24 hours, then the EAF exposure for that replicate is ended

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[22] One effect of exposure to the test substance might be retardation of the rate of development. In certain instances, there might be some replicates that have not achieved ≥90% hatch of the surviving embryos by the time the typical EA test would be ended (i.e., 20 days after ≥90% of the surviving embryos in controls and the other replicates have hatched). At this point in the EA test, the percent hatch of any replicate exhibiting such an extremely retarded rate of development should be recorded and the stage of development of the remaining embryos should be verified (see Section 4.4), and the test terminated.

[23] At this stage, the fish demonstrates the ability to maintain position in the water column, typically rising to the surface and remaining there for extended periods of time. Also at this time, the yolk sac is no longer readily visible and the fry is said to have "buttoned up." However, the resorption of the yolk sac might not be complete, as some yolk might still remain in the abdomen. Therefore, the ability of the fry to exhibit swimming behaviour and the readiness to feed, are more definitive indicators of attaining the swim-up fry stage than is yolk resorption.

[24] If it becomes difficult to judge exactly whether ≥50% of the fish are exhibiting swim-up behaviour while they are confined to the incubation unit, it might be helpful to release the fish into the aquarium when it appears that an increasing number of the fish are exhibiting such behaviour, and their yolk sacs have become less visible. By doing this, the number of fish exhibiting swim-up behaviour in each replicate can be more readily determined. In any case, it is not advisable to release alevins into the aquarium prematurely.

One effect of exposure to the test substance might be retardation of the rate of development. There might be some replicates that have not achieved ≥50% swim-up of the surviving alevins by the time the typical EAF test would be terminated (i.e., 30 days after ≥50% of the surviving alevins in controls and the other replicates have exhibited swim-up behaviour). In such a case, there could be a replicate containing both alevins and swim-up fry, or even embryos, alevins, and swim-up fry. At the end of the EAF test, the percent swim-up for any replicate exhibiting such an extremely retarded rate of development should be recorded, the stage of development of any remaining embryos verified (see Section 4.4), and the test terminated.

[25] Depending on the objectives of the study and the nature of the test substance, subsample(s) of ≥20 surviving fry from each replicate in the EAF test might, in some instances, be removed at this stage. Mean wet and dry (24 hours at 60°C) weights should be measured before the start of feeding for later comparison with fry weight at the end of test. These subsamples might then be frozen or otherwise treated in preparation for analyses of tissue burdens of specific contaminant(s).

and mortalities, abnormalities, and weights of all surviving fish in the replicate must be recorded (see Section 4.4).

4.3.7 Thinning

Successful fertilization and survival through hatching and larval development can vary widely among species and among various batches of gametes. Although it is desirable to have 100% fertilization and control survival, such success is rarely achievable. Particularly because of the uncertainty of fertilization success, and to provide an adequate number of organisms for a statistically valid test, it might be necessary to begin the EA or EAF test with more than 100 eggs per replicate. Therefore, when either the EA or EAF test is being undertaken and appreciable uncertainty exists regarding fertilization success, more than 100 eggs may be placed in each replicate, and subsequently thinned to the minimum required number of embryos within each replicate (i.e., 100). However, thinning should be delayed until the eggs have reached the eyed stage, to minimize damage to embryos from handling.

When the E test is being undertaken, no thinning is possible since the test ends before the eggs have reached the eyed stage. Therefore, the E test is started with the required number of eggs per replicate (i.e., ≥ 40), and the number of unfertilized eggs must be determined at the end of the test (see Section 4.4 and footnote 30). If the average fertilization rate in the controls or any exposure concentration is <70%, then the results of the E test are not valid (see Section 4.6).

If either the EA or EAF test is started with more than the minimum required number of eggs/replicate, percent survival of embryos must be noted and recorded for each replicate from the time of test initiation to thinning. Overall survival of embryos in each replicate (not including unfertilized eggs) is to be calculated as the product of percent survival to the time of thinning, multiplied by percent survival from thinning (divided by 100) through to the end of the test. However, if the average fertilization rate in the controls or any exposure concentration is <70%, the results of the test would not be valid (see Section 4.6). In such a case, the test should be terminated as soon as possible after the eggs have reached the eyed stage (and the total number of unfertilized eggs is determined), and restarted with another batch of newly fertilized eggs.

For any E, EA, or EAF test, an early indication of fertilization success can be obtained one or two days after fertilization by holding additional replicates in control/dilution water under conditions identical to the test treatments, and clearing and examining them microscopically (see footnote 30, Section 4.4) for the incidence of developing embryos. If the fertilization rate in the addditional replicates is <70% at this time, the investigator may choose to end the test.

If the use of more than the minimum required number of eggs/replicate is anticipated in either the EA or EAF test due to fertilization concerns, preliminary studies are recommended. Such studies should determine the maximum number of embryos that can be placed initially in each incubation unit without causing detrimental effects (such as insufficient oxygen exchange or accumulation of metabolic waste) due to crowding. By distributing the embryos only one layer thick on the bottom screen of the incubation unit, efficient recognition and counting of dead or hatching embryos will also be facilitated. The maximum number for an incubation unit should be determined

for the particular species, temperature, flow rate, embryo size, size of the unit, volume of the aquarium, and the expected size of the alevin or fry at the end of the test.[26] In cases where more than one incubation unit is suspended in an aquarium, embryos or alevins may be moved among the incubation units within the same aquarium to distribute them evenly. However, it is not permissable to transfer organisms from one aquarium (i.e., replicate) to another.

4.3.8 Feeding of Swim-up Fry

When ≥50% of the surviving fish in a given replicate exhibit swim-up behaviour in the EAF test, the fish should be released from the incubation unit(s) into the aquarium, and feeding should be initiated.[27] Feeding is continued in the replicate for 30 days after the demonstration of ≥50% swim-up behaviour, then fish are not fed for 24 hours, the test exposure is terminated for that

replicate, and all final observations and measurements (Section 4.4) are made.

A commercial starter feed suitable for the selected species should be used. The fry should be fed 4% of their body weight per day, with approximately equal portions of this ration offered at least four times per day. The ration for each replicate should be based on the product of the mean weight of control fish and the number of fish in the aquarium (ASTM, 1991a). Newly hatched brine shrimp may also be used. Special measures might be necessary to successfully initiate feeding of Atlantic salmon fry (Peterson and Martin-Robichaud, 1989).[28]

4.3.9 Reference Toxicant

For the E test only, the routine use of a reference toxicant or toxicants is practical and necessary to assess, under standardized test conditions, the relative sensitivity of the

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[26] For example, thinning in each replicate (particularly the controls and other replicates in which survival is high) might be required at the eyed egg stage to reduce the number of embryos per replicate to 100 in the EA and EAF tests. Thinning of each replicate after completion of hatching, and/or after all fry have initiated feeding, could be necessary to ensure that the fish-loading density does not exceed the allowable limits.

[27] It is important to synchronize the start of feeding relative to the rate of development in each replicate. However, it is also important not to delay making food available to the fry when they are ready to consume it, to avoid any possibility of stress from starvation. If it becomes difficult to judge exactly whether ≥50% of the fish are exhibiting swim-up behaviour while they are confined to the incubation unit, it might be helpful to release the fish into the aquarium when it appears that an increasing number of the fish are exhibiting such behaviour, and their yolk sacs have become less visible. By doing this, the number of fish exhibiting swim-up behaviour in each replicate can be more readily determined, and the initiation of feeding will not be unnecessarily delayed.

Special considerations regarding initiation of feeding are necessary when exposure to the test substance results in retardation of the rate of development. There might be some replicates which have not achieved ≥50% swim-up of the surviving alevins, yet the swim-up fry are ready to consume food. For example, in an extreme case there could be a replicate containing both alevins and <50% swim-up fry, or even embryos, alevins, and swim-up fry, although the control and other replicates have achieved ≥50% swim-up and initiated feeding. In such a case, it is suggested that feeding of the fry in "retarded" replicates be initiated shortly after control and other replicates have initiated feeding, and continued based on the willingness of the fry to consume food.

[28] The period of transition from yolk utilization to exogenous feeding has often been characterized by high mortality in Atlantic salmon culture. Mortalities >20% are frequently evident for this phase and species. The probable important factors associated with feeding success of swim-up Atlantic salmon fry are: physical characteristics of the rearing troughs and the water supply; time of presentation of first food; feeding frequency; food quality; and rearing temperatures. In performing an EAF test using Atlantic salmon, particular attention should be paid to increasing the feeding frequency, minimizing any motion or disturbance when feed is presented, that might cause a reaction by the fish, and creating a surface flow into which the fry might orient and feed can be presented. Use of freeze-dried brine shrimp (commercially available) has been found to facilitate first feeding of Atlantic salmon fry.

group of embryos used, and the precision and reliability of data produced by the laboratory for that/those reference toxicants (Environment Canada, 1990a). Sensitivity of embryos to the recommended reference toxicant(s) should be evaluated at the time each E test is performed, or at least once each month for laboratories performing numerous E tests on a routine basis (i.e., several per month). Due to the prolonged duration of EA or EAF tests, a concurrent reference toxicant test is normally impractical for these early life-stage tests.

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

- chemical readily available in pure form;

- stable (long) shelf life of chemical;

- highly soluble in water;

- stable in aqueous solution;

- minimal hazard posed to user;

- easily analyzed with precision;

- good dose-response curve for salmonid embryos;

- known influence of pH on toxicity to test organism; and

- known influence of water hardness on toxicity to salmonid embryos.

Reagent-grade phenol and/or zinc (prepared using zinc sulphate) are recommended for use as reference toxicants for this test. Sensitivity of salmonid embryos to one or both of these reference toxicants should be evaluated using standard E test(s), and the ICp or NOEC/LOEC determined for one or both of these chemicals. Conditions and

procedures for undertaking E tests with reference toxicant(s) are to be consistent and as described in this report.

Reference toxicant tests would normally use the control/dilution water that is used at the laboratory for the definitive E, EA, or EAF test. Alternatively, soft reconstituted water (hardness 40 to 48 mg/L as $CaCO_3$, pH 7.2 to 7.5) should be prepared (see footnote 38, Section 5.4) and used as control water and for all dilutions if a greater degree of standardization is desired (U.S. EPA, 1985b; Environment Canada, 1990b).

A warning chart (Environment Canada, 1990a) should be prepared and updated for each reference toxicant used. Successive ICps or NOECs are plotted on this chart and examined to determine whether the results are within ± 2 SD of values obtained in previous tests. The geometric mean NOEC together with its upper and lower warning limits [± 2 SD calculated on a geometric (logarithmic) basis] are recalculated with each successive ICp or NOEC until the statistics stabilize (U.S. EPA, 1989; Environment Canada, 1990a). If the ICps or NOECs fail to show a lognormal distribution, an arithmetic mean and SD might prove more suitable.

If a particular ICp or NOEC falls outside the warning limits, the sensitivity of the embryos and the test system are suspect. Inasmuch as this might occur 5% of the time due to chance alone, an outlying value does not necessarily mean that the sensitivity of the embryos or the precision of the toxicity data produced by the laboratory are in question. Rather, it provides a warning that this might be the case. A check of all pre-test and test conditions and procedures is required at this time.

Use of warning limits does not necessarily indicate that a laboratory is generating

consistent results. A laboratory that produced extremely variable data for a reference toxicant would have wide warning limits; a new data point could be within the warning limits but still represent undesirable variation in results obtained in tests. A coefficient of variation of 20 or 30% is tentatively suggested as a general limit for reference toxicant tests by Environment Canada (1990a).

Stock solutions of phenol should be made up on the day of use. Zinc sulphate (usually $ZnSO_4 \cdot 7H_2O$, molecular weight 4.398 times that of zinc) should be used for preparing stock solutions of zinc, which should be acidic (pH 3 to 4). Acidic zinc solutions may be used when prepared, or stored in the dark at $4 \pm 2°C$ for several weeks before use. Concentration of zinc should be expressed as milligrams of Zn^{++}/L.

Concentrations of reference toxicant in all stock solutions should be measured chemically using 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 ICp or NOEC be atypical (outside warning limits). If stored, sample aliquots must be held in the dark at $4 \pm 2°C$. Both zinc and phenol solutions should be preserved before storage (APHA *et al.*, 1989). 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 ICp or NOEC should be based on the geometric mean measured concentrations if they are appreciably (i.e., ≥20%) different from nominal ones and if the accuracy of the chemical analyses is satisfactory.

4.4   Test Observations and Measurements

In the E, EA, and EAF tests, observations of the number of dead organisms in each replicate must be recorded daily. In the EA and EAF tests, the number of dead alevins and fry exhibiting deformities in each replicate must also be recorded daily. Any dead embryos, alevins, or fry should be removed as soon as they are noted[29]; live individuals, whether deformed or not, are not to be removed. When removing dead individuals, extreme care should be taken not to bump or damage adjacent embryos or alevins, since they are extremely delicate and sensitive (see Section 4.2). To ensure that opaque unfertilized eggs are not considered as dead embryos and included in this count, the stage of embryonic development of all opaque eggs removed from the incubation units before the eyed stage, must be verified.[30]

In the EAF test, the individual weights of all fish surviving in each replicate at the end of the test must be recorded to the nearest

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[29] Death can be discerned in young embryos as a marked loss of translucency and change in colouration caused by coagulation and/or precipitation of protein, leading to a white, opaque appearance. In older embryos, death is the absence of movement and heartbeat. In alevins and fry, death is immobility and lack of reaction to mechanical stimulus, as well as the absence of respiratory movement and heartbeat, and is usually accompanied by a white, opaque colouration of central nervous system.

[30] The dead embryos and/or unfertilized eggs are placed in a saturated salt solution or a solution of glacial acetic acid, methanol, and water (1:1:1, v/v), until they become clear. After clearing, they are examined by the naked eye or, as necessary, under a dissecting microscope, for evidence of cleavage of the germinal disc, or of a white streak which is the embryo.

0.01 g. Dry weight (i.e., after 24 hours at 60°C) must be measured; however, measurement of both wet and dry weight is recommended. Measurement of individual weights of a subsample of early fry from each replicate, after ≥50% of the surviving fish have exhibited swim-up behaviour but before commencement of feeding, is also recommended (see Subsection 4.3.6, and footnote 23).

In both the EA and EAF tests, daily records should be made of the number hatched in each replicate. In the EAF test, the number exhibiting swim-up behaviour in each replicate should be recorded daily. Documentation of abnormal appearance or behaviour is also recommended for both the EA and EAF tests. Abnormal behaviour includes uncoordinated swimming behaviour, atypical quiescence, atypical feeding behaviour, hyperventilation, and loss of equilibrium.

4.5 Test Endpoints and Calculations

Biological endpoints which must be used in this test are increased mortality (E, EA, and EAF tests), increased incidence of deformities (EA and EAF tests), and adverse effect on growth as judged by attained dry weight (EAF test). For each endpoint, the effect is assessed by comparison with the controls. The most sensitive effect (i.e., the endpoint which shows a statistically significant change at the lowest concentration) is taken as the definitive

indication of toxicity (Woltering, 1984; Birge and Black, 1990).

Other recommended endpoints include the following: longer time to ≥90% hatch (EA and EAF tests); longer time to ≥50% swim-up (EAF test); higher incidence of abnormal behaviour (EA and EAF tests); lower percent hatch upon hatching ≥90% of the surviving control embryos (EA and EAF tests); lower percent swim-up upon swim-up of ≥50% of the surviving control fish (EAF test); and lower weight of a subsample of surviving fry upon swim-up of ≥50% of the surviving fish in the same replicate (EAF test) [see Subsection 4.3.6 and Section 4.4]. In each case the comparison is with performance in the controls. A significant deleterious effect in any of these recommended items is taken as a definitive indication of toxicity in the same way as an effect on the required endpoints described in the previous paragraph.

An additional biological endpoint is total biomass per replicate. It could be used to evaluate hatching success, the weight of fry, and the number surviving.[31] This could prove a powerful endpoint and is also recommended.

The *inhibiting concentration for a specified percent effect* (ICp) is recommended for calculating a point-estimate of the concentration causing a certain degree of effect. The percentage is selected by the

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[31] This option for analysis of results would compare total biomass per replicate at the various concentrations. If initial numbers of fish were different in the vessels, there could be a comparison of the statistic: (total biomass surviving in the replicate) divided by (the number that started the test in that replicate). This is, perhaps, a more rational approach, and in particular, combining information on mortality and growth into one number is useful. This method will probably be more widely used in the future but has not found wide use at the time of writing, so cannot yet be considered a standard procedure. Further development of this method might involve correction of experimental mortalities for any mortality in the control. Individual fish that were accidentally killed by the investigator would not, of course, be included in the total number of fish considered to have started the experiment.

investigator, and is customarily 25% (or 20%) reduction in performance compared to the control. In the present test, it would be 25% lower weight, greater mortality, greater incidence of abnormalities, or effect measured by another optional endpoint, compared to the control. The ICp is a useful measure of effect, often more sensitive and more desirable than NOEC/LOEC determined by hypothesis testing (Suter *et al.*, 1987). In particular, confidence limits can be calculated, allowing statistical comparisons to be made with other such values. An analysis could begin with a plot of weight against the logarithm of test concentration, with IC25 (or IC20) read off. The graph would also serve as a check against the results of mathematical computations. A straightforward *linear interpolation* method provides a mathematical estimate of the ICp (U.S. EPA, 1989, Appendix J). The ICp and confidence limits may be estimated by a "bootstrap" method on computer (Norberg-King, 1988; U.S. EPA, 1989).[32] Use of the program BOOTSTRP requires a math co-processor. It should be remembered that an ICp, whether IC25, IC20, IC5, or IC1, is still, by definition, a concentration that causes the specified degree of harmful effect; it is not a no-effect concentration, technically speaking.

No-observed-effect concentrations (NOECs) and lowest-observed-effect concentrations (LOECs) may be derived statistically by the hypothesis-testing approach, and it is recommended that both be calculated. The

methods of calculation and rationale are discussed in detail by the U.S. EPA (1989). This approach has certain limitations with respect to the size of the difference that can be detected. Deciding differences solely on the basis of statistically significant difference from controls might depend largely on sample sizes and variability within replicates. The NOEC is also not a "no-effect" concentration, technically speaking. Rather, it is a "no-statistically-significant-difference" concentration. In addition, a laboratory that has high variation, or that uses few replicates, could obtain a higher LOEC than a laboratory that maintains lower variation and uses more replicates. Advice should be sought from a statistician when analyzing results using this approach.

For weight of fry, the NOEC and LOEC are determined from the final dry weights of the surviving individuals in each replicate of the control and the various concentrations of wastewater or chemical. If there is complete mortality in all replicates at a given concentration, that concentration is excluded from the analysis. For mortality and for number of fish with a deformity or abnormality, NOEC and LOEC are determined separately, in a similar manner. Analyses when using other recommended endpoints also follow the same general procedure. The lowest set of NOEC/LOEC, for weight, mortality, deformity, or other effect, is taken as the overall result of the test.

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[32] Calculation of the ICp assumes that the effect being measured is a reduction in performance compared to the control. However, in some cases there could be a stimulatory effect at low concentrations (i.e., increased growth, survival, etc.), followed by an inhibitory effect with increasing concentrations. It should be noted that a stimulatory effect cannot be assumed to be a strictly positive effect, any more than an inhibitory effect can be assumed to be a strictly negative effect. What is being measured is a change from the norm (i.e., control). Current thinking is split on whether or not to account for stimulatory effects as a sublethal effect when calculating the ICp. It is suggested that when such stimulatory effects occur, the ICp should be calculated two ways: accepting stimulatory effects, along with inhibitory effects, as a true deviation from the control; and ignoring the stimulatory effect in favour of inhibitory effects as the only true deviation from the control (i.e., pooling data showing stimulatory effects from low concentrations with control data).

The statistical procedures to be followed are given in TOXSTAT[33] (Gulley *et al.*, 1989). An up-to-date version of TOXSTAT can be obtained by contacting Environment Canada (see Appendix B). These methods start with a check of normality and homogeneity of data, and provide suitable tests of significance for particular types of distribution. TOXSTAT also provides appropriate tests in cases where the numbers of replicates are unequal because of accidental loss or other cause.

If the data are regular (i.e., random, independent, normal, homogeneous, etc.) or can be made so by suitable transformation, an analysis of variance is carried out. Usually, differences of each concentration from the control will be ascertained by *Williams' test*. Williams' test is available in TOXSTAT and is designed to be sensitive to the association between the degree of effect and the ordering of concentrations by magnitude (Gulley *et al.*, 1989). Williams' test (Williams, 1971; 1972) is recommended as a more powerful tool than *Dunnett's test*. Dunnett's test, a standard multiple-comparison test, provides estimates of the Minimum Significant Difference,

which is the magnitude of the difference in average weights or average mortality, that would have to exist between the control and a test concentration before a significant effect could be concluded for that concentration. Dunnett's test is not a particularly powerful way of discriminating effects in toxicity tests since it ignores the information on the ordering of test concentrations by magnitude (Masters *et al.*, 1991). If there are unequal numbers of replicates, the *Bonferroni t-test* is substituted for Williams' or Dunnett's test.

A geometric average of the NOEC and LOEC is often calculated for the convenience of having one number rather than two. Such a value may be used and reported, recognizing that it represents an arbitrary estimate of an effect-threshold that might lie anywhere in the range between the LOEC and NOEC. The calculated value of the geometric mean is governed by whatever concentrations the investigator happened to select for the test. No confidence limits can be estimated for the geometric mean, and that is also the case for NOEC and LOEC, although they indicate the outer limits of the estimate.

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[33] The methods of TOXSTAT (Gulley *et al.*, 1989) are not detailed here because the instructions are best followed in the written description that accompanies the programs on computer diskette. Briefly, data are tested for normality by the *Shapiro-Wilks* test, and for homogeneity by *Bartlett's test*. If the data do not meet the requirements, it might be possible to transform them to meet the requirements, using logarithms or arc-sine. The transformation, however, might reduce the sensitivity of the analysis and the ability of the toxicity test to detect differences.

Tests of normality and homogeneity of variance might be problematic in themselves. For example, the Shapiro-Wilks test for normality might not be very powerful and Bartlett's test for homogeneity might be very sensitive to departures from normality. The t-test (and its relatives), however, can be very strong against departures from assumptions provided that the sample sizes are equal. Therefore, if equal sample sizes can be maintained, tests of assumptions for normality or homogeneity would not be necessary.

If a set of data cannot meet the requirements for normality or homogeneity, and cannot be transformed to do so, there are non-parametric tests provided in TOXSTAT that may be substituted (*Steel's many-one rank test*, or the *Wilcoxon rank sum test* in the case of unequal replicates). Those non-parametric options may be used, and are powerful tools for data that are not normally distributed. The non-parametric tests are less powerful than parametric tests, however, when used on normally-distributed data, and in that situation they might fail to detect real differences in effect, i.e., an underestimate of sublethal toxicity might result. It should also be remembered that four replicates are required to make use of the non-parametric methods.

The geometric mean of NOEC and LOEC is often called the *chronic value* in the United States, but that might be somewhat misleading, since the EA and EAF tests represent approximately 5 to 10% of a typical salmonid species' lifetime, and therefore would not necessarily be classified as chronic. In such instances, the mean is sometimes called a *subchronic value*. *Threshold-effect concentration* TEC would be an appropriate name for the calculated value. "Threshold" means, in the dictionary sense, a point at which an effect begins to be observed. This does not imply that undetected effects are absent at lower concentrations, since the TEC is merely a point-estimate, derived from the NOEC and LOEC which are the bounds of the threshold for statistically significant difference from the control treatment.

In a single-concentration test, a t-test is normally the appropriate method of comparing data from the test concentration with those of the control. The procedure for a t-test can be taken from any statistics textbook. An effect of the test substance is accepted if weights are significantly lower, or if mortality or deformity is significantly higher than the same statistics for the control (or for optional effects, whichever are relevant for the E, EA, or EAF test carried out). Requirements for homogeneity of variance and normality must be satisfied (Appendix H of U.S. EPA, 1989; Gulley *et al.*, 1989) before using the standard t-test. If the data do not satisfy the requirements, a non-parametric test could be selected with

advice from a statistician; no particular test appears to have become standard practice as yet.

In some cases, the test replicates might not represent various concentrations of a single sample of wastewater or chemical, but rather a set of different samples, such as full-strength effluents from different industries, or samples of surface waters from different places. It might be desired to test not only whether each sample is different from the control, but also whether the samples are different from each other. That can be done using one option in the statistical program TOXSTAT (Tukey's test). Such sets of tests should report the results of each sample tested, not as NOEC/LOEC and/or ICp, but as the mortality (or weight or deformity, etc.) as a percentage of the control(s), and whether that number was significantly different from the control(s).

4.6 Test Validity

Assuming that all recommended procedures and conditions were followed[34], the validity of the test must be based on each of the following: the incidence of unfertilized eggs; the incidence of control mortality; variation in control weight (EA or EAF tests only); stability of temperature; and maintenance of DO levels.

The average number of unfertilized eggs in the controls and in each exposure concentration must be ≤30% of the total number of eggs placed in each treatment at

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[34] More specifically, this assumption is that: all items of apparatus and all substances were identical in each replicate; all treatments were assigned randomly to replicates; all organisms were assigned randomly to replicates; the test was not terminated prematurely; all required physicochemical variables were monitored as prescribed; and all required biological variables were monitored as prescribed.

the start of the test.[35] If the average fertilization rate in the controls or any exposure concentration is <70%, the results of the test would not be valid. In such a case, the test should be terminated as soon as possible after the total number of unfertilized eggs is determined (i.e, at the end of the seven-day E test, or once the eggs have reached the eyed stage during the EA and EAF tests), and restarted using another batch of freshly fertilized eggs.

The average mortality of controls must be ≤30% for embryos until they have hatched (not including unfertilized eggs), and ≤20% post-hatch until the end of the test. If the average mortality of controls is >30% for embryos or >20% post-hatch, then the test must be considered invalid.

The coefficient of variation (CV = 100 × standard deviation divided by the mean) of the overall control weight must be ≤20% at the end of the EA test and ≤30% at the end of the EAF test. If the CV for control weight is greater, then the results of the test are questionable and it must be considered invalid.

At any given time, the difference between the measured temperature in any two test vessels must be ≤2°C. Additionally, the difference between the time-weighted mean temperature for any two vessels must be ≤1°C (ASTM, 1989). Since the development and growth of early life stages are related intimately to temperature, the test must be considered invalid if any deviation in test temperature occurs beyond these limits. Problems with stability of

temperature are usually a direct consequence of inadequacies of test facilities and temperature control equipment.

Except where test objectives allow otherwise (see Subsection 4.3.4), the concentration of dissolved oxygen in each replicate must be between 60 and 100% of the air saturation value, throughout the test. Even temporary deviations outside of this range can affect the development, growth, and survival of embryos, alevins, and fry. If, at any time, DO is outside of this range in one or more replicates, the test is normally considered invalid. However, in instances where the test substance has an oxygen demand sufficiently high to prevent maintaining DO ≥60% in one or more solutions, the results might be considered valid provided that the interaction of stress from low oxygen with stress from exposure to the test substance is accepted as part of the effect being measured (Subsection 4.3.4).

4.7 Legal Considerations

Care must 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 custody; 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 results.

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[35] If the average fertilization rate in the controls or any exposure concentration is ≥70%, a chi-square statistical analysis is recommended to demonstrate that the proportion of fertilized eggs in each treatment is considered equal. Although fertilization rate does not depend on treatment, the number of fertilized eggs per treatment will be important in choosing the appropriate statistical analyses of endpoint data (see Section 4.5).

Specific Procedures for Testing Chemicals

This section gives particular instructions for testing chemicals, in addition to the procedures in Section 4. A multiple-concentration test is usually performed, to determine the NOEC and LOEC and/or ICp. Three replicates, the minimum required for statistical analysis of results, might also be required under regulations for registering a pesticide or similar category of chemical.

5.1 Test Options

Depending on objectives and regulatory requirements, a salmonid early life-stage test to evaluate the toxicity of chemical sample(s) may be undertaken using the embryo (E) test, the embryo/alevin (EA) test, or the embryo/alevin/fry (EAF) test (Subsections 4.3.1 and 4.3.6). Assessments required under regulations for registering a pesticide or similar category of chemical, or for other regulatory assessments of chemical(s), might be most suitably performed as an EAF test. The EAF test might also be used in research studies concerned with providing a definitive assessment of a chemical's toxicity toward salmonid fish. The EA test might be used for comparative screening of several chemicals for relative toxicity to salmonid fish, or for other purposes, while the E test might be used for frequent monitoring purposes. Selection of the most suitable test will require consideration of the physicochemical characteristics, as well as the mode of toxic action, of the substance being tested. Before routine use of the E

test, initial comparison of the sensitivity of the E test with the more definitive EAF test is recommended to confirm that the results of the E test will be sufficiently protective for the purpose intended.

5.2 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. Datasheets on safety aspects of the test substance 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.[36] An acceptable analytical method should also be known for the chemical in water at concentrations intended for the test, together with data on precision and accuracy.

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

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[36] Knowledge of the properties of the chemical will help to identify any special precautions or requirements for handling and testing it (e.g., a well ventilated facility, or the need for solvent). Information regarding chemical solubility and stability in fresh water will also be useful in interpreting results.

operating procedures for chemical handling and storage should be followed.

5.3 Preparing Test Solutions

Solutions of the chemical are usually prepared by adding aliquots of a stock solution made up in control/dilution water. Alternatively, for strong solutions or large volumes, weighed (analytical balance) quantities of chemical may be added to control/dilution water to give the nominal strengths for testing. If stock solutions are used, the concentration and stability of the chemical in the solution should be determined before the test. Stock solutions subject to photolysis should be shielded from light, and unstable solutions must be prepared as frequently as necessary to maintain concentrations for each renewal of test solutions.

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. Ultrasonic dispersion can produce droplets that differ in size and uniformity, some of which might migrate towards the surface of the liquid, or vary in biological availability creating variations in toxicity. Organic solvents, emulsifiers, or dispersants should not be used to increase chemical solubility except in instances where they might be formulated with the test chemical for its normal commercial purposes. If used, an additional control solution should be prepared containing the same concentration of solubilizing agent as in the most concentrated solution of the test chemical. Such agents should be used sparingly, and should not exceed 0.1 mL/L in any test solution. If solvents are used, the following are preferred: dimethyl formamide, triethylene glycol, methanol, ethanol, and acetone (U.S. EPA, 1985b).

5.4 Control/Dilution Water

Control/dilution water may be natural groundwater, surface water, reconstituted water, dechlorinated municipal water (as a last choice, if necessary; see Section 3.4), or a particular sample of receiving water if there is special interest in a local situation. The choice of control/dilution water depends on the intent of the test.[37]

If a high degree of standardization is required (e.g., the measured toxicity of a chemical is to be 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

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[37] Volume requirements, based on which test option (E, EA, or EAF) or type (static-renewal or flow-through) is being used, might also have a bearing on the choice of control/dilution water (see also Section 6.1 and its footnotes).

for all dilutions and as the control water[38] (U.S. EPA, 1985b; Environment Canada, 1990b).

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.[39] Examples of such situations include appraisals of the toxic effect of chemical spills (real or potential) or intentional chemical applications (e.g., spraying of a pesticide) on a particular waterbody. The laboratory supply of natural water may also be used for this purpose, especially where the collection and use of receiving water is impractical. Normal laboratory water is also appropriate for use in other instances (e.g., preliminary or intra-laboratory assessment of chemical toxicity).

5.5 Test Observations and Measurements

In addition to the observations on toxicity described in Section 4.4, there are certain additional observations and measurements to be made during tests with chemicals.

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[38] Because the pH, hardness, and other characteristics of the dilution water can markedly influence the toxicity of the test substance, use of a standard reconstituted water provides results that can be compared in a meaningful way with results from other laboratories. While it is desirable to test the embryos, alevins, and fry in reconstituted water, that is seldom feasible because of the need to make up large volumes of water. However, in some laboratories, this might be feasible for static-renewal tests; therefore, if possible, soft reconstituted water is recommended for this purpose. Soft, reconstituted water is prepared by adding the following quantities of reagent-grade salts to carbon-filtered, deionized water or glass-distilled water (ASTM, 1991b):

	salt	mg/L
Sodium bicarbonate	$NaHCO_3$	48
Calcium sulphate	$CaSO_4 \cdot 2H_2O$	30
Magnesium sulphate	$MgSO_4$	30
Potassium chloride	KCl	2

The reconstituted water should be aged several days (U.S. EPA, 1985b) and aerated intensely before use. It can be expected to have a total hardness of 40 to 48 mg/L and a pH of 7.4 ± 0.2.

[39] Contaminants already in the receiving water might add toxicity to that of the chemical or wastewater being tested. In such cases, uncontaminated dilution water (natural, reconstituted, 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 at the site of interest.

If the intent of the test is to determine the effect of a specific chemical on a specific receiving water, it does not matter if that receiving water modifies sample toxicity by the presence of additional toxicants, or conversely by the presence of substances that reduce toxic effects, such as humic acids. In the case of toxicity added by the receiving water, it is appropriate to include a second control using laboratory water as a minimum and, as a maximum, another series of concentrations using laboratory water as diluent.

While it is desirable to test embryos, alevins, and fry with receiving water for the control and for dilution, that is seldom feasible because of the need to transport large volumes of water. Transport of receiving water to the laboratory might be possible for the E test, and it might be feasible to use receiving water for the EA or EAF tests if they were done adjacent to the site of interest.

An alternative (compromise) to using receiving water as control/dilution water is to adjust the pH and hardness of the laboratory water supply (or reconstituted water) to that of the receiving water. Depending on the situation, the adjustment (using reagent-grade salts; ASTM, 1991b; U.S. EPA, 1985b) might be to seasonal means, or to values measured in the receiving water at a particular time.

During preparation of solutions and at each of the prescribed observation periods during the test, each solution should be examined for evidence of chemical presence and change (e.g., odour, colour, opacity, precipitation, or flocculation). Any observations should be recorded.

It is desirable and recommended that test solutions be analyzed to determine the concentrations of chemicals to which embryos, alevins, and fry are exposed.[40] If chemicals are to be measured, sample aliquots should be taken from all replicates in at least the high, medium, and low concentrations, and the control(s). Separate analyses of these sample aliquots should be performed with samples preferably taken immediately before the start of initial exposure, and at weekly intervals thereafter until the test is completed. On days when sample aliquots are collected, samples should be taken at the beginning and end of the renewal periods in static-renewal tests; and twice/day, not less than 8 hours apart, in flow-through tests. All aliquots should be collected, 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 toxicity of the chemical should be re-evaluated by a test in which solutions are renewed more frequently, using either the static-renewal or flow-through mode. If there is rapid disappearance or decline of toxicant, it might be possible to maintain stable concentrations of chemical in solution (perhaps decreased, but stable) using a flow-through test (McKim, 1985).

Toxicity results for any tests 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 the calculations, each test solution should be characterized by the geometric average measured concentration to which fish were exposed.

5.6   Test Endpoints and Calculations

The statistical endpoint for tests performed with chemicals will usually be the ICp and/or NOEC/LOEC for mortality, weight, or another of the primary endpoints described 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 20%.

When a solvent or other chemical is used to maintain the solubility of the test substance in solution, data for the solvent control should not be pooled with those for the control/dilution water, as permitted in TOXSTAT, since this can bias endpoint calculations. Related data must be compared by Student's t-test or other appropriate statistic, to ensure that no statistically significant, solvent-related effects exist. If there are no effects, then the solvent control is used as the control for other calculations.

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[40]   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 (U.S. EPA, 1985b): the test solutions are aerated; the test substance is volatile, insoluble, precipitates out of solution, or is known to sorb to the material(s) from which the test vessels are constructed; or a flow-through system is used. Some situations (e.g., testing of pesticides for purposes of registration) might require the measurement of chemical concentrations in test solutions.

If there are significant solvent-related effects, then the test is invalid. Pooling of the controls is inappropriate since the control/dilution water is lacking an influence that the other treatment groups have experienced (i.e., the solvent).

Section 6

Specific Procedures for Testing Samples of Effluent, Elutriate, and Leachate

Particular instructions for testing samples of effluent, elutriate, and leachate, in addition to the procedures listed in Section 4, are given in this section.

6.1 Test Options

Routine (e.g., quarterly) tests with effluent samples, performed for monitoring and compliance with regulatory requirements, would normally be rainbow trout E tests (Subsection 4.3.1). Depending on objectives and regulatory requirements, early life-stage tests with samples of elutriate or leachate could also be undertaken using the E test procedure. However, before the E test is adopted for routine use, comparative assessment of this abbreviated test with the more comprehensive EA or EAF tests is recommended to quantify differences in

sensitivity. Depending on the nature of the sample, volume requirements, objectives, etc., any of the three test options might be conducted as either static-renewal or continuous-flow assays. The requirements for volume of wastewater sample should be given serious consideration before undertaking any program.[41]

Samples of effluent, leachate, or elutriate are normally not filtered or agitated during the test. However, the presence of suspended or settleable inorganic or organic solids in a sample can impair the development of embryos, alevins, or swim-up fry, and can cause stress responses, decreased growth, or other sublethal effects in fry and older life stages at concentrations ≤ 100 mg/L (Noggle, 1978; McLeay *et al.*, 1987; Servizi and Martens, 1987). High concentrations of

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[41] The requirements for amount of sample will be approximately equal for static-renewal or flow-through tests but will differ considerably depending on which test option is selected. Some hypothetical examples can be given for testing with rainbow trout. For an E test, we might assume medium-sized rainbow trout eggs each weigh approximately 75 mg. With 40 of these in a replicate, the weight would be approximately 3 g, requiring 6 L/d for each replicate, or 18 L/d for three replicates. Five concentrations in a normal series including full strength would require twice as much test substance as for the 100% concentration alone, and thus the test would require 36 L of wastewater per day.

For an EA test, medium-sized rainbow trout alevin of 130 mg might be assumed (Table 1). With the same 100 individuals, 3 replicates, and 5 concentrations, the sample requirement would be 156 L/d, for either a static-renewal or flow-throught test. For an EAF test, swim-up rainbow trout fry of 100 to 200 mg (Table 1) might grow to 500 mg by the end of the test. To allow for that weight, and assuming 30 swim-ups per replicate are maintained and fed for 30 days, calculations indicate a wastewater requirement of 180 L/d at the end of the test.

Appreciable savings in the required volumes of wastewater could be achieved in the EA and EAF tests by starting with the lower daily volumes of testwater required at first, and increasing in phases, the daily volumes delivered to the aquaria, as required by the actual biomass contained in the aquaria. That procedure may be used as long as replicate groups are kept intact and are not mixed with other replicates.

Given the sample volume requirements of the EA and EAF tests, it might be preferable to undertake such tests on-site, at the source of the effluent, elutriate, or leachate, using a mobile laboratory.

biological solids in certain types of treated effluent can also contribute to sample toxicity due to ammonia and/or nitrite production (Servizi and Gordon, 1986). An additional test should be conducted simultaneously if there is concern about elevated concentrations of suspended or settleable solids in samples of effluent, elutriate, or leachate contributing to toxicity, and if the intent of the study is to quantify the degree to which sample solids contribute to toxicity. The second test should use a portion of the sample, treated by filtering or decanting to remove solids, but procedures should be otherwise identical.

6.2 Sample Collection, Labelling, Transport, and Storage

Containers for transportation and storage of samples of effluent, leachate, or elutriate must be made of nontoxic material. Glass or Teflon™-coated containers are preferred as they are inert and reduce sorption of chemicals. Polyethylene or polypropylene containers manufactured for transporting drinking water are less desirable, but may also be used. The containers must either be new or thoroughly 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.

Some tests with effluent, elutriate, or leachate will be performed "off-site" in a controlled laboratory facility. Fresh samples of effluent or leachate for off-site testing should be collected on at least three occasions each week, at intervals of two to three days, throughout the test period. Testing of effluent and leachate samples should commence as soon as possible after collection. Whenever possible, testing should begin within 24 hours of sampling,

and should commence no later than 72 hours after sampling. Each sample should be used initially within 24 hours of receipt in the laboratory, and replaced with each subsequent consecutive sample within 24 hours of its arrival. If effluents or leachates are tested in on-site laboratories, samples should be collected daily and used within 24 hours (U.S. EPA, 1989).

Samples of sediment or other solid substance collected for aqueous extraction and subsequent testing of the elutriate should be extracted and tested as soon as possible following collection, and no later than ten days following receipt in the laboratory. For the derived elutriates, aliquots of the prepared sample should be used on the same schedule as indicated for samples of effluent or leachate, if possible. Storage of elutriate samples for >24 hours is undesirable because the toxicity of the sample might not be stable. Elutriate tests should commence within 72 hours of sample preparation or as specified in a regulation or prescribed method.

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. In addition, samples arriving in partially filled containers should not be routinely tested, because 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, although records should indicate whether sample containers were only partially filled.

All samples of effluent or leachate should be kept cool (1 to 7°C, preferably 4 ± 2°C) throughout transport. Upon collection,

warm (>7°C) samples should be cooled to 1 to 7°C using ice, frozen gel packs, or refrigeration. As necessary, gel packs or other means of refrigeration should be used to assure that sample temperature remains within 1 to 7°C during transit. Samples must not freeze during transport.

Upon arrival at the laboratory, an aliquot of effluent or leachate required at that time may be adjusted immediately or overnight to 8 to 15°C, depending on the life stage and/or species used in the test. The remaining portion(s) of sample required for subsequent solution renewals should be stored in darkness in sealed container(s) at $4 \pm 2°C$. For elutriates, temperature and storage conditions should be as indicated for effluents and leachates.

6.3 Preparing Test Solutions

Samples in the collection containers must be agitated thoroughly just before pouring to ensure the re-suspension of settleable solids. Subsamples (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 subsample containers and stored (Section 6.2) until used.

Samples that might contain small organisms that could have detrimental effects on embryos or alevins might be filtered through a coarse (e.g., 25-μm mesh) plankton net before use. Such filtration could remove suspended solids that are characteristic of the sample and might otherwise contribute part of the toxicity or modify the toxicity. If there is such a concern, samples should be tested without filtration.

It is recommended that the acute toxicity (i.e., 96-h LC_{50}, or 96-h test for survival in 100% sample) of each sample of effluent, elutriate, or leachate collected from a single source and used in the ongoing EA or EAF test be measured routinely upon receipt, using Environment Canada's (1990b) acute lethality test with rainbow trout fry or fingerlings. Monitoring each sample for acute lethality will detect atypical variations in toxicity due to unusual chemical spills, in-plant process changes, sudden poor performance of an effluent treatment plant, temporal environmental changes (if leachate), or other incidents. Information from concurrent acute toxicity tests will be useful in interpreting time-related toxic effects that occur during the EA or EAF tests.

6.4 Control/Dilution Water

Tests with samples of effluent or leachate, intended to assess compliance with regulations, should use as the control/dilution water, either the laboratory water, or a sample of the receiving water. Because results could be different for the two sources of water, the objectives of the test must be decided before a choice is made. Given the volume requirements, the use of receiving water for dilutions and as control water is largely impractical for routine off-site tests.

The use of receiving water as the control/dilution water might be desirable in certain instances for on-site tests, if site-specific information is required for the potential toxic effect of an effluent, leachate, or elutriate on a particular receiving water. An important example of such a situation would be testing for sublethal effect at the edge of a mixing zone, under site-specific regulatory requirements. Conditions for the collection, transport, and storage of such

receiving-water samples should be as described in Section 6.2.

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 normally used for rearing and testing fish. Endpoints representing weight, mortality rates, and incidence of deformities of fish in the laboratory control water should be compared to that in the sample of receiving water (Section 4.5).

Tests requiring a high degree of standardization may be undertaken using reconstituted water as the control/dilution water. While in some instances it might be desirable to test the embryos, alevins, and fry in reconstituted water, that is seldom feasible because of the need to make up large volumes of water. However, in some laboratories, it might prove both desirable and feasible, in which case the use of soft reconstituted water (hardness 40 to 48 mg/L as $CaCO_3$, pH 7.2 to 7.5) is recommended for this purpose (see Section 5.4). For example, the use of soft reconstituted water would be worthwhile if it were desirable to minimize any modifying influence due to (differing) dilution-water chemistry. Such situations might include 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 was variable.

6.5 Test Observations and Measurements

Observations of the number of dead (E, EA, and EAF tests) and deformed individuals (EA and EAF tests) in each replicate must be recorded daily, and the weight of each

surviving fry in each replicate recorded at the end of the EAF test (see Sections 4.4 and 4.5). Observations on the number hatched (EA and EAF tests) and the number exhibiting swim-up behaviour (EAF test) in each replicate should also be recorded daily, as should abnormal behaviour.

Colour, turbidity, odour, and homogeneity (i.e., presence of floatable material or settleable solids) of the sample of effluent, leachate, or elutriate should be observed at the time of preparing solutions. Precipitation, flocculation, colour change, odour, 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 one or more test aquaria, the embryos and alevins should be inspected by briefly lifting the incubation unit out of each solution. If necessary, the incubation unit could be moved briefly to a container of clear control/dilution water while observations were made on mortality and aberrant appearance or behaviour. All replicates, including controls, must be treated identically if incubation units are moved briefly for inspection of developing fish.

For effluent samples with appreciable solids content, it is desirable to measure total suspended and settleable solids (APHA et al., 1989) upon receipt, as part of the overall description of the effluent, and as sample characteristics that might influence the results of the toxicity test. Additional worthwhile measurements that help characterize each sample of effluent, leachate, or elutriate should also be made. These could include pH, conductivity,

hardness, alkalinity, colour, chemical oxygen demand, biochemical oxygen demand, dissolved oxygen, and concentrations of specific toxic contaminants (e.g., resin and fatty acids, chlorophenolic compounds, dissolved metals, chlorine, chloramine, ammonia) common to the wastewater.

6.6 Test Endpoints and Calculations

Tests for monitoring and compliance with regulatory requirements should normally include, as a minimum, three or more undiluted portions of the sample, and three or more replicate control solutions. Depending on regulatory requirements, tests for compliance might use a single concentration (100% wastewater unless otherwise specified), or might determine the ICp and/or NOEC/LOEC (see Section 4.5).

Biological endpoints which should be considered are increased mortality (E, EA, and EAF tests); increased incidence of deformities (EA and EAF tests); and adverse effect on growth as judged by attained dry weight (EAF test). For each endpoint, the effect is assessed by comparison with the

controls. The most sensitive effect is taken as the definitive indication of toxicity. Increased mortality is the only biological endpoint measured in the E test.

Toxicity tests conducted for other purposes (e.g., determination of in-plant sources of toxicity, treatment effectiveness, effects of process changes on toxicity) might, 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 measurable toxicity or as a method for screening a large number of samples for relative toxicity. Endpoints for these tests would again depend on the objectives of the undertaking, but could include arbitrary "pass" or "fail" ratings, or percent mortality of embryos, alevins, and/or fry at a suitable time period. Items in Section 4.5 provide instructions that are relevant here, on statistical analysis and reporting of results from a set of tests on different samples, each tested at only one concentration.

Specific Procedures for Testing Receiving-water Samples

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

7.1 Test Options

Routine tests with samples of receiving water, if performed for monitoring and compliance with regulatory requirements, would normally be performed as embryo (E) tests (Subsection 4.3.1). More definitive tests with samples of receiving water should include feeding of fry, and should therefore be performed as embryo/alevin/fry (EAF) tests. Comparisons of several samples for the effects on multiple life stages of salmonid fish could be performed using the embryo/alevin (EA) test. Before routine use of the E test, initial comparison of the sensitivity of the E test with the more comprehensive EA or EAF test is recommended to confirm that the results of the E test will be sufficiently protective for the purpose intended. Depending on the nature of the contaminant in the sample, sample-volume requirements, objectives, etc., E, EA, or EAF tests with samples of receiving water may be conducted as either static-renewal or continuous-flow assays.[42]

7.2 Sample Collection, Labelling, Transport, and Storage

Procedures for the labelling, transportation, and storage of samples should be as described in Section 6.2. Testing of samples should commence as soon as possible after collection, preferably within 24 hours, and no later than 72 hours after sampling.

7.3 Preparing Test Solutions

Samples in the collection containers should be agitated before pouring to ensure their homogeneity. Compositing of subsamples should be as described in Section 6.3.

Samples that might contain organisms which could affect developing embryos, alevins, or swim-up fry might be filtered through a coarse (e.g., 25 μm mesh) plankton net before use, as described in Section 6.3. If there is concern that such filtering might reduce toxicity, samples should be tested without filtration.

Depending on the potential for an acutely lethal receiving water, routine assessment of each sample used in an EA or EAF test for acute toxicity might or might not be warranted. Routine characterization for acute toxicity, of receiving-water samples used in an EA or EAF test, would be appropriate if the undiluted receiving water could be acutely toxic to fish at any time during the early life-stage test. In such instances, it is recommended that each sample to which fish are exposed in an EA or EAF test be monitored for acute toxicity (96-h test for fish mortalities in 100% sample, or 96-h LC_{50}) upon receipt, using Environment Canada's (1990b) acute lethality test with rainbow trout fry or fingerlings. Information from concurrent acute toxicity tests will be useful in

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[42] The requirements for volume of sample will differ with the type of test selected. Given the volumes required by the EA and EAF tests, it is recommended that they should be done at the site, using a mobile testing laboratory.

interpreting time-related toxic effects that occur during the EA or EAF tests.

7.4   Control/Dilution Water

For receiving-water samples near 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 (see Section 5.4). This control/dilution water should be collected as close as possible to the contaminant source(s) of concern, but upstream from or outside of the zone of influence. Such surface water should be filtered to remove organisms, as described in Section 6.3.

If "upstream" water is used as control/dilution water, a separate control solution should be prepared using the laboratory water that is normally supplied to the fish. Test conditions and procedures for preparing and evaluating each control solution should be identical, and as described in Sections 4.1 and 5.4.

Logistic constraints, expected toxic effects, or other site-specific practicalities might prevent or rule against the use of upstream water as the control/dilution water. In such cases, the laboratory water normally used for rearing fish should be used as control water and for all dilutions. It could be adjusted to partially simulate upstream water (see Section 5.4).

7.5   Test Observations and Measurements

Observations of sample and solution colour, turbidity, foaming, precipitation, etc. should be made as described in Section 6.5, both during preparation of solutions and

subsequently during the tests. These are in addition to the primary observations on test organisms described in Section 4.4.

Each receiving-water sample received in the laboratory should be characterized chemically. Depending on the nature of the contaminating source(s) under investigation, measurements might include such characteristics as pH, conductivity, hardness, alkalinity, colour, chemical oxygen demand, biochemical oxygen demand, and concentrations of specific toxic contaminants (e.g., resin and fatty acids, chlorophenolic compounds, dissolved metals, chlorine, chloramine, ammonia).

7.6   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. Endpoints would normally be the same items mentioned in Section 4.5.

Tests for monitoring and compliance purposes should normally include, as a minimum, three or more undiluted portions of the sample, and three or more replicate control solutions. If toxicity of receiving-water samples is likely, and information is desired concerning the degree of dilution necessary to permit normal growth and development of embryos, alevins and/or fry, a full test to determine ICp and/or NOEC/LOEC should be conducted as outlined in Sections 4.1 and 4.5, with one or more undiluted samples (100% concentration) in the series tested.

Certain sets of tests might use a series of samples such as surface waters from a number of locations, each tested at full strength only. Statistical testing and reporting of results for such tests should follow the procedures outlined in Section 4.5.

Reporting Requirements

The test report should describe the materials and methods used, as well as the results. A 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 or compliance purposes) and consistent with specifications in this report may be referred to by citation or by attachment of a general report that outlines standard laboratory practice. Where choices exist, the approach selected should be specified. The general report should convey the procedural information included in Sections 8.2 to 8.5. An individual report giving the findings should contain the information indicated in Sections 8.1, 8.6, and 8.7. Specific monitoring programs might require other selected items in the report (e.g., procedures and results for tests requiring pH adjustment or modified aeration/oxygenation). Other details pertinent to the conduct and findings of the test, which are not conveyed by the 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 Substance

- 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 substance;

- 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, percentage of container volume occupied by sample);

- identification of person(s) collecting and/or providing the sample; and

- date and time of sample collection, receipt at test facility, start and end of definitive test.

8.2 Test Organisms

- species;

- source of gametes or broodstock; and

- procedures used for transporting and/or handling gametes, and procedures for fertilization.

8.3 Test Facilities and Apparatus

- name and address of laboratory;

- name of person(s) performing the test;

- description of systems for regulating light and temperature within the test facility;

- description of incubation units and test aquaria (size, shape, type of material, design); and

- description of flow-through apparatus, if used.

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, etc.); and

- measured water quality variables (Sections 3.4; Subsections 4.3.3, 4.3.4, 4.3.5) before and/or at time of commencement of toxicity test.

8.5 Test Method

- brief mention and description (e.g., E, EA, or EAF test, static-renewal or flow-through; aerated or non-aerated) of method used if standard (i.e., as per this report);

- design and description if specialized procedure (e.g., renewal of test solutions at intervals other than daily) or modification of standard method;

- procedure used in preparing stock and/or test solutions of chemicals;

- any chemical analyses of 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 replicate treatment, and number of replicates;

- description of any thinning of embryos (manner, numbers, timing);

- manner and rate of exchange of test solutions;

- photoperiod, light source, and intensity at surface of test solutions;

- statement concerning aeration (if any, give rate, duration, manner of application) of test solutions before and during exposure of fish;

- description of any test solutions receiving pH adjustment or filtration, including procedure;

- 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;

- appearance of test solutions and changes noted during test; and

- conditions and procedures for measuring the ICp and/or NOEC for reference toxicant(s) used in the E test.

8.7 Test Results

- average number and percentage of unfertilized eggs in each treatment;

- number and percentage of dead (E, EA, and EAF tests) and deformed individuals (EA and EAF tests) in each replicate (including controls) as noted during each observation period and at the end of the test;

- weight of surviving individuals in each replicate at end of EAF test;

- the NOEC/LOEC and/or ICp for weight of swim-up fry in EAF test, for mortality of embryos (E, EA, and EAF tests), alevins (EA and EAF tests), and/or swim-up fry (EAF test), and incidence of abnormalities/deformities (EA and EAF tests); Minimum Significant Difference in average weights and weight of control fish; the statistical test(s) used, and any transformation of data that was required;

- optional observations if done: time to ≥90% hatch in each replicate (EA and EAF tests); percent hatch in each replicate upon hatching of ≥90% of the surviving control embryos (EA and EAF tests); time to ≥50% swim-up in each replicate (EAF test); percent swim-up in each replicate upon swim-up of ≥50% of the surviving control fish (EAF test); incidence of abnormal behaviour (EA and EAF tests); individual weights of a subsample of surviving fry in each replicate upon swim-up of ≥50% of the surviving fish (EAF test);

- the results of E tests with the reference toxicant(s), conducted concurrently or within the month that an E test is performed with a test substance, together with the geometric mean value (± 2 SD) for the same reference toxicant(s) as derived at the test facility in previous tests; and

- results of any acute lethality tests conducted concurrently using rainbow trout fry or fingerlings.

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*   The statistical programs of TOXSTAT and BOOTSTRP are available from the Laboratory Division at this
address, by providing a formatted computer diskette. A math co-processor is required to use BOOTSTRP.

Review of Procedural Variations for Undertaking Early Life-stage Tests Using Salmonids*

1. Test Substance and Type of Test

Document	Test Substance	Test Type	Test Duration (days)
Birge *et al.*, 1985	effluents	static-renewal	9
U.S. EPA, 1985a	chemicals	flow-through static-renewal	~ 90
Rexrode and Armitage, 1987	pesticides	flow-through	~ 60
van Aggelen, 1988	effluents receiving waters	recirculating	~ 60
ASTM, 1991a	chemicals	flow-through	~ 90
Birge and Black, 1990	cadmium effluents receiving waters	flow-through static-renewal	28
Hodson *et al.*, 1991	aromatic compounds	flow-through	85
Paine *et al.*, 1991	receiving waters	static-renewal	7 to 10
Neville, 1992	copper sulphate Na-dodecyl sulphate 2,4,5-trichlorophenol	static-renewal	12 to 15
OECD, 1992a	chemicals	flow-through static-renewal	50 to 55
OECD, 1992b	chemicals	flow-through static-renewal	~ 90

*   As specified in Canadian, Provincial, and international methodology documents. Based on reports available to the authors as of June, 1992.

2. Test Species

Document	Species	Life Stage	Age at Test End (days)
Birge *et al.*, 1985	rainbow	eggs[a]	9 (post-fertilization)
U.S. EPA, 1985a	rainbow/brook	eggs/alevins/fry[b]	60 (post-hatch)
Rexrode and Armitage, 1987	various[c]	eggs/alevins[d]	32 (post-hatch)
van Aggelen, 1988	rainbow	eyed eggs/alevins	$\leq$30 (post-hatch)
ASTM, 1991a	various[c]	eggs/alevins/fry[b]	30 (post-swim-up)
Birge and Black, 1990	rainbow	eggs/alevins[a]	4 (post-hatch)
Hodson *et al.*, 1991	rainbow	eggs/alevins/fry[e]	28 (post-swim-up)
Paine *et al.*, 1991	rainbow	alevins[f]	$\leq$12 (post-hatch)
Neville, 1992	rainbow	alevins/fry[g]	5 (post-swim-up)
OECD, 1992a	rainbow	eggs/alevins[h]	20 (post-hatch)
OECD, 1992b	rainbow	eggs/alevins/fry[h]	60 (post-hatch)

[a]   Eggs exposed within 30 minutes of fertilization.
[b]   Eggs exposed within 96 hours of fertilization.
[c]   Rainbow, brook, brown, and lake trout, coho, and chinook salmon.
[d]   The authors discuss both warm water and salmonid species, and indicate that development, survival, and growth of swim-up fry should be monitored. The test duration of approximately 60 days, however, only allows development of salmonids through the alevin stage. Fertilization may be done before exposure to the test substance, or in the test solution. The test should start with eyed eggs selected from a group of which $\geq$70% are fertilized.
[e]   Exposed from day of fertilization through to four weeks of feeding as fry.
[f]   Starting exposure within 24 to 48 hours post-hatch.
[g]   Starting exposure with 11- to 12-day-old sacfry.
[h]   Embryos should be exposed before cleavage of the blastodisc begins, or as soon as possible thereafter.

3. Test Conditions

Document	Test Volume	No./Test Vessel	No. Replicates
Birge et al., 1985	300 mL	50	4
U.S. EPA, 1985a	NI (not indicated)	60	2
Rexrode and Armitage, 1987	15- to 30-cm depth[a]	20(eggs) 30(alevins)	4(eggs) 1(alevins)
van Aggelen, 1988	180 L	100	1
ASTM, 1991a	NI[b]	30	2[c]
Birge and Black, 1990	300 mL	50	2 or 4
Hodson et al., 1991	14 L	200 to 300 eggs[d]	3
Paine et al., 1991	1 L	20	5
Neville, 1992	325 mL	1	12
OECD, 1992a	NI	≥30	≥2
OECD, 1992b	NI	30	≥2

[a] Exposure vessels can vary in size according to the species tested.

[b] Volume of vessel is based on a loading density of 0.5 g·d/L (= 2 L/g·d) for swim-up fry at the end of the test.

[c] For each concentration and control, there must be at least two true replicates in completely separate chambers, not just multiple test containers within one chamber.

[d] Later, when biomass of feeding fry approached the recommended loading rates, half of the fish were removed and discarded.

4. Test System

Document	Exposure Chamber	Test Container	Special Equipment
Birge *et al.*, 1985	deep petri dish	400-mL petri dish with mesh screens	dilution/mixing system
U.S. EPA, 1985a	glass aquaria	screen tray	NI
Rexrode and Armitage, 1987	glass aquaria	glass jar with mesh screen on bottom	oscillating rocker arm or self-starting siphons
van Aggelen, 1988	two 90-L plastic tubs	vertical incubation tray	submersible pump
ASTM, 1991a	glass aquaria	glass jar with mesh screen on bottom	oscillating rocker arm[a]
Birge and Black, 1990	deep petri dish	400-mL petri dish with mesh screens	dilution/mixing system
Hodson *et al.*, 1991	glass aquaria	kitchen sieve with nylon screen bottom	NI
Paine *et al.*, 1991	2-L glass beaker	net plus petri dish	bubble curtains
Neville, 1992	glass jar with four separate sections	glass jar with mesh screen on bottom	balance accurate to 10 µg
OECD, 1992a	glass or other inert chamber	glass or other inert vessel with mesh sides/ends	oscillating rocker arm
OECD, 1992b	glass or stainless steel chamber	glass/steel vessel with mesh sides/ends	oscillating rocker arm

[a] Alternatively, test solutions should flow directly into the cups, or the water level in test chambers should be varied by means of self-starting siphons.

5. Type of Control/Dilution Water

Document	Water Type	Hardness (mg/L)	pH	Min. DO	Renewal Period (h)
Birge et al., 1985	Re[a] or NW[a]	101[b]	7.7[b]	>60% sat.*	12 or 24 (St-Rn[c])
U.S. EPA, 1985a	NW or DW[a]	NI	NI	>90% sat.	<24 (≥6 vol./d)
Rexrode and Armitage, 1987	NW or Re	40 to 48	7.2 to 7.6	>75% sat.	12 (90%)
van Aggelen, 1988	RW equiv.[d]	RW equiv.[d]	RW equiv.[d]	>60% sat.	96 (50%)
ASTM, 1991a	NW, Re, DW	NI	NI	>60% sat.	<24 (5 to 10 vol./d)
Birge and Black, 1990	Re or NW	101[b]	7.7[b]	>60% sat.	1.5 (F-T[c]) 12 or 24 (St-Rn)
Hodson et al. 1991	DW	135	7.8 to 8.1	NI	3 to 5.5 (95%)
Paine et al., 1991	DW and NW	65	6.0 to 8.0	>60% sat.	twice/wk
Neville, 1992	DW or RW[a]	135[e]	NI	>60% sat.	twice/24
OECD, 1992a	NW, DW, Re	NI	NI	>60% sat.	24[f]
OECD, 1992b	NW, DW, Re	NI	NI	>60% sat.	24[f]

[a] DW = Dechlorinated tap water. NW = Natural water (uncontaminated, ground, or surface) Re = Reconstituted water. RW = Receiving water.
[b] Values for reconstituted water.
[c] F-T = Flow-through tests. St-Rn = Static-renewal tests.
[d] Receiving water or equivalent.
[e] Diluted as required for soft-water tests.
[f] Flow-through tests, ≥5 tank volumes per day. Static-renewal tests, ≥0.67 of volume renewed daily.

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* sat. = saturation

6. Temperature, Aeration, Dissolved Oxygen and pH Adjustment During Test

Document	Temp. (°C)	Aeration	DO of Control/Dilution Water Before Test	pH Adjustment
Birge et al., 1985	12 to 13	150 bubbles/min	near saturation	NI
U.S. EPA, 1985a	10 to 12	none	90 to 100% sat.*	NI
Rexrode and Armitage, 1987	10 ± 2	none[a]	near saturation[a]	NI
van Aggelen, 1988	10	must be used	near saturation	NI
ASTM, 1991a	10	gentle[b]	90 to 100% sat.	NI
Birge and Black, 1990	13	150 bubbles/min	near saturation	NI
Hodson et al., 1991	10, 12, 15[c]	NI	NI	NI
Paine et al., 1991	10 to 12	gentle[d]	NI	<6.0, >8.0
Neville, 1992	13.5 ± 1	none	near saturation	NI
OECD, 1992a	10 ± 2 (embryos) 12 ± 2 (larvae)	NI	NI	NI
OECD, 1992b	10 ± 2 (embryos) 12 ± 2 (larvae, juveniles)	NI	NI	NI

[a] Dilution water should be aerated vigorously so that DO is near saturation.
[b] Loss of test substance by aeration is not considered a problem because results are based on measured concentrations.
[c] Temperatures were 10°C for eggs, 12°C for yolk resorption, and 15°C for fry growth.
[d] If DO of test solution(s) <60% saturation before use, pre-aerate until 60% saturation achieved, or for a maximum of 2 hours.

* sat. = saturation

7. Lighting Conditions During Test

Document	Intensity	Type	Photoperiod	Dawn/Dusk
Birge *et al.*, 1985	dark	NI	NI	NI
U.S. EPA, 1985a	dark[a]	NI	14h L/10h D[a]	15 to 30 min[a]
Rexrode and Armitage, 1987	<216 lux[b]	NI	16h L/8h D[b]	NI
van Aggelen, 1988	dark	NI	NI	NI
ASTM, 1991a	<216 lux[c]	Incandescent	NI	15 to 30 min
Birge and Black, 1990	dark	NI	NI	NI
Hodson *et al.*, 1991	NI	NI	NI	NI
Paine *et al.*, 1991	dark	NI	NI	NI
Neville, 1992	low[d]	Fluorescent	16h L/8h D	NI
OECD, 1992a	dark[e]	NI	12 to 16h L[e]	NI
OECD, 1992b	dark[e]	NI	12 to 16h L[e]	NI

[a] Dark during egg incubation and up to one week post-hatch. After that, intensity during light part of photoperiod is 30 to 100 lumens. The dawn/dusk transition is optional.

[b] Intensity is during egg incubation. Photoperiod refers to post-hatch period.

[c] During egg incubation.

[d] ~ 30 lux

[e] Dark until one week after hatching, subdued lighting during the balance of test.

8. Feeding of Swim-up Fry

Document	Feed Type	Feeding Rate
Birge *et al.*, 1985	NA (not applicable)	NA
U.S. EPA, 1985a	starter feed or brine shrimp	3 times/day at 4-h intervals
Rexrode and Armitage, 1987	NA	NA
van Aggelen, 1988	NA	NA
ASTM, 1991a	moist starter diet or brine shrimp	>4% body weight/day[a] (portions fed 4 times/day)
Birge and Black, 1990	NA	NA
Hodson *et al.*, 1991	starter diet	NI
Paine *et al.*, 1991	NA	NA
Neville, 1992	brine shrimp	3 times/day
OECD, 1992a	NA	NA
OECD, 1992b	NI	4% body weight/day (portions fed 2 to 4 times/day)

[a]   Based on mean wet weight of controls, and dry weight of food.

9. Monitoring Water Quality During Test

Document	Variables[a]	Frequency
Birge *et al.*, 1985	T, DO[b], pH, cond, hard, alk, concn	daily
U.S. EPA,1985a	T, DO pH, cond, hard, alk, TOC	daily weekly
Rexrode and Armitage, 1987	DO, pH, cond, hard, alk, concn	weekly
van Aggelen, 1988	T, DO, pH, cond, hard, alk, NH₃, TOC, metals concn PCB, pest	monthly 96 h[c] source-dependent
ASTM, 1991a	DO, pH, cond, hard, alk, NH₃, TOC, concn, part, TDG T	weekly hourly[d]
Birge and Black, 1990	T DO[b], pH, cond, hard, alk, concn	daily
Hodson *et al.*, 1991	T, DO, pH, cond, hard, alk concn	NI daily
Paine *et al.*, 1991	T, DO, pH cond, hard	daily twice/week
Neville, 1992	T, DO, pH, cond, concn, metals, N, NH₃, NO₂, NO₃, hard	daily start/end of test[e]
OECD, 1992a	T DO, concn pH, hard	daily[f] ≥3 times/test start/end of test
OECD, 1992b	T, DO, concn pH, hard	weekly[f] start/end of test

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[a]

alk	=	total alkalinity	NO₂	=	nitrite, NO₃ = nitrate
cond	=	specific conductivity	part	=	particulate material
concn	=	concentration of test substance	PCB	=	polychlorinated biphenyls
DO	=	dissolved oxygen	pest	=	total organophosphorus pesticide
hard	=	total hardness	pH	=	hydrogen ion concentration
metals	=	selected metals	T	=	temperature
N	=	total nitrogen	TDG	=	total dissolved gases
NH₃	=	total ammonia nitrogen	TOC	=	total organic carbon

[b] If necessary in static-renewal tests, DO should be measured at the beginning and end of each renewal interval in at least one chamber for each concentration.

[c] Subsamples taken with every effluent replacement.

[d] Daily maximum and minimum temperatures must be measured. Temperature must be measured concurrently in all test chambers, if possible, near the beginning, middle, and end of the test.

[e] On the second day of exposure, and the next-to-last day, these items are measured in each concentration at the start of the 24-h period, and in each replicate at the end of the 24-h period.

[f] Temperature should preferably be measured continuously in at least one test vessel.

[g] All concentrations should be measured three times, spaced evenly over the test. In static-renewal tests, both the old and new test solutions of all concentrations should be analyzed on at least one occasion.

10. Biological Observations During Test

Document	Variables	Frequency	Endpoints
Birge *et al.*, 1985	mort.[a]	daily	mort.
U.S. EPA, 1985a	mort., def.[a], no. hatch/swim-up[b] wt.[c]	daily end of test	mort./wt.[a]
Rexrode and Armitage, 1987	mort., no. hatch, timed hatch[d]/swim-up[b] pathol./histol./clinical effects wt.[f]	daily weekly[e] end of test	mort./wt.
van Aggelen, 1988	mort., def.	daily	mort.
ASTM, 1991a	mort.[g], def. wt.[h]	daily end of test	mort./wt.
Birge and Black, 1990	mort., def.[i], timed hatch	daily	mort.
Hodson *et al.*, 1991	mort.[j], def., hatching wt. alevin body/yolk weight	daily weekly once	mort./wt.
Paine *et al.*, 1991	mort. body weight, yolk weight	daily start/end test[k]	mort./growth
Neville, 1992	mort., def. growth[f]	daily start/end test	mort./growth
OECD, 1992a	mort., def., no. hatched, timed hatch length	daily end of test	mort.
OECD, 1992b	mort., def., no. hatch, timed hatch/swim-up wt.	daily end of test	mort./wt.

[a] mort. = mortality   def. = deformities/abnormalities   wt.= weight
[b] no. hatch and no. swim-up = number hatched and number of swim-up fry
    timed hatch and swim-up = time to hatching and time to swim-up
[c] Standard length and wet weight. If apparent edema, dry weight is recommended.
[d] Determine when hatching is about 90% completed or 48 hours after first hatch by counting live young fish.
[e] At a minimum, 11, 18, 25, and 32 days after hatching.
[f] Wet weight should be obtained for all live fish. Dry weight should also be used if edema is possible.
[g] Thin at eyed egg stage. Overall survival is product of percent survivals.
[h] Wet weight; add length and dry weight if edema is possible.
[i] Deformed fish alive at the end of the test are counted as dead, in the final tabulation.
[j] To avoid bias, calculate total number of fish-days and express mortality as number per 1000 fish-days.
[k] Preserve 40 alevins at start of test. At end, preserve all alevins for 1 week, then dissect to estimate yolk conversion efficiency (YCE). Wet and dry weights of bodies and yolk sacs.

11. Statistical Endpoint for Test

Document	Endpoint(s)	Criterion
Birge et al., 1985	LC_{50}, LC_{10}, LC_1[a] NOEC, LOEC	sig. diff.* from control
U.S. EPA, 1985a	NI	sig. diff. from control by ANOVA
Rexrode and Armitage, 1987	MATC[b]	sig. or specified diff. from control[c]
van Aggelen, 1988	LT_{50}[d], LC_{50}	sig. diff. from control
ASTM, 1991a	NI	sig. or specified diff. from control[c]
Birge and Black, 1990	LC_{50}, LC_{10}, LC_1[a] NOEC, LOEC	sig. diff. from control
Hodson et al., 1991	IC25, NOEC, LOEC	sig. diff. from control
Paine et al., 1991	Yolk conversion efficiency	compared to control
Neville, 1992	NOEC, LOEC	sig. diff. from control[e]
OECD, 1992a	NOEC, LOEC	sig. diff. from control[f]
OECD, 1992b	NOEC, LOEC	sig. diff. from control[f]

[a] The concentration of a substance in water that is estimated to kill 50%, 10%, and 1% of the test fish, respectively, after a fixed period of exposure.

[b] Maximum acceptable toxic concentration (i.e., the TEC), for quantitative data (length, weight) by ANOVA and multiple comparison test; for quantal data (e.g., no. of fish hatching) using 2 x 2 contingency table.

[c] Deciding on differences solely on the basis of statistically significant difference from controls might depend largely on sample sizes and variability within replicates. An alternative endpoint can be a specified magnitude of difference from the control in some biological attribute.

[d] Median lethal time, the period of exposure estimated to cause 50% mortality in a group of fish held at a particular concentration.

[e] Growth based on individual percent gain in wet weight, using 0% for any mortality that occurred.

[f] One-way ANOVA and multiple comparison can be used in a test without replicate chambers, but it should be shown that chamber-to-chamber variability is acceptably low.

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* Sig. diff. = significantly different

12. Validity of Test

Document	Test Substance, Variation in Conc.	Temperature Variation (°C)	Maximum Control Mortality	Variation in Control Weight
Birge *et al.*, 1985	NI	± 1	≤20%	NA
U.S. EPA, 1985a	≤20%[a]	± 1.5[b]	≤20%, ≤30%[c]	CV ≤40%[d]
Rexrode and Armitage, 1987	NI	≤2	20%	CV ≤40%[d]
van Aggelen, 1988	NI	NI	NI	NI
ASTM, 1991a	≤30%; ≥50%[e]	≤1, 2, or 3[f]	30%[g]	NI
Birge and Black, 1990	NI	NI	≤20%	NI
Hodson *et al.*, 1991	NI	<1	≤20%	CV 28%
Paine *et al.*, 1991	NI	NI	≤20%	NI
Neville, 1992	NI	≤1	<10%	≤15%
OECD, 1992a	± 20% of mean	± 1.5[h]	≤30%[i]	NI
OECD, 1992b	± 20% of mean	± 1.5[h]	≤30%[i]	NI

[a]   Concentration of toxicant should not be more than 20% lower than the mean measured concentration.

[b]   Test temperatures should remain within 1°C of the selected temperature.

[c]   Average mortality of control fish must be ≤20%; mortality in any single control group must be ≤30%.

[d]   Maximum coefficient of variation (CV = 100 times standard deviation divided by the mean) for weights of fish that were alive at the end of the test in any control chamber.

[e]   Unacceptable if measured concentration in any treatment >30% higher than time-weighted average concentration for more than 5% of test duration, or if measured concentration in any treatment <50% of time-weighted average concentration measured in any treatment for >10% of test duration.

[f]   Difference between time-weighted average measurements for any two test chambers ≤1°C. At any one time, difference between any two test chambers ≤2°C. Any individual measurement ≤3°C different from overall mean of time-weighted average temperatures for individual chambers.

[g]   From thinning of embryos to termination of test.

[h]   Difference <1.0°C between test chambers or between successive days.

[i]   Post-hatch. Maximum mortality of control embryos should be 34% to time of hatch.

Distribution, Life History, and Husbandry of Test Species*

Atlantic Salmon *(Salmo salar)*

Distribution. The range of the Atlantic salmon includes most of the North Atlantic Ocean and many of its accessible rivers and streams. In the eastern Atlantic, it is found from above the Arctic Circle, south to Portugal, including the Scandinavian countries, the White Sea area of northwestern Russia, the Baltic Sea, and in Britain and Ireland. It is also found in waters off Iceland and southern Greenland.

In the northwest Atlantic, this species of salmon ranges from Ungava Bay in northern Quebec, to a few rivers in the northeastern United States. Distribution in Canada includes many rivers and streams in New Brunswick, Nova Scotia, Newfoundland, Labrador, Quebec, and a few streams in Prince Edward Island. Surviving natural populations in the United States are confined to a small number of streams in Maine, although one salmon run has been re-established in the Connecticut River.

Life History. Some Atlantic salmon return to fresh water shortly before spawning, and some enter months beforehand. The normal spawning period is from October to January, although some races spawn in February and March in warmer, southern waters. Adult Atlantic salmon weigh between 4 to 6 kg and normally return after two years at sea. Maturing fish that return after only one year at sea are known as "grilse", and typically weigh between 1.5 to 3.5 kg. A small proportion will remain at sea for three years

and these fish weigh 8 to 14 kg. Up to 30% (typically, 10%) survive to spawn again. Spent spawners returning downstream are known as "kelts". Egg production varies directly with fish size, averaging 1500 to 1800 eggs/kg female weight (Farmer *et al.*, 1990).

Eggs are 5.5 to 6.8 mm in diameter when laid in gravel nests (redds). Young fish remain in fresh water from one to three years, until they reach a length of about 12 to 15 cm. The time of smolting depends on the temperature and the length of the summer feeding period. While in fresh water, developing Atlantic salmon feed mainly on insect larvae and zooplankton.

Ocean migrations can be extensive, but Atlantic salmon prefer the colder waters along the Arctic ice-pack and the Labrador and Barents Seas (2 to 9°C). During their marine life, Atlantic salmon initially feed on crustaceans (amphipods and euphausiids), gradually switching to a piscivorous diet (herring, capelin, and sand eels).

Coho Salmon *(Oncorhynchus kisutch)*

Distribution. Coho salmon populate the smaller rivers from Baja California to Norton Sound in Alaska, with the largest populations occurring between the Columbia River in Oregon and Cook Inlet in Alaska. Coho are found in the Shuswap system of the Fraser River and Babine Lake flowing into the Skeena River. On the Asian coast, coho have been found from near the Arctic

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* Based primarily on information from Gordon *et al.*, 1987.

Circle to northern Japan and Korea. Coho have been transplanted successfully into the Great Lakes.

Life History.   Coho enter their spawning rivers shortly before mating in October and November. However, some populations of coho wait until December or January to spawn. Their average size at spawning is 2.7 to 5.4 kg. Most are three years old when they return, but "jacks" returning after one year at sea are relatively common (about 4% of the returning population). The river migration can be short or fairly long. The average fecundity is 2100 to 2800 eggs/ female.

Fertilized eggs, 5.8 to 7.5 mm in diameter, are buried in redds within the gravel, where they incubate over the winter. Coho fry typically emerge from mid-March to late June. They remain in fresh water for one to two years before migrating to sea, feeding primarily on zooplankton, aquatic insect larvae, and terrestrial insects. The-two year residence time for developing coho is typical of northern rivers and other cold, unproductive rivers such as the Pitt or Coldwater Rivers of B.C.

After smolting from mid-April to mid-June, coho migrate to sea. Most coho frequent coastal and estuarine waters for some time (within 40 km of shore), but they can also migrate as far as 1600 km into the Pacific Ocean. During their first year at sea, the bulk of their diet is made up of crustaceans (copepods, amphipods, euphausiids, and larvae of barnacles and crabs). Later, they become almost totally piscivorous, feeding on high-fat shoal fish such as herring and eulachons, but also on rockfish and kelp greenlings.

Rainbow Trout *(Oncorhynchus mykiss)*

Distribution.   Rainbow trout are native to western North America, and are found from Baja California to Alaska. However, the largest numbers of fish are found from northern California into northern B.C., particularly in larger rivers and their tributaries, as well as lakes and streams. Rainbow trout have been introduced successfully around the world, and now frequent waters of all Canadian provinces as a result of intentional or unintentional releases. Populations spend their entire life in fresh water, although they can also frequent estuarine waters as juveniles or adults, and subspecies (i.e., steelhead) on both coasts of Canada run to sea and return to streams for spawning. In Canada and elsewhere, these trout are widely reared in hatcheries for stocking natural waters to support sports fishing. Rainbow trout is among the most common species used in commercial aquaculture and one of the standard species used worldwide for aquatic toxicity tests, particularly in Canada.

Life History.   Rainbow spawn from late winter through the spring. Spawning fish are usually three to four years of age and weigh 1.5 to 4 kg, but repeat spawners can be considerably older and larger in size. Eggs 3.0 to 5.0 mm in diameter are laid in gravel redds, and emerge as swim-up fry in late May or June. Juvenile fish usually feed on insect larvae and zooplankton. Adult fish are known to feed on insects, crustaceans, and other fish.

Husbandry Techniques for Test Species. *Stripping of Broodstock.*   Practical considerations might dictate that gametes for the toxicity test should be obtained from broodstock held and spawned at the test facility. If this approach is being considered,

there are several important factors to take into account. Some of the more fundamental aspects of stripping broodstock fish are described here, but more detailed information on specific procedures should be thoroughly studied before undertaking this approach.

Broodstock are normally sorted to separate males from females and ripe individuals from sexually immature ones. It is straightforward to separate the sexes and determine ripe males, but selecting ripe females for stripping takes experience and practice. If female ripeness is not checked at frequent intervals, there is a high risk of acquiring infertile eggs. Maximum fertility of eggs is achieved within a three- to four-day period, between 4 to 8 days post-ovulation. A period of 8 days or less is optimal for fertilization. Allowing the eggs to over-ripen affects survival adversely, not only at fertilization, but also at the eyed stage through to swim-up fry.

Careful handling of the fish while checking for ripeness is essential; they are easily damaged internally, and broken eggs result in infertility. External signs of ripeness include a soft, enlarged abdomen, swollen and red urogenital papilla protruding from the vent, and spontaneous flow of eggs from the vent. Extruded eggs can be checked for ripeness by clearing them and examining the position of the germinal vesicle and lipid droplets in the yolk. Proper handling of broodstock and checking ripeness requires experienced personnel.

Stripping can be carried out with one or two people, depending on their experience. One holds the fish while the other performs the stripping. Eggs can be removed from the females by various methods, depending on whether the female is to be killed (typical for coho salmon) or anaesthetized (an option for Atlantic salmon or rainbow trout). A mature male can be stripped more than once. If he is to be stripped again, a period of one week should lapse between stripping sessions, otherwise milt quality might be compromised.

Handling of Gametes. The procedure detailed in this report requires the fertilization of eggs just before the start of the test. This necessitates the coordinated and timely procurement and handling of unfertilized eggs and milt. Although gametes can be obtained from sexually mature broodstock held at the laboratory, it is frequently easier and less costly to obtain them from a hatchery, and transport the milt and unfertilized eggs to the test facility (see Section 2.2). Provided that care is taken and conditions are optimal, both milt and unfertilized eggs can be transported and stored for a few days before fertilization.

Milt, if handled and stored properly, normally maintains 70 to 90% fertility for at least five days. Milt from rainbow trout or Atlantic salmon seems to be more amenable to storage than that from Pacific salmon. The sperm in freshly collected milt remains immotile in the seminal fluid, due to the fluid's potassium content. Subsequent quality of sperm is affected during transportation and storage by temperature, depth of milt in container, sterility of the container, and humidity. Lower temperatures (ideally, 0 to 4°C) allow longer storage of sperm. Even if shipped and stored cold, however, more stored sperm are required to fertilize a batch of eggs than if there were no delay in fertilization. Keeping the depth of milt in the container at a minimum (<6 mm) is important to ensure that the sperm receive adequate oxygen. Flushing the milt with oxygen is also desirable. The use of moisture-saturated oxygen or air can significantly increase

storage time, since it helps to prevent drying of the gametes.

Unfertilized eggs can be transported and stored in much the same way as milt. Eggs should be collected as soon after ovulation as possible, since a decreased storage ability occurs as eggs ripen. Eggs should be shipped and stored chilled (0 to 3°C), no more than four layers thick, in insulated containers designed to minimize breakage. Unfertilized eggs, if handled in this manner, should retain normal fertilization rates for about three days. To maximize fertilization, stored eggs should be fertilized with fresh milt.

Fertilization.   Fertilization can take place with or without water. However, fertilization with water requires precise timing since water-activated sperm are only motile for 90 seconds and the micropyle for the egg closes in 3 minutes. Therefore, the dry method of fertilization is recommended. In this method, one to three females are spawned into a dry, clean bucket or plastic tray. The milt, spawned into a plastic or glass container, is then added. It is preferable to fertilize eggs with milt from more than one male, to improve the success of fertilization. Upon addition of milt, the gametes are gently mixed by hand using new, clean surgical gloves. Gametes are left undisturbed for 2 to 4 minutes to allow fertilization, then aerated water is added by running it gently down the side of the container. The eggs are stirred, and the water is poured off to close the micropyle and to remove excess milt and other debris which can serve as substrate for fungal growth. The washing is repeated 2 to 3 times. Thereafter, the freshly fertilized eggs are placed gently and quickly (within 20 min) into incubation units, and the test commenced (see Section 4.2). Eggs should be kept submersed in control/dilution water

during the transfer process, and should not be poured into the incubation units. The entire fertilization process should take place under low levels of lighting.

Incubation and Development of Embryos.
Table D.1 provides guidance on the optimal and lethal temperatures for embryos of the three species of test fish. Water temperature is the major variable governing development of the embryos, and can be used to predict the time when the various stages of development are reached. Values vary between species and even between races of the same species. Table D.2 gives predicted incubation periods for the different species to achieve 50% hatch.

Embryos are especially sensitive to mechanical shock (physical agitation) at certain developmental stages. Embryos cannot be handled, stirred, poured, or transported without significant mortality during these sensitive stages. Sensitivity to mechanical shock has been found to occur at three stages of embryonic development, each successive stage being more sensitive. The first occurs 10 to 45 minutes after the immersion of embryos in water following fertilization. During this time, fusion of the male and female chromosomes takes place. The second stage occurs 2 to 72 hours after the embryos are immersed, at which time the cells are undergoing rapid division. The third and most sensitive stage occurs 4 to 14 days post-fertilization, when the embryo is undergoing rapid cellular differentiation. Sensitivity to mechanical shock decreases thereafter and is no longer detectable at and after the eyed stage is reached.

Since the embryonic development rate depends on temperature and species, the changes in sensitivity will vary for different incubation conditions and species. However, to minimize losses, any handling of embryos

should be completed within 24 hours of immersing the embryos in the test solutions. Although the embryos are sensitive during this time, they are not overly so. Embryos should not be handled at all throughout the period from 24 hours post-fertilization until the eyed stage is reached.

Table D.1   Water Temperatures Affecting Development and Survival of Embryos of Test Species [a]

Species	Lower Limit [b] (°C)	Upper Limit [b] (°C)	Optimum Temperature (°C)
Atlantic salmon	4.0	16.0	8.0 to 10.0
Coho salmon	1.6 to 3.0	13.0 to 13.5	5.0 to 8.0
Rainbow trout	0.5 to 2.25	14.6	10.0 to 12.0

[a]   From Gordon et al., 1987.

[b]   The limit is defined as the temperature causing 50% mortality of the developing embryos, during the incubation period from fertilization to 50% hatch of the survivors.

Table D.2   Predicted Incubation Periods at Constant Temperatures for Test Species

Temperature (°C)	Days from Fertilization to 50% Hatch		
	Atlantic salmon [a]	Coho salmon [b]	Rainbow trout [a]
1	—	—	182
2	—	—	138
3	—	—	107
4	117	120	86
5	98	96	71
6	84	80	59
7	72	69	50
8	62	60	43
9	54	53	37
10	48	48	32
12	38	40	25
14	—	—	20

[a]   From Gordon et al., 1987.

[b]   Based on the observation of personnel at a hatchery of Fisheries and Oceans Canada that coho salmon generally hatch at 480 accumulated thermal units (ATUs) (Schubert, 1992).

Logarithmic Series of Concentrations Suitable for 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 Rochinni *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 can 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 by 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.