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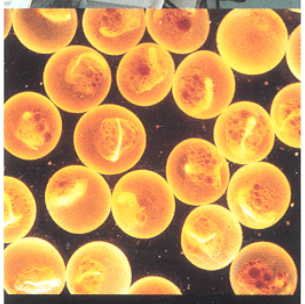

ENVIRONMENTAL
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# Biological Test Method: Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout)

Environment Canada Environnement Canada

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# Biological Test Method:
# Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout)

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## Abstract

*Revised procedures for toxicity tests using salmonid (i.e., rainbow trout) embryos, alevins, and swim-up fry, are recommended by Environment Canada in this report. Three test options are described: an embryo (*E*) test for frequent or routine monitoring; an embryo/alevin (*EA*) test for measuring effects on multiple phases of development; and an embryo/alevin/fry (*EAF*) test for more definitive investigations. All three test options start at 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 seven days after half of the alevins are seen to have hatched in the control. The embryo/alevin/fry test ends after 30 days of feeding swim-up fry. Selection of the most suitable test option 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.*

*Rainbow trout (*Oncorhynchus mykiss*) must be used when performing each of these three test options. Procedures are given for spawning broodstock, handling gametes, and fertilizing eggs of rainbow trout before starting the test, as well as for incubating embryos and alevins and feeding swim-up fry during the tests which include these life stages. General or universal conditions and procedures are outlined for testing a variety of substances for their effects on the early life stages of rainbow trout. Additional conditions and procedures are stipulated, which are specific for testing samples of chemical, effluent, elutriate, leachate, or receiving water. 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, endpoints, methods of calculation, and validation.*

## Résumé

*Dans le présent rapport, Environnement Canada recommande des méthodes révisées pour les essais de toxicologiques employant des embryons, des alevins et des jeunes de salmonidés (c'est-à-dire de la truite arc-en-ciel). On y décrit trois variantes d'essais : un essai (dit E), employant des embryons, destiné aux programmes de surveillance fréquente ou systématique ; un essai (dit EA), employant des embryons et des alevins, pour mesurer les effets sur divers stades du développement ; un essai (dit EAT), employant des embryons, des alevins et des truitelles pour obtenir des résultats plus concluants. Les trois débutent au commencement du stade de l'embryon et mesurent le développement et la survie aux premiers stades du cycle biologique. L'essai E se termine sept jours après la fécondation ; l'essai EA, sept jours après l'éclosion de la moitié des alevins du groupe témoin ; l'essai EAT, 30 jours après le début du stade de la truitelle. Le choix de l'option dépend des objectifs de l'essai et des caractéristiques physico-chimiques de la substance d'essai. Comme, aux premiers stades de leur existence, les poissons sont généralement très sensibles, on devrait considérer ces essais comme des dosages biologiques puissants et significatifs.*

*Pour les trois options, il faut utiliser la truite arc-en-ciel (*Oncorhynchus mykiss*). Le rapport renferme des instructions sur les géniteurs, la manipulation des gamètes et la fécondation des œufs avant le début des essais ainsi que sur l'incubation des embryons et des alevins et l'alimentation des truitelles, le cas échéant, pendant les essais. On expose les conditions et méthodes générales ou universelles de l'évaluation des effets de diverses substances sur les premiers stades du cycle biologique de la truite. On précise aussi d'autres conditions et méthodes propres à l'évaluation d'échantillons de produit chimique, d'effluent, d'élutriat, de lixiviat ou d'eau réceptrice. Le lecteur trouvera des instructions et des marches à suivre concernant l'appareillage, les installations d'essai, la manutention et l'entreposage des échantillons, la préparation des solutions d'essai et la mise en route des essais, les conditions précises dans lesquelles ces essais doivent se dérouler, les observations et les mesures appropriées, y compris les paramètres ultimes, les méthodes de calcul et la validation des résultats.*

# Foreword

*This is the second edition of Environment Canada's three-option biological test method for performing toxicity tests using early life stages of salmonid fish (i.e., EC, 1992) which was published in December 1992. Since that time, investigators within Canadian government and private laboratories as well as scientists in the United States have proposed a number of changes to the test procedures and conditions which simplify and improve the performance of each of the three options (i.e.,* E, EA*, and* EAF *options) that comprise this biological test method. Unlike the first edition (EC, 1992), which recommended three species of salmonid fish (i.e., rainbow trout, coho salmon, or Atlantic salmon) as candidate test organisms, this second edition specifies that* Oncorhynchus mykiss *(rainbow trout or steelhead trout) must be used as the species of test organisms when applying any of the three test options using the revised procedures and conditions described herein.*

*This is one of a series of* ***recommended methods*** *for measuring and assessing the aquatic biological effects of toxic substances or materials. Recommended methods are those that have been evaluated by Environment Canada (EC), and are favoured:*

- *for use in EC 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 protocol or standard reference method.*

*The different types of tests included in this series were selected because 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 the toxicity to aquatic life of specific test substances or materials within the aquatic environment or destined for it. Depending on the biological test method chosen, substances or materials to be tested for toxicity could include samples of chemical, effluent, elutriate, leachate, receiving water or, where appropriate, sediment or similar particulate material.*

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# List of Abbreviations and Chemical Formulae

| | |
|---|---|
| $CaCO_3$ | 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 |
| EC50 | median effective concentration |
| 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) |
| LC50 | 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 internationale d'unités |
| sp. | species |
| TOEC | threshold-observed-effect concentration |
| TIE | toxicity identification evaluation |
| TM (™) | Trade Mark |
| °C | degree(s) Celsius |
| 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* is physiological adjustment to a particular level of one or more environmental conditions 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). Often referred to as a yolk-sac fry, sometimes referred to as a larva. See also *nonviable alevin*.

*Broodstock* are the adult fish which 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 solution's temperature. Conductivity is reported as micromhos per centimetre (μmhos/cm) or as millisiemens per metre (mS/m); 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 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 substance that aids the fine mixing (in the form of small droplets) within water, of an otherwise hydrophobic substance.

*Embryo* is an 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 in which 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, expressed as mg/L calcium carbonate.

*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, 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.

*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 for toxicity of samples of effluent, elutriate, leachate, or receiving water.

*Nonviable alevin* means an egg that has failed to hatch into a normal living, alevin, seven days after 50% hatch is seen to be achieved in the control groups of this toxicity test. The category encompasses failure of egg fertilization, dead embryos, embryos for which development is delayed, and alevins which are obviously deformed or atypical (e.g., two-headed alevins or those with bifurcated tails).

*Nonviable at swim-up* refers to a test organism which has failed to survive or develop normally, at the time when 50% swim-up is seen to be achieved in the control group of this toxicity test. The category includes failure of egg fertilization, failure to hatch, and mortality, delayed development, or abnormal development among embryos, alevins, and early swim-up fry. Any swim-up fry that is alive and apparently normal at the above-mentioned time would be counted as viable.

*Nonviable embryo* means an egg which has failed to survive or develop normally, when observed. The category includes failure of egg fertilization, mortality, delayed development, or abnormal development among embryos. Any embryo that is alive and apparently normal when observed is counted as viable.

*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* describes the durations of illumination and darkness within a 24-h day.

*Precipitation* is the formation of a solid (i.e., precipitate) from some or all of the dissolved components of a solution.

*Pretreatment* means 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.*, 1995). It is usually reported in grams per kilogram or parts per thousand (‰).

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

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

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

*Thinning* refers to the random removal of a number of individual test organisms from one or more replicates, to reduce crowding, maintain an acceptable loading density, and/or minimize the volumes of test solution required during each renewal. Thinning must not be done at any time during an E or EA test, nor during the embryo or alevin stages of an EAF test. Thinning may only be done, if desired, at the time that the final phase of an EAF test is started.

*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 two hours 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 substance that might be mixed with, deposited in, or found in association with water.

*Control* is a treatment in an investigation or study that duplicates all the conditions and factors that might affect the results, except the specific condition that is being studied. In an aquatic toxicity test, the control must duplicate all the conditions of the exposure treatment(s), but must contain no added 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 handling of organisms).

*Control/dilution water* is the water used for diluting the test substance, for the control test, or for 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 a toxicity test.

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

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

*Elutriate* is an aqueous solution obtained after adding water to a solid substance or material (e.g., contaminated soil or sediment, tailings, drilling mud, dredge spoil), shaking the mixture, then centrifuging it, 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 characteristics of pH, alkalinity, and hardness.

*Stock solution* is a concentrated 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* is 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.

## Statistical and Toxicological Terms

*Acute* means within a short period in relation to the life span of the organism, for example a four-day test with 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 exposure to a toxic substance that was either acute or chronic.

*Chronic toxicity* refers to long-term effects that are usually related to changes in such things as metabolism, growth, reproduction, or ability to survive. Because of the long life span of salmonids, early life-stage tests do not measure chronic toxicity, although the intent of this test is to estimate approximately, what such sublethal chronic toxicity might be.

*Chronic value* is a synonym for TOEC *(q.v.)*. TOEC is the recommended term because it can be applied accurately to all sublethal effects whether acute or chronic.

*Continuous-flow* describes tests in which solutions in test vessels are renewed by the continuous inflow of a fresh solution, or by a frequent intermittent inflow. The meaning is the same as *flow-through.*

*EC50* is the median effective concentration. That is, the concentration of substance in water (e.g., mg/L) that is estimated to cause a discernible sublethal or lethal toxic effect to 50% of the test organisms. In most instances, the EC50 (including its 95% confidence limits) is statistically derived by analysis of an observed biological response (e.g., incidence of nonviable embryos or reduced hatching success) for various test concentrations, after a fixed period of exposure. The duration of exposure must be specified.

*Endpoint* means the reaction of the organisms to show the effect which is intended to mark completion of the test, and also means the measurement(s) or value(s) derived, that characterize the results of the test (e.g., 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 is estimated to cause a designated percent impairment in a quantitative biological function such as the size attained by fish during a growth period. For example, an IC25 could be the concentration estimated to cause fish to attain dry weight that is 25% lower than that attained by control fish. This term \

should be used for any toxicological test which measures a quantitative effect or change in rate, such as growth, reproduction, or respiration. (The term EC50 or *median effective concentration* is not appropriate in tests of this kind since it is limited to quantal measurements, i.e., number of exposed individuals which show a particular effect.)

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

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

*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 test concentration at which growth of fish was decreased significantly from that of the control groups. LOEC is generally reserved for adverse sublethal effects but can also be used for mortality, which might sometimes be the most sensitive effect observed.

*Minimum Significant Difference* (MSD) means the difference between values for individual concentrations (e.g., mean percent nonviable embryos; mean percent nonviable alevins) that would have to exist before it could be concluded that there was a significant difference between the groups. The MSD is provided by certain statistical tests including Dunnett's multiple-range test, a standard statistical procedure.

*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 test concentration at which growth was not decreased significantly from that of the control groups. NOEC customarily refers to adverse sublethal effects, and to the most sensitive effect unless otherwise specified.

*Replicate* is a single test chamber 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 test chambers would be used. For each concentration or control, there would be three test chambers or replicates. A replicate is an independent test unit; therefore, any transfer of organisms or solutions from one replicate to another would invalidate the statistical analysis.

*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.

*TOEC* is the threshold-observed-effect concentration. It is calculated as the geometric mean of NOEC and LOEC. A term variously defined in some other countries is the MATC (maximum acceptable toxicant concentration). *Chronic value* or *subchronic value* are alternative terms that might be appropriate depending on the duration of exposure in the test.

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

*Toxicity Identification Evaluation* (TIE) describes a systematic sample pretreatment (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.

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*Section 1*

# Introduction

## *1.1 Background*

Aquatic toxicity tests are used within Canada and elsewhere to measure, predict, and control the discharge of discrete substances or complex mixtures that could adversely affect 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) (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., EC, 1991).

Universal procedures and conditions for conducting early life-stage tests using 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). For guidance on the implementation of this and other Environment Canada biological test methods, and on the interpretation and application of the endpoint data, the reader should consult Environment Canada (1998a).

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

The biological test method presented in this report is based largely on other embryo-larval and early life-stage methods developed in North America and Europe (USEPA, 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; OECD, 1992a;b;c; Neville, 1995a;b; OECD, 1996; 1997). It has been developed following a review of specific procedural variations indicated in existing "methodology" documents (Appendix C) and in other related reports and publications.

This report replaces the biological test method "*Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout, Coho Salmon, or Atlantic Salmon*", which was published by Environment Canada in 1992 (EC, 1992a). Since that time, certain pretest and test conditions and procedures have been simplified, clarified, or otherwise improved, based on (a) the experience of a number of Canadian laboratories performing the "E" or “EA” test options to meet regulatory requirements for monitoring environmental effects (EC, 1991), and (b) controlled studies with the objectives

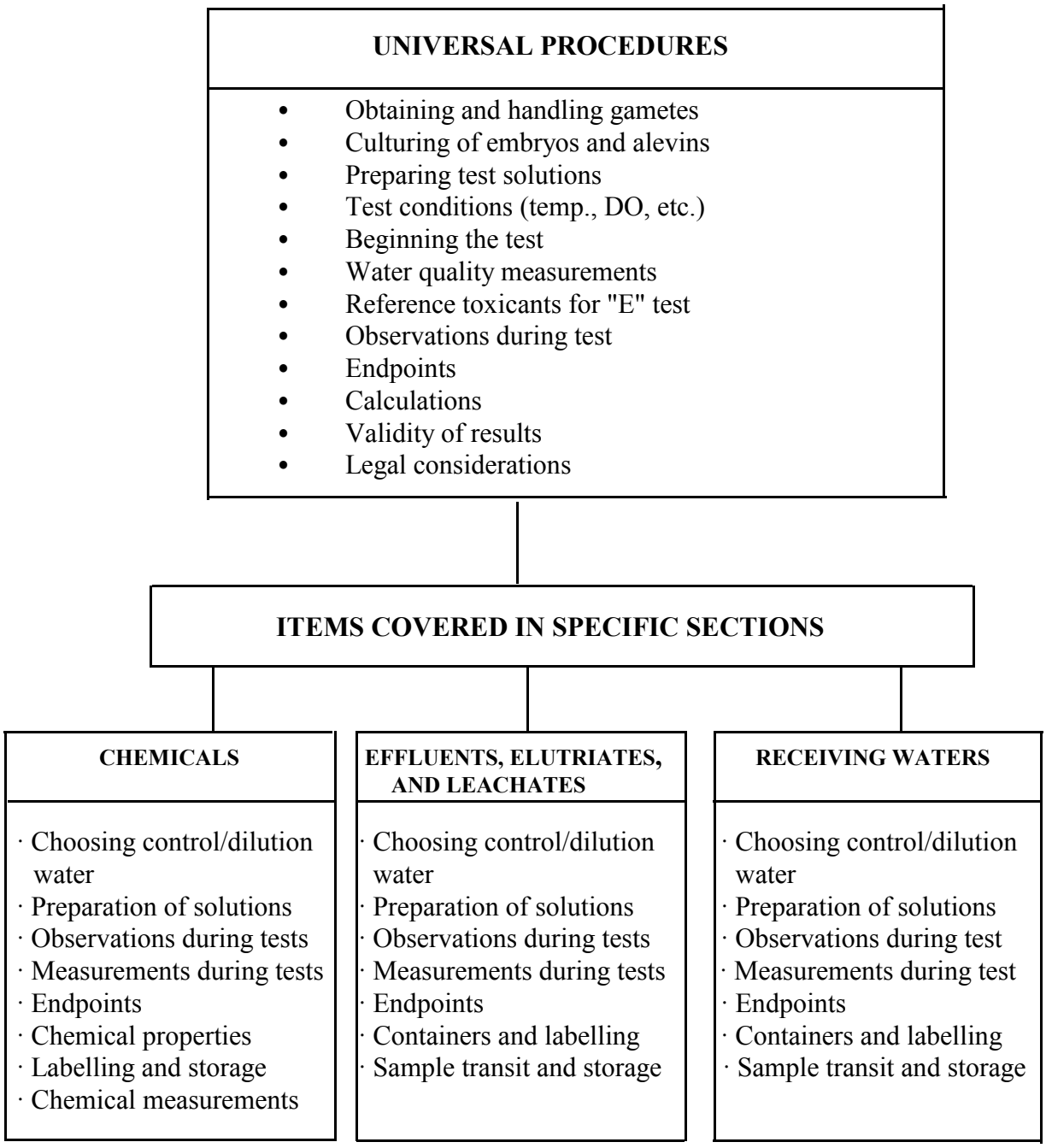


**Figure 1 Considerations for Preparing and Performing Toxicity Tests Using Early Life Stages of Rainbow Trout and Various Types of Test Substances**

of simplifying or improving methods (Canaria *et al.*, 1996; Yee *et al.*, 1996; Fennell *et al.*, 1998). Improvements in procedures, system design, statistical treatments of data, and more explicit or more detailed guidance for the performance of tests, are provided herein.

Three test options 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 test options 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, when 50% hatching success is seen in the control, with no feeding of fish being required. The EAF test ends after 30 days of exposing swim-up fry, with feeding. Any of these three test options may be used to evaluate samples of chemical or chemical product, effluent, elutriate, leachate, or receiving water. Selection of the most suitable test options will depend on the objectives of the test and the nature of the substance being tested (see Sections 4.3.1, 5.1, 6.1, and 7.1).

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 most situations in which they would be applied. It is assumed 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 as a guidance document for this and other uses.

## *1.2 Historical Use of Test*

Chronic toxic effects on fish have been studied with either life-cycle tests (egg-to-egg), or partial life-cycle tests (egg-to-juvenile), depending on the nature of the studies and the fish species used. For salmonids, complete life-cycle studies are largely impractical because it takes two to five years for these fish to reach maturity. 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 (McKim, 1977, 1985; 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, 1995a;b). These procedures are based on the assumption that the highest concentrations which are without sublethal effects in early life-stage tests, will approximate the chronically safe concentrations for the salmonid species tested.[1]

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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.

Early life-stage tests using rainbow trout or other species of salmonids for regulatory and research purposes have initiated toxicant exposures at the onset of embryological 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;b; OECD, 1996). 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 estimate of a sublethally safe concentration. Depending on the species and temperature, and on the number of days that alevins are observed before ending the test, the duration of an EA test with salmonid fish might be from as little as ~30 days (Fennell *et al.*, 1998) to as much as ~80 days. The duration of an EAF test might also vary from as little as 70 days to as much as 120 days or longer, depending on species and temperature and on the number of days that the survival and growth of fry are monitored. 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.

Various short-term tests of 7 to 28 days have been developed using rainbow trout embryos and/or alevins (Birge *et al.,* 1985; Birge and Black, 1990; Paine *et al.*, 1991; OECD, 1992a; 1996; Neville, 1995a;b), or fry (OECD, 1992c; 1997). These tests focus on one or more sensitive transitional periods of development (e.g., early embryo development, alevin development and yolk conversion, or 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 toxicity of various substances to rainbow trout in fresh water, by exposing one or more of the early life stages of this species using one of the three test options herein (i.e., E, EA, or EAF test options). The procedures for salmonid early life-stage toxicity tests in existing Canadian, U.S., and international methodology documents vary in terms of recommended test species, duration of exposure, temperature regime(s), 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,

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[2] Short-term tests with rainbow trout using early embryos (Birge *et al.*, 1985) and late sacfry/early swim-up fry (Neville, 1995a;b) have been shown to be more sensitive than similar tests with 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 (OECD, 1992c) and revised for publication (OECD, 1997). This method shows promise as an abbreviated procedure for measuring toxicity.

elutriate, or receiving water, and the rationale for selecting certain approaches. The three test options (i.e., E, EA, or EAF tests) herein are for use with rainbow trout 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 one or more of these three test options may be varied but includes instances in which 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 or 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; Peterson *et al*., 1988; Hodson *et al*., 1991; Neville, 1995a;b).

Rainbow trout is the test organism recommended for use in each of the three test options (i.e., E, EA, or EAF tests) described in this report. Relevant information on the distribution, life history, and husbandry of rainbow trout is given in Appendix D. The test options described herein might also be applied to steelhead trout (an anadromous subspecies of *Oncorhynchus mykiss*); likely without a need for procedural change. Other species of salmonid fish (e.g., cutthroat trout, brook trout, brown trout, chinook salmon, chum salmon, coho salmon, Atlantic salmon, arctic grayling, or whitefish) might also be used with any of these three test options, although modifications to certain procedures and/or conditions (e.g., temperature and test duration) might be necessary. Investigators wishing to use a species other than *O. mykiss* are advised to carefully consider a temperature regime that is known or likely to be compatible with the early development of the desired species, and to determine in one or more preliminary tests (using control/dilution water) the appropriate temperature regime and related test duration for any E, EA, or EAF test option to be applied to that species using a test substance. In particular, the criteria for test validity described herein for rainbow trout (see Section 4.6) should be demonstrated to be achievable with an alternate test species before it is used for measuring the toxicity of a test substance according to any of the test options herein (with or without modification). Additionally, any test-specific report which identifies one of the test options herein as the biological test method used must identify the species and common name of the test organism as well as any modifications to definitive pre-test or test conditions and procedures described herein (see Section 8).

# Test Organism

## *2.1 Species and Life Stages*

Rainbow trout (*Oncorhynchus mykiss*) is the test organism recommended as the source of gametes for use with any of the three test options (i.e., E, EA, or EAF options) described herein (see Sections 1.3 and 4.3.1). Steelhead trout (an anadromous subspecies of *O. mykiss*) may also be used if available.

The generalized appearance of salmonid early life stages is shown in Figure 2. The test is designed to determine effects on rainbow trout from the onset of embryo development through to a particular stage of development, depending on the test option selected (see Section 4.3.1). Since 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 water temperature (see Appendix D).

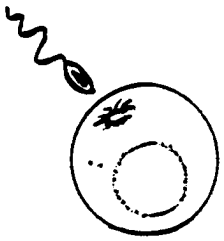
Three major transitions of the different developmental stages in the early life of rainbow trout are important and integral to the test option to be chosen. The first transition is when recently fertilized eggs change to an *embryo*, and develop within an egg membrane that quickly becomes relatively 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 (i.e., the embryo or *E* test option). The second transition is hatching, and the former embryo becomes an *alevin* or yolk-sac fry. This stage is included in the embryo/alevin (*EA*) test option. 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 (*EAF*) test option includes this change and 30 days of exposure for the feeding fry. Further details on the stages and the timing of stages with respect to these three test options are given in Section 4.3.6. Appendix D should be consulted for additional information on the ranges of size, temperature tolerance, and temperature-dependent developmental rates of these life stages.

## *2.2 Source*

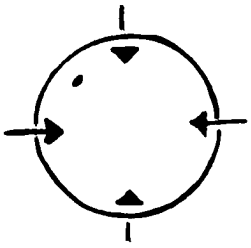
Gametes or broodstock should be obtained from a single population and source of rainbow trout. The best sources are likely to be government hatcheries, government research stations, and private culture facilities that are known to have disease-free fish. To simplify procedures, it is preferable to obtain gametes, since handling, transport, holding and stripping of broodstock require additional holding facilities and experienced personnel. Egg fertility is influenced markedly by the ripeness of female gonads, and timing of stripping for egg procurement should be optimal (Appendix D) to enable good fertilization success.

The pool of eggs to be used in a toxicity test must be obtained from a minimum of four females (see Appendix D, including footnote 46). Additionally, the milt must be obtained from a minimum of three males.

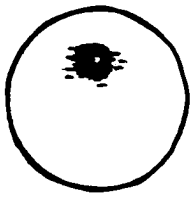
1. Fertilization



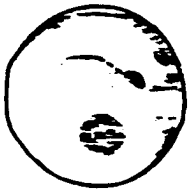
2. Fertilized egg swelling with water uptake



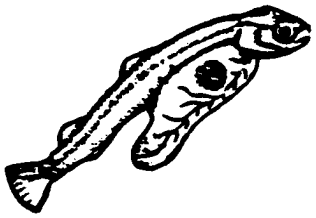
3. Cell division starts



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)

Pre-fertilization screening of milt for sperm mobility must be carried out to improve the likelihood of good fertilization success. The following procedure (Novak, 1996) has proven effective, and is recommended herein. Since experience has shown that inactive milt can be obtained from 25 to 50% of males, samples from three or more sexually mature individuals must be collected and held in separate vials (Novak, 1996; Fennell *et al.*, 1998). At the testing facility, a thin film of milt from each vial must be placed on a dry glass slide, and examined immediately under a compound microscope at ~100 × magnification. The sperm should appear inactive. A small amount of fresh water (or ovarian fluid) is then added, and mixed quickly with the milt on the slide. Sperm should become vigorously active for approximately 20 to 30 seconds, and will be inactive after 60 seconds. Vials that contain inactive sperm must not be used for fertilization. Samples of fresh milt must be obtained if all vials contain inactive sperm.

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. 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 *O. mykiss* is not 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 above-mentioned committee, to the appropriate provincial agency, or the Regional Director General of the Department of Fisheries and Oceans (DFO), depending on procedures in place locally.

# Test System

## *3.1 Facilities and Materials*

The test should 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.

The test facility must be able to maintain the daily mean temperature of all test solutions at 14 ±1.0°C (see Section 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 should be prepared to undertake prompt and accurate analysis of other variables such as hardness, alkalinity, ammonia, and residual chlorine.

Any construction materials or equipment that might contact the test substance, test solutions, or control/dilution water, must not contain any substances that can be leached into the sample, solutions, or water at concentrations that could cause toxic effects. Materials such as borosilicate glass (e.g., Pyrex™), stainless steel, porcelain, nylon, high-density polystyrene, or perfluorocarbon plastics (Teflon™), should be used. Other nontoxic plastics, such as polypropylene or polyethylene, may be used but their re-use should be only after careful and thorough cleaning (e.g., using a phosphate-free detergent wash followed by an acid soak and several rinses with deionized water) to minimize the possible release of sorbed toxicants during a subsequent test. For tests with chemicals or chemical products (see Section 5), glass is the recommended material for containers and apparatus which contact the test solutions before or during the 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 within the range of 100 to 500 lux. Depending upon test requirements and intent, lighting might be provided by overhead full-spectrum fluorescent fixtures.[4]

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[3] Dim incandescent lighting (i.e., <200 lux) may be used for short periods during observations and maintenance (R. Watts, personal communication, Pacific Environmental Science Centre, North Vancouver, BC). Alternatively, the use of red ("dark room") incandescent lighting is recommended (G. van Aggelen, personal communication, Pacific Environmental Science Centre, North Vancouver, BC).

[4] Full-spectrum fluorescent or equivalent lamps, supplemented with natural outdoor illumination if desired, are suitable for simulating the visible range of natural light. However, full-spectrum lights do not emit ultraviolet (UV-B) radiation at intensities approaching that of natural illumination, and the toxicity of certain effluents and chemicals can be altered markedly by photolysis reactions caused by UV-B radiation. For certain tests (e.g., those concerned with photoactivation or photodegradation

The photoperiod should normally be a constant sequence of 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*

In 1996, the test apparatus illustrated in Environment Canada (1992a), which had been used in a number of early life-stage toxicity tests with salmonid embryos or alevins (McLeay and Gordon, 1980; Martens *et al*., 1980; Hodson *et al*., 1991), was re-designed. The modified design was tested and proved effective (Yee *et al.*, 1996). The incubation unit and associated apparatus now recommended (Figure 3) is easily and inexpensively constructed, and enables gentle aeration and/or continuous circulation of test solutions past incubating embryos or alevins and easy renewal of solutions with minimal disturbance. In addition, the unit allows the embryos or alevins to remain bathed in the test solution during the solution renewal process. This incubation unit is made from an 800-mL (or larger) Tri-Pour™ plastic beaker having slightly tapered sides. Unlike the earlier design which required replacing the bottom of the beaker with a plastic mesh floor (EC, 1992a), the bottom of the beaker is left intact, and a series of horizontal slits are cut in the sides, near the bottom, to allow the circulation of test solutions within the beaker (Yee *et al.*, 1996; Figure 3A). A circular hole is drilled in the centre of the bottom of the beaker, and a removable "pressure-fit" 5-cm long standpipe, cut from a standard-supply 10-mL disposable polystyrene volumetric pipette, is inserted through the hole.

The incubation unit can be easily suspended in a test chamber by inserting it through a 10-cm diameter hole cut in the cover of the chamber (e.g., the white plastic lid of a 4-L white plastic pail). A second hole of smaller diameter (~6 cm; Yee *et al.*, 1996) can be cut in the lid to enable monitoring of water quality during the test (Section 4.3). The volume of solution held in each test chamber should not be chosen arbitrarily but should be considered in light of the requirements for amounts of new test solution to be supplied daily (Section 4.3.2), throughout the exposure period involving embryos or alevins. The daily (or more frequent) renewal of each test solution is achieved either by siphoning out ~80% of the old solution and replacing it immediately with a fresh (new) solution prepared to the same strength (i.e., *static-renewal* test), or by the continuous addition of fresh solution to the the test chamber (i.e., a *flow-through* test, see Section 4.3.2).

The test chamber should be adapted to accommodate either static-renewal or flow-through conditions, depending on the

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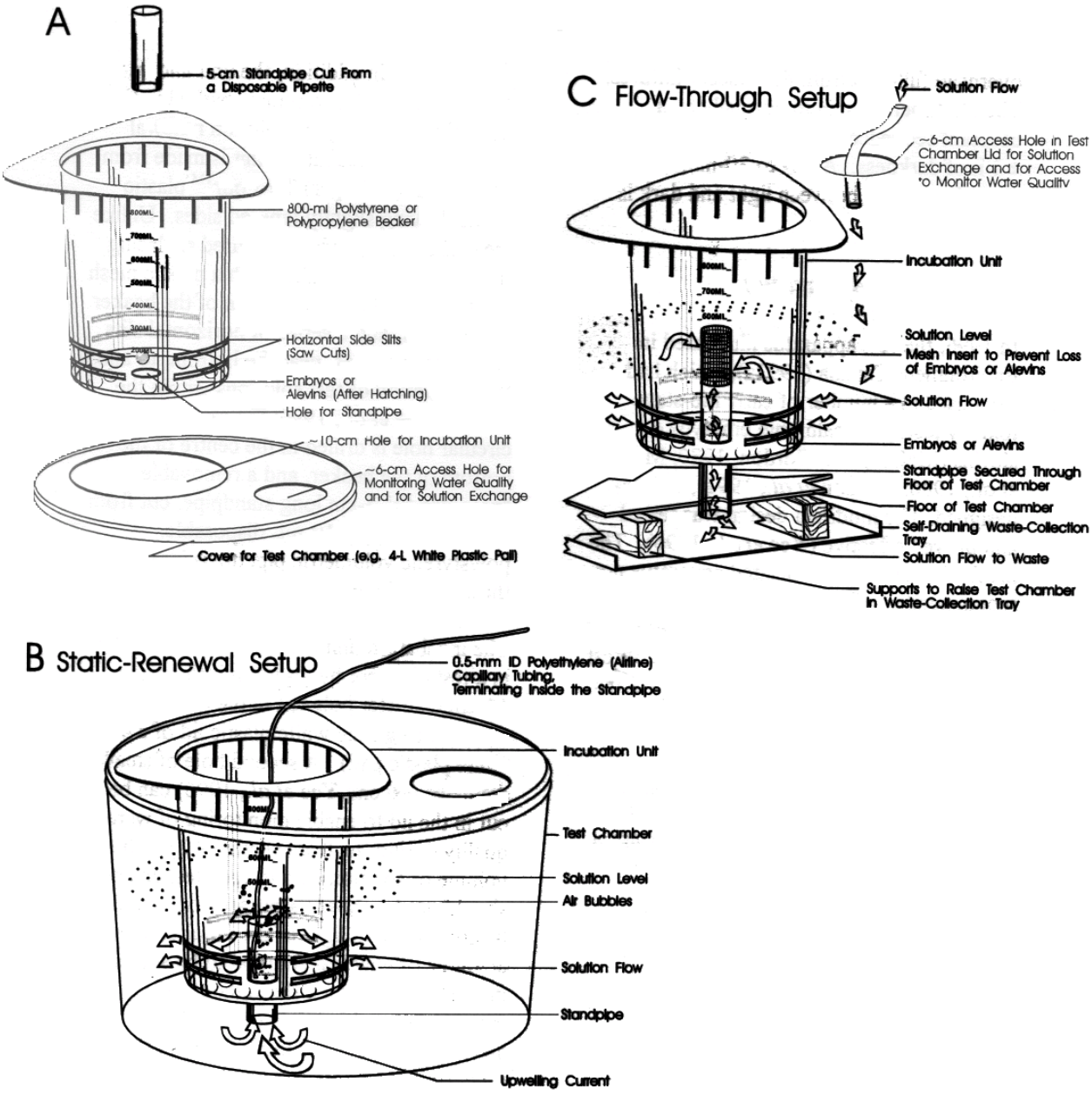
of toxic substances due to ultraviolet radiation), lights with particular spectral qualities may be selected (e.g., high-pressure mercury arc lamps). ASTM (1996) provides useful guidance in this regard.

[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.




A - Exploded view of modified incubation unit and associated apparatus, showing horizontal side slits.

B - Incubation unit suspended in a test chamber, showing aeration and upwelling current.

C - Incubation unit suspended in a test chamber with no aeration, showing standpipe drain and current created by solution flow.

**Figure 3 Recommended Design for Static-renewal or Flow-through Setups for Incubating Embryos or Alevins in Test Solutions**

requirements and objectives of the test.[6] For static-renewal tests (Section 4.3.2), the experimental design shown in Figure 3B is recommended. Using this setup, filtered, oil-free air is bubbled through a suitable length (e.g., 60 cm) of 0.5-mm ID polyethylene capillary tubing, which is inserted in the centre standpipe protruding through the bottom of the incubation unit (Figure 3B).[7] This aeration system, which can also be used if necessary for flow-through tests, provides a continuous current of aerated water past the embryos or alevins.

For flow-through tests (see Section 4.3.2), the experimental design shown in Figure 3C is recommended. This setup may be used with or without aeration.[6,8] A flow of fresh solution passes continuously over the embryos or alevins as it drains from the test chamber (Figure 3C). Using the flow-through design shown in Figure 3C, the incubation unit is suspended around a suitable length of standpipe drain that is secured through both the centre of the incubation unit and the bottom of the test chamber. A plastic mesh insert is placed in the top of the standpipe to prevent loss of embryos or alevins.

Each test chamber, whether it contains one or more incubation units suspended in it, is one replicate. For each test concentration or control, there must be at least three separate test chambers providing true replication, adequate for calculation of experimental error (see Sections 4.1 and 4.5). If hypotheses testing is to be applied, such as estimating NOEC/LOEC, four separate test chambers should be used; that is the minimum number required if it were necessary to use nonparametric statistical tests.

Other apparatus (e.g., Canaria *et al.*, 1996) may also be used as test chambers, 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 for incubating embryos and alevins, and if used will

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[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-renewal tests might also be desirable or necessary when the degradation products of the test substance are of concern. High chemical or biochemical oxygen demand, volatility, or instability of certain substances might necessitate the use of a flow-through test. A flow-through test is also necessary if embryos or alevins are to be incubated in test solutions without aeration, in order to provide a continuous exchange of solution within the incubation unit.

[7] A simple and practical design for aerating several incubation units simultaneously can be quickly assembled by inserting three or four lengths of 0.5 mm ID capillary tubing into one length (e.g., 40 cm) of standard aquarium airline tubing. Clear silicone sealant can be used to fuse the juncture and prevent air leaks (Yee *et al.*, 1996). Other apparatus, such as tubing with Eppendorf tips attached, is also suitable for generating small bubbles and thus for providing gentle aeration through a narrow-bore aperture. Disposable Pasteur pipettes or other air-delivery apparatus used routinely for acute lethality tests with fish (e.g., EC, 1990b) may be used to aerate test solutions with volumes ≥6 L.

[8] Aeration might strip volatile chemicals from solution or increase their rates of oxidation and degradation to other substances. Gentle aeration (Section 4.3.4) might be desirable or even necessary during a flow-through test, to maintain adequate levels of dissolved oxygen when the chemical or biochemical oxygen demand of the test substance is particularly high. If aeration is to be provided using a flow-through setup (e.g., Figure 3C), the end of the airline tubing should be placed in the test solution outside the standpipe drain.

provide a greater degree of standardization of conditions during incubation.

## *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 of the source of contamination, or adjacent to the source but removed from it; or dechlorinated municipal water.[9]

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 to document water quality (e.g., monthly or more frequently if deleterious changes in water quality are suspected or found) (see Table 1). Conditions for the collection, transport, and storage of samples of receiving water, if used as control/dilution water, should be as described in Section 6.2.

If surface water is used as the control/dilution water, it should be filtered and/or sterilized. A conventional sand filter or commercial in-line filter would be suitable. Small quantities might be filtered through a fine-mesh net ($\leq 60$ 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 Section 4.3.3). The total gas pressure (TGP) of this water should not exceed 100%.[10]

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[9] 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. It is difficult to remove the last traces of residual chlorine and chlorinated organic substances, which might be toxic to developing fish. Vigorous aeration of the water followed by the use of activated carbon (bone charcoal) filters can be applied to strip out part of the volatile chlorine gas, and subsequent ultraviolet radiation (Armstrong and Scott, 1974) can be used 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 $\leq 0.002$ 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.

[10] Water entering the test chamber should not be supersaturated with gases (i.e., TGP $\leq 100\%$). In situations where gas supersaturation within the water supply is a valid concern (e.g., groundwater source, without subsequent gas stripping), 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 should be taken (e.g., use of aeration columns or vigorous aeration in an open reservoir) if TGP exceeds 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 1 Recommended 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)<br><0.1 mg/L (pH >6.5) |
| Ammonia (un-ionized) | <5 µg/L (preferably not detectable) |
| Cadmium | <0.3 µg/L (in soft water)<br><0.5 to 0.75 µg/L (in hard water) |
| Chlorine | <2 µg/L |
| Copper | <6 µg/L (in soft water)<br><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)<br><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)<br><103% (total gas pressure) |
| Selenium | <10 µg/L |
| Total suspended solids | <3 mg/L during incubation<br><25 mg/L during larval and fry stages |
| Zinc | <0.03 mg/L (in soft water)<br><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. Available studies of metal toxicity in local water should 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 should be evaluated.

Additionally, its dissolved oxygen concentration (DO) should be 90 to 100% of the air-saturation value, before its use. If necessary, the control/dilution water should be aerated vigorously (oil-free compressed air passed through air stones) immediately before use, and a check made to confirm that a dissolved oxygen concentration of 90 to 100% saturation has been achieved.

# Universal Test Procedures

Procedures described in this section apply to each of the toxicity tests for samples of chemical, wastewater, or receiving water 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 test conditions and procedures in Table 2 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 operating procedures. Control/dilution water should be used as the final rinse water.

For any test that is intended to estimate an EC50, LC50, ICp, or NOEC/LOEC (Section 4.5), a minimum of five concentrations plus a control solution (100% dilution water) must be prepared. For EA and EAF tests, with multiple endpoints based on both lethal and sublethal effects (Section 4.5), more concentrations (e.g., six to eight plus a control) are recommended to improve the likelihood of attaining each endpoint sought. 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). If there was considerable uncertainty about the toxic levels, more concentrations should be used to obtain a wide spread, rather than using a lower factor for dilution. Volume requirements for tests will vary according to the option (E, EA, or EAF) used (see Sections 4.3.2, 5.1, 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 herein, but procedures are evident, and all items apply except for testing a single concentration and a control.

The test must be started with at least three replicates of each concentration including controls. If endpoints are to be calculated using hypothesis tests (i.e., NOEC/LOEC), a

## Table 2 Checklist of Recommended Test Conditions and Procedures

### *Universal*

| | | |
|---|---|---|
| Test options | – | embryo test (*E test*) for frequent or periodic testing<br>– embryo/alevin test (*EA test*) for measuring effects on multiple developmental stages<br>– embryo/alevin/swim-up fry test (*EAF test*) for definitive investigations |
| Test type | – | static-renewal or flow-through |
| Test species | – | rainbow trout (*Oncorhynchus mykiss*) |
| Start of Test | – | within 30 minutes immediately following a period of 5 to 20 minutes for dry fertilization of eggs |
| End of Test | – | *for E test*: seven days after fertilization<br>– *for EA test*: seven days after half of the eggs in the control are seen to have hatched<br>– *for EAF test*: 30 days after half of the surviving fish in the control show swim-up behaviour |
| Control/dilution water | – | ground, surface, reconstituted, or if necessary, dechlorinated municipal water; "upstream" water to assess toxic effect at a specific location |
| Test apparatus, solution renewal | – | for embryos and alevins, an 800-mL plastic beaker with solid bottom and slits in side, suspended in a plastic pail or glass aquarium (the test chamber), with static-renewal or flow-through replacement of test solutions at ≥0.5 L/g·d; for swim-up fry, a plastic pail or glass aquarium with either static-renewal or flow-through replacement of solutions at ≥0.5 L/g·d |
| No. organisms, replicates | – | control plus ≥5 concentrations; *for E test*, ≥120 embryos per concentration including the control, *for EA or EAF test*, 120 to 320 embryos/concentration; ≥3 replicates for standard point-estimation techniques (i.e., at least 40 embryos in each of three replicates in the E test); if hypothesis testing is to be done, ≥4 replicates/concentration would be needed if parametric analysis proved to be invalid and nonparametric analysis were required (i.e., ≥30 embryos in each of four replicates); ≥1 incubation unit/test chamber, the chamber being a replicate |
| Temperature | – | daily mean of 14 ± 1°C throughout the test, for E, EA, or EAF test |
| Oxygen/aeration | – | control/dilution water 90 to 100% DO saturation before use; normally no pre-aeration unless a sample or test solution has DO <60% or >100% upon preparation, in which case pre-aerate sample or all solutions for 30 minutes and if necessary for an additional period of ≤90 minutes, at 6.5 ± 1 mL/min·L; if static-renewal test, gentle aeration; if flow-through test, aerate if necessary or desired to maintain DO at 60 to 100% saturation, and/or increase rate of exchange |
| Lighting | – | dark until one week after hatching is completed, with dim or red light during solution renewals; then controlled at 100 to 500 lux at the water surface, with 16 ± 1 h light : 8 ± 1 h dark, preferably with gradual transition and preferably using full-spectrum fluorescent lights or equivalent |

**Table 2 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 |
| Feeding | – | *for E and EA tests*: no feeding |
| | – | *for EAF test*: feed fry 4% body wt/d with commercial starter feed, ≥4 times/d, starting when when half of the surviving control fish show swim-up behaviour, continuing for a 30-d exposure, but without feed in final 24 h of exposure |
| Observations, each replicate | – | *for E test:* percent nonviable embryos at test end |
| | – | *for EA test:* percent nonviable alevins, and narrative statements on delayed hatching and deformed alevins; |
| | – | *for EAF test:* percent nonviable individuals at swim-up, mortality of fry during final 30 days, average dry weight of surviving fry at test end, and narrative statements on delayed hatching, deformed alevins, delayed swim-up, and abnormal behaviour of fry |
| Measurements | – | temperature, 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 | – | *for E test*: EC50 and/or EC25 for nonviable embryos |
| | – | *for EA test:* EC50 and/or EC25 for nonviable alevins (failure to reach alevin stage); narrative statements on delayed hatching and deformed alevins |
| | – | *for EAF test:* EC50 and/or EC25 for nonviable individuals at swim-up (failure to survive at any stage up to time of early swim-up); LC50 for swim-up fry; IC25 for average dry weight of surviving swim-up fry at test end; narrative statements on deformed alevins, delayed swim-up, and abnormal behaviour of fry |
| Reference toxicant | – | phenol and/or zinc; ; perform as an E test at the time that each E, EA, or EAF test is initiated, using a portion of the same batch of fertilized eggs used to start the definitive test; use procedures described herein for performing an E test with a chemical; determine EC50 |
| Test validity | – | invalid if any of the following occurs:<br>*for E test*: >30% of controls nonviable at end of test<br>*for EA test:* >35% of controls nonviable at end of test<br>*for EAF test:* >40% of controls nonviable at time of 50% swim-up of survivors |

## *Chemicals*

| | | |
|---|---|---|
| Solvents | – | used only in special circumstances; maximum concentration, 0.1 mL/L |
| Concentration | – | recommended measurements: weekly in static-renewal tests in representative high, medium, and low concentrations and control(s), immediately after renewal of test solutions and immediately before renewal, which is usually a 24-h interval; weekly in all replicates of flow-through tests |

**Table 2 Checklist of Recommended Test Conditions and Procedures (cont.)**

### *Effluents, Leachates, and Elutriates*

| | | |
|---|---|---|
| Sample requirement | – | for off-site tests, ≥1 sample(s) collected (effluent, leachate) or prepared (elutriate) weekly; for on-site tests, samples collected daily |
| Transport and storage | – | if warm (>7°C), must cool to 1 to 7°C with regular ice (not dry ice) or frozen gel packs upon collection; transport in the dark at 1 to 7°C (preferably 4 ± 2°C) using regular ice or frozen gel packs as necessary; sample must not freeze during transit or storage; store in the dark at 4 ±2°C; use in testing should begin as soon as possible after collection, and must start within three days of sampling (or extraction, if elutriate) for off-site tests and within one day 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 nonfiltered 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" water |

minimum of four replicates per concentration must be used.[11] 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 analysed with less power (EC, 1998b).

For a given test, the same control/dilution water must be used for preparing the control and all test concentrations. Each test solution must be made up to an identical volume, and well mixed with a clean glass rod, Teflon™ stir bar, or other clean device made of nontoxic material.

The temperature of sample(s) or test solutions (including the control/dilution water) should be adjusted as required for each test option and each life stage (see Section 4.3.3). If necessary, the temperature of samples or test solutions may be adjusted to the test temperature by heating or chilling in a water bath, or by the use of an immersion cooler made of nontoxic material (e.g., stainless steel). Samples or test solutions must not be heated by immersion heaters, since this could alter chemical constituents and toxicity. It might be necessary to adjust the pH of the sample(s) or test solutions (see Section 4.3.5), or to provide preliminary aeration of the solutions (Section 4.3.4).

For site-specific assessments of toxic effect, "upstream" water might 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) must be used.

## *4.2 Beginning the Test*

Eggs must be dry-fertilized (see Appendix D for guidance) to prevent the onset of micropyle closure and water hardening before they are transferred to test solutions.[12]

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[11] Three or more replicates are beneficial for point estimates of ICp as an endpoint. The ICp could still be calculated with two replicates, but power would be lost and wider confidence limits would ensue. Replicates are not required for quantal point estimates (LC50, EC50, and EC25), since results are combined for each concentration. The three replicates are convenient, however, for handling and providing suitable conditions for the numbers of eggs and embryos involved in the test, and there is some security of results in case one replicate is accidentally damaged or lost.

If hypothesis testing is to be done as an extra endpoint (see Section 4.5), a minimum of three replicates per concentration must be available for statistical analysis by the standard parametric analyses. More replicates would provide more power for the statistical analysis. If irregularities in the data made those methods invalid, four replicates would be required to allow the use of nonparametric statistics (USEPA, 1994; EC, 1998b). For instance, Dunnett's test (parametric) requires a minimum of three replicates per concentration, whereas Steel's nonparametric "Many-One-Rank test" needs at least four replicates (Steel and Torrie, 1960). Accordingly, if it were desired to estimate NOEC/LOEC, it would be prudent to use at least four replicates.

[12] To maximize test sensitivity and comparability of results, the start of the test must be standardized to ensure that water hardening occurs during exposure to test solutions. Preferably, the gametes would be transported to the laboratory, fertilization would be carried out, and the 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 test 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 a period of relative sensitivity to shock in the first

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). Any eggs distinguished visually as under- or oversized should be discarded. A minimum of 120 embryos per concentration must be used for the E test; 120 to 320 embryos per concentration are recommended for an EA or EAF test (Section 4.3.1). Each treatment (concentration) including the control(s) must include a minimum of four replicate test chambers if statistics using hypothesis tests are intended; and a minimum of three replicates per treatment if point-estimation techniques (e.g., EC50, ICp) are intended (see Sections 4.1 and 4.5).

Identical numbers of embryos should be added to each test chamber. Using 40 embryos per replicate[13], a test with three replicates (including five concentrations plus a control) requires 720 eggs. Similarly, a test which uses 80 embryos per replicate, three replicates, and five concentrations plus a control, requires 1440 eggs. The eggs must be obtained from a batch of eggs stripped from four or more females of similar size (see Section 2.2 and Appendix D).

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 (Hubert, 1991). 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.

Fertilization must be accomplished by the dry mixing of eggs and milt for a minimum of 5 minutes (Fennell *et al.*, 1998) and a maximum of 20 minutes (Birge *et al.*, 1985). Following this mixing, groups of freshly fertilized eggs *(embryos)* should be transferred as quickly as possible to test solutions (see Appendix D for guidance). This should be accomplished within the 10-minute period immediately following the 5- to 20-minute interval for fertilization, and must be completed within the 30-minute period immediately after this interval. A brief (i.e., no more than 10 seconds) rinse of each group of embryos being transferred might be necessary to wash off debris and excess milt. Either control/dilution water or an aliquot of the respective test solution to which the embryos are to be transferred should be used for this purpose. If water is used, embryos must not contact it for more than a few seconds from time of fertilization until their introduction to the test solutions. Eggs that appear abnormal in any way (e.g., opaque or milky-white in colour), or which

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few hours during and after water hardening, and great care must be taken during this period when pouring or handling the eggs (see Appendix D).

[13] Each test chamber represents one replicate of a given concentration. There may be one or more incubation units suspended in a test chamber, but all of the units together in one chamber represent one replicate.

are noticeably under- or oversized in relation to the other eggs, must 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.

Care must be taken to avoid unnecessary handling of freshly fertilized eggs, or bumping or dropping them as they are transferred into the incubation units. Within the units, embryos need adequate space to ensure sufficient oxygen exchange and removal of metabolic wastes. The embryos must be distributed evenly on the bottom of each unit so that they are only one layer thick and are not clumped together or piled on top of one another. This distribution will also facilitate efficient recognition and counting of nonviable or hatching embryos.[14]

At the start of the test, the number of embryos transferred to each incubation unit needs to be counted or recounted to ensure that the required number is present and to make any necessary adjustments. During this counting procedure, the incubation unit may be raised gently to just below the surface of the test solution if this is necessary for observation. The number of embryos in the incubation unit should be adjusted as necessary by removing excess embryos using egg-picking tweezers or a large-bore pipette with rubber bulb, and by supplementing any missing embryos with the required number transferred gently and carefully from the remaining group(s) of eggs fertilized for use in the test. The appearance of all embryos in each incubation unit should also be examined at this time, and any embryos appearing atypical in size, shape, or colour should be discarded and replaced.

In addition to these procedures, there must be formal random assignment of the group of embryos in each incubation unit to particular concentrations and replicates. The test concentrations must also be in randomized positions in the test facility. Each test chamber 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 test chambers should be checked and adjusted, if required/permitted, to acceptable levels (see Sections 4.3.3, 4.3.4, and 4.3.5) before adding test organisms.

It is recommended that the conductivity of each newly prepared test solution be measured before dispensing it to the test vessels, as a pre-use check on concentration. The temperature, dissolved oxygen concentration, and pH of each newly prepared test solution should also be checked as necessary before its use.

Salmonid embryos are extremely sensitive to any disturbance or mechanical shock until they reach the "eyed" stage (see Appendix D). Therefore, throughout an E test and during the "pre-eyed" stage of an EA or EAF test, any routine maintenance procedures (e.g., renewal of test solutions in static-renewal tests) must be performed with extra care. Before embryos reach the eyed stage, any removal of obviously dead (i.e., opaque) embryos or unfertilized eggs to control

[14] Depending on the surface area of the bottom of the incubation unit and the size of eggs, it might be necessary to suspend more than one incubation unit in a test chamber to have the required number of embryos distributed only one layer thick. If more than one incubation unit is suspended in a test chamber, the embryos should be distributed equally among them.

fungal infection should be done very carefully (without disturbing any of the surviving embryos) using a large-bore pipette (7 to 10 mm) and rubber bulb.

## 4.3 Test Conditions and Procedures

### 4.3.1 Test Options

One or more of the following three test options may be used: an embryo (E) test for frequent or periodic 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 options start with the onset of embryo development, and measure the development and survival of early life stages. The E test must be started with ≥120 embryos per concentration (e.g., three replicates of 40 embryos each, per concentration), and normally ends seven days after fertilization. However, the duration of an E test may be extended to as much as ten days after fertilization (Birge, 1996). The longer exposure might be warranted for obtaining clear results, if previous tests showed slow development of embryos. The EA test normally starts with 120 to 320 embryos per concentration[15], and is terminated seven days after 50% hatching is seen to be achieved among the surviving embryos of the control, with no feeding of fish. The EAF test also normally starts with 120 to 320 embryos per concentration[15], and ends 30 days after 50% of the surviving alevins in the control are seen to have exhibited swim-up behaviour (see Section 4.3.6). The swim-up fry are fed daily during all but the last of the 30 days of the EAF test. Average survival and average dry weight of surviving fry are measured at the end.

Any of these three test options may be used to evaluate samples of chemical, effluent, elutriate, leachate, or receiving water, depending on the objectives of the test. The duration must be ≥7 days for the E test, and is ~30 days for the EA test, and ~70 days for the EAF test, according to the test conditions and procedures herein.

The E test uses only one biological endpoint (nonviability of rainbow trout embryos). This test option is convenient for frequent or periodic monitoring, but in some situations, an initial comparison with or use of the more definitive EA or EAF test is recommended (see Sections 5.1, 6.1, and 7.1). Such a comparison with or use of an EA or EAF test could be appropriate for certain test substances with unusual modes of action, for programs monitoring the environmental effects of particular types of effluents, or for a particular leachate or effluent.

### 4.3.2 Test Type and Solution Replacement

Tests may be run in either a static-renewal or a flow-through mode. With many types of substances, static tests with 12- or 24-hour

[15] For an EA or EAF test, the number of embryos used per replicate and concentration will depend on a number of considerations including anticipated rates of fertilization and hatching success. This will in turn be influenced by the past experience of the laboratory using the same sources of gametes and control/dilution water. The initial number of embryos will also be influenced by such items as the available volume of test sample, volume and type of test chamber, the endpoints to be monitored, and whether organisms are needed for extra measurements such as contaminants in the body (see footnote 25). The minimum number of three replicates per concentration and, accordingly, 40 embryos per replicate, requires 120 embryos per concentration; whereas three replicates and 80 embryos per replicate requires 240 embryos per concentration.

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 biochemical oxygen demand, volatility, or instability, use of a flow-through test with rapid replacement of test solutions might be necessary.

In static-renewal tests, solutions are changed daily or more frequently, and there are two procedures for doing that:

1. prepare new solutions in clean test chambers, 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 renewed by siphoning 80%, then replacing it to the original volume.

The latter procedure should be used in static-renewal E tests, and during the first two weeks or so in the EA or EAF tests. Old solutions should be siphoned out cautiously and new solution added slowly, 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.

Flow-through tests require a system that continually delivers a series of pre-mixed concentrations of the wastewater or other test substance to the test chambers, at a controlled rate. Various devices might create successive dilutions of a stock solution or test substance by means of metering pumps or proportional diluters.

The flow rates of test solutions, or stock solutions and control/dilution water, should be checked daily throughout the test, and should not vary by more than 10%.

The minimum amount of test solution for each replicate is governed by one of two requirements; calculations must be done for both, and the one requiring the most new test solution must be adopted. The first requirement is based on biomass of organisms in the replicate; the amount of new test solution required each day increases in direct proportion to greater biomass. For this requirement, amounts of test solution needed will be the same in a continuous-flow test as in a static-renewal test. The second requirement is that every 24 hours, most of the old test solution in a container must be replaced with new test solution. The relative volumes required in continuous-flow and static-renewal tests will depend on the standing volume in the test container. As the standing volume becomes greater, the second requirement (for replacing it) tends to dominate, and the first requirement based on biomass tends to become less important. Accordingly, the investigator should not choose the size of container arbitrarily, but should make the calculations and decide on a suitable standing volume in the container, keeping in mind the amount of sample or test chemical that is available.

These two absolute requirements for amount of new test solution are minima. Use of minimum replacement might, in some cases, result in lower measured toxicity than would be found with a more generous supply of test solution. It is also quite possible that other items could come into play and increase the needed amounts of new solution. For example, more test solution might be required if the tests showed signs of oxygen depletion.

The biomass requirement is that there should be at least 0.5 L/g of embryo or alevin, every day (i.e., ≥0.5 L/g·d), and for swim-up fry there must be ≥0.5 L/g·d. This can be estimated for the maximum biomass expected during the test, or adjusted periodically through the longer tests. For instance, in an EA test using rainbow trout, 40 alevins of medium size (say 125 mg, as indicated in Appendix D) would represent 5 g in a replicate vessel. Therefore, at least 2.5 L of new test solution should be provided every day, in either a static-renewal or flow-through test. The flow rate to each replicate should be set to deliver that amount if it is the governing requirement.

The second requirement is to replace at least 80% of the test solution in each container every day. In static-renewal tests, a chamber would normally contain a volume of solution that equalled or exceeded the required daily supply for biomass, of 0.5 L/g·d. Every 24 hours or less, 80% of that standing volume must be renewed, according to the methods previously described. More frequent renewal of static solutions might be necessary, depending on the nature of the substance being tested.[16] In a continuous-flow test, to achieve 80% molecular replacement of the old test solution, the daily volume of inflow to the test chamber must equal or exceed 1.6 times the volume of standing liquid in the chamber, assuming that there is complete mixing within the chamber (Sprague, 1973).

Some examples can be given. If the container is set to hold 2 L of test solution in a static-replacement mode, then every day, 80% of that volume would be replaced, i.e., 1.6 L. This is less than the 2.5 L required to satisfy biomass in the previous example, but that would not necessarily always be the case. In a continuous-flow test with 2 L in each container, the daily inflow would have to be 1.6 × 2 L = 3.2 L, more than that for a static-renewal test and more than that in the biomass example. If 1 L within the container were satisfactory for covering the embryos, then the amount could be adjusted and the required daily inflow would only be 1.6 L. A tactic of reducing the standing volume and having a relatively large continuous inflow might be desirable if volatile toxicants were present in the test substance.

### *4.3.3 Temperature*

The rate of early development of rainbow trout and other species 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 E test, the daily mean temperature must be 14 ± 1.0°C for rainbow trout embryos (Fennell *et al.*, 1998); and the instantaneous temperatures of the replicate groups must not vary by more than 3°C at any time. This temperature range, although higher than the optimum for the embryos, is still within the acceptable range for successful 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 (Yee *et al.*, 1996).

Throughout an EA or EAF test, daily mean temperature to which each life stage is exposed (i.e., embryos and alevins in EA

[16] Test solutions of substances which are highly volatile or rapidly degrade will need to be renewed more frequently, perhaps at 12-h or even 6-h intervals. Tests involving unstable wastewaters might best be performed on-site, using flow-through conditions for frequent renewal of each test solution.

test; embryos, alevins, and fry in EAF test) must be 14 ± 1°C (Fennell *et al.*, 1998). Additionally, instantaneous temperatures for the replicate groups must not vary by more than 3°C at any time.

Sample/solution temperature must be adjusted as required to attain an acceptable value for each solution (14 ± 1°C). Samples or test solutions must not be heated by immersion heaters, since this could alter chemical constituents and toxicity. Temperature must be determined by measurements in representative test chambers (i.e., in at least the high, medium, and low concentrations plus control solutions if a multi-concentration test). For a static-renewal test, measurements must be made and recorded at the beginning and end of each 24-h (or earlier, if used) period of exposure, in both the fresh test solution and the used solution just before it is changed. For a flow-through test, measurements must be made and recorded daily. In addition, it is recommended that the temperature of at least one test solution be measured continuously throughout the test.

#### *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, the water should be aerated vigorously to achieve this.

Pre-aeration (before exposure of test organisms) or aeration (during exposure) of each test solution might be required or appropriate, depending on the test substance, type, and objectives (see Sections 3.3, 4.3.2, 5.3, 6.3, and 7.3). Apparatus for exposing embryos and alevins to test solutions, with or without aeration, is described in Section 3.3.

If pre-aeration is done (see Sections 5.3, 6.3, and 7.3), each aliquot of sample or solution used for renewal should be pre-aerated[17] for 30 minutes at a rate of 6.5 ± 1 mL/min·L. Immediately thereafter, the dissolved oxygen content of the sample or solutions should be measured. If (and only if) the measured value in one or more solutions is <60% or >100% of air saturation, the pre-aeration of either sample or all test solutions (including the control) should be continued at the same rate (i.e., 6.5 ± 1 mL/min·L) for an additional period not to exceed 90 minutes. This additional period of pre-aeration must be restricted to the lesser of 90 minutes and attaining 60% saturation in the highest test concentration (or 100% saturation, if supersaturation is evident).[18] Immediately thereafter, fish must be exposed to each test solution, regardless of whether 60 to 100% saturation was achieved in the sample or all test solutions. Any pre-aeration must be reported, including the duration and rate (Section 8).

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[17] A volume of sample or of each test solution, adequate to prepare or renew all replicate groups (see Section 4.3.2), should be pre-aerated in a nontoxic container of a suitable size. Pre-aeration should use oil-free compressed air dispensed through a narrow-bore pipette, capillary tubing, or a commercial air diffuser. 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 the sample or test solutions, or might increase their rate of oxidation and degradation to other substances. However, pre-aeration of sample or test solutions before exposure of test organisms could be necessary due to the oxygen demand of the test substance (e.g., oxygen depleted in the sample during storage). If it is necessary to pre-aerate any test solution, *all* solutions must be treated in an identical manner. Similarly, if it is necessary to aerate within any test chamber during the test, *all* solutions including the controls must be treated in the same manner.

For a static-renewal test (see Section 4.3.2), each test solution including the controls should be aerated continuously throughout the test to ensure an ongoing exchange of solution across the developing embryos or alevins. The rate of aeration of each test solution must be minimal and controlled, to avoid undue stripping of volatile toxicants and/or excessive and uncontrolled detoxification of oxidizable toxic constituents. If a group of test organisms is exposed to a discrete container of solution having a volume ≥6 L, an aeration rate of 6.5 ± 1 mL/min·L can and should be provided using conventional air-control valves and aeration apparatus (see footnote 7). If the volume of test solution is <6 L, this low rate of aeration cannot be achieved or controlled with conventional air-control valves. Accordingly, such low volumes of test solution should be aerated gently through a narrow-bore (e.g., 0.5 mm ID) aperture at a rate which does not exceed 100 bubbles per minute (EC, 1992b; USEPA, 1994). Section 3.3 describes an appropriate apparatus for aerating a static-renewal setup (see footnote 7 and Figure 3B).

A flow-through test (Section 4.3.2) can be performed with or without aeration of the test solutions, since the continuous flow of fresh solution to each test vessel provides an ongoing exchange of solution across the developing embryos or alevins. Section 3.3 describes and illustrates (Figure 3C) a suitable apparatus for conducting a flow-through test with or without aeration. The nature of the test substance (e.g., volatility, oxygen demand, stability) should be considered when deciding if a flow-through setup is appropriate and whether or not to aerate. Depending on the oxygen demand, gentle aeration of each test solution might be necessary during flow-through tests to maintain dissolved oxygen at adequate levels of 60 to 100% saturation (see Section 6.3). If aeration is used, each replicate solution (including the controls) must be aerated at a similar and controlled rate, as previously described. Alternatively or additionally, more rapid renewal of solutions might be required to maintain DO at 60 to 100% 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, a flow-through setup would be used (see Sections 3.3 and 4.3.2), and no aeration of test solutions would be provided during the test.

Dissolved oxygen (DO) 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 the control(s) and the high, medium, and low concentrations. In flow-through tests, DO must be measured in each replicate at the start of the test, as well as daily thereafter in at least the control(s) and the high, medium, and low concentrations.

Oxygen in the test vessels should not fall below 60% of saturation. If it does, the investigator should be aware that the test is not measuring the toxic quality, *per se*, of the substance being tested. Rather, such a test would measure the total effect of the substance (e.g., effluent) including its deoxygenating influence.[19] Initial

[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

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 or continuous 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.

### *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 immediately before and immediately after each renewal in static-renewal tests, and 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 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 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 minutes 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

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(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 or >8.5 might be detrimental in terms of mortality, abnormal behaviour, and poor growth in alevins and older life stages (Gordon *et al.*, 1987).

the toxicant(s) and their susceptibility to detoxification (USEPA, 1991a; b).

### *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 semipermeable 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).

The transition from newly fertilized egg to an embryo in its initial stages of development, before the egg membrane becomes relatively impermeable (until ~2 h 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 should start as soon as possible after fertilization has taken place, and must start within the 30-minute period immediately after the complete dry-mixing of eggs and milt, for which a minimum of 5 minutes and a maximum of 20 minutes are allowed (Section 4.2).

For the transition stage from embryo to alevin (EA or EAF test only), the start of the alevin stage is defined as the time when 50% of the initial number of eggs have hatched. The observer is not likely to record a time for exactly 50% hatch; in practice, a time is adopted when the hatch is first seen to include at least one-half of the embryos, and fairly close to 50%. In the EA test, when 50% of the initial number of control eggs are first seen to have hatched, it is considered that the alevin stage has started, and the test ends after a further seven days. At the end, a complete count is made of successful alevins in each replicate, in order to deduce the number and percentage of nonviable alevins (i.e., those which were unfertilized, died as embryos, failed to hatch, or developed abnormally).[22]

The start of the swim-up fry stage is defined as the time when 50% of the surviving fish exhibit swim-up behaviour.[23] In the EAF test, one phase of the test ends and the final phase begins, when 50% of the surviving control fish are seen to exhibit swim-up behaviour. At that time, there must be a

[21] Some toxic substances might also diffuse through the membrane after water hardening, and could thus exert a toxic effect on the developing embryo beyond this critical period.

[22] Other observations might be made, particularly the percent hatch in all replicates at the time of 50% hatch in the control. Replicates with <50% hatch might be monitored to record the time at which 50% hatch is achieved. Those observations would be useful in a narrative statement on delayed hatching which is part of the test documentation. At the end of the test, the developmental stage of remaining embryos might be ascertained (see Section 4.4), which would allow more detailed comparisons of development rate among concentrations.

[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.

count of the total numbers of alevins, deformed alevins, and swim-up fry in each replicate, after which the alevins are discarded.[24] Some or all of the surviving swim-up fry in each replicate are released from the incubation unit(s) into the test chamber. The number of fry to be used, and the possibility of thinning, is discussed in Section 4.3.7.[25] Feeding of fry is initiated (see Section 4.3.8) and continued for 29 consecutive days. Then fish are not fed for 24 hours, the exposure is ended, and mortalities, abnormalities, and average weight of fish surviving in the replicate are documented (see Section 4.4).

### *4.3.7 Fertilization Success and Thinning*

For any E, EA, or EAF test, an early indication of fertilization success and control viability can be obtained a few 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 28, Section 4.4) for the incidence of nonviable embryos. If the mean percentage of nonviable control embryos (including unfertilized eggs) is >30% at this time, the investigator must end the test, and restart it using another population of freshly fertilized eggs.

Successful fertilization, survival through hatching, and larval development can vary widely among various batches of gametes. Although it is desirable to have 100% fertilization and 100% control survival, such success is rarely achieved.

*Thinning* refers to the random removal of a number of individual test organisms from one or more replicates, to reduce crowding, maintain an acceptable loading density, and/or minimize the volumes of test solution required during each renewal (Section 4.3.2). Thinning must not be done at any time during an E or EA test, or during the embryo or alevin stages of an EAF test. It might seem desirable to start with an excess number of eggs, and select equal numbers of viable embryos when it is possible to distinguish them from apparently infertile eggs because of the possibility of poor fertilization success in all concentrations including the control. This must not be done, however, because it could compromise the validity of statistical tests. Exposure to the toxicant before thinning could influence the viability in some concentrations, creating a bias in the choice of organisms. There is only one time in the EAF test when thinning can be done if desired, i.e., when starting the final phase of this test for survival and growth of fry (see Section 4.3.8).

When preparing for an EA or EAF test, preliminary studies are recommended. Such studies should determine the maximum

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[24] Since it can be difficult to judge 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 test chamber for easier observation. It is not advisable to release alevins into the test chamber prematurely.

[25] Numbers of swim-up fry per replicate used for this phase of the study will depend on a number of considerations, including the minimum daily solution-renewal rate (i.e., ≥0.5 L/g·d), fish size at this stage, sample/solution volume requirements, and numbers of fish surviving in each replicate. The use of more than 10 fry per replicate (e.g., 15 to 30), if manageable, should improve test sensitivity.

Depending on the objectives of the study and the nature of the test substance, subsample(s) of ≥10 surviving fry from each replicate in the EAF test might, in some instances, be removed at this stage. These subsamples might then be frozen or otherwise treated in preparation for analyses of tissue burdens of specific contaminant(s). Random selection should be maintained, following the guidance for thinning in Section 4.3.7.

number of embryos that can be placed initially in each incubation unit without causing detrimental effects from crowding (such as insufficient oxygen or accumulation of metabolic waste). By distributing the embryos only one layer thick on the bottom of the incubation unit, efficient recognition and counting of viable versus nonviable embryos (including unfertilized eggs) will also be facilitated. The maximum number for an incubation unit should be determined for the embryo size, flow rate, dimensions of the incubation unit, amount of test solution provided to the test chamber, and the expected size of the alevin or fry at the end of the test. In cases where more than one incubation unit is suspended in a test chamber, embryos or alevins may be moved among the incubation units within the same test chamber to distribute them evenly. However, organisms must not be transferred from one test chamber (i.e., replicate) to another.

In the EAF test, thinning of swim-up fry may be done before starting the final 30-day exposure, i.e., at the time when 50% of the control organisms are seen to exhibit swim-up behaviour. Thinning might be done to maintain the biomass requirement and to minimize the associated sample and solution volume requirements (Section 4.3.2). Thinning might also be done to achieve better balance in numbers, e.g., to attain an identical number of individuals per replicate for the final phase of an EAF test. The extent of thinning may be independent for each replicate, and it is not required that the degree of thinning be balanced among replicates or concentrations. The number of fry within a given replicate must, however, be reduced in a random manner. Thinning cannot be done during the 30-day exposure; that would render the test invalid.[26]

There are advantages in retaining all the fry, rather than thinning, if facilities and amount of test substance allow. Other factors being equal, larger numbers of test organisms produce narrower confidence limits on endpoints, for example on the LC50 for fry. If thinning is done, ideally it should result in the same large number of fry in each replicate. Preferably, each replicate should retain ≥10 fry, but lower numbers could be used if necessary, as long as they meet the minimum requirements listed in Section 4.3.8.

### *4.3.8 Final Phase of EAF Test*

When 50% of the surviving fish in the control of an EAF test are seen to have attained swim-up status in the EAF test, one phase of the test ends and the final 30 days of exposure commences. A count is made of the numbers of alevins and deformed alevins in each replicate, after which the alevins are terminated. Those data are used for narrative statements on the results of the EAF test (see Section 4.4). The number of individuals in each replicate that are

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[26] Also, thinning cannot be done at any earlier time, or in the E or EA tests. Thinning part-way through an exposure is most unlikely to be possible in a balanced and unbiased manner, because the cumulative effects of exposure would differ in the different concentrations. Accordingly, the investigator must ensure that the minimum daily solution-renewal rate per gram of biomass (i.e., ≥0.5 L/g·d) will be met as the organisms develop. Thinning is allowable just at the start of the 30-day exposure of fry because that time marks the end of one phase of the test, with its observations of effect, and the beginning of a separate phase of the test. There might be some carryover of effects from the first to the second phase of the test, through the relative health of the swim-up fry from the various concentrations, and that is accepted as part of the result.

*nonviable at swim-up* (see Section 4.4) is also determined and recorded at this time, as the first endpoint of the EAF test (Section 4.4).

The swim-up fry existing in all replicates should be released from the incubation unit into their test chamber, and counted. These are the fish that are used for the last phase of the test. There could be thinning of these fry if necessary or desired (Section 4.3.7). Thereafter, these groups of fish are used for subsequent observations of mortality, behaviour, and growth. At least five swim-up fry must be present in a replicate; if not, that replicate is excluded from the final (30-day) phase of the EAF test. At least two replicates must be available for a given concentration; if not, that concentration is excluded from the exposure. There must be at least two replicates of the control, each with ≥5 fry; without that, this final 30-day part of the EAF test cannot be done. Fry must not be transferred among replicates to make up the requirements. The test may proceed with unbalanced numbers of replicates and/or unbalanced numbers of fry per replicate.[27]

Feeding is initiated in each replicate and continued for 29 days, then fish are not fed during the final day, the 30-day exposure is terminated, and all final observations and measurements are made (Section 4.4).

A commercial starter feed suitable for rainbow trout swim-up fry 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. Newly hatched brine shrimp may also be used.

The bottom of each test chamber should be siphoned daily to remove any excess food or faeces that have accumulated. For static-renewal tests, this procedure can be combined with the daily siphoning and replacement of each test solution. Care should be taken during siphoning to avoid any injury to the fish. The inlet to the siphon tube should be screened to avoid drawing fish into the tube during this procedure.

### *4.3.9 Reference Toxicant*

The routine use of a reference toxicant or toxicants is practical and necessary to assess, under standardized conditions, the relative sensitivity of the group of embryos that are used, and the precision and reliability of data produced by the laboratory for the reference toxicants (EC, 1990a). Sensitivity of embryos to the recommended reference toxicant(s) must be evaluated at the time that each E, EA, or EAF test is performed, using a portion of the same group of freshly-fertilized eggs used to start that test. The concurrent reference toxicity test undertaken at the start of either of these tests should be an E test because of the long duration of an EA or EAF test.

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

- chemical readily available in pure form;
- stable (long) shelf life of chemical;

[27] For the final (30-day) phase of an EAF test, there may be unequal numbers of fry in the replicates and in the concentrations, as well as unequal numbers of replicates. That is less desirable than having balanced numbers of individuals and balanced numbers of replicates, but results can still be used to estimate an endpoint. Allowing unequal numbers of fry per replicate (each with ≥5 fry) and/or unequal numbers of replicates per concentration (≥2 replicates/concentration) will, in some tests, enable this phase of the test to include certain (high) concentrations which might otherwise be excluded.

- highly soluble in water;
- stable in aqueous solution;
- minimal hazard posed to user;
- easily analyzed with precision;
- good dose-response curve for salmonid embryos;
- knowledge of the degree and type of any influence of pH on toxicity to test organism; and
- knowledge of the degree and type of any influence of water hardness on toxicity to rainbow trout embryos.

Reagent-grade phenol and/or zinc (prepared using zinc sulphate) are recommended for use as the reference toxicant(s) for this test. Sensitivity of rainbow trout embryos to one or both of these reference toxicants should be evaluated using E test(s), and the EC50 determined for one or both of the chemicals (see Section 4.5).

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

The same procedures and conditions (e.g., static-renewal or flow-through test; same source of control/dilution water) should be used within a testing facility each time that the reference toxicity test is performed. Embryo tests with one or more reference toxicants would normally use the control/dilution water that is used at the laboratory for the definitive E tests. Alternatively, if a greater degree of standardization is desired, soft reconstituted water should be prepared (hardness 40 to 48 mg/L as $CaCO_3$, pH 7.2 to 7.5; see footnote 37, Section 5.4). This should be used for controls and dilutions (USEPA, 1985b; EC, 1990b).

A warning chart (EC, 1990; 1998b) must be prepared and updated for each reference toxicant used. Successive ICps are plotted on this chart and examined to determine whether the results are within ±2 SD (= warning limits) of values obtained in previous tests using the same reference toxicant and test procedure. The mean and standard deviation of available log EC50s is recalculated with each successive test until the statistic stabilizes (EC, 1990; 1998b). The warning chart should plot logarithm of EC50 on the vertical axis against date of the test (or test number) on the horizontal axis.

The logarithm of concentration (log EC50) should be used in all calculations of mean and standard deviation, and in all plotting procedures. This simply represents continued adherence to the assumption by which each EC50 was estimated on the basis of logarithms of concentrations. The warning chart may be constructed by plotting the logarithms of the mean and its limits on arithmetic paper, or by plotting arithmetic values on the logarithmic scale of semi-log paper. If it were definitely shown that the EC50s failed to fit a log-normal distribution, an arithmetic mean and limits might prove more suitable.

Each new EC50 for the reference toxicant should be compared with the established warning limits of the chart; it is considered acceptable if it falls within the warning limits.

If a particular EC50 falls outside the warning limits, the sensitivity of the embryos and the performance and precision

of the test are suspect. Since this might occur 5% of the time due to chance alone, an outlying EC50 does not necessarily mean that the sensitivity or precision 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.

Results that remained within the warning limits would not necessarily indicate that a laboratory was generating consistent results. Extremely variable data for a reference toxicant would produce wide warning limits; a new data point could be within the warning limits but still represent undesirable variation. A coefficient of variation of no more than 30% is tentatively suggested as a reasonable limit by Environment Canada (1990).

Stock solutions of phenol should be made up on the day of use. Stock solutions of zinc should either be made up just before their use, in which instance preservation is unnecessary; or acidified with nitric acid to pH <2 if stored (APHA *et al.*, 1995). If stored, acidic zinc solutions should be held in the dark at 4 ± 2°C, and in that state they may be stored for several weeks before 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. The concentration of zinc should be expressed as mg $Zn^{++}$/L.

Concentrations of reference toxicant in all stock solutions should be measured chemically using appropriate methods (e.g., APHA *et al.*, 1995). 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 if the EC50 was found to be outside warning limits. If stored, sample aliquots must be held in the dark at 4 ± 2°C. Both zinc and phenol solutions should be preserved before storage, following the appropriate guidance given in APHA *et al.* (1995). 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 EC50 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 all tests, any obviously dead (i.e., opaque) embryos, alevins, or fry should be removed as soon as they are noted, and their numbers recorded. Live individuals must not be removed, whether or not they are deformed. In particular, developing embryos which are not obviously dead but appear atypical, should not be disturbed or removed for microscopic examination until the end of the test (if an E test) or at least until the eyed stage is reached (if an EA or EAF test). 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). In particular, extreme care must be taken until the eyed stage, to avoid disturbing the other embryos.

In all tests, daily tabulations should be made of any individuals removed from each replicate. In the longer tests, daily tabulations would also include the number hatched, the number exhibiting swim-up behaviour, the numbers of alevins and fry

with deformities, and the number of fry showing abnormal behaviour. Abnormal behaviour includes uncoordinated swimming behaviour, atypical quiescence, atypical feeding behaviour, hyperventilation, and loss of equilibrium.

Routine measurements of test conditions must be carried out as outlined in other sections. Temperature and dissolved oxygen must be measured daily in representative test chambers (Sections 4.3.3 and 4.3.4). Dissolved oxygen must also be measured in each sample and/or test solution before the test, and pre-aeration applied if required or appropriate (Section 4.3.4). The pH must be measured in the control solutions and in representative test chambers at the beginning of the test before embryos are added, and should be measured daily thereafter (Section 4.3.5). For a multi-concentration test, measurements of temperature, dissolved oxygen, and pH must include at least the high, medium, and low concentrations plus the control solution(s). Certain measurements of conductivity are recommended in Section 4.2.

For the E test, observations of the number and percentage of nonviable embryos, including unfertilized eggs, and living but obviously deformed embryos (e.g., those with two heads), must be recorded in each replicate at the end of the test, normally seven days after fertilization. Upon completion of the exposure, the group of embryos and unfertilized eggs remaining in each incubation unit should be transferred together to a pre-labelled vial containing a fixative/clearing solution.[28] After clearing, the contents of the vial should be transferred to a shallow container such as a weighing boat, and examined carefully under a dissecting stereo-microscope (Yee *et al.*, 1996).

At the end of the E test, each embryo or unfertilized egg must be scored as *viable* or *nonviable*. Viable embryos appear to have developed normally to the stage typical for the controls. Those scored as nonviable would include eggs that were apparently unfertilized, embryos with marked retardation in rate of development, and obviously deformed or otherwise atypical embryos, including twins. Any unfertilized eggs, or embryos which turned opaque and were removed before the end of the test, must be included in the count of nonviable embryos. If the count indicates some missing individuals compared to the starting number, they must also be included in the nonviable category. Observations should start with the control groups, to gain familiarity with the appearance of normal, developing embryos. Yee *et al.* (1996) provide a series of colour photomicrographs

[28] Embryos and unfertilized eggs should be placed in one of the following solutions until they become clear: a saturated salt (NaCl) solution; Stockard's solution (an 85:6:5:4 mixture of water, glycerin, formalin, and glacial acetic acid; ASTM, 1991a); or a 1:1:1 mixture of glacial acetic acid, methanol, and water. After clearing, they are examined under a dissecting microscope for evidence of cleavage of the germinal disc, or of a white streak which is the embryo. If vials containing cleared eggs or embryos become cloudy during storage, they can be "re-cleared" before microscopic examination by adding an additional quantity of salt.

The use of vital staining might prove advantageous for monitoring embryonic development, differentiating viable from nonviable embryos, and confirming the exact stage of development at test end. This procedure could be implemented with one or more replicates per concentration, set aside for this purpose, if it were desired to monitor development. It might require some "clearing" of the chorion by dissection, before examining the embryos (Birge, 1992).

to assist in distinguishing viable from nonviable embryos, and further information is given in Vernier (1969) and Velsen (1980).[29]

In the EA test, the number and percentage of nonviable alevins in each replicate must be determined and recorded seven days after 50% hatch is seen to be achieved in the control, marking the end of the test. All individuals are classified as *viable alevins* or *nonviable alevins*. Nonviability includes failures at any stage: non-fertilization of eggs; mortality as an embryo or alevin; failure to hatch by the end of the test; and obviously deformed or otherwise atypical embryos or alevins (e.g., two-headed individuals). If the count indicates some missing individuals compared to the number which started in the replicate, they must also be included in the *nonviable alevin* category.

For each replicate, observations are made seven days after 50% hatch is seen to be achieved in the control. There are counts for each replicate, of the number of apparently unfertilized eggs, number of dead embryos, number of live embryos, number of dead alevins, number of "living but deformed or otherwise atypical" alevins, and number of "living and apparently normal" alevins.

Narrative statements must be made for one or both of the following two categories of effect during an EA test, for which there are no formal endpoints. In each case, there must be a brief narrative statement describing apparent differences from the control, or lack of difference. Approximate numerical data on differences should be given in the statement, or in tabular form if appropriate.

- *Delayed hatching:* useful comparisons with the control could be: (a) approximate times to median hatch in each concentration; and/or (b) approximate percentage judged to have hatched in each concentration at the time of median hatch in the control.

- *Deformed alevins:* approximate percentage of deformed alevins in each concentration including control. Tally deformities throughout test and sum to obtain the total number of deformed alevins (living plus dead). Express as a percentage of the number that hatched in that concentration.

Other observations could be made and reported, if it were desired to increase the kinds of information provided by the test. These might include the proportions of nonviable embryos, and the mortality of alevins after hatching. When an E test is done in parallel with an EA test, it would provide information for the earlier stages of development.

In the EAF test, the first phase provides an endpoint based on nonviability at time of swim-up, and the second phase provides discrete endpoints on mortality and growth of fry.

For each replicate in an EAF test, the number and percentage of test organisms

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[29] During the initial few days of the test, it is difficult to distinguish unfertilized eggs from developing embryos, even after their clearance and microscopic examination (see footnote 28). Beyond this brief period, 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 (eyed) 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 the central nervous system.

that are *nonviable at swim-up* must be determined and recorded when 50% swim-up is seen to be achieved in the control groups. Scoring as *nonviable at swim-up* includes failure at any stage until early swim-up: non-fertilization of eggs; mortality as an embryo, alevin, or early swim-up fry; failure to hatch; and obviously deformed or otherwise atypical embryos, alevins, or early swim-up fry. If the count indicates some missing individuals compared to the number which started in the replicate, they should also be included in the *nonviable at swim-up* category.

The observations are made when 50% swim-up is seen to be achieved in the control. There are counts for each replicate, of the number of apparently unfertilized eggs, number of dead embryos, number of live embryos, number of dead alevins, number of "living but deformed or otherwise atypical" alevins, number of "living and apparently normal" alevins, number of dead swim-up fry, number of "living but deformed or otherwise atypical" swim-up fry, and number of "living and apparently normal" swim-up fry. All alevins are then discarded, marking the end of the first phase of the EAF test.

The second phase of the EAF test begins at this time. This phase is a discrete 30-day exposure that includes feeding, and measures mortality and weight of fry. If thinning is to be done on the number of fry, it is performed at this time, before the 30-day exposure (see Section 4.3.7). The fry may either be held in the same open test chambers used for the first phase of the test, or transferred to other (larger) test chambers if necessary to prevent the biomass requirement (i.e., $\geq$0.5 L/g·d; see Section 4.3.2) from being exceeded as the fish feed and grow throughout this phase of the test.

After 29 consecutive days of feeding, the exposure continues for another day without feeding. The number of fry that died in each replicate during the 30 days is then tabulated. The total dry weight (after 24 hours at 60°C) of the group of surviving fry in each replicate must be recorded to the nearest 0.01 g. Average dry weight of surviving fry is calculated.

Observations during an EAF test must enable narrative statements on the following three categories of effect, which do not have formal endpoints. In each case, there must be a brief narrative statement describing apparent differences from the control, or lack of difference. Approximate numerical data on differences should be given in the statement, or in tabular form if appropriate.

- *Deformed alevins:* approximate percentage of deformed alevins in each concentration including control, as in the EA test.

- *Delayed swim-up:* useful comparisons with the control could be: (a) approximate times to median swim-up in each concentration; and/or (b) percentage judged to exhibit swim-up behaviour in each concentration at the time of median swim-up in the control.

- *Abnormal behaviour of fry:* report degree and type of abnormal behaviour.

Other observations could be made and reported, if it were desired to increase the kinds of information provided by the test. These might include the proportions of nonviable embryos, delayed hatching (as in the EA test), and mortality of alevins after hatching.

## 4.5 Test Endpoints and Calculations

### 4.5.1 Biological Endpoints

Biological endpoints to be estimated in this test depend on the option chosen (E, EA, or EAF), as indicated in the following tabulation.

- The E test assesses *nonviable embryos*, i.e., developmental failure in embryos which occurs sometime during the exposure period which starts immediately after fertilization. One or both of the following two endpoints are estimated for the same effect: (1) *effective concentration for 25% nonviable embryos* (EC25)*;* and (2) *median effective concentration for nonviable embryos* (EC50).

- The EA test is based on *nonviable alevins*, i.e., failure to reach the alevin stage in a timely and normal manner because of deterioration at any previous stage, including failure of egg fertilization, mortality as an embryo or alevin, failure to hatch by the end of the test, and abnormal development. One or both of the following two endpoints are obtained for the same effect: (1) *effective concentration for failure of 25% of individuals to develop normally to the alevin stage* (EC25); and (2) *median effective concentration for failure to develop normally to the alevin stage* (EC50).

The EAF test has the following three endpoints. The most sensitive effect (i.e., the endpoint with the lowest concentration) is taken as the definitive indication of toxicity (Woltering, 1984; Birge and Black, 1990).

- *Nonviable at swim-up* includes failure to survive at this or any previous stage: failure of egg fertilization; mortality of embryos; failure to hatch; mortality of alevins, mortality of early swim-up fry; and obviously deformed or otherwise atypical embryos, alevins, or early swim-up fry. One or both of the following two endpoints are estimated for this effect: (1) *effective concentration for25% failure to develop normally to the early swim-up stage* (EC25); and (2) *median effective concentration for failure to develop normally to the early swim-up stage* (EC50).

- *Mortality of fry* measures mortality within the 30-day exposure of fry in the final phase of the test. Mortality preceding that time is not included. One endpoint is estimated, the *median lethal concentration for fry* (LC50).

- *Weight of fry* measures the average dry weight of surviving fry, compared to the control, at the end of the test, after they have been exposed for 30 days in the final phase of the test. This is essentially a measurement of successful growth, except that no measurements of initial weight are made. One endpoint is estimated, the *inhibiting concentration for 25% less dry weight than the controls*, among surviving fry at the end of the test (IC25). An extra endpoint could be obtained by estimating the NOEC and LOEC and calculating the TOEC, if desired and if enough replicates were available.

Several narrative reports must be made on additional observations during the EA and EAF tests, as listed in the following text. These are not formal endpoints of the tests and do not require rigorous counting and statistical procedures. Nevertheless, the

statements are required as part of the documentation of the test (see Section 8). In each case, there must be a brief narrative statement describing apparent differences from the control, or lack of difference. Approximate numerical data on differences should be given in the statement, or in tabular form if appropriate. Some of these observations might help explain the results for the formal endpoints, previously listed. An apparent difference from the control in any of these items is taken as an indication of toxicity, but cannot be considered definitive in the absence of formal statistical analysis.

- *Delayed hatching* (EA test). Useful comparisons with the control could be: (a) times to median hatch in each replicate or concentration; and/or (b) percent hatching at the time of median hatch in the control.

- *Deformed alevins* (EA and EAF tests). This is the number of deformed alevins throughout test (living plus dead individuals), as a percentage of the number that hatched in that concentration.

- *Delayed swim-up* (EAF test). Useful comparisons with the control could be: (a) times to median swim-up in each replicate or concentration; and/or (b) percent swim-up at the time of median hatch in the control.

- *Abnormal behaviour of fry* (EAF test). The prevalence and type(s) of abnormal behaviour are to be reported.

Other observations could be made if more information were desired, but these also would not be considered formal endpoints of the test. The other items could include proportions of nonviable embryos (EA and EAF tests), delayed hatching (EAF test), and mortality among alevins after hatching, as discrete observations within the alevin phase of development (EA and EAF tests).

### *4.5.2 Effective and Lethal Concentrations*

For toxic effects using EC50, EC25, or LC50 (Section 4.5.1), the following steps apply when calculating the endpoint.

- Count the affected (or missing) individuals by replicates, but combine the numbers from the replicates at a given concentration.

- The EC50/LC50 cannot be estimated unless one concentration results in an effect $\geq$50%. The EC25 cannot be estimated unless one concentration results in an effect $\geq$25%.

- For EC50/EC25, use Abbott's formula on the combined numbers, to allow for a reasonable control effect (see the following text). This applies to endpoints that incorporate success of fertilization.

- Use probit analysis to calculate EC50 and EC25, or LC50, and their 95% confidence limits.

- If results do not satisfy the requirements for probit analysis, use the binomial method to calculate the EC50/LC50, and the range that would encompass the 95% confidence limits. Estimate the EC25 less formally by interpolation or by other acceptable quantal statistics.

Comments on each of those steps follow.

*Replicates:* the affected (or missing) individuals are counted by replicates, but then the numbers at a given concentration

are combined. The procedure uses three replicates for convenience in handling and achieving desired loading during the test, and as insurance in case of accidental loss or other problem in one test chamber. The best use of the ensuing data is to combine the replicates to obtain larger numbers of individuals in a single analysis, which provides narrower confidence limits.

*Restrictions on data:* it is not valid to estimate the endpoints by extrapolation from low levels of effect. The EC50/LC50 cannot be estimated unless at least one concentration results in an effect ≥50%. Similarly, the EC25 cannot be estimated unless one concentration results in an effect ≥25%.

*Abbott's formula* (see Finney, 1971, or EC, 1998b)*:* unless indicated otherwise in Environment Canada (1998b), this formula should be used when calculating ECx in the E and EA tests, and for nonviability at swim-up in the EAF test. The formula corrects the effect in each test concentration for the percent effect in the controls, helping to adjust for the variable and gamete-dependent differences in fertilization from test to test (Yee *et al.*, 1996). The formula is applied after the data from replicates have been combined.

A limit of 30% failure of fertilization must be met for validity of each of these tests (Section 4.6). The same value applies to the control results in the E test for nonviable embryos, and Abbott's formula should be used for any effect up to 30% in the control. The longer EA test for nonviable alevins allows up to 35% effect in the controls at the end of the test, before it is considered invalid (Section 4.6), and Abbott's formula should be used to correct for the effect. The still longer EAF test for viability as swim-up fry allows up to 40% effect in the control before it is considered invalid (Section 4.6), and Abbott's formula should be used to correct for the control effect.

Abbott's formula must not be used to correct for control mortality in the second phase of the EAF test with swim-up fry unless advised otherwise in Environment Canada (1998b). If control mortality exceeds 20% during that 30-day phase of the test, the test is invalid (Section 4.6). There would be little advantage in using Abbott's formula for corrections up to 20%, because it would not greatly influence the value calculated for the LC50.[30]

*Probit analysis:* the choice of statistical procedures is the same for each analysis to determine ECx or LC50. General instructions on statistical approaches are provided here; further advice is found in Environment Canada (1998b).

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[30] The rationale for use of Abbott's formula is given in an Environment Canada statistics guidance document (EC, 1998b). Briefly, Abbott's correction can be used to adjust for a reasonable effect in the control, for those endpoints which include success of fertilization. The rate of fertilization might be poor in some of these salmonid tests (i.e., up to 30% failure, beyond which the test is considered invalid). However, fertilization occurs before the start of exposure to the test substance, and so fertilization success is not related to conditions during the test. Furthermore, fertilization success is unknown at the start of exposure, so no selection or adjustment can be done before running the toxicity test. Correction (using Abbott's formula) for a reasonable rate of failure in fertilization, therefore, is allowed in the endpoints involving fertilization.

Use of Abbott's formula is not allowed, however, in the test for lethality to swim-up fry. That is a straightforward quantal test with 30 days of exposure, and mortality in the control should be slight under good conditions.

Provided that a suitable range of test concentrations was selected, and partial effects occurred at two concentrations, probit analysis can be used. If the effect in at least one concentration does not attain 50% after use of Abbott's correction for control effect, the EC50 or LC50 cannot be estimated. Similarly, EC25 cannot be estimated unless at least one concentration achieved 25% effect. If there is no effect at a certain concentration, that information is used, being an effect of zero percent. However, if successive concentrations yield a series of 0% effects, only one such value should be used in estimating the EC50 or LC50, and that should be the highest concentration of the series, i.e., the zero-effect that is "closest to the middle" of the distribution of data. Similarly, if there were a series of successive complete effects (e.g., 100% unhatched embryos at the high concentrations in the test), only one value of 100% effect would be used, again the one "closest to the middle", i.e., the 100% effect at the lowest of those concentrations. Using additional values of 0% and/or 100% effect would likely distort the estimate of EC50 or LC50.

TOXSTAT™ (West and Gulley, 1996) or other commercial software packages can be used for standard probit analysis. They estimate EC50/LC50 and 95% confidence limits. The programs will also estimate EC25 and its confidence limits, or any other selected ECx.

A statistical program in BASIC language, adopted from Stephan (1977) and available from Environment Canada (see Appendix B), is simple to use for calculating the EC50 or LC50 with 95% confidence limits by the probit method. The program also estimates EC50/LC50 by the binomial method. The EC50 with 95% confidence limits is also estimated by the method of moving averages, but this has no advantage over probit analysis.

The EC25 should be calculated in addition to the EC50, to provide a somewhat more sensitive endpoint. Some monitoring programs, regulations, or experiments might require calculation of another endpoint such as the EC20. Commercial computer programs including TOXSTAT can be used to calculate the EC25 and its 95% confidence limits. The investigator should be aware, however, that precision decreases progressively when determining such "lesser-effect" values, and confidence limits become correspondingly wider. Estimates below EC20 are not recommended (EC, 1998b).

*The binomial method* must be used to estimate EC50/LC50 if the data do not provide at least two partial effects (i.e., between 0% and 100% response). The program of Stephan (1977), previously mentioned, is the only computer program known to be available at present, that provides the binomial method. The program accompanies that with conservative (wide) outer limits for the EC50/LC50, within which the true confidence limits would lie. Unfortunately, the Stephan program does not estimate the EC25.

A simple equivalent of the binomial estimate of EC50/LC50 can be done by hand calculation for those cases in which one concentration produces 0% effect and the next higher concentration produces 100% effect. The geometric mean of the two concentrations is a best estimate of the EC50. The two concentrations almost always represent conservative estimates of the confidence limits, but that is not invariably the case and some caution should

be expressed in offering them as probable limits. (The usefulness of the Stephan computer program is that it calculates probabilities and selects concentrations that will definitely have a wider span than the true confidence limits.) Hand calculation of the geometric mean can be done as the mean of the logarithms of the concentrations, converted back to an arithmetic value. The geometric mean can also be calculated as the square root of the product of the two concentrations that produce zero and complete effects.

The EC25 can also be calculated by hand, in those cases for which probit analysis is not valid. Calculate it using probits of the observed proportions, to interpolate to the expected probit for 25% effect. Use logarithms of concentration for the calculations. Convert the logarithm obtained for EC25 to an arithmetic value. Alternatively, estimate the EC25 graphically on logarithmic-probability paper. Log-probit paper can be purchased at some university or technical bookstores, or copied from the figure in Environment Canada (1998b). Percentages can be converted to probits from tables in Finney (1971), Newman (1995), or some handbooks of statistics.

No confidence limits on the EC25 are provided by the current binomial/hand calculation methods.

### *4.5.3 Inhibiting Concentration for a Specified Percent Effect*

The *ICp*, and in particular the IC25 is recommended as a point-estimate of the concentration causing a certain degree of effect on quantitative (graded) biological functions, such as weight of swim-up fry attained in the EAF test (Section 4.5.1). The percentage "*p"* is selected by the investigator, but is customarily 25% (or 20%) lower performance than in the control (EC, 1998b). IC25 is a formal endpoint which must be calculated in the EAF test, for average dry weight of fry after 30 days of exposure with feeding. The 95% confidence limits must also be calculated and reported for each ICp, to allow statistical comparisons with other such values.

An analysis to determine the IC25 for attained dry weight of fry should begin with a hand plot of percent lower weight compared to the control, against the logarithm of test concentration. The purpose of the hand plot is to check for reasonable results from later mathematical computations. The percent lower weight is calculated for a given test replicate from the average dry weight of fry surviving in that replicate, in relation to the overall average weight attained in the control replicates. The percent "deficit" for each test replicate should be plotted separately. The approximate IC25 should be read from an eye-fitted line. Any major disparity between the approximate graphic IC25 and the subsequent computer-derived IC25 must be resolved. The graph would also show whether a positive and logical relationship was obtained between concentration and effect, a desirable feature of a valid test (EC, 1998b).

At present, the standard computerized method for estimating the ICp with 95% confidence limits is based on smoothing and interpolation, using the program *ICPIN* (Norberg-King, 1993; USEPA, 1994; EC, 1998b). This modification of *BOOTSTRP* (Norberg-King, 1988) is included in the latest version of TOXSTAT™ (West and Gulley, 1996). ICPIN first smooths the data as necessary, then estimates the ICp by simple interpolation, and obtains the

confidence limits by a "bootstrap" method of many random resamplings from the actual observations (USEPA, 1994, Appendix M; or EC, 1998b). To use this program, Canadian investigators must either (a) enter concentrations as logarithms, or (b) if a logarithmic transformation is offered in a software package, make sure that it is actually retained for analysis. At time of writing, ICPIN appears to be the only method routinely used for obtaining an ICp with confidence limits, but linear or general-purpose regression would provide better estimates (EC, 1998b).[31] Investigators should be alert for improved methods which might become available as computerized packages for environmental toxicology.

Some common-sense limitations should be applied to estimates of the IC25. It should not be derived from an extrapolation. To estimate the IC25, there should be at least one concentration causing more than 25% lower performance than the control, and at least one concentration causing less than 25% lower performance (but still lower than the control, i.e., not 0% effect).[32] Variability is greater near the extremes of the relationship, and in particular, observed impairments of 0% and 100% would add little information for an accurate estimation of ICp.

Calculation of the ICp assumes a reduction in performance compared to the control. In some cases there could be a stimulatory effect at low concentrations (e.g., increased growth), but with an inhibitory effect at higher concentrations. Stimulation cannot be assumed to be a strictly positive or beneficial effect, any more than inhibition can always be assumed to represent a strictly negative effect. What is being measured is a difference from the norm (i.e., the control). Current thinking is divided on whether to consider stimulatory effects at low concentrations *(hormesis)* as a sublethal effect when calculating the ICp, whether to regard it as some kind of parallel "control" performance, or whether to combine it with the control performance (as is automatically done in the smoothing of the ICPIN program. The latter option is not recommended for growth of fry in the EAF test. It is suggested here, that if a stimulatory effect occurs, the test results should be reported in two ways. First, the stimulation should be treated as a deleterious deviation, and a narrative statement should be made on the degree of stimulation and the concentration(s) associated with it. Second, when entering data into the program for calculation of the IC25, the concentrations

[31] At present, ICPIN's method of smoothing and linear interpolation appears to be the only method in common use, for obtaining confidence limits on an ICp. There are some undesirable features of linear interpolation, such as a requirement that "the responses are monotonically non-increasing" (USEPA, 1989; 1994), e.g., in an EAF test, a larger size of fry should not prevail at a high concentration than at a lower concentration. That is not always the case in toxicity assays based on growth, and the correction by smoothing can bias the estimate of ICp in linear interpolation. Second, the ICp is interpolated between two bracketing concentrations, but the rest of the relationship between concentration and effect is not used in the final estimate. Third, the interpolation to estimate the ICp is done on an arithmetic basis of concentration instead of a logarithmic one, which would introduce a slight bias in deriving the ICp.

[32] The quality and distribution of other data in the test also influence the value of the estimate of an extreme ICp, and no firm guideline can be given for the required closeness of an observed data-point to the effect of interest. The spread of the confidence limits will always indicate the reliability of the ICp.

showing a stimulatory effect should be ignored by not entering them. That way, the control performance will not be changed upwards in the calculations.

### *4.5.4 NOEC and LOEC*

The hypothesis-testing approach can be used, if desired, by estimating the *no-observed-effect concentration* (NOEC) and *lowest-observed-effect concentration* (LOEC). They can be derived statistically from the same quantitative (graded) data used for estimating the IC25 for weight of fry (see Section 4.5.3). If NOEC is used, the *Minimum Significant Difference* must also be calculated and reported (see the following text).

Using NOEC/LOEC as an endpoint has certain limitations. The NOEC is not a "no-effect" concentration, but rather, it is a "no-statistically-significant-difference" concentration. The concentration that became designated as the NOEC might depend largely on sample size, number of replicates, and variability within replicates. A laboratory that had high variation, or that used few replicates, could obtain a higher NOEC than a laboratory with lower variation and more replicates.

NOEC and LOEC could be determined for the average dry weight of surviving individuals in each replicate, following the final 30 days of exposure. If there were complete mortality in a replicate, that replicate would be excluded, leading to an unbalanced analysis. Similarly, if there were complete mortality in all replicates of a given concentration, that concentration would be excluded from the analysis.

The statistical procedures to be followed are given in TOXSTAT™.[33] The 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 normally distributed 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*, which 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. This test (Williams, 1971; 1972) is recommended as a more powerful tool than *Dunnett's test*, which ignores 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' test. All of these are multiple-comparison tests, which provide estimates of the Minimum Significant Difference, the magnitude of the difference in averages that would have to exist between the control and a test concentration before a significant effect could be concluded for that

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[33] The methods of TOXSTAT™ (West and Gulley, 1996) are not detailed here because the instructions are best followed in the written description that accompanies the programs on computer disk. An up-to-date (i.e., 3.5 or later) version of TOXSTAT on disk can be purchased by contacting WEST, Inc. (2003 Central Avenue, Cheyenne, WY, 82001). 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 with logarithms or arc-sine to meet the requirements. The transformation can reduce the sensitivity of the analysis and the ability of the toxicity test to detect differences.

concentration (discussed in USEPA, 1989; 1994; and EC, 1998b).

If a set of data cannot meet the requirements for normality or homogeneity, and cannot be transformed to do so, there are nonparametric 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 nonparametric options may be used, and are powerful tools for data that are not distributed normally. The nonparametric 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 nonparametric methods.

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

The meaning of "threshold" in TOEC is in the dictionary sense, a point at which an effect begins to be observed. Undetected effects might be present at lower concentrations. The geometric mean of NOEC and LOEC is often called the *chronic value* in the United States, but that term would be somewhat misleading here. The E, EA, and EAF test options herein represent less than 10% of the anticipated life span of rainbow trout, and therefore should not be classified as chronic.

### *4.5.5 Student's t-test*

In a single-concentration test, *Student's 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 the effect measured in a standard endpoint is significantly different than the same statistic for the control (i.e., percent nonviable embryos, nonviable alevins, nonviable individuals at swimup, mortality of fry, and average weight of fry). The test could also be applied to those effects recommended for additional observations and associated narrative statements, if the effect was firmly and numerically documented (i.e., delayed hatching, deformed alevins, mortality of alevins, delayed swim-up, or abnormal behaviour of fry). Requirements for homogeneity of variance and normality must be satisfied (USEPA, 1994, Appendix B; EC, 1998b) before using the standard t-test. If the data do not satisfy the requirements, a nonparametric test could be selected with advice from a statistician; no particular test appears to have become standard practice as yet.

### *4.5.6 Tukey's Test*

In some cases, the treatments in a test 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 *Tukey's test* (one option in the statistical program TOXSTAT™; West and Gulley, 1996). Such sets of tests should report the results of each sample tested, as the percent effect for the endpoint(s) selected, expressed as a percentage of the control(s), and should determine (using Tukey's test) whether that number was significantly different from the corresponding value for 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: stability of temperature; maintenance of DO levels; the incidence in the control of nonviable embryos (E test), nonviable alevins (EA test), or nonviable individuals at swim-up (EAF test); the incidence of control mortality among fry (EAF test); and variation in control weight (EAF test).

For all tests, a failure rate greater than 30% for fertilization invalidates the test (Yee *et al.*, 1996). Direct measurement of the fertilization rate might not be available since it is not required, but the limit is implicit in the following validity requirements, with some adjustments for the longer tests.

For an E test to be valid, the average percentage of nonviable control embryos must be ≤30%. Unfertilized eggs are included in the count of nonviable control embryos, and in fact, the criterion is the same rate as allowed for fertilization failure.

For an EA test, the average percentage of nonviable alevins in the control at the end of the test must not be greater than 35%.

For an EAF test, the average percentage of nonviable control individuals at the time of 50% swim-up of survivors, must not be greater than 40%.

For a valid EAF test, there is an additional requirement that mortality of control fry during the final 30-day period of exposure must not be >20%.

## *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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[34] More specifically, it is assumed that: all items of apparatus and all substances were identical in each replicate; all concentrations 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.

*Section 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 endpoints of the E, EA, or EAF test.

## *5.1 Test Options*

Depending on objectives and regulatory requirements, a rainbow trout 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 (Sections 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 chemicals, 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 rainbow trout. The EA test might be used for such purposes as comparative screening of several chemicals for relative toxicity to rainbow trout, while the E test might be used for frequent monitoring. 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.

At the time that an EA or EAF test is set up, it is recommended that a multi-concentration E test be established and run concurrently using the test chemical and fertilized eggs from the same pool of test organisms. The findings of the E test will provide insight into the fertilization success rate for controls in the EA or EAF test, and will be useful in appraising the relative sensitivity to the test substance for the acute (E) and longer (EA or EAF) test options.

Before any frequent or "routine" use of the E test for regulatory or other programs involving the screening of chemicals for toxicity, initial comparison of its sensitivity with that of the more definitive EAF test is recommended, to confirm that results 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.[35] 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 upon receipt (e.g., chemical name, supplier, date received). Storage conditions (e.g., temperature, protection from light) are frequently dictated by the nature of the chemical. Standard operating procedures should be followed for handling and storage of a chemical, or else those recommended by the manufacturer, by a Material Safety Data Sheet, or by similar advisory information.

## *5.3 Preparing and Aerating 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 must 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 (USEPA, 1985b).

Upon preparation of each test solution including the control(s), its dissolved oxygen content should be measured. Thereafter, the test organisms should be exposed to the solutions, or else each test solution should be pre-aerated (see Section 4.3.4). In most instances, the pre-aeration (before fish exposure) and aeration (during fish exposure) of chemical solutions is not necessary nor warranted, and should be avoided unless dissolved oxygen levels go outside the range 60% to 100% saturation at any time (see Section 3.3 including footnotes 6 to 8).

Any test performed without aeration should use a flow-through setup (see Sections 3.3 and 4.3.2, and Figure 3C), to enable a continuous circulation of test solutions

[35] 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.

around the developing embryos or alevins. If pre-aeration or aeration is appropriate (e.g., dissolved oxygen in one or more test solutions is <60% or >100% of air saturation), the guidance given in Section 4.3.4 should be followed.

## *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.[36]

If a high degree of standardization is required for comparative purposes, soft reconstituted water should be prepared and used for the control and all dilutions (hardness 40 to 48 mg/L as $CaCO_3$, pH 7.2 to 7.5)[37] (USEPA, 1985b; EC, 1990b).

If the toxic effect of a chemical in a particular body of wateris 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.[38] Examples of such

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

[37] 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 for other chemicals and from other laboratories. It is desirable to test in reconstituted water, although that requires making up large volumes of water. In some laboratories, that might be feasible, at least for static-renewal tests. Soft, reconstituted water is recommended, and 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 | $CaSO_4.2H_2O$ | 30 |
| Magnesium sulphate | $MgSO_4$ | 30 |
| Potassium chloride | KCl | 2 |

The reconstituted water should be aged several days (USEPA, 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.

[38] Contaminants already in the receiving water might add toxicity to that of the chemical 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 chemical of concern, but not necessarily of the total impact at the site.

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 would be 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.

Tests using receiving water for the control and dilution would require transport of large volumes of water to the laboratory. That might be reasonable for the E test, but it might not be feasible for the EA or EAF tests. If not, consideration should be given to conducting EA or EAF tests adjacent to the site of interest, by pumping upstream receiving water into a mobile testing facility.

A compromise would be to adjust the pH and hardness of the laboratory water supply, or reconstituted water, to that of the receiving water, using reagent-grade salts (ASTM, 1991b; USEPA, 1985b). Depending on the situation, the adjustment might be to seasonal means, or to values measured at a particular time.

situations include appraisals of the toxic effects of real or potential chemical spills or intentional applications of spray or pesticide on a particular waterbody. The laboratory supply of water may be used for this purpose, especially if the collection and use of receiving water is impractical. Normal laboratory water is also appropriate for preliminary or intralaboratory assessment of a chemical's 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.

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.[39] 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 the aliquots should be performed, preferably on samples taken immediately before the start of the initial exposure, and at weekly intervals thereafter until the test is completed. On sampling days, separate aliquots should be taken from static-renewal tests at the beginning and end of the renewal periods; in flow-through tests, aliquots should be taken twice per day, at least six hours apart.

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 use a high-volume flow-through test to maintain stable concentrations of chemical in solution (perhaps decreased, but stable) (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. The intent of sampling intervals and averaging should be

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[39] 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: 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 if a flow-through system is used (USEPA, 1985b). Some situations (e.g., testing of pesticides for purposes of registration) might require the measurement of chemical concentrations in test solutions.

to obtain a realistic and time-balanced average concentration to which organisms were exposed.

## *5.6 Test Endpoints and Calculations*

The endpoints for tests performed with chemicals will usually be the standard ones, i.e., the EC50 and EC25 for nonviability at various stages of development in the E, EA, and EAF tests, and additionally in EAF, the 30-day LC50 and 30-day IC25 for average attained weight of swim-up fry. Other narrative statements on delayed development, deformities, and behaviour are required in the longer tests, and additional (optional) observations can be detailed, as described in Section 4.5.

If a solvent control is used to maintain the test substance in solution, there must be assurance that the solvent itself does not cause undue effects. Such a test is rendered invalid if the solvent control (or the untreated control) does not meet the criteria for test validity specified in Section 4.6.

When a solvent or other chemical is used, then it becomes the control for assessing the effects of the toxicant. Data for the solvent control must not be pooled with those for the control/dilution water. Pooling of the controls would be inappropriate since that could bias endpoint calculations; the control/dilution water lacks an influence that could act on organisms in the other concentrations (i.e., the solvent).

# 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*

Periodic tests with effluent, elutriate, or leachate (i.e., wastewater) samples for monitoring and compliance with regulatory requirements might use either the E, EA, or EAF test option (Section 4.3.1). Before one of these test options is adopted for periodic or frequent use (e.g., as part of an environmental effects monitoring program) with a particular wastewater, comparative assessment of these test options is recommended to quantify differences in sensitivity. Any of the three test options might be conducted as either static-renewal or continuous-flow assays, depending on the objectives, nature of the sample, volume needed, etc.

At the time that an EA or EAF test is set up, it is recommended that a multi-concentration E test be established and run concurrently, using the samples or subsamples of wastewater used for the first week of the test, and fertilized eggs from the same pool of test organisms. The findings of this E test will provide insight into the fertilization success rate for controls in the EA or EAF test, and will be useful in appraising the relative sensitivity to the test substance for the acute (E) and longer (EA or EAF) test options. A series of multi-concentration E tests might also be performed weekly with the samples or subsamples of wastewater as the EA or EAF test progresses, to provide information on the relative toxicity of the test substance used for each week of the test (Fennell *et al.*, 1998).

Regulatory testing programs might require test designs and endpoints other than the standard ones described herein. For example, regulations might require a single-concentration test with three or more undiluted portions of the sample, and three or more replicate control solutions. A required endpoint might be based on the results for a single concentration, usually 100% wastewater. See Section 6.6 for further guidance.

The requirements for volume of wastewater sample should be given serious consideration before undertaking any program. Approximately equal amounts of sample would be required for static-renewal and flow-through tests, but the amount might differ considerably for the different test options.[40] Appreciable savings in the

[40] Some hypothetical examples can be given for testing with rainbow trout. For an E test, it might be assumed that medium-sized rainbow trout eggs each weigh approximately 75 mg. With 40 of these in a replicate, the weight would be approximately 3.0 g, requiring about 1.5 L/d for each replicate, or ~4.5 L/d for three replicates. Seven concentrations plus a control (see Section 4.1) in a geometric series including full strength (e.g., 100, 46, 22, 10, 4.6, 2.2, 1.0, 0%) would require approximately twice as much test substance as for the 100% concentration alone, and thus the test would require ~9 L of wastewater per day.

required volumes of wastewater could be achieved in the EA and EAF tests by starting with the lower daily volumes of new testwater required at first, and increasing the volume in phases, as required by the actual biomass in a test chamber. Given the requirements for large volumes of sample in certain EA or EAF tests, especially those with ≥50 alevins per replicate and/or large individuals, it might be preferable to undertake such tests at the source of the wastewater, using a mobile laboratory. Any strategy for minimizing the sample volume requirements must, of course, keep replicate groups intact and separate from other replicates.

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 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.

Measurement of acute lethality to rainbow trout fry or fingerlings is recommended upon receipt of each sample to be used in an EA or EAF test. The lethal test should determine the 96-h LC50 or mortality in 100% sample during 96 hours, following the methods of Environment Canada (1990b, with 1996 amendments). Monitoring each sample for acute lethality might detect atypical variations in toxicity from chemical spills or other incidents, in-plant process changes, performance of an effluent treatment plant, or temporal environmental changes (if leachate). Information from concurrent acute toxicity tests will be useful in interpreting time-related toxic effects that occur during the EA or EAF tests.

## *6.2 Sample Collection, Labelling, Transport, and Storage*

Containers for transportation and storage of samples or subsamples of effluent, leachate, or elutriate must be made of nontoxic material. Collapsible polyethylene or polypropylene containers manufactured for transporting drinking water are recommended (e.g., Reliance™), since their volume can be reduced to fit into a cooler

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Medium-sized rainbow trout alevins might be assumed to average 130 mg (Appendix D). If there were 50 alevins/replicate in an EAF test, 3 replicates, and 7 concentrations as above, the sample requirement at this stage of the test would be ~20 L/d, for either a static-renewal or flow-through test. If those fish weighed an average of 150 mg at time of swim-up (Appendix D), the daily requirement for sample might be ~23 L/d at that time. At the end of an EAF test with swim-up fry weighing on average 500 mg/individual, and assuming 10 fry per replicate and three replicates were maintained in the same concentrations, a wastewater requirement of ~15 L/day might be anticipated at the end of the test. Sample requirements would differ if the fish were larger or smaller, or if the concentration series differed.

for transport, and air space within kept to a minimum when portions are removed in the laboratory for the toxicity test or for chemical analyses. 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.

Upon collection, each sample container must be filled, sealed, and labelled or coded. Labelling should include at least sample type, source, date and time of collection, and name of sampler(s). Unlabelled or uncoded containers arriving at the laboratory should not be tested. Nor should samples arriving in partially filled containers be routinely tested, 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.

An effort must be made to keep samples of effluent or leachate cool (1 to 7°C, preferably 4 ± 2°C) throughout their period of transport. Upon collection, warm (>7°C) samples must be cooled to 1 to 7°C with regular ice (not dry ice) or frozen gel packs. As necessary, ample quantities of regular ice, gel packs, or other means of refrigeration must be included in the transport container in an attempt to maintain sample temperature within 1 to 7°C during transit. Samples must not freeze during transport or storage.

Upon arrival at the laboratory, the temperature of the sample or, if collected, one of the subsamples (with the remaining subsamples left unopened and sealed), must be measured and recorded. An aliquot of effluent or leachate required at that time may be adjusted immediately or overnight to 14°C, and used in the test. The remaining portion(s) of sample or subsamples required for subsequent solution renewals must be stored in darkness in sealed containers, without air headspace, at 4 ± 2°C. For elutriates, as well as for samples intended for aqueous extraction and subsequent testing of elutriate, transport and storage conditions should be as indicated for effluents and leachates.

Tests with effluent, elutriate, or leachate may be performed "off-site" in a controlled laboratory facility. Each off-site E, EA, or EAF test must be conducted using one of the following two collection procedures and approaches.

1. A single sample of wastewater may be used for performing an E test, provided that it is divided into three separate containers upon collection, for transport and storage. If this collection procedure is followed, each of the three subsamples must be used to prepare all test solutions of the sample during two or three consecutive days of the test.[41] Similarly, for an off-site EA or EAF test, a single sample of wastewater may be used for each 7-day interval provided that it is subdivided into three full, sealed containers upon collection and used in the same manner.

2. If changes in toxicity of the wastewater are known or anticipated during 7 to 10 days of storage before use, fresh samples for an off-site E test should be collected on at least three separate occasions with sampling intervals of two to three days or less. These samples must be used

[41] For example, the first subsample could be used for Days 1 and 2 of the test, the second for Days 3 and 4, and the third for Days 5, 6, and 7.

consecutively during the test.[42] Similarly, for off-site EA or EAF tests involving wastewater samples known or anticipated to be unstable during storage, this sampling and testing regime (involving ≥3 discrete samples per week) should be implemented throughout each week of the test.

An alternative approach for unstable wastewater is to perform these tests on-site, using fresh wastewater and either flow-through or static-renewal conditions (see Sections 4.3.2 and 6.1, including footnote 40).

Testing of effluent and leachate samples should commence as soon as possible after collection. Use of any sample in a test should begin within one day whenever possible, and must begin no later than three days after sampling. If effluents or leachates are tested in on-site laboratories, samples should be used in the test within one day or less following their collection[43] (USEPA, 1989).

Samples of sediment, soil, or other solid material collected for aqueous extraction and subsequent testing of the elutriate should be extracted and tested as soon as possible following their 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. The prolonged storage of elutriate samples is undesirable because the toxicity of the sample might not be stable. Elutriate tests must commence within three days of sample preparation, unless specified otherwise in a regulation or prescribed method.

## *6.3 Preparing and Aerating Test Solutions*

Each sample or subsample in a collection or storage container must be agitated thoroughly just before pouring, to ensure the re-suspension of settleable solids. The dissolved oxygen content and pH of each sample or subsample must be measured just before its use. As necessary, each test solution should be pre-aerated (see Section 4.3.4) before aliquots are distributed to replicate test chambers.

Filtration of samples or subsamples is normally not required nor recommended. However, if they contain organisms which might be confused with the test organisms, attack them, or compete with them for food, the samples or subsamples must be filtered through a sieve with 60µm mesh openings before use (USEPA, 1989; 1994). Such filtration could remove suspended solids that are characteristic of the sample or subsample, and might otherwise contribute part of the toxicity or modify the toxicity. If there is such a concern, a second and concurrent test should be conducted using an unfiltered portion of the sample/subsample.

During E, EA, or EAF tests with samples of effluent, each solution including the controls should normally be gently aerated within the chamber. A decision to test without aeration might be made, however, because of

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[42] For instance, if three samples were collected during a one-week interval (e.g., on Monday, Wednesday, and Friday), the first could be used for Days 1 and 2 of the test, the second for Days 3 and 4, and the third for Days 5, 6, and 7.

[43] On-site testing might use the schedule and procedures described here for off-site tests. Alternatively, certain on-site tests might require fresh wastewater which is fed continuously (flow-through test) or at intervals of ≤12 h to each test chamber.

regulatory requirements, a very low oxygen demand of the wastewater, or a particular test objective such as including the oxygen demand as part of the overall toxic effect. In such a case, use of a flow-through test is recommended (see Sections 3.3, 4.3.2, and 4.3.4).

## *6.4 Control/Dilution Water*

Tests with samples of effluent or leachate, intended to assess compliance with regulations, should use either the laboratory water or a sample of the receiving water as the control/dilution 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 might be impractical for off-site tests.

The use of receiving water as the control/dilution water might be desirable for some on-site tests, if site-specific information were desired. An important example would be testing for sublethal effect at the edge of a mixing zone, under site-specific regulatory requirements. 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 must be prepared using the laboratory water supply that is normally used for rearing and testing fish. Measured biological endpoints (e.g., embryo viability, mortality rates for alevins or swim-up fry, incidence of deformed fish, weight of fry at test end) of fish in the laboratory control water must be compared to that in the sample of upstream receiving water (Section 4.5).

Tests requiring a high degree of standardization may be undertaken with reconstituted water for the control and for dilution. This requires relatively large volumes of water, but might be feasible and desirable in some cases. In such a case, the use of soft reconstituted water is recommended (hardness 40 to 48 mg/L as $CaCO_3$, pH 7.2 to 7.5, see Section 5.4). For example, the use of soft reconstituted water would be worthwhile if it were desired to minimize any modifying influence of the dilution water. Such situations might include studies intended to interrelate toxicity data from various types and sources of wastewater, from a number of test facilities, or from a single facility where water quality was variable. It is not recommended that the hardness of the reconstituted water be adjusted higher than values typical of the waterbody receiving a particular wastewater, nor that pH be adjusted outside the normal range, since such practice can reduce (or increase) the toxicity of the test substance and provide a misleading test result.

## *6.5 Test Observations and Measurements*

Observations of the number of obviously dead (E, EA, and EAF tests) and deformed individuals (EA and EAF tests) in each replicate should be recorded daily. Complete counts for the relevant stages, including "missing" individuals, should be made at the end of the major stage in each test (i.e., seven days after fertilization in the E test, seven days after 50% hatch in the control in the EA test, and at the time of 50% control swim-up in the EAF test). For

the EAF test, there are measurements of mortality and average dry weight of surviving fry after 30 days of exposure with feeding (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.

Various measurements besides those specified in Sections 4.3.3, 4.3.4, 4.3.5, and 4.4 should be done on the characteristics of the wastewater and conditions during the test. When solutions are prepared from the sample of wastewater, there should be observations of its colour, turbidity, odour, and homogeneity (i.e., presence of floatable material or settleable solids). Upon dilution with water, records should be made of precipitation, flocculation, colour change, odour, or other reactions. During the test, observations should be made on any changes in appearance of solutions, such as foaming, settling, flocculation, increase or decrease in turbidity, and colour change.

For tests with highly coloured or opaque solutions, or for samples producing foam in one or more test chambers, 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 for any such inspection manoeuvres.

For effluent samples with appreciable solids content, it is desirable to measure the total suspended and settleable solids upon receipt (APHA *et al.*, 1995), as part of the overall description of the effluent, and as sample characteristics that might influence the results of the toxicity test. Additional measurements that would help to 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 acids, chlorophenolic compounds, dissolved metals, chlorine, chloramine, ammonia).

## *6.6 Test Endpoints and Calculations*

The endpoints for tests performed with samples of wastewater will usually be the standard ones, i.e., the EC50 and EC25 for nonviability at various stages of development in the E, EA, and EAF tests, and additionally in EAF, the 30-day LC50 and 30-day IC25 for average attained weight of swim-up fry. Other narrative statements on delayed development, deformities, and behaviour are required in the longer tests, and additonal (optional) observations can be detailed, as described in Section 4.5.

Tests for monitoring or regulating effluents, leachates, or elutriates must use the standard options and endpoints defined in Section 4. In the EAF test, with three standard endpoints, the most sensitive effect would be taken as the definitive indication of toxicity. The standard methods of analysis would apply (see Section 4.5).

Tests for monitoring and compliance with regulatory requirements should normally include, as a minimum, three or more

replicates of the undiluted sample/subsamples (or a specified dilution thereof), and three or more replicate control solutions. Depending on regulatory requirements, tests for compliance might be restricted to a single concentration (100% wastewater unless otherwise specified), or might require a series of concentrations (i.e., a multi-concentration test) including 100% wastewater (see Section 4.5). Single-concentration tests are often cost-effective for determining the presence of measurable toxicity, and also for screening a large number of samples.

Specific adaptations of the standard toxicity test could be adopted for special purposes such as locating in-plant sources of toxicity, or assessing the effectiveness of in-plant process changes or of effluent treatment. The tests could be multi-concentration or single-concentration (100% or an appropriate dilution, plus a control). Endpoints would depend on the objectives of the undertaking, but could include arbitrary "pass/fail" limits such as a maximum percent nonviable embryos or maximum percent mortality of alevins at a suitable time period. Section 4.5 provides relevant instructions on statistical analysis and reporting for sets of tests on different samples, each tested at only one concentration.

*Section 7*

# 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*

Periodic tests with receiving water, for monitoring and compliance with regulatory requirements, would normally use the embryo (E) or embryo/alevin (EA) test options (Section 4.3.1). Tests to measure effects on diverse life stages of developing salmonid fish would use the EA or embryo/alevin/fry (EAF) test option. Definitive tests including effects on survival and growth of developing fry would use the EAF test option. Before the E or EA test is adopted for periodic or frequent use (e.g., as part of an environmental effects monitoring program), comparative assessment of either of these test options with the more comprehensive EAF test is recommended, to quantify differences in sensitivity. Any of the three test options might be conducted as either static-renewal or continuous-flow assays, depending on the objectives, nature of the sample, volume needed, etc.[44] A test and its associated endpoints might be restricted to three or more undiluted portions of the sample and three or more replicate control solutions (i.e., a single-concentration test), or might involve three or more replicates of each of a series of concentrations of the sample including 100%. See Section 7.6 for further guidance.

At the time that an EA or EAF test is set up, it is recommended that an E test be established and run concurrently, using the samples or subsamples of receiving water used for the first week of the test, and fertilized eggs from the same pool of test organisms. The findings of this E test will provide insight into the fertilization success rate for controls in the EA or EAF test, and will be useful in appraising the relative sensitivity to the test substance for the acute (E) and longer (EA or EAF) test options. A series of E tests might also be performed weekly with the samples or subsamples of receiving water as the EA or EAF test progresses, to provide information on the relative toxicity of the test substance used for each week of the test (Fennell *et al.*, 1998).

The routine assessment of acute lethality of each sample which is to be used in an EA or EAF test might or might not be warranted. This would be appropriate if it were suspected or anticipated that the undiluted receiving water might be lethal at any time during the early life-stage test. The information should be useful for interpreting toxic effects that occurred at particular times during the EA or EAF tests. The lethal test should be done on a portion of the sample, upon receipt, to determine the acute lethality

[44] The requirements for volume of sample will differ with fish life stage and size (biomass), and with the type of test selected. For certain EA and EAF tests (see Section 4.3.2 and footnote 40 of Section 6.1), the need for large volumes of sample, together with a decision to use upstream water as control water in the test, might make it preferable or more practical to undertake such tests at the site, using a mobile laboratory or existing facilities.

to rainbow trout fry or fingerlings (96-h LC50 or mortality in 100% sample during 96 hours), following the methods of Environment Canada (1990b, with 1996 amendments).

## *7.2 Sample Collection, Labelling, Transport, and Storage*

Procedures for labelling, transporting, and storing samples are found in Section 6.2. Toxicity tests should commence as soon as possible, preferably within 24 hours of sampling, and no later than three days.

## *7.3 Preparing and Aerating 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 salmonids should be filtered through a sieve with 60 µm mesh openings (Section 6.3) before use. If there is a concern that such filtering might change toxicity, a second unfiltered test should be run concurrently.

Dissolved oxygen content of each test solution including the control(s) must be measured upon preparation. Thereafter, either the organisms should be exposed to the solutions, or else each test solution should be pre-aerated (before organisms are exposed). In most instances, pre-aeration or aeration during the test will not be necessary or warranted (see Section 3.3, including footnotes 6 to 8), and should be avoided. A test performed without aeration should use a flow-through setup, to create a continuous circulation of test solutions around the developing embryos or alevins (see Section 3.3 including Figure 3C, and Section 4.3.2). If dissolved oxygen is below 60% of air saturation or above 100% saturation, pre-aeration or aeration should be used in either a static-renewal or flow-through test, following the guidance in Section 4.3.4.

## *7.4 Control/Dilution Water*

For samples of surface water collected near a wastewater discharge, chemical spill, or other point-source of 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, but upstream of it or outside its zone of influence. Surface water should be filtered to remove organisms, as described in Section 7.3.

If "upstream" water is used as control/dilution water, there must be a separate control solution of the laboratory water normally used for rearing and testing fish. Procedures for preparing and evaluating each control solution should be identical, and as described in Sections 4.1 and 5.4. Results of test exposures should be compared with those of the control in which receiving water was used (Section 4.5).

It might be unreasonable to use upstream water for a control because of logistical constraints, expected toxic effects, or other site-specific practicalities. 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*

The primary observations on test organisms should be as described in Section 4.4.

In addition, there should be observations of sample and solution colour, turbidity, foaming, precipitation, etc., as described in Section 6.5, both during preparation of solutions and subsequently during the test.

Each receiving-water sample should be characterized chemically. Depending on the suspected nature of the toxicants, measurements might include pH, conductivity, hardness, alkalinity, colour, chemical oxygen demand, biochemical oxygen demand, and concentrations of specific toxicants (e.g., resin acids, chlorophenolic compounds, dissolved metals, chlorine, chloramine, ammonia).

## *7.6 Test Endpoints and Calculations*

Endpoints for tests with receiving water should normally be the standard ones described in Section 4.5. The use of options and approaches should be consistent with those identified in Sections 4.5 and 6.6.

The tests could be multi-concentration or single-concentration (100% or an appropriate dilution, plus a control). Tests of regulatory compliance would often include three or more undiluted portions of the sample, and three or more replicate control solutions. Regulatory tests might use only a single concentration, usually the undiluted receiving water. Single-concentration tests are often cost-effective for determining the presence of measurable toxicity, and also for screening a large number of samples (e.g., from various locations within the receiving water). Statistical testing and reporting of results for such tests should follow the procedures outlined in Section 4.5.

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 rainbow trout, a multi-concentration test should be conducted as outlined in Sections 4.1 and 4.5, to determine the appropriate standard endpoints; i.e., the EC50 and EC25 for nonviability at various stages of development in the E, EA, and EAF tests, and additionally in EAF, the 30-day LC50 and 30-day IC25 for average attained weight of swim-up fry. The series tested should include one or more undiluted samples. Narrative statements on delayed development, deformities, and behaviour (EAF test only) are required when reporting the findings of an EA or EAF test, and additional (optional) observations can be detailed (see Section 4.5).

*Section 8*

# Reporting Requirements

Each test-specific report must indicate if there has been any deviation from any of the "must" requirements delineated in Sections 2 to 7 of this biological test method, and, if so, provide details as to the deviation. The reader must be able to establish from the test-specific report whether the conditions and procedures preceding and during the test rendered the results valid and acceptable for the use intended.

Section 8.1 provides a list of the items that must be included in each test-specific report. Section 8.2 gives a list of items that must either be included in the test-specific report, provided separately in a general report, or held on file for a minimum of five years. Specific monitoring programs or related test protocols might require selected test-specific items listed in Section 8.2 to be included in the test-specific report, or might relegate certain test-specific information as "data to be held on file" (e.g., details about the test substance and/or explicit procedures and conditions during sample collection, handling, transport, and storage).

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 document, may be referred to by citation or by attachment of a general report which outlines standard laboratory practice.

Details on the conduct and findings of the test, which are not conveyed by the test-specific report or general report, must be kept on file by the laboratory for a minimum of five years, so that the appropriate information can be provided if an audit of the test is required. Filed information might include:

- a record of the chain-of-continuity for samples tested for regulatory or monitoring purposes;
- a copy of the record of acquisition for the sample(s);
- certain chemical analytical data on the sample(s);
- bench sheets for the observations and measurements recorded during the test;
- bench sheets and warning chart(s) for the reference toxicity tests;
- detailed records of the source and health of the broodstock;
- all pertinent information on the procedures for stripping, handling, packaging, and storage of gametes and on the subsequent fertilization process; and
- information on the calibration of equipment and instruments.

Original data sheets should be signed or initialled and dated by the laboratory personnel conducting the tests.

## *8.1 Minimum Requirements for a Test-specific Report*

Following is a list of items that must be included in each test-specific report.

### *8.1.1 Test Substance*

- brief description of sample type (e.g., chemical or chemical product, effluent, elutriate, leachate, or receiving water), if and as provided to the laboratory personnel;
- information on labelling or coding, for each sample or subsample;
- date of sample/subsample collection; date and time sample(s)/subsample(s) received at test facility;
- dates or days during test when individual samples or subsamples used;
- for effluent or leachate, measurement of temperature of sample or, if multiple subsamples, one only of these subsamples, upon receipt at test facility;
- measurements of dissolved oxygen and pH of sample or subsample of wastewater or receiving water, just before its preparation and use in toxicity test; and
- date of elutriate generation and description of procedure for preparation; dates or days during an elutriate test when individual samples or subsamples are used.

### *8.1.2 Test Organism*

- species and common name;
- source of gametes or broodstock; number of female and male broodstock used for fertilization;
- brief description of procedure for checking sperm motility;
- brief description (including time interval) of procedure for fertilization of gametes;
- time interval from completion of fertilization until exposure of all groups of eggs to test solutions; and
- any unusual appearance or treatment of gametes or eggs, before their use in the test.

### *8.1.3 Test Facilities and Apparatus*

- name and address of test laboratory;
- name of person(s) performing the test; and
- brief description of test chamber(s) and associated apparatus (e.g., incubation units; pumps or other apparatus if flow-through test).

### *8.1.4 Control/Dilution Water*

- type(s) and source(s) of water used as control and dilution water; and
- type and quantity of any chemical(s) added to control or dilution water.

### *8.1.5 Test Method*

- citation of biological test method used (i.e., as per this document);

- brief mention and description of test options chosen (e.g., E, EA, or EAF test; static-renewal or flow-through test);
- design and description if specialized procedure (e.g., renewal of test solutions at intervals other than daily; preparation and use of elutriate; preparation and use of solvent and, if so, solvent control);
- brief description of procedure(s) in those instances in which a sample, subsample, or test solution has been filtered or adjusted for hardness or pH;
- brief description of frequency and type of all observations and all measurements made during test; and
- name and citation of program(s) and methods used for calculating statistical endpoints.

### *8.1.6 Test Conditions and Procedures*

- design and description if any deviation from or exclusion of any of the procedures and conditions specified in this document;
- number and concentration of test solutions, including controls; volume and depth of solution in each test chamber;
- number of individuals per test chamber, and number of replicates per concentration;
- brief statement concerning presence or absence of pre-aeration or aeration; if any, give rate and duration used to aerate sample or test solutions before and during exposure of test organisms;
- manner and rate of exchange (i.e., L/g·d) of test solutions;
- dates when test was started and ended; and
- all required measurements of temperature, pH, and DO (mg/L and percent saturation) in sample and test solutions including controls, before and during the test.

### *8.1.7 Test Results*

- average number and percentage of nonviable embryos in each replicate and concentration, including the controls (E test), seven days after fertilization; EC50 and 95% confidence limits, and EC25;
- average number and percentage of nonviable alevins in each replicate and concentration, including the controls, seven days after 50% hatch in the controls (EA test); EC50 and confidence limits, and EC25;
- average number and percentage of nonviable individuals at time of 50% control swim-up, in each replicate and concentration, including the controls (EAF test); EC50 and confidence limits, and EC25;
- number of dead fry in each concentration after 30 days of exposure with feeding, and number that started the exposure (EAF test); LC50 and confidence limits;
- average dry weight of fry surviving the 30-d exposure with feeding in each replicate and concentration (EAF test); IC25 and 95% confidence limits;

- narrative statement(s) on delayed hatching and deformed alevins in each concentration (EA test); description of any apparent differences from control;

- narrative statement(s) on deformed alevins, delayed swim-up, and abnormal behaviour of fry in each concentration (EAF test); description of any apparent differences from control;

- the results of E tests with the reference toxicant(s), conducted concurrently, together with the geometric mean value (± 2 SD) for the same reference toxicant(s) as derived at the test facility in previous tests; and

- anything unusual about the test, any deviation from these procedures, any problems encountered, any remedial measures taken.

## *8.2 Additional Reporting Requirements*

Following is a list of items that must be either included in the test-specific report or the general report, or held on file for a minimum of five years.

### *8.2.1 Test Substance*

- identification of person(s) who collected and/or provided the sample or subsamples;

- records of sample/subsample chain-of-continuity and log-entry sheets; and

- conditions (e.g., temperature, in darkness, in sealed container) of samples/subsamples upon receipt and during storage.

### *8.2.2 Test Organism*

- history of broodstock (e.g., fecundity; records of any disease and treatments);

- procedures and observations during gamete collections (e.g., degree of ripeness of females; motility of sperm); and

- conditions and procedures during transport and storage of gametes, motility of sperm preceding fertilization, details regarding procedures for fertilization.

### *8.2.3 Test Facilities and Apparatus*

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

- description of system for providing air and regulating air flow to test chambers;

- detailed description and/or illustration of test chambers and associated apparatus (e.g., size, shape, type of material, design); and

- description of procedures used to clean or rinse test apparatus.

### *8.2.4 Control/Dilution Water*

- sampling and storage details if the control/dilution water was "upstream" receiving water;

- details on any pre-treatment of water (e.g., filtration, sterilization,

chlorination/dechlorination, temperature adjustment, de-gassing, aeration); and

- ancillary water-quality variables (e.g., dissolved metals, ammonia, pesticides, suspended solids, humic and fulvic acids) measured before and/or during the toxicity test.

### *8.2.5 Test Method*

- description of laboratory's previous experience with the test option chosen and associated procedures herein;

- procedure used in preparing and storing stock and/or test solutions of chemicals; description and concentration(s) of any solvent used;

- methods used for chemical analyses of sample or test solutions, with citations; details concerning sampling, sample preparation and storage, before chemical analyses; and

- use and description of preliminary or range-finding test.

### *8.2.6 Test Conditions and Procedures*

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

- description of any thinning of swim-up fry to start the second phase of an EAF test (manner, numbers, timing);

- description of food source, type, and ration (quantity and frequency of feeding) used to feed swim-up fry during the EAF test;

- any other chemical measurements on sample, stock solutions, or test solutions (e.g., chemical concentration, suspended solids content, conductivity, hardness, and alkalinity), before and/or during the test;

- appearance of sample or test solutions; changes in appearance noted during test; and

- conditions and procedures for measuring the EC50 for reference toxicant(s) used in the E test.

### *8.2.7 Test Results*

- additional observations and numerical data supporting narrative statements of effect during an EA or EAF test for which there are no formal observations (see Sections 4.4 and 8.1.7), including information pertaining to: proportions of nonviable embryos (EA and EAF tests); delayed hatching (EA and EAF tests); mortality of alevins after hatching (EA and EAF tests); deformed alevins (EA and EAF tests); delayed swim-up (EAF test); and abnormal behaviour of fry (EAF test);

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

- warning chart showing the most recent and historic results for toxicity tests with the reference toxicant(s);

- graphical presentation of data; and

- results of any acute lethality tests conducted concurrently using rainbow trout fry or fingerlings and a portion of the sample or test solutions.

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*Appendix A*

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*Appendix B*

# Environment Canada, Environmental Protection Service, Regional and Headquarters Offices

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**Western and Northern Region**
Room 210, Twin Atria No. 2
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**Quebec Region**
105 McGill Street
14th Floor
Montreal, Quebec
H2Y 2E7

**Pacific and Yukon Region**[45]
224 Esplanade Street
North Vancouver, British Columbia
V7M 3H7

[45] A BASIC computer program for calculating LC50 is available from the Aquatic Toxicology Section, Pacific Environmental Science Centre, 2645 Dollarton Highway, North Vancouver, BC, V7H 1V2, by providing a formatted computer diskette.

*Appendix C*

# Review of Procedural Variations for Undertaking Early Life-stage Tests Using Salmonid Fish*

## 1. Test Substance and Type of Test

| Document | Test Substance | Test Type | Test Duration (days) |
|---|---|---|---|
| Birge *et al*., 1985 | effluents | static-renewal | 9 |
| USEPA, 1985a | chemicals | flow-through<br>static-renewal | ~ 90 |
| Rexrode and Armitage, 1987 | pesticides | flow-through | ~ 60 |
| van Aggelen, 1988 | effluents<br>receiving waters | recirculating | ~ 60 |
| ASTM, 1991a | chemicals | flow-through | ~ 90 |
| Birge and Black, 1990 | cadmium<br>effluents<br>receiving waters | flow-through<br>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<br>Na-dodecyl sulphate<br>2,4,5-trichlorophenol | static-renewal | 12 to 15 |
| OECD, 1992a | chemicals | flow-through<br>static-renewal | 50 to 55 |
| OECD, 1992b | chemicals | flow-through<br>static-renewal | ~ 90 |

* As specified in Canadian, Provincial, and international methodology documents. Based on documents 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) |
| USEPA, 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 | ≤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] | ≤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 warmwater 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 ≥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 commences, 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 |
| USEPA, 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/L per day (= 2 L/g per day) 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 |
| USEPA, 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 | vert. 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 4 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]) |
| USEPA, 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 h (FT[c])<br>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] FT = 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.

* 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 |
| USEPA, 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)<br>12 ± 2 (larvae) | NI | NI | NI |
| OECD, 1992b | 10 ± 2 (embryos)<br>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 h.
* sat. = saturation

## 7. Lighting Conditions During Test

| Document | Intensity | Type | Photoperiod | Dawn/Dusk |
|---|---|---|---|---|
| Birge *et al*., 1985 | dark | NI | NI | NI |
| USEPA, 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 |
| USEPA, 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 |
| USEPA, 1985a | T, DO<br>pH, cond, hard, alk, TOC | daily<br>weekly |
| Rexrode and Armitage, 1987 | DO, pH, cond, hard, alk, concn | weekly |
| van Aggelen, 1988 | T, DO, pH, cond, hard, alk, $NH_3$, TOC, metals<br>concn<br>PCB, pest | monthly<br>96 h[c]<br>source-dependent |
| ASTM, 1991a | DO, pH, cond, hard, alk, $NH_3$, TOC, concn, part, TDG<br>T | weekly<br>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<br>concn | NI<br>daily |
| Paine *et al*., 1991 | T, DO, pH<br>cond, hard | daily<br>twice/week |
| Neville, 1992 | T, DO, pH, cond<br>concn, metals, N, $NH_3$, $NO_2$, $NO_3$, hard | daily<br>start/end of test[e] |
| OECD, 1992a | T<br>DO, concn<br>pH, hard | daily[f]<br>$\geq$3 times/test[g]<br>start/end of test |
| OECD, 1992b | T, DO, concn<br>pH, hard | weekly[f]<br>start/end of test |

[a] alk = total alkalinity; cond = specific conductivity; concn = concentration of test substance; DO = dissolved oxygen; hard = total hardness; metals = selected metals; N = total nitrogen; $NH_3$ = total ammonia nitrogen; $NO_2$ = nitrite, $NO_3$ = nitrate; part = particulate material; PCB = polychlorinated biphenyls; pest = total organophosphorus pesticide; pH = hydrogen ion concentration; T = temperature; TDG = total dissolved gases; 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. |
| USEPA, 1985a<br>mort./wt.[a] | mort, def[a], no. hatch/swim-up[b] | daily | |
| | wt.[c] | end of test | |
| Rexrode and Armitage, 1987 | mort, no. hatch, timed hatch[d]/swim-up[b]<br>pathol./histol./clinical effects<br>wt.[f] | daily<br>weekly[e]<br>end of test | mort/wt |
| van Aggelen, 1988 | mort, def | daily | mort |
| ASTM, 1991a | mort[g], def<br>wt[h] | daily<br>end of test | mort/wt |
| Birge and Black, 1990 | mort, def[i], timed hatch | daily | mort |
| Hodson *et al*., 1991 | mort[j], def, hatching<br>wt<br>alevin body/yolk weight | daily<br>weekly<br>once | mort/wt |
| Paine *et al*., 1991<br>mort/growth | mort | daily | |
| | body weight, yolk weight | start/end test[k] | |
| Neville, 1992<br>mort/growth | mort, def | daily | |
| | growth[f] | start/end test | |
| OECD, 1992a | mort, def, no. hatched, timed hatch<br>length | daily<br>end of test | mort |
| OECD, 1992b | mort, def, no. hatch, timed hatch/swim-up<br>wt | daily<br>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 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 | LC50, LC10, LC1[a]<br>NOEC, LOEC | sig. diff.* from control |
| USEPA, 1985a | NI | sig. diff. from control by ANOVA |
| Rexrode and Armitage, 1987 | MATC[b] | sig. or specified diff. from control[c] |
| van Aggelen, 1988 | LT50[d], LC50 | sig. diff. from control |
| ASTM, 1991a | NI | sig. or specified diff. from control[c] |
| Birge and Black, 1990 | LC50, LC10, LC1[a]<br>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.

* 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 |
| USEPA, 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.

*Appendix D*

# Distribution, Life History, and Husbandry of Rainbow Trout

## 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 British Columbia, particularly in larger rivers and their tributaries, as well as lakes and streams. In central British Columbia, these fish are sometimes referred to as Kamloops trout. Rainbow 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. *O. mykiss* is among the most common species used in commercial aquaculture and is 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. Fecundity is approximately 1000 to 1400 eggs/kg spawning female. Eggs 3.0 to 5.0 mm in diameter are laid in gravel redds. After hatching, alevins ranging from 80 to 175 mg (wet weight) remain in the redds until their yolk is absorbed, and emerge as 0.1 to 0.2 g swim-up fry in late May or June. Young hatched in streams commonly remain there for the first winter, after which they migrate to lakes. Fry and juvenile fish usually feed on insect larvae and zooplankton (e.g., daphids). Adults are known to feed on insects, crustaceans, and other fish (Carl *et al.*, 1973); Gordon *et al.*, 1987).

Young (to ~12 cm) have 9 to 13 dark oval parr marks along the lateral line, which are overlain by fine black spots on back and sides. A series of 5 to 10 median parr marks lie along the mid-dorsal line ahead of the dorsal fin. The dorsal fin has a dark leading edge in small fish (fry) and a series of distinct black bars or spots in older fish. Distinct white or pale orange tips appear on the dorsal and anal fins. One or two black spots are common on the adipose fin; with few or no spots on the tail. No red dashes are found on the underside of the lower jaw (Carl *et al.*, 1973).

## Husbandry

**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 stripping.

The seasonal availability of mature gametes depends on local situations and populations, and on hatchery practices (i.e., seasonal water temperatures and photoperiods). Certain hatcheries have successfully selected and manipulated different populations of broodstock to provide gametes year round.

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. For optimal fertilization success, eggs taken for toxicity tests should be stripped during this 4- to 8-day period following ovulation. 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. Typically, 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 or anaesthetized. Excess force during stripping of eggs should be avoided. 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. Minimizing the storage period to as

brief as possible (ideally, <24 h) is desirable, though, to enhance the likelihood of good fertilization success.

Milt, if handled and stored properly, normally maintains 70 to 90% fertility for at least five days. 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 4°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.** Although fertilization can take place with water, this technique must be avoided since it triggers closure of the micropyle before the freshly fertilized eggs are exposed to test solutions. Accordingly, the dry method of fertilization must be used. Using this method, it is recommended that the eggs from four or more females[46] (see Section 2.2) be spawned into a dry, clean bucket or plastic tray. The milt, from one or more vials containing motile sperm when activated (see Section 2.2), is then added. It is preferable to fertilize eggs with milt from more than one male, to improve fertilization success. However, sperm used to fertilize the eggs must be taken from only those vials (one to four, depending on sperm motility) for which sperm have been demonstrated to be active when mixed with fresh water or ovarian fluid (Section 2.2). Upon addition of milt, the gametes are gently mixed (e.g., by hand using clean surgical gloves; or using a goose-wing feather). A period of five minutes for mixing and fertilization is recommended (Fennell *et al.*, 1998); although a period of up to 20 minutes for mixing and fertilization may be used (Birge *et al.*, 1985). Fertilization should take place under low lighting intensity. Immediately after fertilization, groups of fertilized eggs should be transferred as quickly as possible to the test solutions. The transfer of all groups of freshly-fertilized embryos to the test solutions is normally achieved within 10 minutes or less per test (Yee *et al.*, 1996), and must be completed within 30 minutes per test.

Various techniques have been used for transferring groups of freshly fertilized eggs to test solutions. Choice of technique is left to the discretion of the investigator, provided that it limits the pre-test exposure of newly fertilized eggs to water (for the purpose of washing off any

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[46] Use of fewer females increases the likelihood of an invalid test. For instance, if three females were used and the eggs from one proved entirely infertile, the likely fertilization success rate would be ≤67% and the criterion for a valid E, EA, or EAF test defined in Section 4.6 would not probably not be met.

unwanted debris or excess milt) to no more than 10 seconds, and enables all groups of eggs to be transferred quickly (i.e., as soon as possible, and within 30 minutes) to test solutions after fertilization (see Section 4.2). A proven and recommended technique (Canaria *et al.,* 1996; Yee *et al.*, 1996; Fennell *et al.*, 1998) is to partially fill labelled weighing boats with each test solution, and then transfer groups of freshly fertilized eggs to the boats (one group per boat). A plastic scoop (e.g., for measuring coffee) or spoon is useful for this purpose, to enable the quick transfer of the approximate number of eggs required per replicate to each weighing boat. An initial count of eggs is made following each transfer. Once all groups of eggs have been distributed to the weighing boats, the number of eggs per boat is recounted and adjusted as required. Following an additional period of no more than 30 minutes (to ensure water hardening and minimize stress during that period; Fennell *et al.*, 1998), the contents of each boat must then be transferred gently to its assigned test chamber.

Another recommended technique (Birge, 1996) is to transfer each group of freshly fertilized eggs from the fertilization chamber directly to the test chamber, using a suitably sized container (e.g., plastic scoop or graduated beaker with small holes drilled to enable draining or brief rinsing with water). To remove debris or excess milt, the fertilized eggs can be washed by dipping the scoop in control water which has been previously adjusted to the test temperature. Upon completion of all transfers, the number of eggs placed in each incubation unit can be counted and adjustments for numbers or appearance (e.g., size uniformity) of eggs made as necessary (see Section 4.2).

**Incubation and Development of Embryos.** Table D.1 provides guidance on the optimal and lethal temperatures for rainbow trout embryos. 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 races of the same species. Table D.2 gives predicted incubation periods 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 developmental rate depends on temperature, the changes in sensitivity will vary for different incubation conditions. 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**[a]

| Lower Limit[b] (°C) | Upper Limit[b] (°C) | Optimum Temperature (°C) |
|---|---|---|
| 0.5 to 2.3 | 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**

| Temperature (°C) | Days from Fertilization to 50% Hatch[a] |
|---|---|
| 1 | 182 |
| 2 | 138 |
| 3 | 107 |
| 4 | 86 |
| 5 | 71 |
| 6 | 59 |
| 7 | 50 |
| 8 | 43 |
| 9 | 37 |
| 10 | 32 |
| 12 | 25 |
| 14 | 20 |

[a] From Gordon *et al.* (1987).

*Appendix E*

# 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 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.