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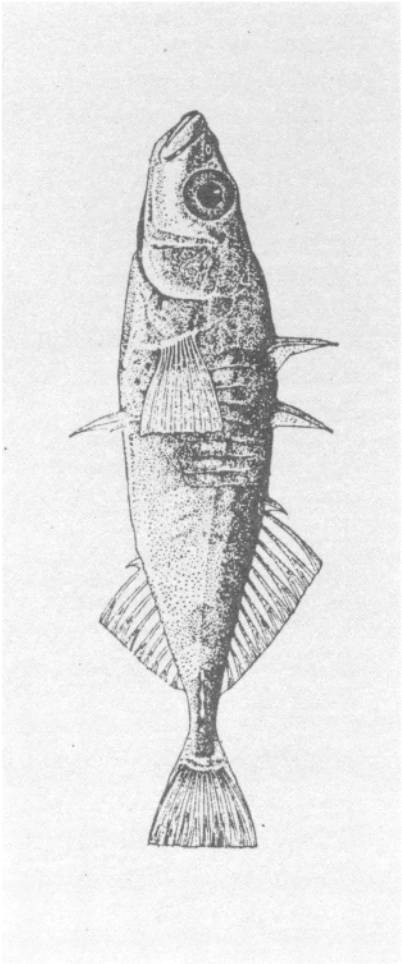

Biological Test Method:

Acute Lethality Test Using Threespine Stickleback *(Gasterosteus aculeatus)*

Report EPS 1 /RM/10
July 1990
(including March 2000 amendments)

Canada

Environment Canada Environnement Canada

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Method Development and Applications Section
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Abstract

Methods recommended by Environment Canada for performing acute lethality tests with seawater-acclimated threespine stickleback (Gasterosteus aculeatus) *are described in this report.*

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

Résumé

Le présent document expose les méthodes recommandées par Environnement Canada pour l'exécution d'essais de létalité aiguë sur l'épinoche à trois épines (Gasterosteus aculeatus) *acclimatée à l'eau de mer.*

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

Foreword

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

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

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

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

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Glossary

°C . . . . . degree(s) Celsius
cm . . . . . centimetre
d . . . . . day
DO . . . . . dissolved oxygen (concentration)
g . . . . . gram
h . . . . . hour
HCl . . . . . hydrochloric acid
L . . . . . litre
LC50 . . . . . median lethal concentration
LT50 . . . . . time to 50% mortality
mg . . . . . milligrams
min. . . . . minute
mL . . . . . millilitre
N . . . . . normal
NaOH . . . . . sodium hydroxide
O_2 . . . . . oxygen
SD . . . . . standard deviation
SI . . . . . Système international d'unités
TIE . . . . . Toxicity Identification Evaluation
™ . . . . . trade mark
µ . . . . . micro
µg . . . . . microgram
> . . . . . greater than
< . . . . . less than
≥ . . . . . greater than or equal to
≤ . . . . . less than or equal to
‰ . . . . . parts per thousand (salinity)
± . . . . . plus or minus

Terminology

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

Grammatical Terms

Must is used to express an absolute requirement.

Should is used to state that the specified condition or procedure is recommended and ought to be met if possible.

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

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

General Technical Terms

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

Compliance means in accordance with governmental permitting or regulatory requirements.

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

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

Euryhaline is the ability to tolerate a wide variation in salinity without stress.

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

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

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

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

pH is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions on scale from 0 to 14, with 7 representing neutrality, numbers less then 7 signifying increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic of alkaline reactions.

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

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

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

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

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

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

Terms for Test Materials

Artificial seawater is fresh water to which commercially available dry ocean salt or hypersaline brine has been added in a quantity that provides the salinity (and pH) desired for the water in the test.

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

Control is a treatment in an investigation or study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific condition that is being studied. In an aquatic toxicity test, the control must duplicate all the conditions of the exposure treatment(s), but must contain no test material. The control is used to determine the absence of measurable toxicity due to basic test conditions (e.g., quality of the control/dilution water, health or handling of test fish).

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

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

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

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

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

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

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

Estuarine water is brackish seawater, residing in a coastal body of ocean water that is measurably diluted with fresh water derived from land drainage.

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

Marine water is seawater residing in or obtained from the open ocean and without appreciable dilution by natural fresh water derived from land drainage.

Receiving water is, in this report, natural seawater (e.g., in a marine or estuarine waterbody) that has received a discharged water, or else is about to receive such a waste. Further description must be provided to indicate which meaning is intended.

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

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

Upstream water is natural seawater (e.g., in a marine or estuarine waterbody) that is not influenced by the test material, by virtue of being removed from it in a direction against the current or sufficiently far across the current.

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

Toxicity Terms

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

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

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

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

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

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

Overt means obviously discernible under the test conditions employed.

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

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

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

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

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

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

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This document was co-authored by Don McLeay (McLeay Associates Ltd., West Vancouver, B.C.) and John B. Sprague (J.B. Sprague Associates Ltd., Guelph, Ontario).

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Members of the Inter-Governmental Aquatic Toxicity Group (IGATG) (Appendix A) participated actively in the development and review of this document and are thanked accordingly. Special acknowledgement is made of the technical contributions provided by the IGATG subcommittee members (K. Doe, R. Parker, R. Scroggins, G. Sergy, R. Watts) responsible for initial and final review. The laboratory testing support of Environment Canada is also acknowledged.

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Introduction

1.1 Background

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

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

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

1.2 Species Distribution and Historical Use in Tests

The threespine stickleback *(Gasterosteus aculeatus)*, a common anadromous and freshwater species, is tolerant of marine estuarine, and fresh waters, occupying mainly the shallow-water areas. Almost circumpolar in coastal habitats, it prefers the temperate and subarctic zones of the northern hemisphere. It is widely distributed in the northern hemisphere on all coasts, with the exception of those of cold, arctic seas. Threespine stickleback occur on the Pacific coast from California to northwestern Alaska and on the Atlantic coast from Nova Scotia to northern Labrador (Hart, 1973; Scott and Scott, 1988).

G. aculeatus have been used for several years in Environment Canada (Appendix A) and other Canadian laboratories by investigators concerned with evaluating the acute toxic effects of effluents discharged to the estuarine or marine environment. Environment Canada (EPS, 1985) and Fisheries & Oceans Canada (Wong, 1982) have published procedures for conducting acute lethal toxicity tests using seawater-acclimated threespine stickleback for evaluations of the toxic effects of oil-drilling fluids discharged to the Canadian marine environment. Canadian researchers have reviewed the literature reporting the findings of acute lethal toxicity tests performed with this species (EVS, 1976)

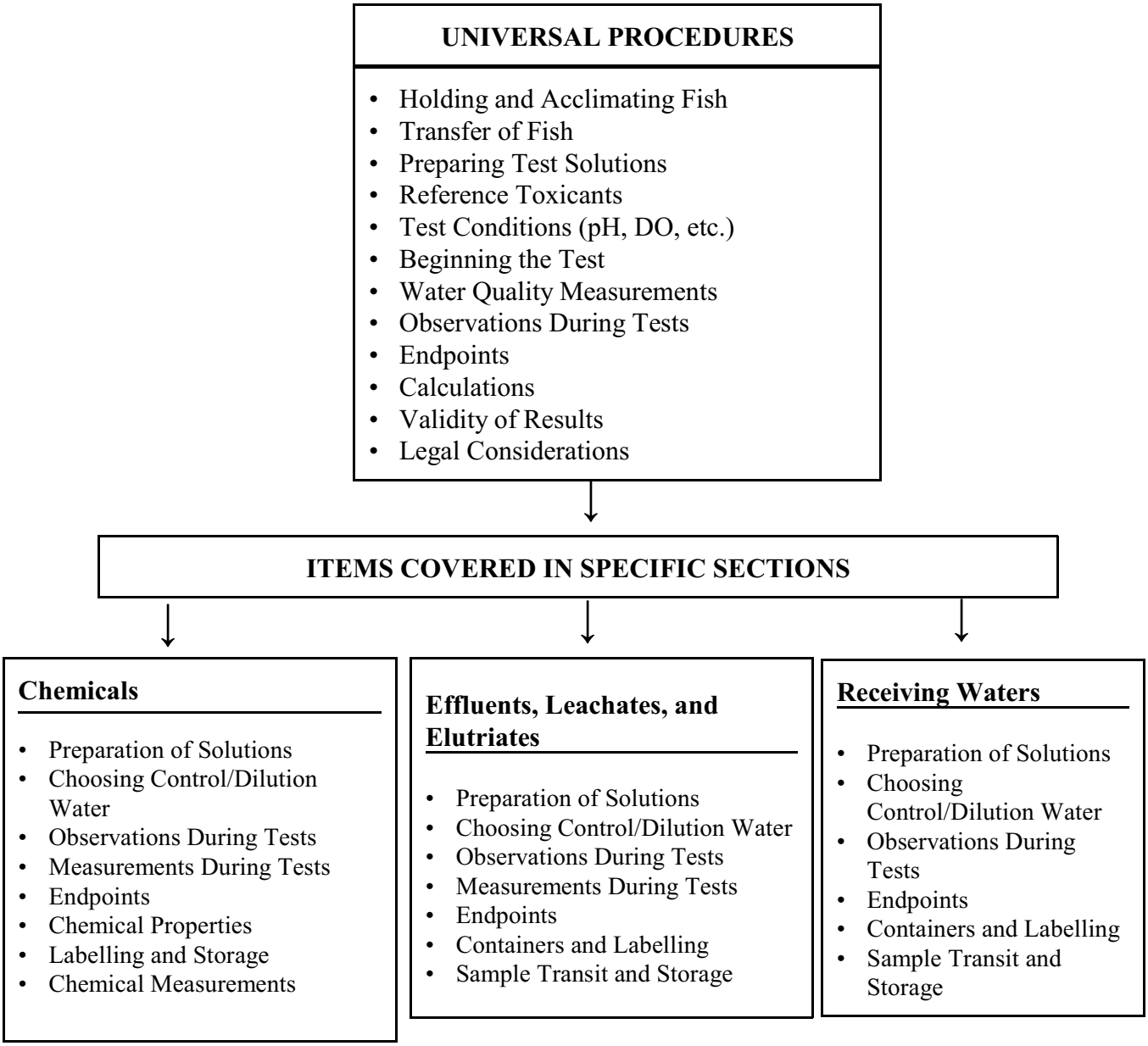

Figure 1 Diagram of Approach Taken in Delineating Test Conditions and Procedures Appropriate to Various Types of Materials

and, based upon comparative seawater toxicity tests using this and other marine fish species, have recommended threespine stickleback as a suitable species for seawater tests with aquatic contaminants (EVS, 1977). This fish species has also been recommended as an appropriate marine/estuarine toxicity test organism by both the United States Environmental Protection Agency (USEPA, 1975; 1985a) and the American Society for Testing and Materials (ASTM, 1980).

Reasons for the choice of threespine stickleback as a suitable test organism for seawater toxicity test include:

– much of the biology and life history of threespine stickleback is well documented (Hart, 1973; Wootton, 1976; Purcell, 1979; Coad, 1981; Allen and Wootton, 1982);

– this species is widely distributed in Canadian coastal (Atlantic, Pacific, and arctic) waters, marine, and estuarine;

– threespine stickleback are easily captured from coastal waters and acclimate readily to laboratory conditions;

– the species is euryhaline, and thrives at a range of salinities under laboratory conditions;

– fish size is suitable for performing acute lethal toxicity at economical costs; and

– this species has been used previously in tests of the toxicity of chemicals and wastewaters in both seawater and fresh water (van den Dikkenberg *et al.*, 1989), and has been cited previously by Canadian and U.S. researchers and environmental regulatory personnel as a suitable test organism for seawater toxicity tests.

The methodology presented in this report was developed for use with seawater-acclimated fish and seawater as the dilution and control water. Depending on the test objectives, this seawater may be artificial or natural, and brackish (e.g., salinity 10 to 20 ‰) or full-strength. Other tests, using freshwater-acclimated fish or other sensitive freshwater life, are available for evaluating the acute lethal toxic effect of chemicals or wastewaters destined for, discharged to, or within the freshwater environment. A review of procedural variables and approaches specific to existing methodology documents for conducting acute lethality tests using threespine stickleback is provided in Appendix B.

Test Organisms

2.1 Test Species

Threespine stickleback *(Gasterosteus aculeatus)* are to be used as the test species.

2.2 Life Stage and Size

Underyearling or juvenile life stages may be used as test fish. The average wet weight of test fish should be between 0.2[a] and 3g. The length of the largest fish should not be more than twice that of the smallest in the same test. Mean (± SD) fork lengths and wet weights should be measured routinely for a representative sample of fish (e.g., weekly measurements of ≥10 fish taken from the holding tank or measurements of controls at the end of the test), to ensure adequate loading rates and uniformity of size in tests.

2.3 Source

All fish used in a test should be derived from the same population and source, and must be free of known diseases. Fish may be cultured, or captured from coastal marine or estuarine waters and acclimated subsequently to laboratory conditions. Beach seines, cast nets, or minnow traps are suitable for capturing these fish. Procurement and shipment of fish should be approved by the Federal Department of Fisheries and Oceans (DFO).

The blackspotted stickleback, *Gasterosteus wheatlandi*, resembles the threespine stickleback and, in coastal Atlantic waters, can be captured together with *Gasterosteus aculeatus* (Scott and Scott, 1988). Accordingly, populations of stickleback captured from Atlantic waters must be examined carefully to distinguish these two species (Figure 2) and to ensure that only *G. aculeatus* are used in this test.

2.4 Holding and Acclimation

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

2.4.1 Facilities

Fish may be reared and acclimated in troughs or tanks. These must be made of nontoxic materials (e.g., stainless steel, porcelain, fibreglass-reinforced polyester, polyethylene, acrylic, or polypropylene. Troughs or tanks used for holding and acclimating test fish should be located away from any physical disturbances and preferably in a location separate from the test tanks.

Holding (rearing) troughs or tanks may be outdoors or indoors; tanks for acclimating fish to laboratory lighting and other test conditions should be indoors or, if outdoors, covered with lids fitted with photoperiod-controlled lights.

2.4.2 Lighting

Depending on test requirements and intent, lighting during acclimation may be natural or as provided by overhead full-spectrum[b] fluorescent

[a] Young fish with mean weight ≥0.2 g are allowable for toxicity tests provided that they have been actively feeding in the laboratory for a minimum of two weeks and have been acclimated for that period to the lighting, temperature, and salinity conditions for the test.

[b] Fluorescent or other tubes with a full-spectrum wavelength lamp, supplemented if desired with natural outdoor illumination, should be used to simulate the visible range of natural light. However, it should be noted that full-spectrum lights do not emit the intensity of ultraviolet (UV-B) radiation approaching that of natural illumination, and that the toxicity of certain effluents and chemicals can be altered markedly by photolysis reactions caused by UV-B radiation. For certain tests (e.g.,

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

2.4.3 Water

Depending on the nature and intent of the test (see Sections 5 to 7), fish may be held and acclimated in either and uncontaminated supply of natural seawater or "artificial" seawater. The seawater used should previously have been demonstrated to consistently and reliably support good survival, health, and growth of fish. The water supply should be monitored and assessed as frequently as required to document its quality. Analyses of variables including salinity, temperature, total dissolved gases, ammonia, nitrogen, nitrite, metals, total organophosphorus pesticides, and total organochlorine content (if municipal water), are recommended.

Artificial *(reconstituted)* seawater should be prepared by adding hypersaline brine *(HSB)* to a suitable fresh water, in quantities sufficient to provide the desired salinity. The use of HSB derived from an uncontaminated source of natural seawater is recommended (EC, 1997). Hypersaline brine may also be prepared using commercially available dry ocean salts (e.g., *Forty Fathoms*™, *Instant Ocean*™, or *Rila Marine Mix*™) or reagent-grade salts (i.e., *modified GP2*; see Table 8 in USEPA, 1993).

If ocean salts are used to prepare HSB, the suitability and consistency of a particular batch of these salts should be verified beforehand by testing, since some investigators feel that specific batches of sea salt can produce unwanted toxic effects or sequester test substances. A preliminary, 96-h test is recommended which monitors the survival, appearance, and behaviour of one or more replicate groups of stickleback held in control/dilution water that is adjusted for salinity using HSB derived from the batch of commercial ocean salts in question. Depending on the findings, the batch of sea salts would be suitable for preparing HSB to be used for any salinity adjustments required.

Ocean salts may also be added to natural seawater to obtain a desired (higher) test salinity.

Sources of water used for preparing artificial seawater may be de-ionized water or distilled water; or an uncontaminated supply of natural surface water or groundwater, or dechlorinated municipal drinking water. If municipal or natural freshwater sources are used, this water should also be monitored and assessed as appropriate to document its quality. The analysis of variables such as total residual chlorine (if municipal water), pH, salinity, suspended solids, dissolved oxygen, total dissolved gases, temperature, ammonia nitrogen, nitrite, metals, total organophosphorus pesticides, and total organochlorine content in batches of artificial seawater is recommended.

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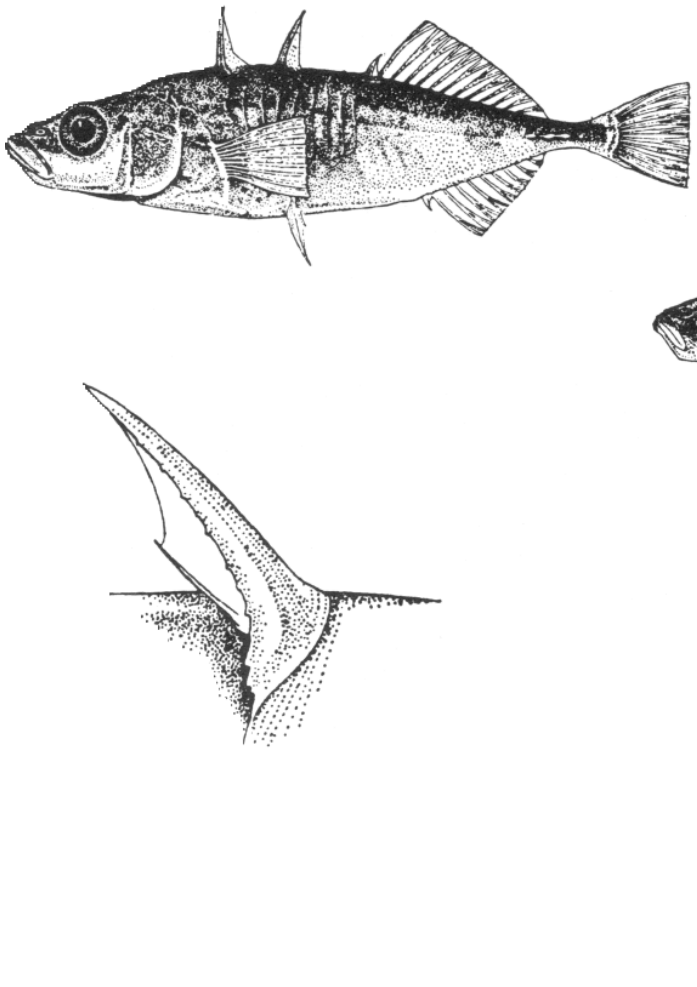
photoactivation or photodegradation of toxic materials due to ultraviolet radiation), special lights (e.g., high-pressure mercury-arc lamps) with differing spectral qualities may be used. ASTM (1996) provides useful guidance in this regard. Studies wishing to determine the influence of lighting conditions on toxicity could conduct concurrent side-by-side comparisons with replicate solutions held under different light (e.g., full-spectrum versus mercury arc).

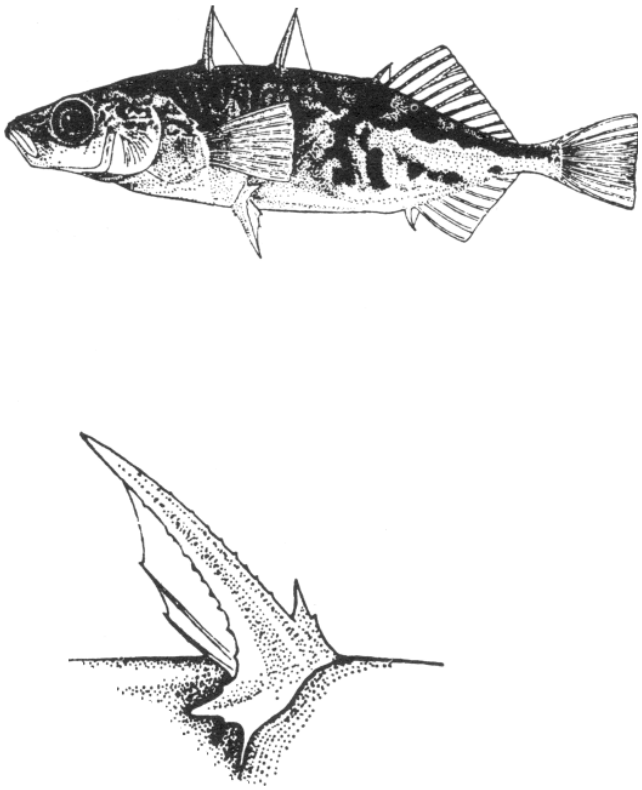
[c] A "dawn/dusk" transition period is recommended because abrupt changes in intensity startle and stress fish. Automated control systems are available for dimming and brightening the intensity of fluorescent lights, although they are costly. Alternatively, a secondary incandescent light source, regulated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

THREESPINE STICKLEBACK



Dorsal spines three (rarely four) last spine short; pelvic fin of one spine and one soft ray, spine with one pointed cusp at base; caudal peduncle with a keel; body without round black spots; colour in life green, blue silvery.

BLACKSPOTTED STICKLEBACK



Dorsal Spines three (rarely two); Pelvic fin of one spine with two Soft rays, spine with two well-developed pointed cusps at base; Caudal peduncle keel-less; many round black spots along sides; Colour in life lemon-yellow.

Figure 2 Key for Distinguishing Threespine Stickleback *(Gasterosteus aculeatus)* from Blackspotted Stickleback *(G. wheatlandi)* (from Scott and Crossman, 1973; and Scott and Scott, 1988).

Table 1 Checklist of Recommended Conditions and Procedures for Holding and Acclimating Threespine Stickleback

Source of fish	–	cultured or captured from estuarine or marine waters; free of known diseases; procurement and transport approved by Fisheries & Oceans Canada
Water	–	uncontaminated natural seawater or artificial seawater; holding volume and flow, 1.0 L/10 g of fish and 1.4 L/g fish per day, respectively; ideally, salinity within 5 ‰ of value for control/dilution water, for ≥2 weeks
Temperature	–	holding temperature within the range compatible with good fish health; acclimation temperature achieved at rate ≤3° C/d and held at 10 ± 2° C for ≥2 weeks
Oxygen/aeration	–	dissolved oxygen 80 to 100% saturation; maintained by aeration (filtered, oil-free air) if necessary
Lighting	–	broad spectrum (fluorescent or equivalent), 100–500 lux at surface, 16 ± 1 h light:8 ± 1 h dark, preferably gradual transition between light and dark
Feeding	–	at least once a day with standard commercial pelleted or flaked food, ground fresh fish and/or brine shrimp; feed stored from or according to manufacturer's recommendations
Cleaning	–	siphoning of debris daily, or as required; transfer to clean, disinfected tanks as necessary
Disease	–	mortalities monitored daily and moribund fish removed; mortality rate for group to be used in tests, ≤ 1%/day for each of seven days preceding test; if treated for disease, not to be used within two weeks thereafter
Measurements/	–	temperature, dissolved oxygen, pH, salinity, flow rate, and mortality records should be measured and recorded, preferably daily

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

residual chloramine and other chlorinated organic compounds[d].

A constant flow of seawater through the holding and acclimation tanks is necessary. To prevent buildup of metabolic wastes, at least one litre per minute of new seawater should flow into the tank for every kilogram of fish being held (equals 1.4 L/g fish · d or 0.69 g fish · d/L)[e]. Additionally, to prevent overcrowding, a tank should contain at any given moment at least one litre of water for every 10 grams of fish held (Sprague, 1973). Circumstances such as acclimation of fish to artificial seawater or a limited seawater supply might require the filtration and recirculation of water, or its periodic renewal in static systems. In such cases, ammonia and nitrite should be measured frequently to check that they do not reach harmful levels.

Water entering holding and acclimation tanks must not be supersaturated with gases. In situations where gas supersaturation within the water supply is a realistic possibility, total gas pressure within this water supply should be frequently checked (Bouck, 1982). Remedial measures (e.g., use of aeration columns or vigorous aeration in an open reservoir) should be taken if dissolved gases exceed 100% saturation.

Ideally, fish should be acclimated for a minimum of two weeks to seawater with salinity within 5 ‰ of that for the control/dilution water to be used in the test. Recent unpublished data (Environment Canada, Atlantic Region; see Appendix A for address), derived using reference toxicants and a range of test salinities differing form that to which the fish were acclimated, indicate that this may not be critical for acute lethality tests.

Water, temperature, dissolved oxygen, pH, and flow should be monitored for each holding or acclimation tank, preferably daily. Weekly or more frequent monitoring levels of ammonia and nitrite in holding or acclimation tanks is recommended.

2.4.4 Temperature

The water temperature for holding populations of fish for subsequent test purposes may be outside the acceptable limits for the test provided that it is compatible with good fish health. When preparing a group of fish for the acclimation period, water temperature may be changed at a rate not exceeding 3° C per day, until an acclimation temperature of 10 ± 2° C[f] is achieved. Fish are to be acclimated to 10 ± 2° C for a minimum of two weeks, and preferably ≥3 weeks, before the test is started.

2.4.5 Dissolved Oxygen

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

2.4.6 Feeding

Threespine stickleback will eat a variety of food under laboratory conditions. Fish may be fed a daily ration of ground fresh fish, tropical fish food flakes, or other standard fish feeds available commercially. This diet may be supplemented

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[d] Thiosulphate or other chemicals effective in removing residual chlorine from water should not be used in preparing artificial seawater to be used as dilution water in toxicity tests. Such chemical(s) could alter sample toxicity.

[e] If necessary (e.g., fish are being acclimated to artificial seawater or a particular receiving-water source for which volumes of water are restricted), water-volume requirements for fish acclimation may be decreased substantially by recirculating the flow to the fish tank through a filter suitable for removing metabolic wastes.

[f] A lower (e.g., 5° C) or higher (e.g., 15° C) acclimation/test temperature may be appropriate in instances where the toxicity of materials entering or at risk of entering very cold or more temperate marine waters is under investigation.

with brine shrimp (EPS, 1985). Depending on water temperature and fish size, feeding should be one or more times daily. A daily ration (dry food basis) approximating 1 to 5% of wet body weight is recommended as a guideline, although this may be changed due to feed type and fish-feeding behaviour. The method and maximum duration for storing commercial food should be as recommended by the manufacturer.

2.4.7 Cleaning of Tanks

Troughs and tanks used for holding and acclimating fish should be kept clean. Siphoning of excess food and faeces should be undertaken once a day or as frequently as necessary to eliminate buildup of excess food or faecal material. Tank designs that provide partial self-cleaning (e.g., those with central, double standpipes) are recommended as they reduce maintenance requirements.

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

2.4.8 Fish Morbidity, Mortality, and Treatment

Fish should be inspected daily for signs of disease[g]. Dead and moribund individuals should be removed immediately. Mortalities in the stock tank(s) from which test fish are to be taken must not exceed 1% per day during the seven-day period preceding the test. If mortalities during this period are higher, the acclimation period must be extended for at least another seven days, until mortalities no greater than 1% per day for each of the seven days immediately preceding their use in the test.

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

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[g] Symptoms of unhealthy fish include loss of appetite, abnormal distribution in the tank, lethargy, erratic or atypical swimming behaviour, darkened colouration, pale gills, eroded or frayed fins, and external lesions.

Test System

3.1 Facilities

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

A test facility is required that will maintain the temperature of all test solutions within the range specified (10 ± 2° C, unless otherwise indicated). This may be achieved using various types of equipment such as a thermostat-controlled air conditioning unit or a series of temperature-controlled water baths in which test vessels are immersed.

3.2 Lighting

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

3.3 Test Vessels

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

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

3.4 Control/Dilution Water

Depending on the test material and intent (see Sections 5 to 7), the control/dilution water may be: an "uncontaminated" supply of natural seawater; artificial seawater or natural seawater with added hypersaline brine for salinity adjustment (see Sections 2.4.3 and 4.1); or a sample of receiving water collected from a point adjacent to but removed from the influence of the contaminant source of concern. If the latter is selected, conditions for sample collection , transport, and storage should be as described in Section 6.1.

Control/dilution water must be adjusted to the test temperature before use. Supersaturation of this water with excess gases should be prevented.

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

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

Universal Test Procedures

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

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

4.1 Preparing Test Solutions

All test vessels, measurement devices, stirring equipment, and fish-transfer pails must be thoroughly cleaned and rinsed in accordance with standard operational procedures. Each test vessel should be rinsed with control/dilution water just prior to use (rinsing is not needed if disposable polyethylene liners are used).

The test concentrations and numbers of test solutions to be prepared will depend on the purpose of the test. For tests intended to estimate a 96-h LC50, at least five test concentrations plus a control solution (100% dilution water) are to be prepared[i]. An appropriate geometric dilution series may be used, in which each successive concentration is about 50% of the previous one (e.g., 100, 50, 25, 12.5, 6.3). This may be used to assist in the precise calculation of the LC50 and its 95% confidence limits. Test concentrations may be selected from other appropriate logarithmic dilution series (see Appendix C).

When receiving water is used as dilution and control water, a second control solution should be prepared using the laboratory water to which fish have been acclimated for two or more weeks. Receiving water cannot be used if it is clearly toxic according to the criteria of the test for which it was intended.[j] In such cases, artificial seawater (Section 2.4.3) or the laboratory's supply of uncontaminated natural seawater should be used as the control water and for all dilutions.

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

If artificial seawater is to be used as the dilution and control water, it should be prepared using either hypersaline brine (HSB) derived from an uncontaminated source of natural seawater, or HSB prepared using commercial sea salt or reagent-grade salts (see Sections 2.4.3 and 4.3.3). Any HSB which is prepared using commercial sea salt or reagent-grade salts must be filtered ($\leq$ 1µm), aerated overnight, and then capped and stored in the dark at 4 ± 2° C for at least one week before use (EC, 1997). Additionally, laboratory personnel must be able to demonstrate that they meet the test-specific criteria for a valid test (see Section 4.3) using aged artificial

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

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

Table 2 Checklist of Recommended Test Conditions and Procedures

Universal	
Test type	– static, 96-h duration*
Control/dilution water	– "uncontaminated" laboratory seawater; artificial seawater if requiring a high degree of standardization; "upstream" receiving water to assess toxic effect at a specific location,** dissolved oxygen (DO) content 90 to 100% saturation at time of use; ideally, salinity within 5 ‰ of acclimation
Fish	– underyearlings or juveniles, mean weight 0.1 to 3 g; normally a minimum of 10/test solution; fish-loading density ≤0.5 g/L, for at least four days
Solution depth	– ≤15 cm
Temperature	– 10 ± 2° C (unless otherwise specified)
Oxygen/aeration	– upon preparation, pre-aerate each test solution for 30 min. at 6.5 mL/min · L if required or necessary (see Sections 5.2, 6.2, and 7.2); thereafter, and only if necessary, pre-aerate each test solution at 6.5 mL/min · L for the lesser of 90 additional minutes or achieving ≥70% saturation in the highest test concentration. Aerate solutions at this rate throughout the test
pH	– no adjustment if pH of test solution within the range 6.5 to 8.5***; a second (pH-adjusted) test may be required or appropriate if sample/solution pH beyond this range
Lighting	– full-spectrum fluorescent, 100–500 lux at surface, normally 16 ± 1 h light : 8 ± 1 h dark; preferably gradual transition
Feeding	– do not feed for the 16- h period immediately preceding the start of the test, nor during the test
Observations	– at least 24, 48, 72, and 96 h; for fish death, appearance, and behaviour
Measurements	– solution temperature, pH, and DO; at least at beginning and end (preferably daily); salinity at least at start
Endpoints	– as specified and/or depending on test objectives and test material; may be 96-h LC50 (requiring 95% confidence limits) or single-concentration test (% mortality at 96 h or earlier; LT50)
Reference toxicant	– phenol and/or zinc (as zinc sulphate); determine static 96-h LC50 upon acclimation and at least monthly thereafter
Test validity	– invalid if >10% of control fish die or exhibit atypical/stressed behaviour

Chemicals

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

Effluents and Leachates

Transport and storage	– transport at ambient temperature (>1° C, <30° C) or at 1 to 8° C if transit time >2 d; sample should not freeze during transit; store in the dark at 1 to 8° C (preferably 4 ± 2° C); test within three days of sampling if possible; must be tested within five days of sampling
Control/dilution water	– as specified and/or depending on intent; laboratory seawater or "upstream" receiving water for monitoring and compliance
High solids or floatables	– may choose to recirculate test solutions
Salinity	– normally not adjusted; if sample is essentially fresh water (salinity <5 ‰) and it is desired to understand the contribution of salinity towards sample toxicity, conduct a second (salinity-adjusted) test; for this (second) test, adjust salinity of the sample to within 5 ‰ of that of the control/dilution water

Elutriates

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

Receiving Water

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

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

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

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

seawater, before artificial seawater is used to prepare HSB or control/dilution water (EC, 1997).

4.2 Beginning the Test

Each test vessel placed within the test facility must be clearly coded or labelled to identify the test substance, the concentration, and the date and time of starting the test. The vessels should be positioned for easy observation of fish behaviour and mortalities. The test solutions should be placed in random order (Sprague, 1973). It is recommended that, if necessary, test vessels be covered by clean, nontoxic screens or glass to prevent fish from escaping. The latter material should be used if concern exists with respect to contaminants entering the test solution from other sources, or the loss of volatiles from solution. Temperature, dissolved oxygen, and pH levels in the vessels should be checked and adjusted, if required/permitted, to acceptable levels prior to the introduction of fish.

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

Fish in the acclimation tank must not be fed for at least the 16-h period immediately preceding the test. To minimize stress, transfer of fish from the acclimation tank to test vessels should be done as quickly as possible. Any fish dropped or injured during transfer are to be discarded. Dip nets should be rinsed (dilution water) between transfers if contact is made with a test solution. Seawater within fish-transfer pails should be aerated if necessary to maintain dissolved oxygen levels at 80 to 100%

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

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

[l] The total wet weight of fish in any test solution (including the control) must be no greater than 0.5 g/L, for this four-day static test. A lower loading of fish could be used routinely when feasible, to reduce the buildup of metabolic wastes and the depletion of toxicant(s) from the water by the fish. A favourable density of 0.125 g/L has been suggested, to last for a four-day test (Sprague, 1973).

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

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

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

of air saturation during the period required for introduction of fish to test vessels.

4.3 Test Conditions

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

The test should be conducted at 10 ± 2° C unless specified otherwise[f].

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

Each test solution, including the control(s), is to be aerated at the rate of 6.5 ± 1 mL/min · L.

Fish are not to be fed during the test.

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

4.3.1 Dissolved Oxygen and Aeration

Depending on the test material or study objectives, pre-aeration of each test solution (including the controls) under defined conditions just before the addition of test fish might be recommended or required (see Sections 5.2, 6.2, and 7.2). For those instances where pre-aeration is recommended or required, each solution including the control(s) is to be aerated gently for a period of 30 minutes at a rate of 6.5 ± 1 mL/min · L. Immediately thereafter, the dissolved oxygen content of each test solution should be measured. If (and only if) the measured value in one or more solutions is < 70% or > 100% of air saturation, the pre-aeration of all solutions should be continued at the same rate for an additional period not to exceed 90 minutes[m]. This additional period of pre-aeration should cease if and when oxygen 70 % saturation in the highest test concentration (or 100 % saturation, if supersaturation is evident). Immediately after 90 minutes or attaining 70 % saturation, the test must be initiated by introducing fish.

At the start of the test, the aeration of test (and control) solutions should be commenced or continued (see Sections 5.2, 6.2, and 7.2), at a rate of 6.5 ± 1 mL/min · L. This aeration should be maintained throughout the test period. Any aeration (or pre-aeration) of test solutions should be provided by bubbling compressed air, previously filtered so as to be free of oil, through a clean silica-glass air diffuser** or disposable glass pipette. The aeration rate should be verified and monitored at least daily using a suitable gas flow meter.

If, using the prescribed aeration rate, the dissolved oxygen levels to which fish are exposed become depressed below 60 % saturation (OECD, 1984; USEPA, 1985a) and the intent of the test is to distinguish the degree to which oxygen depletion may contribute to fish deaths, a second test may be conducted with the sample (or a portion thereof) using a higher aeration rate sufficient to maintain dissolved oxygen values ≥ 70 % saturation.

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

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

** 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 no. (819) 842-2890).

Alternatively, the second test may be conducted using compressed oxygen gas bubbled at a controlled rate (6.5 ± 1 mL/min · L) into each test solution.

4.3.2 pH

Toxicity tests should normally be carried out without adjustment of pH. In instances where the chemical, wastewater, or receiving-water sample causes the pH of any test solution to be outside the range 6.5 to 8.5, and it is desired to assess toxic chemicals rather than the lethal or modifying effects of pH, then the pH of the test solutions or sample should be adjusted before adding the fish, or a second (pH-adjusted) test should be conducted concurrently[n,o]. For this (second) test, the initial pH of the sample, or of each test solution[p] may (depending upon the test objectives) be neutralized (adjusted to pH 7.0) or adjusted to within ±0.5 pH units of that of the dilution water, prior to fish exposure. Another acceptable approach for this second test is to adjust each test solution (including the control) to pH 6.5 to 7.0 (if test sample has/causes pH <6.5) or to pH 8.0 to 8.5 (if sample has/causes pH >8.5). Solutions of hydrochloric acid (HCl) or sodium hydroxide (NaOH) at strengths ≤ 1 *N* should normally be used for all pH adjustments. Some situations (e.g., effluent samples with highly buffered pH) may require higher strengths of acid or base.

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

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

4.3.3 Salinity

The salinity of an aqueous sample should be measured prior to the start of the test. Toxicity tests should normally be carried out without adjustment of salinity.

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[n] The pH of natural, uncontaminated seawater is normally within the range of 7.5 to 8.5. Seawater solutions with pH values beyond the 6.5 to 8.5 range are atypical of the estuarine or marine environment. In this context, such pH values are considered as (environmentally) atypical.

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

Notwithstanding, test materials causing shifts in the pH of seawater solutions beyond the range 6.5 to 8.5 may cause toxic effects due to adverse pH alone. A second (pH-adjusted) test, if conducted concurrently under otherwise identical test conditions, would enable a distinction of the degree to which pH contributed to sample toxicity, and the mitigation of toxicity due to pH adjustment.

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

In instances where the chemical, wastewater, or receiving-water sample is essentially fresh water (salinity < 5 ‰), and it is desired to understand the contribution of salinity towards the sample toxicity, a second (salinity-adjusted) test should be conducted concurrently. For this (second) test, the salinity of an aliquot of the sample should be adjusted to within 5 ‰ of that of the control/dilution water. The minimal quantity of hypersaline brine (HSB; see Sections 2.4.3 and 4.1) necessary to enable this adjustment should be added to the sample or each test solution. If any HSB is added to the test sample/solutions to adjust salinity, the toxicity test must include a control prepared using only this HSB and deionized water, adjusted to the test salinity. Additionally, any test using dilution water which differs from this *HSB control* in any respect (e.g., natural seawater with or without HSB added; natural fresh water with HSB added, etc.) must include a separate control prepared using this same dilution water.

4.4 Test Observations and Measurements

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

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

Measurements of dissolved oxygen, pH, and temperature must be made in each test solution including the control(s), at the start and end of the test as a minimum and preferably at the start of each 24-h period of exposure. Final measurements should be done after biological observations are complete. The salinity of each test solution should be measured at the start of the test as a minimum.

Mean (±SD) length and wet weight of control fish must be determined at the end of the test.

4.5 Test Endpoints and Calculations

In multi-concentration tests, record the percentages of fish killed in ≤96 h for each test solution of the wastewater or chemical. Calculate the 96-h LC50 and its 95% confidence limits, and report the method used for those calculations.

To estimate an LC50, mortality data are combined from all test tanks at a given concentration. If mortality is not ≥50 % in at least one concentration, the LC50 cannot be estimated. If there are no mortalities at a specific concentration, that information is used as an effect of 0 % mortality. However, if successive concentrations yield a series of 0 % mortalities, only the highest concentration of the series should be used in estimating the LC50 (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 mortalities at the high concentrations in the test, only one value of 100 % effect would be used, the one at the lowest concentration. Use of only one 0 % and one 100 % effect applies to any form of statistical analysis and to hand plotting on a graph.

Various computer programs may be used to calculate the LC50. Stephan (1977) developed a program to estimate LC50s using probit,

moving average, and binomial methods. This program in the BASIC language is recommended and is available on diskette (through the courtesy of Dr. Charles E. Stephan, USEPA, Duluth, Minn.) from Environment Canada (address in Appendix A). Other satisfactory computer and manual methods may be used (e.g., USEPA, 1985a; Hubert, 1987; APHA *et al.*, 1989; EC, 2000). Programs using the trimmed Spearman-Kärber method (Hamilton *et al.*, 1977) are not recommended because divergent results might be obtained by operators who are unfamiliar with the implications of trimming ends of the dose-response data (EC, 2000).

The recommended program of Stephan (1977) estimates the LC50 by each of its three methods if there are at least two partial mortalities. For smooth or regular data, the three results will likely be similar [q], and values from the probit analysis should be taken as the preferred ones and reported. The probit analysis also gives the slope of the line, which should be reported. The binomial estimate might differ somewhat from the others, and this estimate should only be used as a last resort. If the results do not include two partial mortalities, only the binomial method can be used to provide an estimate of the LC50. It does not estimate formal confidence limits; instead it provides outer limits of a range, within which the LC50 and the true confidence limits would lie.

Any computer-derived LC50 should be checked by examining a hand plot of percent mortalities for the various test concentrations, on logarithmic-probability scales (see Figure 3 and footnote q) (APHA *et al.*, 1989; EC, 2000). Any major disparity between the estimated LC50 derived from this plot and the computer-derived LC50 must be resolved. A computer-generated plot could be used if it were based on logarithmic-probability scales. If there had been an error in entering the data, however, a computer-generated plot would contain the same error as the mathematical analysis, and so the investigator should carefully check for correct placement of points.

For single-concentration tests, the endpoints depend on the objective of the test. Appropriate endpoints may include: a). determination of percent mortality upon exposure of fish to the undiluted sample for 96 h; b). percent mortalities at various times for toxicity comparisons; or c). measurement of times to death for individual fish in each solution.

If successive measurements are made (items b or c), the median time to death (LT50) may be estimated if desired, by plotting in similar fashion to Figure 3 except that the horizontal

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[q] Figure 3 was based on concentrations of 1.8, 3.2, 5.6, 10, and 18 mg/L, with mortalities of 0, 2, 4, 9, and 10 fish, out of 10 per concentration. The eye-fitted line estimated the LC50 as 5.6 mg/L.

Computer programs gave very similar estimates to the graphic one, for the regular data of Figure 3. The LC50s (and 95 % confidence limits) were as follows:

Probit analysis of Hubert (1987): 5.56 (4.28–7.21)

Stephan (1977): probit analysis 5.58 (4.24–7.37)
moving average 5.58 (4.24–7.33)
binomial 6.22 (1.8–10)

Spearman-Kärber method:
0% trim 5.64 (4.38–7.26)
10% trim 5.73 (4.34–7.58)
20% trim 5.95 (4.34–9.80)

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

In fitting a line such as that in Figure 2, relatively more significance should be assigned to points that are near 50% mortality. If successive concentrations yield a series of 0 % mortalities, only one such value should be used in fitting the line (i.e., the one that is “closest to the middle” of the distribution of data). Similarly, only the first of a series of successive 100% values should be used.

axis is the logarithm of time instead of concentration. The 95 % confidence limits may be estimated and compared by carrying the graphic analysis a stage further (Litchfield, 1949). It should be recognized that neither an LT50 nor percentage survival at short exposures is a dependable method of judging ultimate toxicity; therefore, comparisons based on those endpoints give only semi-quantitative guidance.

4.6 Reference Toxicant

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

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

– chemical readily available in pure form;

– stable (long) shelf life of chemical;

– highly soluble in seawater;

– stable in aqueous solution;

– minimal hazard posed to user;

– easily analyzed with precision;

– good dose-response curve for test organism;

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

– known influence of water hardness on toxicity to test organism.

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

Seawater (natural or artificial[r]) is to be used as the dilution and control water. To provide a high degree of standardization for the reference toxicant tests, the salinity of the dilution and control water should be adjusted to a consistent value (e.g., 28 ± 1 ‰ or 14 ± 1 ‰) for all intra-laboratory determinations. The salinity chosen should be based upon that for which existing intra-laboratory toxicity data have been previously derived, and on the value that most

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The same principle applies to computer programs; only one successive 0 % or 100 % should be entered; additional ones may distort the estimate of LC50. Logarithmic-probability paper ("log-probit") as shown in Figure 3 may be purchased in or ordered through good technical bookstores.

If it is desired to estimate LT50, a graph such as Figure 3 can be plotted using logarithm of time as the horizontal axis. Individual times to death of fish could be used but they are seldom available since tests are not inspected continuously. The cumulative percent mortality at successive inspections is quite satisfactory for plotting, and an eye-fitted line leads to estimates of confidence limits following the steps in Litchfield (1949).

[r] The use of artificial seawater as dilution water for tests with reference toxicants is recommended, as it reduces the likelihood of test-by-test variability in results due to daily or seasonal changes in seawater chemistry (if natural seawater were used).

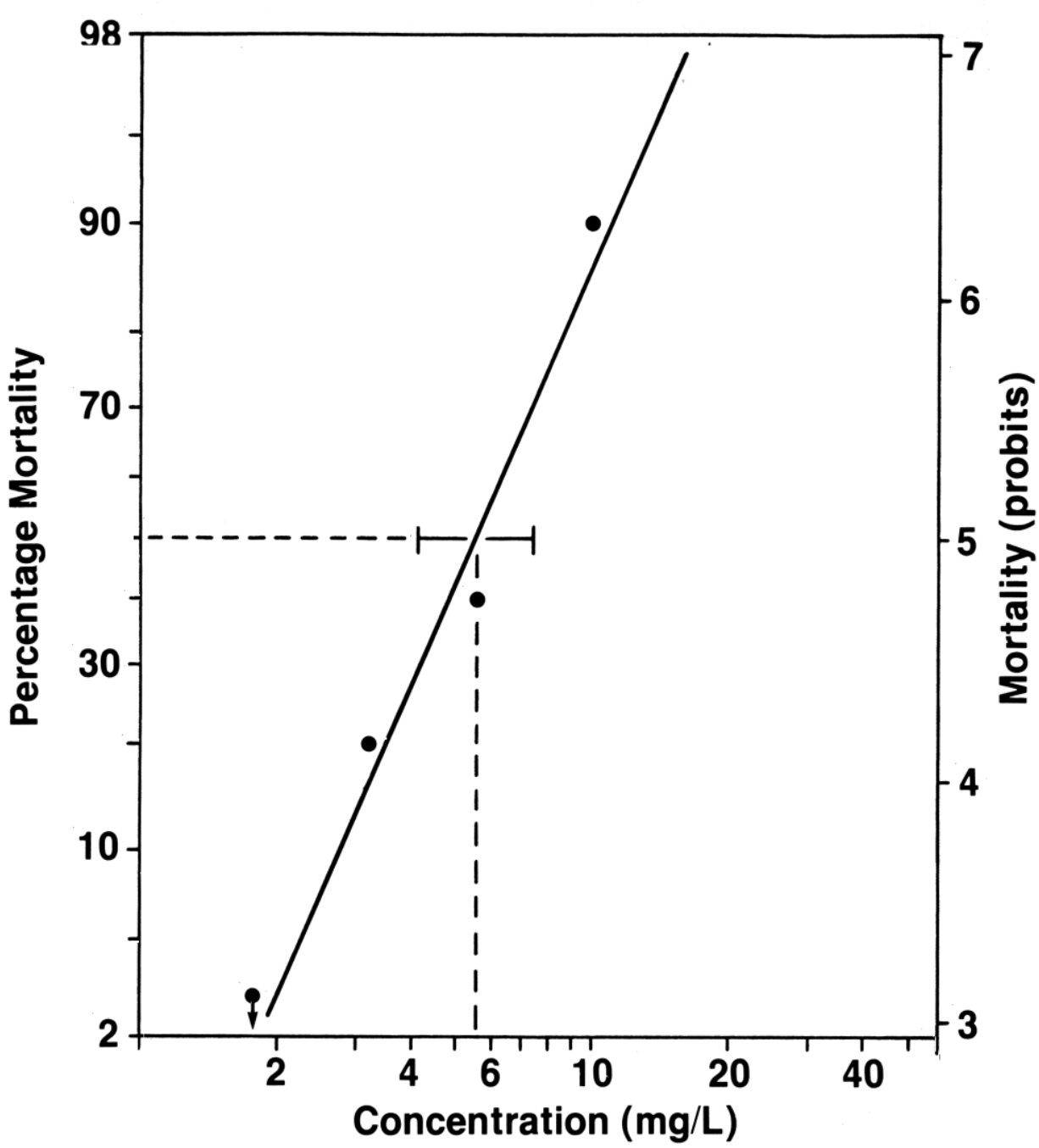

Figure 3 **Estimating a Median Lethal Concentration by Plotting Mortalities on Logarithmic-probability Paper**. In this hypothetical example, there were ten fish tested at each of five concentrations. The line was fitted by eye[q]. The concentration expected to be lethal to 50% of the fish may be read by following across from 50 % (broken line) to the intersection with the fitted line, then down to the horizontal axis for an estimated LC50 (5.6 mg/L).

closely approximates the salinity of the solutions of chemical, effluent, elutriate, leachate, or receiving-water sample being studied. Additionally, the salinity selected for the reference toxicant tests must be within 10 ‰ of that to which the population of stickleback are acclimated. As merited and depending on the availability of separate groups of stickleback acclimated to each of these salinities, a static test to determine the 96-h LC50 of the reference toxicant may be performed at each of these salinities.

Once sufficient data are available (EC, 1990), a warning chart should be prepared and updated for each reference toxicant used. Successive LC50s for the reference toxicant are plotted on this chart and examined to determine whether they are within ± 2 SD of the mean of previous values; the new LC50 is acceptable if it falls within the warning limits. To create the chart, the mean of available values of log(LC50), together with the upper and lower warning limits (±2 SD) should be recalculated with each successive LC50 for the reference toxicant, until the statistics stabilize (USEPA, 1985a, 1993; EC, 1990, 2000). The mean and ±2 SD should be plotted on the logarithmic vertical axis, against date of the test (or test number) on the horizontal axis.

The logarithm of concentration (including LC50) must be used in all calculations of mean and standard deviation. This represents continued adherence to the assumption by which each LC50 was estimated on the basis of logarithms of concentrations. The warning chart may be constructed by plotting the logarithmic values of the mean and ±2 SD on arithmetic paper, or by converting them to arithmetic values and plotting those on the logarithmic scale of semi-log paper. If it were demonstrated that the LC50s failed to fit a log-normal distribution, an arithmetic mean and SD might prove more suitable.

If a particular LC50 falls outside the warning limits, the sensitivity of the test organisms and the performance and precision of the test would be suspect. Since this might occur 5 % of the time due to chance alone, an outlying LC50 would not normally indicate abnormal sensitivity of the test organisms or unsatisfactory precision of toxicity data. Rather, it would provide a warning that there might be a problem. A check of all holding and test conditions is required at this time.

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

Concentrations of reference toxicant in all stock solutions should be measured chemically by appropriate methods (e.g., APHA *et al.,* 1989). Upon preparation of the test solutions, aliquots should be taken from at least the control, low, middle, and high concentrations, and analyzed directly or stored for future analysis should the LC50 be atypical (outside warning limits). If stored, sample aliquots must be held in the dark at 4 ± 2° C. Both zinc and phenol solutions should be preserved (APHA *et al.,* 1989) before storage. Stored aliquots requiring chemical measurement should be analyzed promptly upon completion of the toxicity test. It is desirable to measure concentrations in the same solutions at the end of the test, after completing biological observations. Calculations of LC50 should be based on the average measured concentrations if they are appreciably (i.e., ≥20 %) different from nominal ones and if the accuracy of the chemical analyses is reliable.

4.7 Legal Considerations

Complete and detailed specifications for acute lethality tests undertaken for legal purposes are

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

Specific Procedures for Testing Chemicals

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

5.1 Properties, Labelling, and Storage of Sample

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

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

5.2 Preparing Test Solutions

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

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

Organic solvents, emulsifiers, or dispersants should not be used to increase chemical solubility except in instances where these substances might be formulated with the test chemical for its normal commercial purposes. If used, an additional seawater control solution must be prepared containing the same concentration of solubilizing agent as that present in the most concentrated solution of the test chemical. Such agents should be used sparingly and should not exceed 0.5 mL/L in any test solution (USEPA, 1985b). If solvents are used, the following are

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

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

preferred (USEPA, 1985b): dimethyl formamide, triethylene glycol, methanol, acetone, and ethanol.

The dissolved oxygen should be measured upon preparation of each test solution including the control(s). Thereafter, the test should be initiated by introducing fish (see Section 4.2), or else each test solution should be pre-aerated (see Section 4.3.1) before adding fish. In most instances, the pre-aeration of test solutions is not necessary nor warranted (see footnote m). For those situations where pre-aeration is appropriate, the guidance for pre-aeration of solutions given in Section 4.3.1 should be followed.

5.3 Control/Dilution Water

Control/dilution water may be artificial seawater, the laboratory's supply of natural "uncontaminated" seawater, or (if there is special interest in a local situation) a particular estuarine of marine receiving-water sample taken adjacent to but without influence from the contaminant of concern. The choice of control/dilution water depends on the intent of the test.

If a high degree of standardization is required (e.g., the measured toxicity of a chemical is to be compared and assessed relative to values derived elsewhere, for this and/or other chemicals), artificial seawater adjusted to one or more salinities common to all tests should be used as the control/dilution water. Test salinities of 28 ‰ (representing full-strength seawater) and 14 ‰ (representing brackish water for estuarine conditions) are recommended for comparative tests.

If the toxic effect of a chemical on a particular marine estuarine or receiving water is to be assessed, sample(s) of the receiving water collected "upstream" (i.e., from a place that was isolated from influences of the chemical), could be used as the control/dilution water[u, v, w]. An example of such a situation would be an appraisal of the toxic effect of a chemical spill (real or potential) on a particular estuarine or marine water body. The laboratory supply of natural seawater may also be used for this purpose, especially where logistical constraints make the collection and use of receiving water impractical. This supply of natural seawater is also appropriate for use in other instances (e.g., preliminary or intra-laboratory assessment of chemical toxicity).

If information is desired regarding the influence of salinity on the toxicity of the chemical under investigation, separate tests should be conducted concurrently at three or more salinities (e.g., 10, 20, and 28 ‰). Control/dilution water required for such comparative tests should be from the same source. This source may be artificial seawater (Section 2.4.3) or a supply of natural, full-strength seawater (salinity ≥28 ‰) diluted to the desired salinity values using "uncontaminated" fresh water.

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

[v] While it would be desirable to acclimate a group of fish to the receiving water before using them in a test with that water used for dilution and control, this is often not feasible because of the need to transport large volumes of water to the laboratory. If possible and appropriate, tests using receiving water could be carried out near the site of interest, in which case acclimation should last at least five days.

[w] An alternative (compromise) to using receiving water as dilution and control water is to use artificial seawater or the laboratory seawater supply, adjusted to the salinity and pH of the receiving water. Depending on the situation, the adjustment may be to seasonal means, or to values in the receiving water at a particular time.

5.4 Test Observations and Measurements

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

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

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

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

5.5 Test Endpoints and Calculations

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

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

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

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

Specific Procedures for Testing Effluent, Elutriate, and Leachate Samples

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

6.1 Sample Labelling, Transport, and Storage

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

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

Testing of effluent and leachate samples should commence as soon as possible after collection. The test should begin within three days and must commence no later than five days after termination of sampling. Samples collected for extraction and subsequent testing of the elutriate should be tested within ten days of receipt. Elutriates should be tested within three days of sample preparation or as specified.

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

Samples must not freeze during transport. Upon arrival at the laboratory, effluent and leachate samples may be adjusted immediately or overnight to the test temperature ($10 \pm 2°$ C)[f], and testing commenced. If more prolonged sample storage is needed, sample containers must be stored in darkness at $4 \pm 2°$ C.

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

6.2 Preparing Test Solutions

Samples in the collection containers must be agitated thoroughly just prior to pouring, to ensure the re-suspension of settleable solids. Sub-samples (i.e., a sample divided between two or more containers) must be mixed together to ensure their homogeneity. If further sample storage is required, the composited sample (or a portion thereof) should be returned to the sub-sample containers and stored (Section 6.1) until used. If necessary, the temperature of samples or test solutions may be adjusted to the test temperature by heating or chilling in a water bath, or by the use of an immersion cooler made of non-toxic material (e.g., stainless steel).

Samples or test solutions must not be heated by immersion heaters, since this could alter chemical constituents and toxicity.

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

The adjustment of sample or solution salinity is normally not required for this test. However, if it is desired to understand the contribution of salinity towards sample toxicity, a second (salinity-adjusted) test should be conducted concurrently (Section 4.3.3).

In instances where the intent of the test is to compare the relative toxicity of a particular effluent, leachate, or elutriate source or type with that for other effluent, leachate, or elutriate samples derived and/or tested elsewhere, the salinity of all test and control solutions should be adjusted to the same value. Test salinities of 28 ‰ or 14 ‰ are recommended as appropriate values where comparative toxicity results representative of marine or estuarine environmental conditions are required. Procedures described in Sections 2.4.3 and 4.1 for the preparation of salinity-adjusted dilution and control water, using hypersaline brine (HSB), as well as those described in Section 4.3.3 for adjusting the salinity of an aliquot of the sample or the test solutions if necessary, must be followed in preparing test solutions with the desired salinity.

6.3 Control/Dilution Water

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

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

Leachates of concern can, at some times, be found flowing directly into the estuarine environment. In other instances, leachates can flow into and mix with coastal streams or rivers before entering the marine environment. Depending on these situations and the study objectives, receiving-water samples taken immediately upstream of or adjacent to but removed from the influence of the leachate may be used as the control/dilution water. The supply of seawater to which fish have been acclimated for two or more weeks may also be used for this purpose, and is an appropriate control/dilution water when testing leachates for routine monitoring and compliance requirements.

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

Tests requiring a high degree of standardization may be undertaken using reconstituted water as

the dilution and control water. Situations where the use of artificial seawater is appropriate include investigative studies intended to interrelate toxicity data for various effluent, leachate, or elutriate types and sources, derived from a number of test facilities or from a single facility where water quality is variable. In such instances, it is desirable to minimize any modifying influence due to (differing) dilution-water chemistry.

6.4 Test Conditions

Samples of effluent, leachate, or elutriate are normally not filtered or agitated during the test. However, the presence of high concentrations of suspended solids in a sample may be stressful to exposed fish, and can be acutely lethal if present in sufficiently high strengths (e.g., ≥2000 mg/L, Noggle, 1978; McLeay *et al.,* 1987; Servizi *et al.,* 1987). High concentrations of biological solids in certain types of treated effluent may also contribute to sample toxicity from ammonia and/or nitrite production (Servizi and Gordon, 1986). If concern exists about a contribution to toxicity from elevated concentrations of suspended or settleable solids in samples of effluent, elutriate, or leachate, an additional test may be conducted by maintaining solids in suspension throughout the period of fish exposure. Test vessels with vertical sides and steeply sloped, conical-shaped bottoms (Noggle, 1978; McLeay *et al.,* 1983) may be used for this purpose. Using this or similar apparatus, test suspensions can be continuously agitated during the test by aeration from the conical bottom or by use of a pump which draws from the bottom and redistributes to the surface. The insertion of a basket into each test vessel will permit their periodic inspection and protection from the recirculating apparatus. A third test, using a portion of the sample treated by filtering or decanting to remove solids, may also be performed using otherwise identical procedures if the intent of the study is to quantify the degree to which sample solids contribute to acute lethal toxicity.

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

6.5 Test Observations and Measurements

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

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

6.6 Test Endpoints and Calculations

Tests for monitoring and compliance with regulatory requirements should normally include, as a minimum, one or more undiluted portions of the samples and one or more control solutions. Depending on specified regulatory requirements, tests for regulatory compliance may use a single concentration (100% wastewater unless otherwise specified) or may determine the 96-h LC50 (see Section 4.5).

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

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

Specific Procedures for Testing Receiving-water Samples

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

7.1 Sample Labelling, Transport, and Storage

Procedures for the labelling, transportation, and storage of samples should be as described in Section 6.1. Testing of samples should commence as soon as possible after collection. The test should begin within three days and must commence no later than five days after termination of sampling.

7.2 Preparing Test Solutions

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

7.3 Control/Dilution Water

For tests with samples of estuarine or marine receiving waters taken within the vicinity of a wastewater discharge, chemical spill or other point-source of possible contamination, a suitable dilution and/or control water may be seawater collected concurrently from the same receiving-water body[v, w]. A seawater sample taken for this purpose should be collected as close as possible to the contaminant source(s) of concern, but be isolated from the zone of influence. Water current of dispersal tracer studies may be required to establish an acceptable location for sampling receiving water to be used as control and dilution water.

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

Logistic constraints, expected toxic effects, or other site-specific practicalities may prevent or rule against the use of an appropriate receiving-water sample as the control/dilution water. Other situations ruling against its use include insufficient information regarding the pattern of mixing and dispersal of the contaminant of concern, or an inability to find a suitable source of unpolluted seawater isolated from the influence of the contaminating source. In such cases, the laboratory water supply to which fish have been acclimated should be used as the control water for all dilutions. It could be adjusted to partially simulate "upstream" receiving water[w].

Upon preparation of each test solution including the control(s), its dissolved oxygen content should be measured. Thereafter, the test should be initiated by introducing fish (see Section 4.2), or else each test solution should be pre-aerated (see Section 4.3.1) before adding fish. In most instances, the pre-aeration of test solutions is not necessary nor warranted (see footnote m). For

those situations where pre-aeration is appropriate, the guidance for pre-aeration of solutions given in Section 4.3.1 should be followed.

7.4 Test Observations and Measurements

Observations of sample and solution colour, turbidity, foaming, precipitation, etc. should be made as described in Section 6.5, both during preparation of test solutions and subsequently during the tests. These are in addition to the preliminary observations on fish described in Section 4.4.

7.5 Test Endpoints and Calculations

Endpoints for tests with samples of receiving water should be consistent with the options and approaches identified in Sections 4.5 and 6.5.

Tests for monitoring and compliance purposes should normally include, as a minimum, one or more undiluted portions of the sample and one or more control solutions. Endpoints for tests with receiving-water samples may be restricted to a determination of percent fish mortality at 96 h in the undiluted sample, together with time-to-death data where applicable.

In instances where toxicity of receiving-water samples is likely and information is desired concerning the degree of dilution necessary to permit short-term survival of fish, a test to determine the 96-h LC50 should be conducted. One or more undiluted (100 % sample) concentrations and at least four dilutions should be included in this test, together with one or more control solutions. Assuming that data permit, the LC50 and its 95 % confidence limits should be computed.

Reporting Requirements

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

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

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

8.1 Test Material

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

8.2 Test Organisms

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

8.3 Test Facilities and Apparatus

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

8.4 Control/Dilution Water

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

8.5 Test Method

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

8.6 Test Conditions

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

8.7 Test Results

- appearance of test solutions and changes noted during test;
- fish behaviour, appearance, number and percentage of mortalities in each test

solution (including control) as noted during each observation period; number and percentage of control fish showing atypical/stressed behaviour;

- results for range-finding test (if conducted);
- any 96-h LC50 or LT50 values (including the associated 95% confidence limits) determined, including reference to the statistical method used for their calculation; and
- the 96-h LC50 and 95% confidence limits for the reference toxicant(s) determined within one month of the test using the same group of fish as those from which the test fish were selected, together with the mean value (±2 SD) for the same reference toxicant as derived at the test facility in previous tests.

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Appendix A

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

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

Federal (Environment Canada)

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

B. Moores
St. John's, Newfoundland

K. Doe
Dartmouth, Nova Scotia

W. Parker
Dartmouth, Nova Scotia

N. Bermingham
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C. Blaise
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G. Elliot
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Western and Northern Region
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Edmonton, Alberta
T6B 2X3

Pacific and Yukon Region*
224 Esplanade Street
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V7M 3H7

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

Appendix B

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

1. Type of Test and Test Material

Document	Test Type	Test Material
EPS 1985	static	drilling fluid
Wong 1982	static	drilling fluid

2. Acclimation Conditions for Fish

Document	Duration (weeks)	Water Exchange (L/g)	Loading Density (g/L)
EPS 1985	≥2	≥1.4	≤10
Wong 1982	≥3	>1.4	≤10

3. Feeding Conditions

Document	Feed Type	Pre-test	During Test
EPS 1985	choice of brine shrimp, ground fresh fish, or tropical fish flakes	feed daily; do not feed 24 h pre-test	do not feed
Wong 1982	food containing 30 to 40 % protein and some vegetable substances	feed at least twice daily; 3 to 5 % body weight	do not feed

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

4. Type of Control/Dilution Water

Document	Recommended Type and Treatment
EPS 1985	natural or artificial seawater
Wong 1982	natural or artificial seawater

5. pH Adjustment Prior to and During Test

Document	Pre-test Adjustment	During Test
EPS 1985	as required, adjust test solution to PH 7.0 to 9.0	as required, adjust test solution daily to pH 7.0 to 9.0
Wong 1982	sample pH adjusted to within 0.5 pH units of acclimation water	not indicated

6. Temperature Prior to and During Test

Document	Acclimation Rate (° C/day)	Acclimation Temperature (° C)	Test Temperature (° C)
EPS 1985	gradual	10 ± 1	10 ± 1
Wong 1982	≤5	10 ± 1	10 ± 1

7. Aeration Prior to and During Test

Document	Acclimation DO	Test Aeration (mL/min · L)
EPS 1985	> 80 % saturation	≤7.5
Wong 1982	7 mg/L	5 to 7.5 for ≤2 h

8. Salinity Prior to and During Test

Document	Acclimation Salinity (‰)	Salinity of Control and Dilution Water
EPS 1985	not indicated	30 to 35 ‰
Wong 1982	20 to 25* or 27 to 32**	not indicated

9. Lighting Conditions Prior to and During Test

Document	Photoperiod (L:D)	Intensity (lux)	Dawn/Dusk (min.)
EPS 1985	14h:10h	20 to 30	≥15
Wong 1982	10h:14h	20 to 30	≥15

10. Weights of Test Fish

Document	Weight Range (g)
EPS 1985	0.2 to 3.0
Wong 1982	0.5 to 1.0

11. Starting the Test

Document	No. Fish per Solution	No. Test Solutions	Depth of Solutions (cm)	Fish Loading Density (g/L)
EPS 1985	≥10	≥6	≥15	≤0.5
Wong 1982	≥5	≥6	≥15	not indicated

* Salinity for testing Beaufort Sea samples
** Salinity for testing Davis Strait samples

12. Test Endpoints

Document	Test Duration	NOEC	96-h LC50
EPS 1985	96 h	not determined	yes
Wong 1982	96 h	not determined	yes

13. Requirements for Test Validity

Document	Percent Survival During Acclimation	Percent Survival of Controls
EPS 1985	not indicated	≥90%
Wong 1982	≥99 % /day for 2 weeks pre-test	≥90%

14. Reference Toxicant

Document	Chemical
EPS 1985	not indicated
Wong 1982	not indicated

Appendix C

Logarithmic Series of Concentrations Suitable for Use in Toxicity Tests*

Column (Number of Concentrations Between 100 and 10, or between 10 and 1)**

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

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

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

Appendix D

Terms Suitable for Describing Fish Appearance and Behaviour

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

* modified from USEPA 1985a