



Freshwater Fish *(Gobiomorphus cotidianus)*

Acute Toxicity Test Protocol

Appendix to:

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***(Gobiomorphus
cotidianus)***

**Acute Toxicity
Test Protocol**

Abstract

The method prepared by the National Institute of Water and Atmospheric Research (NIWA) for determining the acute toxicity of whole effluents to the freshwater common bully *Gobiomorphus cotidianus*, is described. Included are details on collection and holding conditions and requirements for the test species, 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 data analyses, including statistical procedures, and the use of reference toxicants.

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List of Abbreviations and Chemical Formulae

°C	degree(s) Celsius
cm ² /L	square centimetre(s) per litre
d	day
DO	dissolved oxygen (concentration)
g	gram
h	hour
H ₂ O	water
L	litre
LC ₅₀	median lethal concentration
LOEC	lowest observed effect concentration
m	metre
mg	milligram
min	minute
MgSO ₄	magnesium sulphate
mL	millilitre
MSD	minimum significant difference
NaHCO ₃	sodium bicarbonate
NaOH	sodium hydroxide
NOEC	no observed effect concentration
SD	standard deviation
^{TN}	Trade Name
ZnSO ₄ .7H ₂ O	zinc sulphate
μL	microlitre
μm	micrometre
μmol m ⁻² s ⁻¹	micro moles per metre square per second
>	greater than
<	less than
≥	greater than or equal to
≤	less than or equal to

Terminology

Note:

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

Grammatical Terms

<i>Must</i>	is used to express an absolute requirement.
<i>Should</i>	is used to state that the specified condition or procedure is recommended and ought to be met if possible.
<i>May</i>	is used to mean “is (are) allowed to”.
<i>Can</i>	is used to mean “is (are) able to”.

General Technical Terms

<i>Acclimation</i>	means to become physiologically adapted to a particular level of one or more environmental variables such as temperature. The term usually refers to controlled laboratory conditions.
<i>Hardness</i>	is the concentration of cations in water that will react with a sodium soap to precipitate an insoluble residue. In general, hardness is a measure of the concentration of calcium and magnesium ions in water, and is expressed as mg/L calcium carbonate or equivalent.
<i>Monitoring</i>	is the routine (e.g., daily, weekly, monthly, quarterly) checking of quality or collection and reporting of information. In the context of this protocol, 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.
<i>Percentage (%)</i>	is a concentration expressed in parts per hundred parts. One percent represents one unit or part of material (e.g., effluent, elutriate, leachate, or receiving water) diluted with water to a total of 100 parts. Concentrations can be prepared on a volume-to-volume or weight-to-weight basis, and are expressed as the percentage of test material in the final solution.
<i>pH</i>	is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions on a scale from 0 to 14, with 7 representing neutrality, numbers less than 7 signifying increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.
<i>Photoperiod</i>	is the duration of illumination and darkness within a 24-h day.

<i>Precipitation</i>	is the formation of a solid (i.e., precipitate) from a solution.
<i>Pre-treatment</i>	is the treatment of a sample or dilution thereof, prior to exposure of fish.
<i>Tail-base length</i>	is the length of a fish, measured from the tip of the nose to the base of the tail.

Terms for Test Materials

<i>Carbon filtered water</i>	tap water that is passed through a carbon filter cartridge to remove residual chloramines and other chlorinated organic compounds.
<i>Control</i>	is a treatment in an investigation or study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific condition that is being studied. In an aquatic toxicity test, the control must duplicate all the conditions of the exposure treatment(s), but must contain no test material. The control is used to determine the absence of measurable toxicity due to basic test conditions (e.g., quality of the control/dilution water, health or handling of test organisms).
<i>Control/Dilution water</i>	is the water used for diluting the test material, or for the control test, or both.
<i>Dechlorinated water</i>	is a chlorinated water (usually municipal drinking water) that has been treated to remove chlorine and chlorinated compounds from solution.
<i>Deionised water</i>	is water that has been passed through resin columns to remove ions from solution and thereby purify it.
<i>Dilution water</i>	is the water used to dilute a test material in order to prepare different concentrations for the various toxicity test treatments.
<i>Distilled water</i>	is water that has been passed through a distillation apparatus of borosilicate glass or other material, to remove impurities.
<i>Receiving water</i>	is surface water that has received a discharged waste, or else is about to receive such a waste (e.g., it is away from the discharge point). Further descriptors must be provided to indicate which meaning is intended.
<i>Reconstituted water</i>	is deionised or glass-distilled water to which reagent-grade chemicals have been added. The resultant synthetic fresh water is free from contaminants and has the desired pH and hardness characteristics.
<i>Reference toxicant</i>	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 that can be stored. Measured volumes of a stock solution are added to dilution water in order to prepare the required strengths of solutions.

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

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

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

Toxicity Terms

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

End point means the variables (i.e., time, reaction of the organisms, etc) that indicate the termination of a test, and also means the measurement(s) or value(s) derived, that characterize the results of the test (LC_{50} , 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.

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

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

LOEC lowest observed effect concentration. The lowest concentration tested causing a statistically measurable effect to the test system.

MSD minimum significant difference. The difference between values for individual treatments that would have to exist before it could be concluded that there was a significant difference between the

groups. MSD is provided by certain statistical tests including *Dunnnett's multiple-range test*, a standard statistical procedure.

NOEC no observed effect concentration. The highest concentration tested causing no statistically measurable effect to the test system.

Overt means obviously discernible under the test conditions employed.

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

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 a living organism.

Toxicity test is a determination of the effect of a material on a group of selected organisms under defined conditions. An 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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1.0 Introduction

No single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection (Environment Canada, 1990a). When used as a component in a suite of well-defined toxicity tests where a variety of endpoints are measured and species are tested, the results can contribute to an holistic interpretation of toxic impacts. Whole effluent toxicity testing as opposed to single chemical testing enables a greater correlation between the toxicity test results and the likely impacts in the actual environment. The relevance of laboratory toxicity testing to the environment is also enhanced through the use of native species such as the common bully (*Gobiomorphus cotidianus*). The acute lethality test using the common bully is one of several “core” aquatic toxicity tests selected for standardization.

1.1 Principles of the Test Method

Common bully (*G. cotidianus*) are exposed in a static system, to a dilution series of a test substance, an effluent or zinc reference toxicant under defined conditions. The survival of the fish exposed to the test substance is compared with the survival of the fish in an appropriate control over a fixed period of time. A test substance is considered toxic when a statistically significant, dose-dependent mortality of fish occurs.

1.2 Historical Use of the Test

Test procedures with freshwater fish have been evaluated over a long period of time and have been adopted as a standard approach for determining the toxic effect of complex effluents on fish. Fish species such as Rainbow Trout (*Oncorhynchus mykiss*) and Fathead Minnow (*Pimephales promelas*) have been used extensively in Canada and United States of America over the last 20 years (Environment Canada, 1990a, 1990b; USEPA, 1993). The use of the common bully (*G. cotidianus*) in toxicity testing in New Zealand has been limited due to the lack of economic importance of this species, but is now being recommended because it showed

to be relatively sensitive to a variety of toxicants. This species has recently been used to determine the toxicity of ammonia and pH to native fish (Richardson, 1997; West, 1997)

1.3 Summary of the Test

Fish collected from the field are held for 4 days prior to conducting a 96 h test with a 16:8 h L:D photoperiod at 15°C. The test system is static and non-renewable whereby the fish are exposed to the same test solution for the duration of the test. The 4 L test solutions are aerated and the temperature, pH and dissolved oxygen (DO) are measured. Once fish of a similar size have been placed into the test solutions, observations of mortality and overt sub-lethal behaviour are made at 24, 48, 72 and 96 h intervals. The mortality is recorded and used to calculate of the LC₅₀ which is the percentage concentration of effluent causing mortality in 50% of the fish. The head to tail-base length and weight of fish from one replicate of each treatment is recorded at the end of the experiment.

1.4 Application, Advantages, and Limitation of the Test System

The experimental design consists of three to five replicates of each test concentration, including the control, with each replicate containing 10 fish. It may be necessary to reduce the number of fish per replicate in situations where either the number of fish or the volume of test solution is insufficient. A reduction in the number of fish per test solution from ten to seven will result in a minimal loss of precision in the LC₅₀ (Environment Canada, 1990a).

The static non-renewal system is simple and inexpensive to operate. Limited resources (technical staff, space and equipment) are required and a smaller volume of effluent is necessary as compared to a static renewal system or flow-through system where the test solution is replaced daily (USEPA, 1993). Dissolved oxygen levels should be maintained at least at 70% saturation in the highest

effluent concentration to support fish survival. There is the possibility of a loss of toxicants through degradation of the test solution over the test duration thereby making the test less sensitive as compared to solution renewal systems. If the test material is known to be highly degradable, static-renewal or flow-through tests are recommended.

2.0 Test Organism

2.1 Species

The common bully, *G. cotidianus* is a native fish species that is widespread throughout New Zealand freshwater systems. Populations can be found near lake shores and gently flowing streams. Lake populations are found in nearly all coastal and lowland lakes, and also in many inland and alpine lakes. Habitat preferences of the common bully in lowland streams are intermediate of fish preferring fastwater and those that are edge-dwelling. They have been found to occur in high abundance where other native migratory fish occur (Jowett and Richardson, 1996). They are less cover-seeking than other bullies and as juveniles, occur in abundant numbers on open shores in either sandy, gravelly or rocky-bedded lakes. As the fish grow they become bottom-living, and move into the shallows during the summer. Later they move into deeper water, where the large adults mostly occur. In rivers the common bully is found more often hiding amongst marginal cover - overhanging banks, logs, large rocks and debris (McDowall, 1990).

The male common bully is darker in colour than the female and has a blunter head and larger fins. The fish are known to reach a maximum length of 150 mm and commonly reach 110 mm. Lake fish tend to be much smaller, 70-80 mm being the common adult size (McDowall, 1990).

2.2 Source

The fish should be collected from the same source each time as fish of the same species from a different source may have different sensitivity and be of a different genetic stock (ASTM, 1997). Collection of the fish should coincide with their most prolific feeding time which commences at dusk and continues throughout the night. It is at this time that they will move into shallow water making it easier to collect them in large numbers. Seine fishing is used to capture the required number of fish which are then transported back to the laboratory as soon as possible after collection.

The technique of seine fishing involves extending a lead weighted net (4.76 mm size mesh) between two people who then walk parallel to the shore at a steady pace through the water. Any fish caught in the wall of netting are guided into the fabric pocket (1.59 mm size mesh) located in the centre of the net. Plastic scoops consisting of perforated ice cream containers are used to sort and transfer the fish from the net to 45 L insulated bins (e.g. chilly bins) which are already half full of water from the collection site. A density of no more than 150 fish per chilly bin is permitted in order to prevent stress to the fish due to over crowding. Malachite green may be added (10 mL/100 L collection water) to the water in the chilly bin to help prevent the spread of fungal diseases that may already be present in some of the fish. An aloe vera gel formulated for aquaculture use may also be added (0.26 mL/L collection water) to provide protection for the fish against skin abrasion during transportation. The water in the chilly bins must be aerated continuously during the trip to the laboratory. In the laboratory, the fish must be gently transferred to the holding tanks using scoop containers or hand dip nets.

2.3 Holding and Acclimation

The fish should be held for 4 days prior to commencing the test in large plastic or glass tanks, which should be free of contaminants. A density of no more than 2.5 fish per L is permitted to prevent stress to the fish due to over crowding and metabolic waste build up. Fifty percent of the water in the holding tanks should be replaced daily, and sodium bicarbonate (NaHCO_3) should be added to maintain a concentration of 0.157 g/L NaHCO_3 . The NaHCO_3 acts to prevent disease and buffer the pH reduction caused by respiration of the fish. Floating and benthic debris should be removed by siphoning or using a vacuum pump. During the holding period, the fish should be fed once a day on a variety of live invertebrate species such as *Daphnia* sp. and mosquito larvae that can be

collected by sieving (70 µm) water from an artificial pond set up for the purpose of culturing live freshwater fish food. The live food is added *ad libitum* to the fish tanks. An alternative to this form of food is a recognized standard commercial pelleted fish food. The pellets must be of a small size and dispensed as 1 - 5 % of wet body weight (Environment Canada, 1990a). Over-feeding with dry food must be avoided to prevent fouling the water thereby encouraging bacterial and fungal growth. The fish must not be fed 24 h before test initiation or during the 96 h of the test to prevent fouling the test solutions.

Fish that are held for longer than 4 days under these conditions must be observed closely for signs of stress (Appendix 9.1).

2.3.1 Holding Water

Water used in the holding tanks can be carbon filtered tap water or receiving water. The filtered tap water should be stored at 15 °C in plastic drums (or other suitable holding tanks) and aerated for at least 2 days prior to use. The carbon filter removes residual chloramine and other chlorinated organic compounds. Sodium bicarbonate (NaHCO₃) should be added (0.157 g/L) to the water in the holding tanks to discourage fungal growth and maintain pH at 6 - 9. After the daily replacement of 50% of the water, NaHCO₃ is again added to the holding tank to maintain the desired concentration (0.157 g/L NaHCO₃). NaHCO₃ is not added to the water used in the test. An alternative to using NaHCO₃ to control disease is to add uncontaminated filtered seawater (1 µm) 10% (v/v).

2.3.2 Physicochemical Conditions

Holding tanks must be maintained at 15 ± 2 °C with a 16:8h L:D photoperiod. Light intensity at the water surface should be ≤ 6 µmol m⁻² s⁻¹ using full spectrum fluorescent lights. The pH of the holding water must be within the range of 6 - 9 pH units. Dissolved oxygen must be >70 % saturation obtained through aeration with dust filtered oil-free air.

2.4 Quality of Test Organism

All fish used in the test should be uniform in size. The length of the fish should be 20 - 35 mm in length. Generally, the length of the largest fish should not be more than twice that of the smallest in the same test (Environment Canada, 1990a). The wet weight of the fish should be between 0.1 - 1.0 g. Fish must be observed during the holding time and rejected from the test if considered to be showing signs of stress such as loss of buoyancy control and orientation, lethargy or erratic swimming behaviour (a detailed description of stressed fish behaviour can be found in Appendix 9.1). Fish must also be examined for signs of disease at the time of collection and throughout the holding time. Symptoms include loss of appetite, abnormal distribution in the tank, lethargy, erratic or atypical swimming behaviour, darkened colouration, pale gills, eroded or frayed fins, external lesions and white growths around the gills and on the skin (Environment Canada, 1990a). Chemical treatment of fish with diseases is not desirable and should be avoided if possible. It is recommended that fish stocks showing signs of disease be discarded rather than treated as the treatment may influence the organisms sensitivity during the test (Environment Canada, 1990a). Also, systemic bacterial infections cannot be treated effectively and spread rapidly throughout the remaining fish.

A limit is placed on the loading (weight) of organisms per litre of test solution to minimize the depletion of dissolved oxygen, the accumulation of injurious concentrations of metabolic waste products, and/or stress induced by overcrowding. At a temperature of 15°C, the loading of animals in the test solution must not exceed the wet weight of 2.6 g/L (NIWA unpublished). Either the solution volume or the number of fish per container must be altered to accommodate this loading value. In high loading situations analyse the control replicate solutions for detrimental increases in un-ionized ammonia and nitrite.

Procedures involving the handling and testing of animals must obtain approval from a

designated Animal Ethics Committee (AEC) before testing commences. To prevent excessive disturbance and stress being caused to the fish the following conditions and handling procedures are recommended:

- During seining, the duration of the trawl should be adjusted such that all of the fish caught can be placed into water within five minutes of the net coming onto the shore.
- The fish should be transported from the collection site in suitable containers e.g. non-contaminated insulated with water taken from the collection site. The container volume should be sufficient to prevent sudden temperature increases or depletion of dissolved oxygen, especially in during high temperatures. Chilling equipment such as packed ice and compressed air or oxygen should be available to maintain adequate temperature and dissolved oxygen concentrations.
- The fish should be handled as little as possible during the holding time and the actual test to reduce stress. When handling is necessary, it should be done gently and quickly. Any fish that are dropped during handling or come into contact with dry surfaces should not be used in the test and must be maintained separately from other fish if not contaminated. Otherwise it must be disposed of as described in section 4.5.
- All holding tanks should be covered where ever possible and where not, staff should be careful to make no sudden movements over the top of the tanks, especially if they cause shadows to fall across the water.

Performance and fish sensitivity must be evaluated by routinely measuring the survival and relative sensitivity of *G. cotidianus* to a reference toxicant (Section 3.6.2).

3.0 Test System

3.1 Summary of Test System

The static non-renewal test system consists of three to five replicate 4 L test chambers of each test concentration with each replicate containing 10 fish. There must be a minimum of 15 cm solution depth in each test chamber with a base area of 290 cm². A reference toxicant (zinc sulphate), control and 5 effluent dilutions should be prepared. Throughout the period of the test, the fish mortality and any observations of stress induced behaviour is recorded. At the completion of the test, the temperature, pH and DO are recorded from one replicate of each test treatment. The average fish size should be obtained by measuring and weighing all the fish in one replicate of each treatment.

3.2 Facilities

The test should be conducted in a facility isolated from general laboratory disturbances. This can be achieved by conducting the test in a separate room or surround the test area with an opaque curtain (e.g. black plastic) to minimise stress to the fish during testing. A constant temperature room set at 15 °C is ideal. If this is not available a series of temperature controlled water baths can be used to immerse the test chambers. Dust should be minimised.

3.3 Equipment

All equipment (Table 1) coming in contact with the test water must be contaminant free and non-toxic.

Physical and physiochemical parameter measuring equipment should be calibrated regularly. Records must be kept of calibration changes made so interpolations can be calculated for data recorded between calibrations. Calibrations should also be traceable to international standards.

Table 1: Equipment and reagents required to perform a freshwater *G. cotidianus* 96 h test

Equipment for collection of fish

- seine net (mesh size = 4.76 mm for the wings and 1.59 mm for the pocket, total length of net = 7.5 m)
- 45 L insulated container e.g. chilly bins
- plastic container scoops (2 L)
- Malachite Green
- Aloe vera gel protection for fish abrasion (optional)
- aeration pump (battery operated or adapted for a vehicle cigarette lighter)
- aeration tubing (5mm diameter), air valves and air stones
- head lamps with standby batteries
- waders
- hand dip nets
- Life jackets

Equipment for holding fish

- thermostatically controlled room or water bath set to 15 ± 2 °C
- dilution water (carbon filtered tap water or receiving water that has been aerated for at least 2 days)
- carbon filter (Amtek C1 carbon filter cartridge with carbon impregnated paper or equivalent)
- sodium bicarbonate (NaHCO₃)
- plastic or glass holding tanks (suggested dimensions: length x height x width = 87cm x 30cm x 61cm)
- clear polythene plastic to line the holding tanks (optional, to be used if tanks are not contaminant-free)
- source of clean live freshwater invertebrates, e.g. artificial pond, animal drinking trough or commercial pelleted fish food
- sieve (70-100 µm)
- 10 L bucket to collect live food

Equipment required to perform a 96 h test

Non-consumable Equipment

- 4.5 L plastic or glass test chambers (if they are plastic use a polythene plastic bag liner can be used to avoid the need of decontaminating the chambers after each test). Suggested dimensions: length x height x width = 18cm x 20cm x 18cm
- 2 L and 3 L glass volumetric flasks
- 50 mL, 250 mL and 1 L glass measuring cylinders
- 2 mL pipette
- 500 mL glass beakers
- 1 L glass beaker
- pH meter
- dissolved oxygen meter
- thermometer
- analytical balance
- magnetic stirrer or ultrasound bath
- safety glasses

Consumable Equipment

- polythene bags to line plastic test chambers (optional)
- plastic covered wire ties to secure the openings of the bags
- Aeration tubing (5mm diameter), air valves and air stones or pipette tips (0.5ml)
- non-absorbent gloves
- face mask for volatile effluent
- disposable plastic spoons

Reagents required to perform 96 h test

- zinc sulphate (analytical grade) (ZnSO₄·7H₂O)
- nitric acid (analytical grade)
- dilution water (carbon filtered tap water or receiving water that has been aerated for at least 2 days)
- anaesthetic (Acros 2-phenoxyethanol 99%)

3.4 Test Conditions

Table 2: Test conditions for freshwater *G. cotidianus* 96 h test

<ul style="list-style-type: none"> • water temperature: 15 ± 1 °C • lighting: continuous “cool-white” fluorescent (≤ 6 μmol m⁻² s⁻¹) 16:8h L:D photoperiod • aeration: gentle aeration at > 70% saturation • loading rate of 2.6 g/L wet weight of fish • pH 6 - 9
--

3.5 Cleaning Procedure

Air stones or pipette tips, tubing and plastic bag liners must be disposed of at the completion of the test. All contaminated glassware and non-disposable plastic must undergo a complete wash according to the following method:

- Wash with a non-phosphate and non-ionic detergent solution
- Rinse ten times with tap water
- Rinse with acid solution (10 % HNO₃ v/v)
- Rinse three times with tap water
- Rinse with acetone
- Rinse three times with tap water
- Rinse three times with deionised water
- Place in oven to dry
- Cover openings of glassware with cling wrap or other cap as necessary, and store

Equipment made of any material other than glass, and which can withstand the recommended washing treatment, must be washed using this method.

It is recommended that test chambers and holding tanks be lined with plastic liners. If test chambers or holding tanks become contaminated (for example if plastic liners are perforated), perform an initial wash with a sterilizing compound such as an aquarium disinfectant followed by a complete wash as described.

Laboratory benches and floors that become contaminated should be washed with disinfectant such as a hypochlorite solution (200 mg/L) (ASTM, 1997), and thoroughly washed with tap water afterwards. The aeration fittings should be set aside for washing as per the other fish handling and analytical equipment.

3.6 Preparation of Reagents

3.6.1 Dilution Water

Control/dilution water consists of carbon filtered tap water that is aerated and stored in the dark for at least 2 days in polythene tanks

or equivalent at the temperature of the experiment (i.e. 15°C).

Tests conducted with effluent and reference toxicant samples should use uncontaminated carbon filtered tap water as the control/dilution water unless the objective is to assess the toxic effect of a sample on a particular receiving water, then the receiving water should be used as the control/dilution water. Receiving water containing debris or indigenous organisms, that may be confused with or attack the test organisms, should be filtered through a sieve with a mesh of 60 µm prior to use (USEPA, 1993). A standard control with the standard carbon filtered tap water control/dilution was must also be included. Receiving water must be transported and stored as in section 3.6.3.

Fish should be acclimated for 4 days to the receiving water before the test. This will involve transporting large volumes of receiving water, which could be difficult, so that conducting the test in close proximity to the collection site may be desirable.

3.6.2 Reference Toxicant

Reference toxicant tests are used to assess the reproducibility and reliability of results using a given test organism and test procedure over a specific period of time.

Zinc sulphate (ZnSO₄·7H₂O) is the recommended reference toxicant. It is stable in aqueous form, has a stable and good shelf life and is easy to measure analytically. The reference toxicant dilution series is prepared from a 100 mg/L Zn²⁺ stock solution (1.3194 g ZnSO₄·7H₂O made up in 3000 mL of carbon filtered tapwater) on the day of test initiation. Ensure that the salts is fully dissolved by immersing the bottle of stock solution in an ultrasound bath or using a magnetic stirrer. Chemically verify the stock solution concentration by analysing a sub-sample preserved with 0.2% HNO₃ (v/v). The source and purity of the reference toxicant must be reported.

A logarithmic series of test concentrations is used (Appendix 9.1). The control/dilution

water for use in the reference toxicant test is carbon filtered tapwater as described in section 3.6.1. The control/dilution water used in the reference test may differ from the control/dilution water used in the toxicity test depending on whether a receiving water has been provided with the sample.

Toxicity testing with a zinc reference toxicant should occur each time a test is performed. The test results should be plotted according to a mean chart where the vertical axis represents the endpoint concentration (e.g., LC₅₀ 96 h), and the horizontal axis represents the test date or test number (Figure 1). With a sufficiently large data set (i.e. more than 20

data points) the chart can be used to assess the validity of results from subsequent tests with that reference toxicant. If the LC₅₀ for a recently completed test does not fall within the $\pm 2SD$ range of the mean, it is highly probable that the test is unacceptable. It may indicate a change in test organism health or genetic sensitivity, a procedural inconsistency, or a combination of these factors. In this situation the test should be repeated with all aspects of the test being carefully scrutinized (Environment Canada, 1990c).

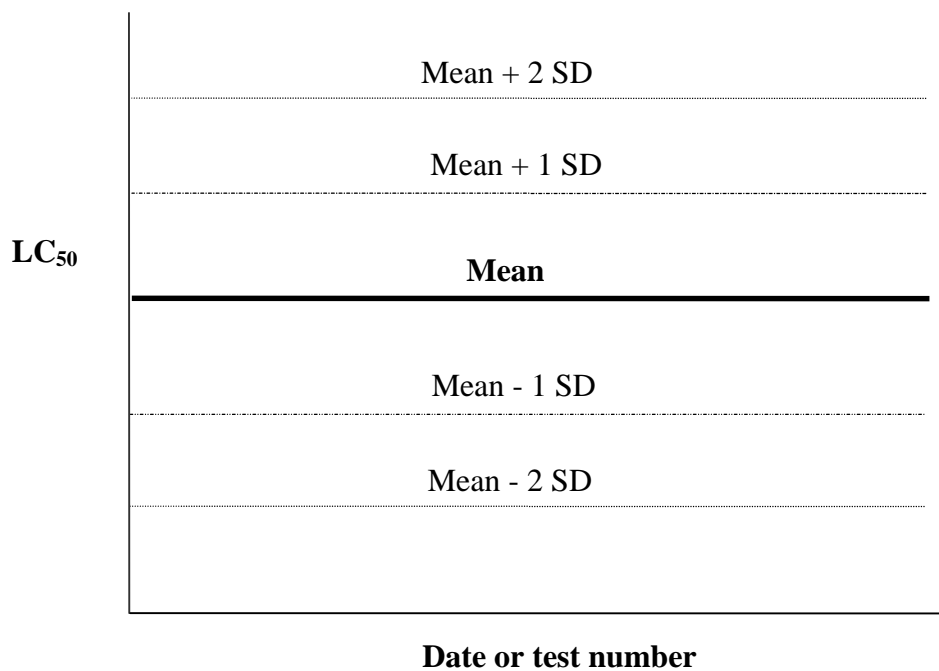


Figure 1: Analytical quality control chart with mean \pm 2 standard deviations (taken from Environment Canada, 1990b).

3.6.3 Effluent

Aqueous samples must be collected in a manner that ensures that they adequately reflect the true nature of the effluent or leachates. Generally, a sample volume of 20 - 30 L is sufficient for testing. The containers for transport and storage should be new or thoroughly cleaned (section 3.5). Rinse the container with the sample prior to filling. Fill to the brim to minimise headspace to prevent volatiles escaping into the air and seal the container. Clearly label with the type of sample, source and/or sample location, sample identification, date and time of collection, and name of sampler(s). The chain of custody must be maintained throughout. Transport the sample in a chilled but unfrozen condition by placing the container on ice in a chillybin.

Once the sample has reached the laboratory, the sample should be stored in the dark and at 4 °C. Samples should be tested as soon as possible i.e. within 36 of the last sample being collected and must be tested within 72 h after collection (USEPA, 1993). The

temperature, DO and pH of the sample must be recorded before testing commences.

It may be desirable to conduct chemical analyses of the sample or measure total suspended solids and total settled solids in effluents characterized with appreciable amounts. Removal of these fractions of the effluent could influence the results of the toxicity tests.

All safety precautions associated with effluent and leachates must be taken when handling and working with these samples. This includes wearing gloves, a laboratory coat and safety glasses and using a face mask or fume hood if the sample is particularly volatile. Any of the test solution that comes in contact with the skin should be washed off immediately.

3.7 Preparation of Test Solutions

The test solutions and number of concentrations 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) should be prepared. A preliminary range-finding test may be conducted prior to the actual test to assist in determining the appropriate dilutions. When using a range-finding test a broader concentration range is used and the test is frequently terminated in 24 h or less. An appropriate geometric dilution series may be used, in which each successive concentration is about 10% of the previous one (e.g., 100, 10, 1.0, 0.1). For the definitive test, the concentrations may be elected from other appropriate logarithmic dilution series (Appendix 9.2).

Agitate the sample thoroughly to ensure homogeneity and to resuspend any particulates. Sub-samples of the effluent (i.e., a sample divided between two or more containers) must be mixed together to ensure their homogeneity.

The pH of the sample should be between 6 and 9. If it does not read within these limits, adjust by using either NaOH or HCl solutions. Adjust to 6.5 or 8.5, whichever is closest to the initial pH of the sample (USEPA, 1993). A pH adjusted test may have to be performed concurrently. If the DO level in the undiluted sample is < 4.0 mg/L the sample should be aerated for a few minutes until the DO level is at an acceptable level (USEPA, 1993).

After the correct volumes of dilution water have been added to the test chambers, the reference toxicant or effluent can be measured using a measuring cylinder and/or pipette and dispensed into the appropriate test chamber. The total volume of solution in each test chamber should be 4 L but is also dependent on the fish loading requirements. If a receiving water is used as the control/dilution water prepare another control series with it. Prepare test solutions from the lowest concentration (control) to the highest concentration to minimise contamination. Preferably, replicate chambers should be placed in random order. Test solutions should be at test temperature before introducing the organism.

4.0 Test Procedure

4.1 Summary of Test Procedure

Table 3: Summary of recommended test conditions for freshwater *G. cotidianus* 96 h test.

Test Parameter	Test Condition
Test Organism:	<i>Gobiomorphus cotidianus</i> (common bully)
Source:	Lake Karapiro or other
Test Type:	Static non-renewal 96h duration
Temperature:	15 ± 1 °C
Light intensity/quality:	Full spectrum fluorescent lights ≤ 6 μmol m ⁻² s ⁻¹ ; 8:16h L:D
Test chambers:	4.5 L plastic containers lined with polyethylene plastic
Test solution volume:	4 L
Dilution water:	Carbon filtered tap water or receiving water, aerated for at least 2 days (0.157g/L NaHCO ₃ in acclimation tanks - but NOT in test dilution water)
Age of test organisms:	Juvenile (30 - 40 mm in length, 0.1 - 1.0 g wet weight)
Number of test organisms per chamber:	10
Number of replicate chambers per treatment:	3 - 5
Dissolved oxygen:	>70 % saturation DO
Aeration:	Gentle (100 bubbles/min)
Observation:	Fish behaviour and mortality
Chemical data:	Temperature, DO, pH, hardness
Effect:	Lethality
Endpoint:	LC ₅₀
Test acceptability criteria:	Mean control mortality no greater than 10%

4.2 Preparation for the Test

Five days prior to the commencement of the test

1. Set up holding tanks for the fish in the 15 °C constant temperature room and ensure that there is enough dechlorinated dilution water.
2. Collect the necessary number of fish and transfer them to the holding tanks.

While holding fish

1. Change 50% of the water in holding tanks in the morning and feed fish in the afternoon.
2. Monitor and record fish behaviour. Remove and count any dead fish.

24 h before test commencement

1. Change 50% of the water in the holding tanks in the morning but do not feed the fish.
2. Monitor and record fish behaviour. Remove and count any dead fish. Make sure that mortality during the acclimation period did not exceed 2%.

4.3 Beginning the Test

T₀ Day of test initiation

1. Place liner bags inside the labelled test chambers and add the required volume of dilution water to each test chamber.
2. Prepare stock solutions of reference toxicants and dispense the required volumes into the test chambers. Dispense the required volume of effluent into the appropriate test chamber. When pouring the test solutions into the test chambers ensure that no cross-contamination occurs between chambers through splashing.
3. Set up the aeration system and allow aeration of the solutions for 30 minutes.
4. Measure and record the temperature, DO and pH from one replicate each test concentration from each toxicant being tested.
5. Prepare fish for transfer into the test chambers by using a hand net to collect 10 fish and placing them in a clean 1L transfer beaker with approximately 700

mL dilution water in it. Pour fish gently through a hand net before placing them into the test solution to prevent water from the transfer container diluting the test solution. The netting must not be allowed to contact the test solution to prevent contamination to fish in the holding tank. If contamination of the net does occur, another net must be used. The contaminated hand net must be washed in non-phosphate detergent and rinsed thoroughly in tap water and distilled water prior to re-use. Fish should be added randomly to the test chambers.

6. Close the plastic bag liners of the test chambers by using a twist tie or some other easily removable attachment to enclose openings of the bags around the aeration tubing. This prevents the fish from escaping.
7. Record the time when the first fish were transferred into the test solutions. This will be the time when the test is monitored 24, 48, 72 and 96 h later.

4.4 During the Test

Day 1 - Day 3

1. Monitor the fish at 24, 48, 72 and 96 h \pm 1h after commencing the test by opening the plastic bag liner and observing whether fish mortality has occurred firstly in the control solutions and then within the replicates from the lowest concentration to the highest concentration for each test sample. Fish are considered dead when they fail to show evidence of opercular or other activity, and do not respond to gentle prodding.
2. Dead fish should be removed using a disposable plastic spoon that can be used for one test sample and then discarded to prevent cross-contamination. The dead fish are then disposed of in a hygienic manner by sealing them in a plastic bag and placing in a waste disposal bin.
3. Where all 10 fish have died in a replicate record the temperature, DO and pH of the test solution.

4.5 Ending the Test

Day 4

Measure the temperature, DO and pH of one replicate from each test sample and the control. Fish are not released back into the environment to prevent contamination from the test samples. They are euthanised with an overdose of anaesthesia (17 mL/L Phenoxyethanol) to dispose of them quickly and humanely. Retain fish from one replicate of each treatment to measure the length from head to tail-base and wet weight. The remaining fish and contaminated disposable equipment such as plastic liner bags must be disposed of in a hygienic manner. All remaining equipment that can be used again, for example, test chambers, should be subjected to the complete cleaning procedure described in Section 3.5. If a plastic polythene liner has not been used in the holding tanks, the tanks must be cleaned with an aquarium disinfectant before being thoroughly rinsed with water, dried and stored.

4.6 Recording Data and Observations

Fish mortality must be recorded on the appropriate form (Appendix 9.3) at 24, 48, 72 and 96 h intervals from the time that fish are first added to the test solutions. A record must also be made of any observed overt sublethal toxic effects (e.g., increased respiratory rates, erratic swimming behaviour, surfacing, discolouration, loss of equilibrium). Any differences from control fish should be noted. After the 96 h fish observation, record the temperature, DO and pH in one replicate of each test solution if it has not been done previously e.g., in the case of 100% mortality at an earlier stage during the test. Mean (\pm SE) head to tail-base lengths and wet weights of fish from one replicate of each treatment should be determined to provide an estimate of the size range of fish used.

5.0 Acceptability of Test Data

For the results of a fish lethality test to be acceptable and the test to be considered valid, the following conditions must be satisfied:

- Survival of the fish in the holding tanks prior to test initiation must be ≥ 98 %.
- Overall survival in the control replicates must be ≥ 90 %
- Culture sensitivity assessment with a reference toxicant must satisfy the criteria for acceptability (Section 3.6.2).
- Physiochemical conditions must fall within the range of acceptability (Section 3.4).

If the conditions of validity are not satisfied, the reasons why should be investigated and the test should be repeated.

6.0 Data Analysis

6.1 Test Endpoints and Calculations

Various computer programs for calculating the LC₅₀ and confidence limits are available. The software used by NIWA is Toxcalc^{TN} version 5.0 from Tidepool Scientific Software (1994). This software is used to produce a database for all toxicity test results and offers a full suite of parametric and nonparametric statistical methods of analysis that meet with standards required by United States Environmental Protection Agency. The decision of which statistical test to use is outlined in the flow chart (Figure 2).

Once the raw data has been entered as the number of fish alive per replicate after 96 h, hypothesis testing is used to detect statistically significant differences between treatments. This requires that the basic assumptions of hypothesis testing i.e. that the observations within treatments are independent and normally distributed and that the variance is homogenous across all concentrations and controls, be validated. Normality is tested using the Shapiro-Wilks test and homogeneity of variances is tested with the Bartlett's test. If these two assumptions are violated then the data must be transformed using an arcsin square root transformation and the assumptions tested again. If the tests fail with transformed data then a non-parametric method such as Steel's Many One Rank test or Wilcoxon Rank Sum test should be performed. A one tailed hypothesis test with $P > 0.05$ is conducted.

The LC₅₀ is an estimate of the percentage of effluent that will cause 50% mortality in the test species. It is calculated using a probit regression (Finney, 1971) with 95% confidence limits. This analysis consists of transforming the observed proportion of mortalities with a Probit transformation, and transforming the treatment concentrations to log₁₀. The relationship between the above transformed variables is close to linear and from this a regression is used to determine the LC₅₀ and 95% confidence limits. The use of Abbott's

correction adjusts the data for mortality in the control group and should be applied before the probit transformation of the data (USEPA, 1995). An example of the standard printout of test results using Toxcalc^{TN} are shown in Appendix 9.4. Note that the EC₅₀ in the Toxcalc^{TN} printout is equivalent to the LC₅₀.

No Observed Effect Concentrations (NOEC) and Lowest Observed Effect Concentrations (LOEC) are based on hypothesis testing of the organisms response at the concentrations used in the toxicity test. Therefore an *a priori* determinant of the NOEC and LOEC is the experimenter's choice of test concentrations (Grothe *et al.*, 1996). Caution must be exercised when using NOEC and LOEC values and they must be viewed in conjunction with another endpoint e.g. LC₁₀.

To limit the degree of test variability, the minimum significant difference (MSD), or amount of effect "allowable" at the NOEC has been introduced by USEPA (USEPA, 1995; Grothe *et al.*, 1996). The MSD is a measure of the within-test variability and represents the amount of difference from the control that can be detected significantly. It incorporates a level of significance (e.g. $\alpha = 0.05$), number of experimental units, as well as an estimate of test variability (within-test mean square error). The MSD is often expressed as a percentage of the effect in the control response ($\%MSD = MSD / \text{control mean} \times 100$) (Grothe *et al.*, 1996). Toxcalc^{TN} calculates the MSD in transformed units if the data has been transformed (Appendix 9.4). It also calculates the MSD in untransformed units (MSDu) and as a percentage of the control response (MSDp).

The MSD should be presented with the endpoint and calculated as a proportion of the mean control response.

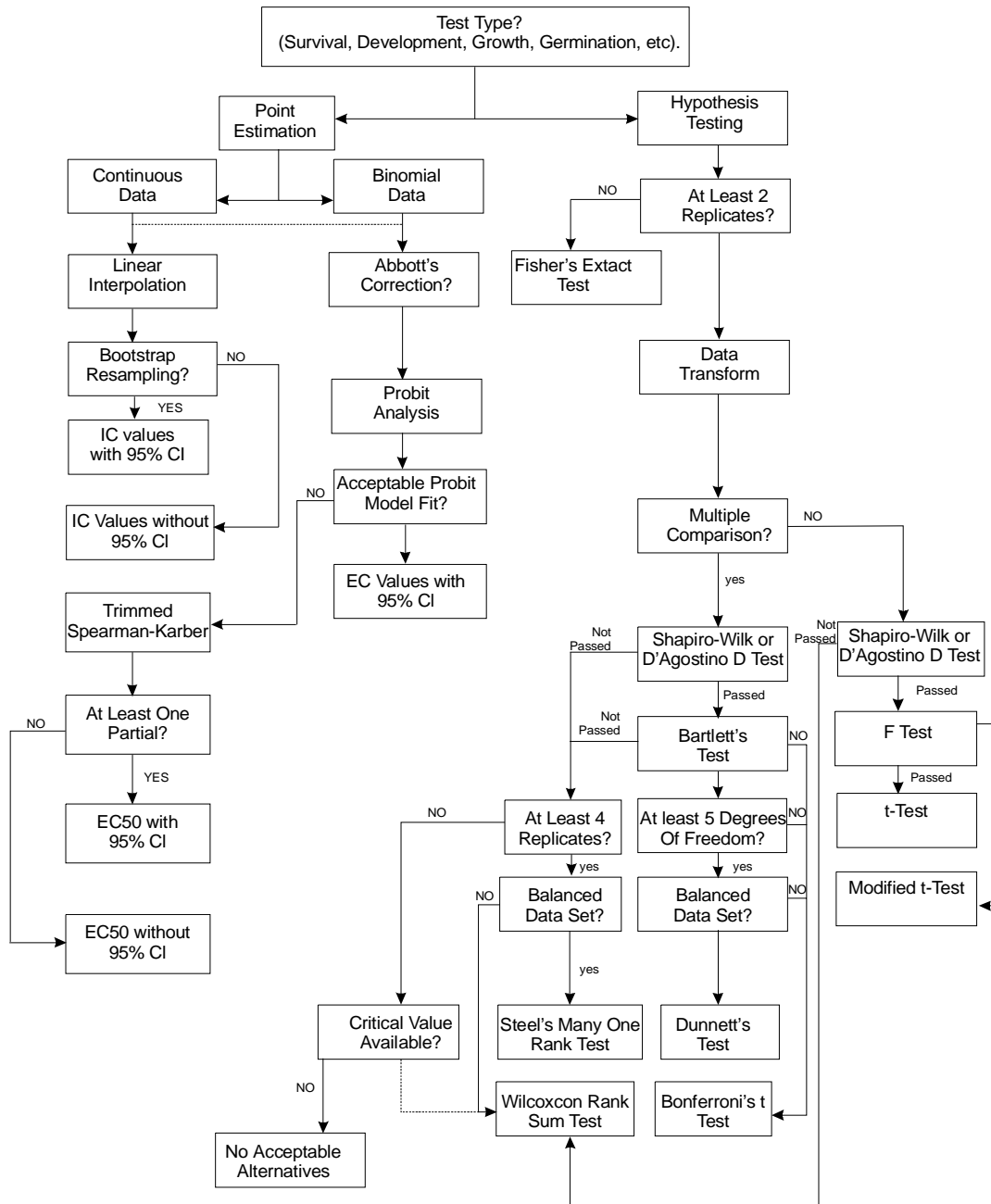


Figure 2: Flow diagram of USEPA approved statistical analysis used by Toxcalc^{TN} (Tidepool, 1994).

7.0 Reporting of Results

The test report should describe the materials 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 or compliance purposes) and consistent with specifications in this document may be referred to by citation or by attachment of a general report which outlines standard laboratory practice. Where choices exist, the approach selected should be specified. Specific monitoring programs may require selected items (e.g., procedures and results for tests requiring pH adjustment, modified aeration or oxygenation) in the test report. Other details pertinent to the conduct and findings of the test, which are not conveyed by the reports, should be kept on file by the test laboratory, so that the appropriate information can be provided if an audit of the test is required.

The following should be included in the report:

7.1 Test Material

- sample type, source and description (chemical, effluent, elutriate, leachate or receiving water; sampling location and method; information 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.

7.2 Test Organisms

- species origin, age, method of attainment and source;
- description of holding and acclimation conditions (facilities, light intensity, temperature, water source and quality, water pre-treatment, water exchange rate and method, density of organism in holding and acclimation tanks, temperature during holding and acclimation, acclimation period, food type, ration and frequency of feeding, disease incidence and treatment);
- percentage of mortalities in test population during acclimation; and
- average length, and wet weight of fish used in the test, with ranges and sample size.

7.3 Test Facilities and Apparatus

- name and address of test laboratory;
- name of person(s) performing each stage of the sample handling and testing;
- description of holding/acclimation and test facilities, including light, aeration and temperature regulating systems; and
- description of testing containers.

7.4 Control/Dilution Water

- type(s) and source(s) of water used as control and dilution water;
- measured water quality variable before and/or at the time of commencement of toxicity test;
- type and quantity of any chemical(s) added to the control/dilution water;
- sampling location and storage details if the control/dilution water was receiving water from an area not affected by the effluent or leachate discharge; and
- water pre-treatment (adjustment of temperature, pH, DO).

7.5 Test Method

- if a standard method is used, cite the document;
- describe procedure if modifications or changes to specific experimental design occur;
- method of preparing and storing stock and test solution(s);
- description of pH adjustment procedure, if applicable;
- any chemical and physical analyses of test solutions and reference to analytical method(s) used;
- composition of the test medium;
- frequency and type of observations made during the test;
- use of preliminary or range-finding test; and
- method for establishing survival of the organism.

7.6 Test Conditions

- number of concentrations, volume and depth of test solutions including controls, number of replicates per treatment;
- population from which the test fish were selected, together with the mean fish length and weight value
- number of organisms per solution and loading density;
- photoperiod, light source, and intensity at surface of test solutions;
- 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).

7.7 Test Results

- pH of test solutions at the beginning and at the end of a test;
- 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;
- mean fish survival in the control and individual test concentrations with corresponding coefficient of variation ($CV = 100 \times \text{standard deviation} / \text{mean}$);
- report the MSD value as a proportion of the control for untransformed data for any analyses done;
- graphical representation of the dose-response relationship (percentage growth inhibition values against concentration);
- results for range-finding test (if conducted);
- any 96 h LC₅₀ values (including the associated 95% confidence limits) determined, including reference to the statistical method used for their calculation;
- the 96 h LC₅₀ and 95% confidence limits for the reference toxicant(s) determined within one month of, or concurrently to the test using the (± 2 SD) for the same reference toxicant as derived at the test facility in previous tests;
- if the LC₅₀ is greater than the highest concentration tested it should be reported as $> X\%$ test substance where X is the concentration tested; and
- anything unusual about the test, any problems encountered and remedial measures taken.

8.0 References

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9.0 Appendices

Appendix 9.1:	Definitions of fish 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 mucus; especially evident at the gills
Haemorrhaging	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	Characterised 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 flaring 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

* Taken from Environment Canada (1990a)

Appendix 9.2: Logarithmic series of concentrations suitable for use in toxicity tests*

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

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

* Taken from Environment Canada (1990a).

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

Appendix 9.4 Example of ToxcalcTM results

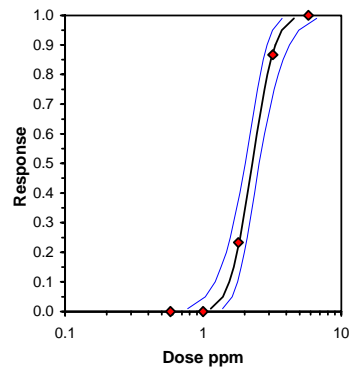
Acute Fish Test-4 days						
Start Date:	14/02/98	Test ID:	AQC373	Sample ID:	REF-Ref Toxicant	
End Date:	18/02/98	Lab ID:	LG & MM	Sample Type:	ZNSO-Zinc sulfate	
Sample Date:	14/02/98	Protocol:	NIWA, 1998	Test Species:	GC-Gobiomorphus cotidianus	
Comments:						
Conc-ppm	1	2	3	4	5	6
D-Control	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
0.58	1.0000	1.0000	1.0000			
1	1.0000	1.0000	1.0000			
1.8	0.6000	0.8000	0.9000			
3.2	0.0000	0.1000	0.3000			
5.8	0.0000	0.0000	0.0000			

Conc-ppm	Mean	N-Mean	Transform: Arcsin Square Root				N	Rank Sum	1-Tailed Critical	Number Resp	Total Number
			Mean	Min	Max	CV%					
D-Control	1.0000	1.0000	1.4120	1.4120	1.4120	0.000	6			0	60
0.58	1.0000	1.0000	1.4120	1.4120	1.4120	0.000	3	15.00	6.00	0	30
1	1.0000	1.0000	1.4120	1.4120	1.4120	0.000	3	15.00	6.00	0	30
*1.8	0.7667	0.7667	1.0808	0.8861	1.2490	16.925	3	6.00	6.00	7	30
*3.2	0.1333	0.1333	0.3534	0.1588	0.5796	60.049	3	6.00	6.00	26	30
5.8	0.0000	0.0000	0.1588	0.1588	0.1588	0.000	3			30	30

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.01) Equality of variance cannot be confirmed	0.73005	0.858	0.11823	2.575402
Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU
Wilcoxon Rank Sum Test	1	1.8	1.34164	

Parameter	Value	SE	95% Fiducial Limits		Maximum Likelihood-Probit						
			Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter		
Slope	7.68898966	1.31915117	5.1034533	10.274526	0	0.16829	11.3449	0.98	0.35553	0.13006	3
Intercept	2.26631477	0.50603328	1.2744895	3.25814							
TSCR											

Point	Probits	ppm	95% Fiducial Limits	
EC01	2.674	1.12973532	0.7738204	1.3805494
EC05	3.355	1.38550848	1.043057	1.6227383
EC10	3.718	1.54475227	1.2195328	1.773822
EC15	3.964	1.66240929	1.3525453	1.8872892
EC20	4.158	1.76227818	1.4661482	1.9858467
EC25	4.326	1.85272487	1.5687839	2.0776661
EC40	4.747	2.1017599	1.8439342	2.3491113
EC50	5.000	2.26742243	2.0157801	2.5497989
EC60	5.253	2.44614265	2.1884034	2.7869026
EC75	5.674	2.7749422	2.474921	3.2748991
EC80	5.842	2.91736262	2.5895282	3.503918
EC85	6.036	3.09262264	2.7249235	3.797988
EC90	6.282	3.32817394	2.899392	4.2119931
EC95	6.645	3.7106985	3.1695094	4.9243572
EC99	7.326	4.55080485	3.7257336	6.6373445



Dose-Response Plot

