



Freshwater Amphipod *(Paracalliope fluviatilis)*

Acute Toxicity Test Protocol

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(Paracalliope fluviatilis)

**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 amphipod *Paracalliope fluviatilis*, 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

CaSO ₄	calcium sulphate
°C	degree(s) Celsius
d	day
DO	dissolved oxygen (concentration)
EC ₅₀	effective median concentration
g	gram
h	hour
H ₂ O	water
KCl	potassium chloride
L	litre
LC ₅₀	median lethal concentration
LOEC	lowest observed effect concentration
MgSO ₄	magnesium sulphate
m	metre
mg	milligram
min	minute
mL	millilitre
MSD	minimum significant difference
NaBr	sodium bromide
NaHCO ₃	sodium bicarbonate
NOEC	no observed effect concentration
ppb	parts per billion
ppm	parts per million
ppt	parts per thousand
SD	standard deviation
^{TN}	Trade Name
ZnSO ₄ ·7H ₂ O	zinc sulphate
µL	microlitre
µm	micrometre
>	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

- 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

- Hardness* is the concentration of cations in water that will react with a sodium soap to precipitate an insoluble residue. In general, hardness is a measure of the concentration of calcium and magnesium ions in water, and is expressed as mg/L calcium carbonate or equivalent.
- Monitoring* 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.
- Percentage (%)* is a concentration expressed in parts per hundred parts. One percent represents one unit or part of material (e.g., effluent, elutriate, leachate, or receiving water) diluted with water to a total of 100 parts. Concentrations can be prepared on a volume-to-volume or weight-to-weight basis, and are expressed as the percentage of test material in the final solution.
- pH* is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions on a scale from 0 to 14, with 7 representing neutrality, numbers less than 7 signifying increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.
- Photoperiod* is the duration of illumination and darkness within a 24-h day.
- Precipitation* is the formation of a solid (i.e., precipitate) from a solution.
- Pre-treatment* is the treatment of a sample or dilution thereof, prior to exposure of amphipods.

Terms for Test Materials

<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>Deionised water</i>	is water that has been passed through resin columns to remove ions from solution and thereby purify it.
<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>	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 amphipods 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.
<i>Stock solution</i>	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.
<i>Upstream water</i>	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.
<i>Wastewater</i>	is a general term which includes effluents, leachates, and elutriates.
<i>Whole Effluent</i>	is any liquid waste (e.g., industrial, municipal) discharged to the aquatic environment.

Toxicity Terms

<i>Acute toxicity</i>	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.
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<i>EC₅₀</i>	is the effective concentration (i.e., the concentration of material in water that is estimated to produce a specifically quantified effect to 50% of the test organisms). The EC_{50} and its 95% confidence limits are usually derived by statistical analysis of a quantal, “all or nothing”, response (such as death, fertilization, germination, or development) in several test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 48-h EC_{50}).
<i>End point</i>	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 (EC_{50} , LC_{50} , etc).
<i>LOEC</i>	lowest observed effect concentration. The lowest concentration tested causing a statistically measurable effect to the test system
<i>MSD</i>	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 <i>Dunnett’s multiple-range test</i> , a standard statistical procedure.
<i>NOEC</i>	no observed effect concentration. The highest concentration tested causing no statistically measurable effect to the test system.
<i>Static</i>	describes toxicity tests in which test solutions are not renewed during the test.
<i>Toxicity</i>	is the inherent potential or capacity of a material to cause adverse effects on a living organism.
<i>Toxicity Test</i>	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 amphipod (*Paracalliope fluviatilis*). The acute lethality test using the amphipod is one of several “core” aquatic toxicity tests selected for standardization.

1.1 Principles of the Test Method

Juvenile freshwater amphipods (*P. fluviatilis*) 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 *P. fluviatilis* exposed to the test substance is compared with the survival of the *P. fluviatilis* in an appropriate control over a fixed period of time. A test substance is considered toxic when a statistically significant, dose-dependent mortality of *P. fluviatilis* occurs.

1.2 Historical Use of the Test

Traditionally the survival of amphipods has been assessed under various test conditions (Burnet, 1972; Hunt 1974; Borgmann *et al.*, 1989; Borgmann and Munawar, 1989). Amphipods have been found to be one of the most sensitive invertebrate species to toxic substances in acute tests (Anderson 1982; Green *et al.*, 1985; Williams *et al.*, 1985). Borgmann and Whittle (1983) found contaminant accumulation in the North American amphipod *Hyaella azteca* with persistent contaminants such as polychlorinated biphenyls and DDT. Borgmann *et. al.* (1989) found *H. azteca* as

sensitive as *Daphnia magna*, to cadmium, and more sensitive to PCP.

The amphipod *P. fluviatilis* is potentially one of the most useful freshwater species in New Zealand for survival studies in the laboratory. The amphipod was found to be 5 times more sensitive to the reference toxicant zinc than the cladoceran *D. magna* (NIWA, unpublished data). Hickey and Vickers (1994) found *P. fluviatilis* to be highly sensitive to un-ionised ammonia. This sensitivity increased 1.7 fold with exposure time.

Burnet (1972) investigated the effects of 2 ppm paraquat applied to a Canterbury stream and found increased numbers of dead *P. fluviatilis* in drift samples taken on the day of application. Furthermore Hunt (1974) found a short exposure period of paraquat (within 10 hours) to be sufficient for *P. fluviatilis* to accumulate a lethal dose. This contaminant accumulation, along with their relative sensitivity to toxicants, suggests that amphipods may be one of the most vulnerable groups of organisms to chemical pollution.

The methodology presented in this report details standardised procedures for performing static-acute survival tests with the freshwater amphipod *P. fluviatilis*.

1.3 Summary of the Test Technique

This test involves exposing *P. fluviatilis* juveniles aged 0 - 7 days, to a test material in a static system. Ten juvenile amphipods are added to each test cup containing 25 mL of test solution. A minimum number of five juveniles is required per test replicate (ASTM, 1997). Each cup receives one square centimetre of 250 µm nylon mesh. The test is conducted at 20 ± 1 °C and kept in constant darkness. After 48 h, the number of surviving amphipods in the test concentrations is compared with the number of surviving amphipods in the control dilution water.

1.4 Application, Advantages, and Limitations of the Test System

Advantages of the amphipod test are;

- Gravid females are available all year round.
- The test can easily accommodate a number of test concentrations as well as replicates per test concentration.
- A large number of samples can be processed in a relatively short time.
- The test is relatively simple to perform.
- The equipment used is relatively cheap and disposable.

Limitations of the amphipod test may be;

- Adsorption of the test substance to the test cup or to the 250 μm nylon mesh inserted in each cup may mask toxicity by reducing

the bioavailability of the compound to the amphipods.

- pH shifts in test solutions might be concentration dependent and affect toxicity of the test substance.
- Dissolved oxygen content may be low in high concentrations and pre-aeration and/or aeration of a test may affect sample toxicity.
- Decreased survival due to cannibalisation can occur, if animals are not uniform in size.

Despite these limitations the acute amphipod test is considered an effective technique to assess the toxicity of chemicals and effluents. Wherever possible, suggestions and recommendations are included to minimise the effects of the test limitations.

2.0 Test Organisms

2.1 Species

The widespread eusirid amphipod *Paracalliope fluviatilis* (Thomason) is abundant in New Zealand freshwaters. It is a freshwater amphipod that is found in lakes and flowing waters (Chapman and Lewis, 1975). Winterbourn and Lewis (1975) noted its presence amongst lake macrophytes and found it to be a common member of the fauna of sand-dune and brackish coastal lakes. It is regarded as an important member of the community in the largest brackish-water lake in New Zealand, Lake Ellesmere (Winterbourn and Lewis, 1975).

The slender body is gray and marked with dark spots and frequently covered with circular markings. Adult males are 4.0 - 4.5 mm long, the females are smaller, around 3.0 - 3.5 mm.

This amphipod can be difficult to culture in the laboratory and appropriate culture conditions have not been identified. Preliminary trials regarding the culturing of *P. fluviatilis* in the laboratory found difficulties in satisfactorily maintaining the amphipods. High mortalities occurred after three to four weeks and a successful breeding rate was not achieved. These trials failed to effectively determine the food preferences of *P. fluviatilis*.

The North American amphipod *Hyaella azteca* is successfully cultured in overseas laboratories (Nebeker *et. al.* 1986; Mackie, 1989; Borgmann *et. al.*, 1991; Borgmann and Munawar, 1989; ASTM, 1997). *H. azteca* is the most common and widely distributed amphipod in North America. It is an important component of shallow water ecosystems, and can be dominant in the diets of fish and waterfowl (Borgmann, 1996).

2.2 Source

Organisms can be obtained from the Waikato River, either in the river itself found amongst the willow roots or among the aquatic

vegetation such as *Nitella* sp. beds. Animals are collected either by a dip net or by the removal of the surrounding vegetation. This is subsequently washed to displace the amphipods. The amphipods are usually found in dark places such as underneath willow roots and in dense *Nitella* sp. beds.

2.3 Culturing/Holding/Acclimation

Gravid females can be collected from the field one week before use and held in the laboratory. Juveniles subsequently released are uniform in size (1.0 - 2.0 mm) and similar in age. Alternatively, juvenile amphipods may be collected from the field and maintained in the laboratory for a short period of time.

Gravid females should be held in aerated glass aquaria with 250 µm nylon mesh on the bottom as substrate along with *Nitella* sp. Culturing in the absence of sand or sediment is preferable because this simplifies collection and counting of the juvenile amphipods. *Nitella* sp. taken from the collection site should be an adequate food supply for the short period of time that the animals are kept in the laboratory.

Animals should be acclimated to test temperatures (20 ± 1 °C) by holding the amphipods in a temperature controlled room or similar for 24 h.

Further research on the culturing methods of *P. fluviatilis* (e.g. food preferences, brood numbers, growth rates and life span) are recommended.

2.3.1 Holding Water

The dilution water for holding *P. fluviatilis* prior to test initiation is the same as the control/dilution water (Section 3.6.1).

2.3.2 Physicochemical Conditions

For static tests, the concentration of dissolved oxygen must be from 60 to 100 % of saturation during the first 48 hours of the test (ASTM, 1997).

Test solutions may be gently aerated during static tests if the concentration of DO in the test material does not meet the above requirements. Turbulence should be avoided as it may increase stress on test organisms, resuspend faecal matter, and greatly increase volatilisation. If aeration is necessary, it should be the same in all test chambers including the control (ASTM, 1997). Temperature must be 20 ± 1 °C. The pH

should be within the natural range for fresh water, 6.0 - 9.0 (ASTM, 1997).

2.4 Quality of Test Organisms

The test organisms should be identified to species under a microscope (x10) and confirmed by referring to the key given in Chapman and Lewis (1976). Juvenile amphipods of a uniform size (1 - 2 mm in length) should be randomly selected by the use of a wide mouthed pipette.

Amphipods should be handled as little as possible during the experimental setup to prevent causing unnecessary stress to the animals. Care must be taken not to damage the organisms when sieving and sorting prior to test commencement. Ensure that the 250 µm nylon mesh sieve is at subsurface water level when separating juveniles from adults. Transfer amphipods using a wide mouthed pipette taking care to dispense them into solution with the pipette tip underwater to prevent a shock when the animals contact the water surface and also to prevent air becoming trapped under the carapace.

Performance and amphipod sensitivity should be evaluated by routinely measuring the survival and relative sensitivity of the amphipod to a reference toxicant (Section 3.6.2).

3.0 Test System

3.1 Summary of Test System

This test involves exposing juvenile *P. fluviatilis* to a test material for 48 hours in a static system. A definitive test is performed which consists of a series of five test concentrations to determine a 48 hour EC₅₀ value. A control consisting of dilution water only is included with every test. Each test replicate requires the addition of one square centimetre of 250 µm nylon mesh to provide an attachment surface and deter flotation of the amphipods. Each test must have a minimum of five concentrations plus a control (EVS, 1995). Each test must have a minimum of 3 but preferably 5 replicates (ASTM, 1997) per treatment except the control which must have a minimum of five replicates. An additional replicate for each treatment is required for daily water quality measurements. Measurements of survival are made after 48 hours when the total number of surviving amphipods is assessed.

3.2 Facilities

The static acute amphipod test should be conducted in a facility where the temperature can be controlled and monitored continuously. A temperature controlled room or incubator is recommended.

3.3 Equipment

All instruments for the routine measurements of basic chemical, physical, and biological variables must be maintained and calibrated regularly. Any equipment that comes in contact with the test organisms, dilution water, nutrient solutions, or test solutions must be made of inert material (e.g., glass, stainless steel, porcelain, teflon) and be clean and free of substances that might interfere with the test (Section 3.5). Equipment not previously used in tests should be preconditioned and tested for toxicity prior to use. Table 1 lists the consumable and non-consumable equipment, and the reagents required to perform the *P. fluviatilis* acute toxicity test.

Table 1: Equipment and reagents required to perform 48 h *P. fluviatilis* test

Non-consumable Equipment

- constant temperature room or incubator at 20 ± 1 °c
- thermometer
- pH meter
- meter
- 1L graduated cylinders
- clear cover sheets with aeration holes
- examination light lamp
- nylon mesh sieve (250 µm)
- holding trays
- pipette with volume adjustment (0.1-1.0 ml capacity)
- pipette tips
- volumetric flasks 100 mL, and 1000 mL capacities
- light source or microscope with at least 6x magnification

Consumable Equipment

- polystyrene cups 55 mL capacity
- deionised water
- pipette tips
- 250 µm nylon mesh
- black plastic bags
- wide mouthed disposable plastic pipettes

Reagents required to perform 48 h test

- zinc sulphate (analytical grade) (ZnSO₄·7H₂O)
 - nitric acid (analytical grade)
 - stock nutrient solutions (section 3.6.1)
 - freshwater
 - deionised water
-

3.4 Test Conditions

Test conditions (Table 2) must be uniform and monitored routinely throughout the test. The test is currently run in constant darkness to reduce stress on organisms associated with loss of natural habitat.

Table 2: Test conditions for 48 h *P. fluviatilis* test

pH:	6.0 - 9.0 throughout the test
DO:	≥ 60 % saturation in all test concentrations throughout the 48 h of the test
Temperature:	20 ± 1 °C
Lighting:	darkness

3.5 Cleaning Procedure

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. If polystyrene cups are used for test, they should be disposed of in an appropriate manner.

3.6 Preparation of Reagents

3.6.1 Control/Dilution Water

Control/dilution water is prepared by the addition of nutrient solutions to a glass fibre filtered (GF/C) solution of 20% fresh water, collected from a non-contaminated site (confirmed by metal and organic chemical analysis) and 80% deionised water aged for at least 2 days. Control/dilution water is aerated and stored in large low density polythene tanks or equivalent at ambient temperature.

Prepare stock nutrient solutions in 1 L volumetric flasks using reagent grade chemicals and deionised water (Table 3). To prepare the control/dilution water, add 40 mL per litre of fresh water of each of the five stock nutrient solutions, NaHCO₃, KCl, CaSO₄, NaBr and MgSO₄ to make moderately hard reconstituted water (USEPA, 1993; Borgmann, 1996).

Table 3: Stock nutrient solutions for dilution water

Stock Nutrient Solution	Amount of reagent required per litre of deionised water
NaHCO ₃	2.48 g/L
KCl	0.1 g/L
CaSO ₄	1.5 g/L
NaBr	1.5 g/L
MgSO ₄	2.93 g/L

NB: pH=7.8 ± 0.2

Tests conducted with samples of effluent, elutriate, or leachate should use the standard dilution water described above as the control/dilution water. To assess the potential impact of a sample on a particular receiving water, the receiving water may be used as the control/dilution water. Receiving water containing debris or indigenous organisms, that may be mistaken for or attack test organisms, should be filtered through a 60 µm mesh sieve (USEPA, 1993). A standard control with moderately hard reconstituted dilution water must also be included in the test. Receiving water must be transported and stored as in section 3.6.3.

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 (219.8

mg ZnSO₄·7H₂O made up in 500 mL of deionised water) which can be stored in the dark at fridge temperature (4 °C). Chemically verify the stock solution concentration every six months 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 the moderately hard reconstituted water 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 48 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 ± 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, 1990b).

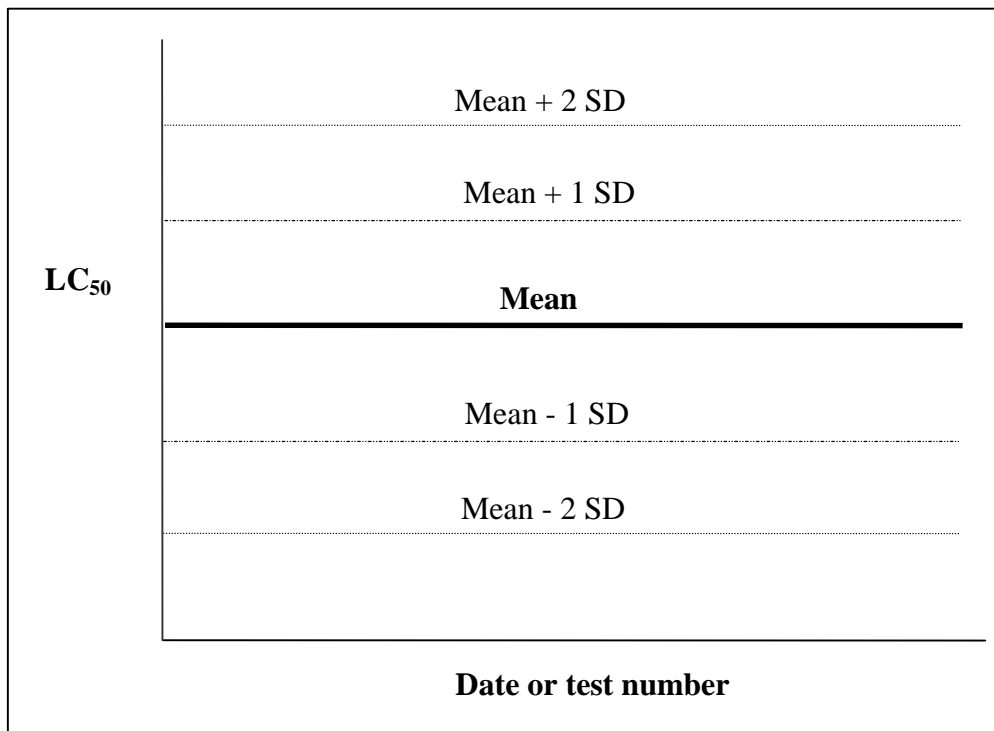


Figure 1: Analytical Quality Control Chart with Mean ± 2 Standard Deviations (taken from Environment Canada, 1990b)

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 4 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, pH and salinity 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 48 h EC₅₀, at least five test

concentrations plus a control solution (100% control/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 9.1).

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). It may be necessary to aerate the sample throughout the test period to ensure adequate levels of DO are maintained. Caution must be exercised to avoid excessive aeration. Turbulence caused by aeration should not result in a physical stress to the test organism.

Prepare stock solutions of the desired concentrations using glass volumetric flasks. 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. Once the test solutions are prepared, they are put into the temperature control room to acclimate to the test temperature.

4.0 Test Procedure

Table 4: Summary of Recommended Test Conditions for the 48 h *Paracalliope fluviatilis* Acute Toxicity Test

Test Parameter	Test Condition
Test Organism:	<i>Paracalliope fluviatilis</i>
Source:	Waikato River or other
Test Type:	Static non-renewal
Temperature:	20 ± 1 °C
Light intensity/quality:	None - darkness
Test vessel size	55 mL polystyrene cups or glass beakers
Test solution volume:	25 mL
Dilution water	20% Freshwater, 80% deionised water + stock nutrient solutions
Renewal of test concentrations:	None
Size of test organisms	Approximately 1-2 mm, aged 0 - 7 d
Number of test organisms per chamber:	10, minimum 5
Number of replicate chambers per treatment:	at least 3 preferably 5, 5 controls
Feeding regime:	None
Aeration:	None
Number of concentrations:	5 plus a control
Test duration:	48 hours
Chemical data	Temperature, pH, dissolved oxygen
Effects measured:	Survival
Test acceptability criteria:	Mean control survival ≥ 90% Survival in each control replicate must be ≥ 80%

4.2 Preparation for the Acute Test

Ensure that there is enough control/dilution water available. This must be done at least the day prior to test initiation. Store at test temperature, aeration is not required.

Label all test containers (polystyrene cups) with the test ID, concentration and replicate numbers. An EC₅₀ test uses a minimum of 26 polystyrene cups, five for the control, three per concentration and one as water quality for each treatment. Treatments consist of five test concentrations and a control. More test concentrations may be required, for example if the test is unable to be repeated within a suitable time frame.

4.3 Test Initiation

Test initiation time depends on when the samples were collected and received.

1. Isolate a minimum of 200 juveniles 0-1 week old from cultures by sieving from the holding water through a 250 µm nylon mesh screen that is sitting below the water surface of the sieving basin. Transfer the animals that passed through the mesh to a sorting tray containing water at the holding temperature and select juveniles by eye with the use of a wide mouth pipette and leave at room temperature in holding dishes.
2. Prepare test solutions as outlined in Section 3.7 by diluting the sample with the appropriate control/dilution water in volumetric flasks.
3. Place a one centimetre square 250 µm nylon mesh into each control and test replicate container.
4. Dispense at least 25 mL of each of the five test solutions into 3 labeled replicate test cups. Have a separate set of cups for measuring the water quality of the five test solutions and control. Leave them in a constant temperature room or incubator for adjustment to the test temperature.
5. Using a light source or otherwise, randomly select active juvenile amphipods from the sorting tray, by use of a clean wide mouth plastic pipette. Count 10 animals each into a set of polystyrene cups containing control/dilution water (one per cup, and same subsequently until each cup contains 10 animals). From these cups the amphipods can be transferred to the test solutions.
6. Place 10 animals into each test solution starting with the control and then increasing in concentration. The test organisms must be dispensed gently, by inserting the pipette tip underwater, to prevent a shock of the animals at the water surface.
7. Observe each replicate with a light source, or otherwise, to ensure that there are 10 animals in each cup.

8. Measure temperature pH, and DO in each water quality cup and record (Appendix 9.2).
9. Place a clear perspex cover sheet with holes drilled through for aeration over the test tray.
10. Cover each test tray with black cover sheets ensuring the tray is completely covered and no light gets through.
11. Place tray in temperature controlled room or similar at 20 ± 1 °C
12. Test initiation time is taken as when the animals are first placed into the test concentrations.

4.5 Test Termination

The test is terminated after 48 h by removing the tray from the temperature controlled room and counting, over a light source or otherwise, the number of live and dead amphipods. Mortality is defined as no observed cardiac movement.

Measure final water quality parameters, temperature, DO, and pH in each water quality cup.

4.6 Data Recording and Observations

Preparation of test solutions, mortality data and water quality data must be recorded on the appropriate sheets (Appendix 9.2).

Note if there are too few or too many animals in a replicate. Care should be taken not to confuse a moult with a dead animal. Record any spills or if any amphipods have been killed or lost as a result of handling. Mean survival in the controls must be ≥ 90 % and each control replicate must have ≥ 80 % survival. For effluent testing where the highest concentration is 100 %, mortality at 100 % concentration will indicate little or no toxicity.

5.0 Test Acceptability

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

- Overall survival in the controls must be $\geq 90\%$.
- Survival in any one of the control replicates must be $\geq 80\%$
- 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 2.3.2).

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

Review the survival data before proceeding with the analysis. If any organisms were accidentally killed or lost as a result of handling, those numbers are deducted from the initial number of organisms in that replicate. If an organism is missing and the reason can not be explained, do not automatically deduct it from the initial count.

Various computer programs for calculating the EC₅₀ and confidence limits are available. The software used by NIWA is ToxcalcTM 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 non-parametric statistical methods of analysis that meet United States Environmental Protection Agency standards. A flow diagram of the appropriate statistical methodology used is shown in Figure 2 and an example of the ToxcalcTM printout for a reference test is given in Appendix 9.3.

The EC₅₀ 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 EC₅₀ 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, 1994).

Calculation of the No Observed Effect Concentration (NOEC) value for survival is calculated by transforming the survival data using an arc-sine-square-root transformation (Figure 2). The arc-sine-square-root transformation is commonly used on proportionality data to stabilise the variance

and satisfy the normality requirement (USEPA, 1993). If data meet the assumptions of normality and homogeneity of variance, parametric analysis can be used to conduct hypothesis testing for statistically significant differences between treatments and the control. It consists of a Shapiro-Wilk or D'Agostino D Test and a multiple comparison test such as Dunnett's test.

If the data do not meet the assumption of normality, then the non-parametric test, Wilcoxon Rank Sum test, can be used to analyse the data. If the data meet the assumption of normality, the F-test for equality of variances is used to test the homogeneity of variance assumption. Failure of the homogeneity of variance assumption leads to the use of a modified *t*-test, where the pooled variance estimate is adjusted for unequal variance, and the degrees of freedom for the test are adjusted. A non-parametric analysis involves a Steel's Many-one rank sum test or a Wilcoxon rank sum test.

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. EC₁₀.

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 / control mean x 100) (Grothe *et al.*, 1996). ToxcalcTM 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.

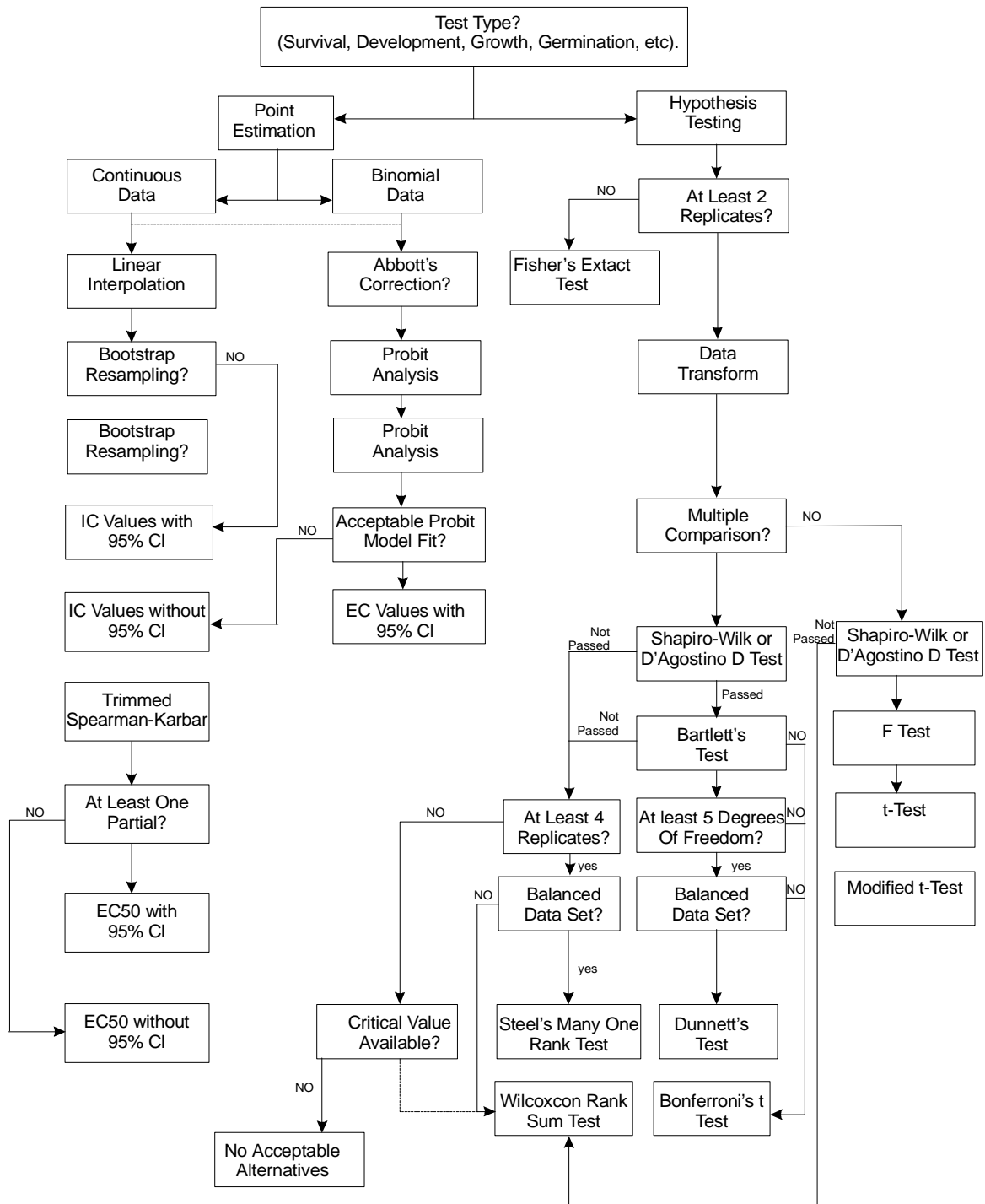


Figure 2: Flow diagram of USEPA approved statistical methods performed by ToxcalcTM (Tidepool, 1994).

7.0 Reporting 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, method of attainment and source;
- description of holding and culture facilities, including light, aeration and temperature regulating systems; and
- estimated percent mortality in holding containers prior to test,
- age and length of test organisms at beginning of test.

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, culturing 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

- date, times, and duration of tests;
- concentrations tested;
- number of concentrations, volume and depth of test solutions including controls, number of replicates per treatment;
- number of organisms per treatment;
- photoperiod, light source, and intensity at surface of test solutions;
- 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 48h EC₅₀ 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;
- mean amphipod survival in the control and individual test concentrations with corresponding coefficient of variation (CV = 100 x standard deviation / 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);
- amphipod behaviour; number and percentage showing mortality or immobility in each test solution including control at each observation time, number and percentage of controls showing atypical/stressed behaviour;
- results for range-finding test (if conducted);
- any 48 h EC₅₀ values (including the associated 95% confidence limits) determined, including reference to the statistical method used for their calculation;
- the 48 h EC₅₀ 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 EC₅₀ 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.

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9.0 Appendices

Appendix 9.1 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 9.3 Example of Toxcalc Data Sheet

Acute 48h Survival Test-48 hours				
Start Date: 13/03/98	Test ID: aqc413	Sample ID: REF-Ref Toxicant		
End Date: 15/03/98	Lab ID: SAR	Sample Type: ZNSO-Zinc sulfate		
Sample Date 22/10/97	Protocol: -NIWA	Test Species: PF-Paracaliope fluviatilis		

Conc-mg/L	1	2	3	4	5
B-Control	1.0000	1.0000	0.8000	1.0000	1.0000
0.058	0.8000	0.8000	0.6000		
0.1	0.8000	1.0000	0.8000		
0.18	1.0000	0.8000	0.6000		
0.32	0.2000	0.2000	0.4000		
0.58	0.0000	0.0000	0.2000		
1	0.2000	0.0000	0.0000		

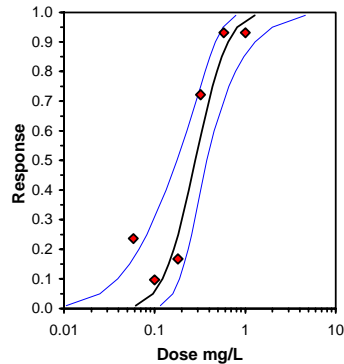
Conc-mg/L	Mean	N-Mean	Transform: Arcsin Square Root				N	t-Stat	1-Tailed Critical	MSD	Number Resp	Total Number
			Mean	Min	Max	CV%						
B-Control	0.9600	1.0000	1.2977	1.1071	1.3453	8.207	5				1	25
0.058	0.7333	0.7639	1.0335	0.8861	1.1071	12.350	3	2.521	2.673	0.2801	4	15
0.1	0.8667	0.9028	1.1865	1.1071	1.3453	11.587	3	1.060	2.673	0.2801	2	15
0.18	0.8000	0.8333	1.1128	0.8861	1.3453	20.637	3	1.764	2.673	0.2801	3	15
*0.32	0.2667	0.2778	0.5373	0.4636	0.6847	23.753	3	7.256	2.673	0.2801	11	15
*0.58	0.0667	0.0694	0.3049	0.2255	0.4636	45.094	3	9.474	2.673	0.2801	14	15
*1	0.0667	0.0694	0.3049	0.2255	0.4636	45.094	3	9.474	2.673	0.2801	14	15

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.94461	0.881	0.09292	-0.76441
Bartlett's Test indicates equal variances (p = 0.95)	1.65954	16.8119		

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Bonferroni t Test	0.18	0.32	0.24		0.20333	0.21929	0.61998	0.02059	7.4E-08	6, 16

Parameter	Value	SE	95% Fiducial Limits		Maximum Likelihood-Probit						
			Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter		
Slope	3.53754976	0.88939785	1.7943299	5.2807696	0.04	7.21126	13.2767	0.13	-0.5524	0.28268	11
Intercept	6.95421859	0.48860268	5.9965573	7.9118799							
TSCR	0.1051506	0.04925804	0.0086048	0.2016964							

Point	Probits	mg/L	95% Fiducial Limits	
EC01	2.674	0.06165423	0.0104743	0.1163093
EC05	3.355	0.09607463	0.024732	0.1589773
EC10	3.718	0.12170475	0.0389251	0.1886399
EC15	3.964	0.14275717	0.0526994	0.2123644
EC20	4.158	0.16205697	0.0668732	0.233936
EC25	4.326	0.18068108	0.0818269	0.2548278
EC40	4.747	0.2376632	0.133791	0.3214945
EC50	5.000	0.28027121	0.1763563	0.3770317
EC60	5.253	0.33051795	0.2268858	0.4530334
EC75	5.674	0.4347547	0.3213789	0.6597477
EC80	5.842	0.48471815	0.3605105	0.7839179
EC85	6.036	0.55024879	0.4071226	0.9703429
EC90	6.282	0.64543039	0.4681087	1.2862571
EC95	6.645	0.81761379	0.5659986	1.986683
EC99	7.326	1.27407261	0.7867076	4.6130415



Dose-Response Plot

