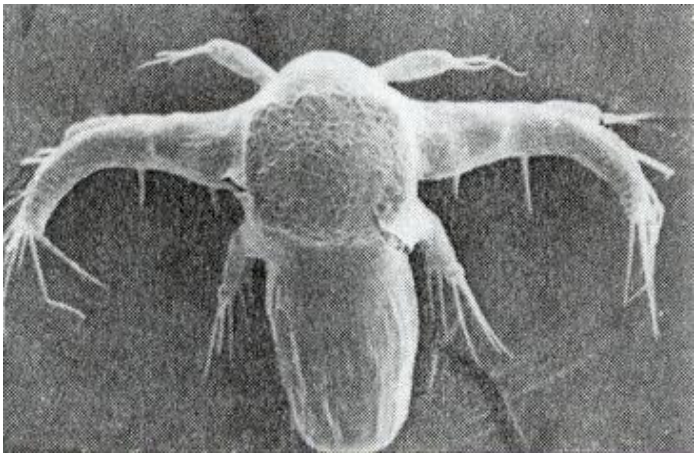
THAMNOTOXKIT F

Crustacean Toxicity Screening Test

for Freshwater



STANDARD OPERATING

PROCEDURE

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INTRODUCTION TO THE THAMNOTOXKIT F

Origin :
This acute toxicity test was developed by the research teams of Prof. Dr. G. Persoone at the Laboratory for Biological Research in Aquatic Pollution (LABRAP*) at the Ghent University in Belgium.

* *The Laboratory has been renamed Laboratory for Environmental Toxicology and Aquatic Ecology (LETAE)*

Scope :
TOXKITS are microbiotests in kits containing all necessary materials, including the test organisms to perform simple, rapid, sensitive and reproducible toxicity tests at low cost. Toxkit microbiotests are suited for toxicity testing of chemicals and wastes, released in aquatic as well as terrestrial.

Principle :
A 24 h LC_{50} bioassay is performed in a multiwell test plate using instar II-III larvae of the fairy shrimp *Thamnocephalus platyurus*, which are hatched from cysts.

Advantages of TOXKIT tests :
The major advantage of TOXKIT microbiotests, in comparison to "conventional" bioassays, is that the test organisms are incorporated in the kits in a "dormant" or "immobilized" form from which they can be hatched or activated prior to the performance of the toxicity tests. This eliminates one of the major bottlenecks in aquatic toxicology which is the need for continuous stock culturing of test organisms.

Features :
Each Thamnotoxkit F contains all the (disposable) materials to perform 6 complete screening toxicity tests (range finding or definitive 24h LC_{50}) or 5 bioassays and one quality control test with a reference toxicant. Using larvae of the fairy shrimp *Thamnocephalus platyurus* hatched from cysts, an acute toxicity test is executed in 24 hours. The only equipment needed is an incubator (25°C), a dissection microscope (magnification 10-12x) and conventional laboratory glassware.

Sensitivity :
The sensitivity of *Thamnocephalus platyurus* compares favorably with that of many invertebrates currently used in aquatic toxicology.

Precision :
Since all Thamnotoxkits contain the same standard test (bio)materials, and test media, the repeatability of this bioassay is very high.
The precision of the *Thamnocephalus platyurus* test has been determined on a reference chemical in an International Interlaboratory Comparison involving 23 laboratories from 14 countries. The outcome of this ringtest revealed the high intra- and interlaboratory reliability of this microbiotest.

Cysts viability :
Optimal viability of the cysts is maintained by storing the tubes with the cysts in the refrigerator at 5 °C (+/- 2 °C in darkness. The hatching success of cysts kept in such conditions is guaranteed for several months.

Representativity :
Although fairy shrimp are found only rarely in permanent water bodies, they could in principle thrive very well in such waters, like other planktonic crustaceans. Freshwater anostracans, however, like their halophic homologs, do not resist predation pressure, and for this reason have retracted into ecologically more extreme habitats, in this case "temporary" water bodies subject to cyclic desiccation.

Standardization
The acute toxicity test with *Thamnocephalus platyurus* has been endorsed by the International Standardization Organization (ISO) as an acute toxicity test. This International Standard is published by the ISO as ISO/14380 under the name “Water quality - Determination of the acute toxicity to *Thamnocephalus platyurus* (Crustacea, Anostraca).

CONTENTS OF THE THAMNOTOXKIT F

Vials with *Thamnocephalus* cysts
Six 1 ml plastic vials containing cysts of the fairy shrimp *Thamnocephalus platyurus*, which should be stored in a refrigerator at 5 °C (± 2 °C) until use. If the hatching procedure is followed properly, the number of larvae obtained will exceed by far the number of test organisms needed for the toxicity test.

Multiwell test plates
Six polystyrene plates (9x13 cm) with 24 wells (3 ml) which will serve as test containers.

Parafilm strips
Six 10x15 cm strips of Parafilm for sealing the multiwell plate to minimize evaporation during the incubation period.

Petri dishes
Two polystyrene petri dishes (5 cm diameter) with cover, to be used for the hatching of the *Thamnocephalus* cysts.

Concentrated salt solutions
Five small glass bottles, each containing a concentrated solution of one particular salt, to make up one liter Standard Freshwater (moderately hard synthetic water, US EPA formula) with deionized water. The Standard Freshwater is used for cyst hatching and the toxicant dilution preparation.
Composition :
Vial 1 : $NaHCO_3$ (96 mg - dissolved in 1 l. = 96 mg/l)
Vial 2 : $CaSO_4.2H_2O$ (60 mg - dissolved in 1 l. = 60 mg/l)
Vial 3 : $CaSO_4.2H_2O$ (60 mg - dissolved in 1 l. = 60 mg/l)
Vial 4 : $MgSO_4.7H_2O$ (123 mg - dissolved in 1 l. = 123 mg/l)
Vial 5 : KCl (4 mg - dissolved in 1 l. = 4 mg/l)

Micropipettes
Six polyethylene micropipettes for transferring the larvae.

Standard Operational Procedure manual
A detailed brochure with all the instructions for the performance of the toxicity tests on pure chemicals or effluents.

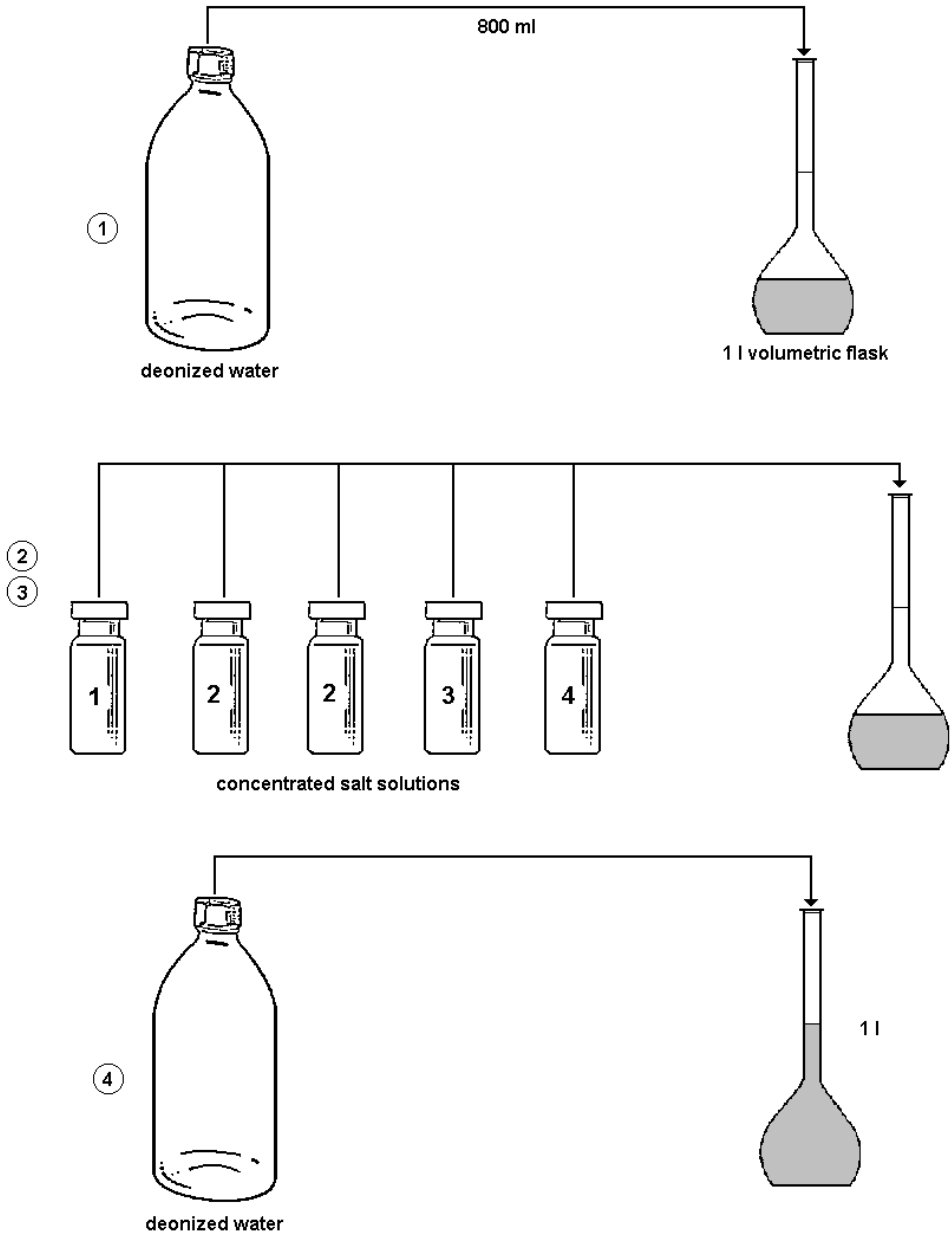
Bench protocol
An abbreviated version of the extended Standard Operational Procedure manual.

Results sheets
Six sheets for scoring the results and calculation of the mean effect percentages.

Specification sheet
A sheet indicating the batch number and shelf life of the cysts, the batch number of the concentrated salt solutions, the expiry date of the Thamnotoxkit and the 24 EC_{50} values for the reference chemical potassium dichromate.

All the non-biological materials provided in the Thamnotoxkit F are made of inert, non-toxic products.
These materials are disposable and should only be used once.

PREPARATION OF STANDARD FRESHWATER



1. PREPARATION OF STANDARD FRESHWATER

General remark : The solutions described hereunder are prepared with deionized water or distilled water. To avoid repetition, only the wording "deionized water" will be used further on.

The vials with concentrated salt solutions provided in the kit are used to prepare one liter Standard (artificial) Freshwater. The Standard Freshwater solution is a "moderately hard water", prepared according to the US EPA formula and is used as hatching medium for the cysts and for the toxicant dilution series preparation.

Procedure (see figure)

1. Fill a 1 liter volumetric flask with approximately 800 ml deionized water.
2. Uncap the vial with concentrated salt solution labeled number 1 ($NaHCO_3$), and pour the contents in the flask.
3. Repeat step 2 for the other vials with concentrated salt solutions, i.e. two vials number 2 ($CaSO_4.2H_2O$), one vial number 3 ($MgSO_4.7H_2O$) and one vial number 4 (KCl), respecting this sequence.
4. Add deionized water up to the 1000 ml mark and shake to homogenize the medium.

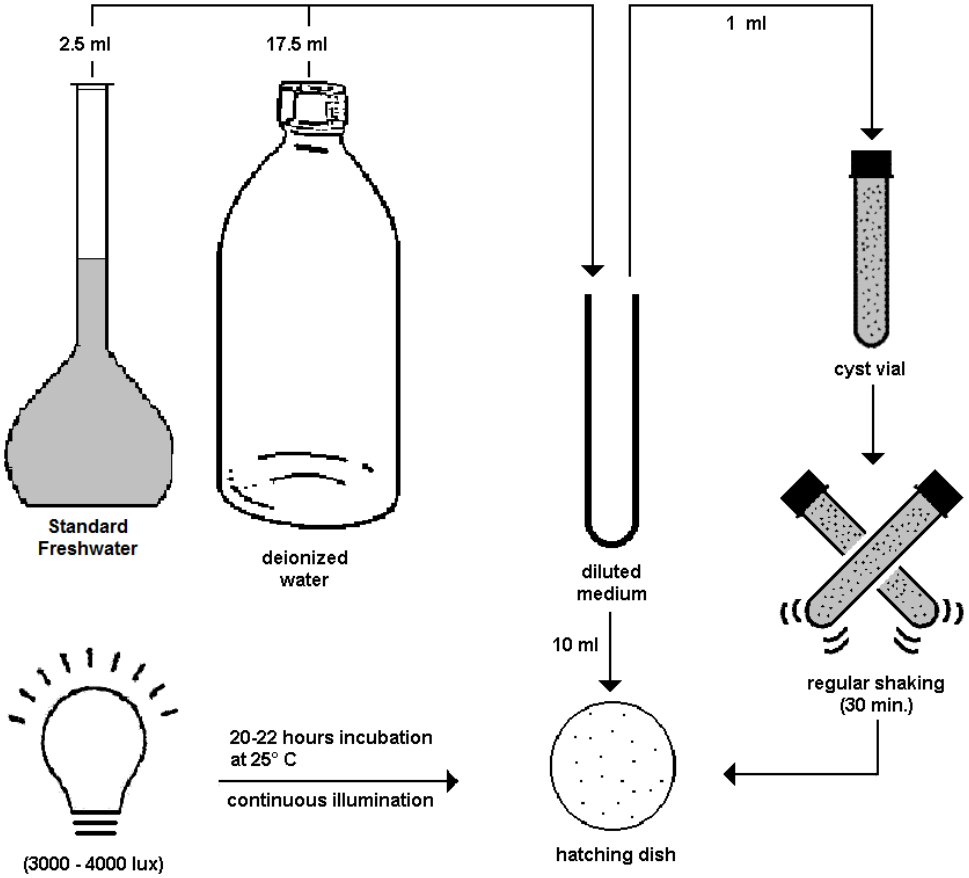
2. PRE-AERATION OF THE STANDARD FRESHWATER

The Standard Freshwater must be aerated for at least 15 minutes prior to use it for the hatching of the cysts and for the preparation of the toxicant dilutions. Pre-aeration can be performed very easily by air bubbling through a tube connected to an aquarium air pump.

3. STORAGE OF THE STANDARD FRESHWATER

The 1 liter solution of Standard Freshwater suffices for the 6 bioassays of each Toxkit. If all 6 tests are not to be carried out within a few days after preparation of the medium, store the Standard Freshwater in the refrigerator in darkness.
Take care to bring the cooled medium (gradually) back to room temperature prior to use.

HATCHING OF THE CYSTS



4. HATCHING OF THE CYSTS

Cyst hatching should be initiated **24 hours** prior to the start of the toxicity test.

Procedure (see figure)

Hatching is performed in diluted Standard Freshwater (dilution 1:8 with deionized water).

Note : Dilution of the Standard Freshwater decreases the osmotic pressure, which results in higher hatching success of the *Thamnocephalus platyurus* cysts.

Pre-hydration of the cysts

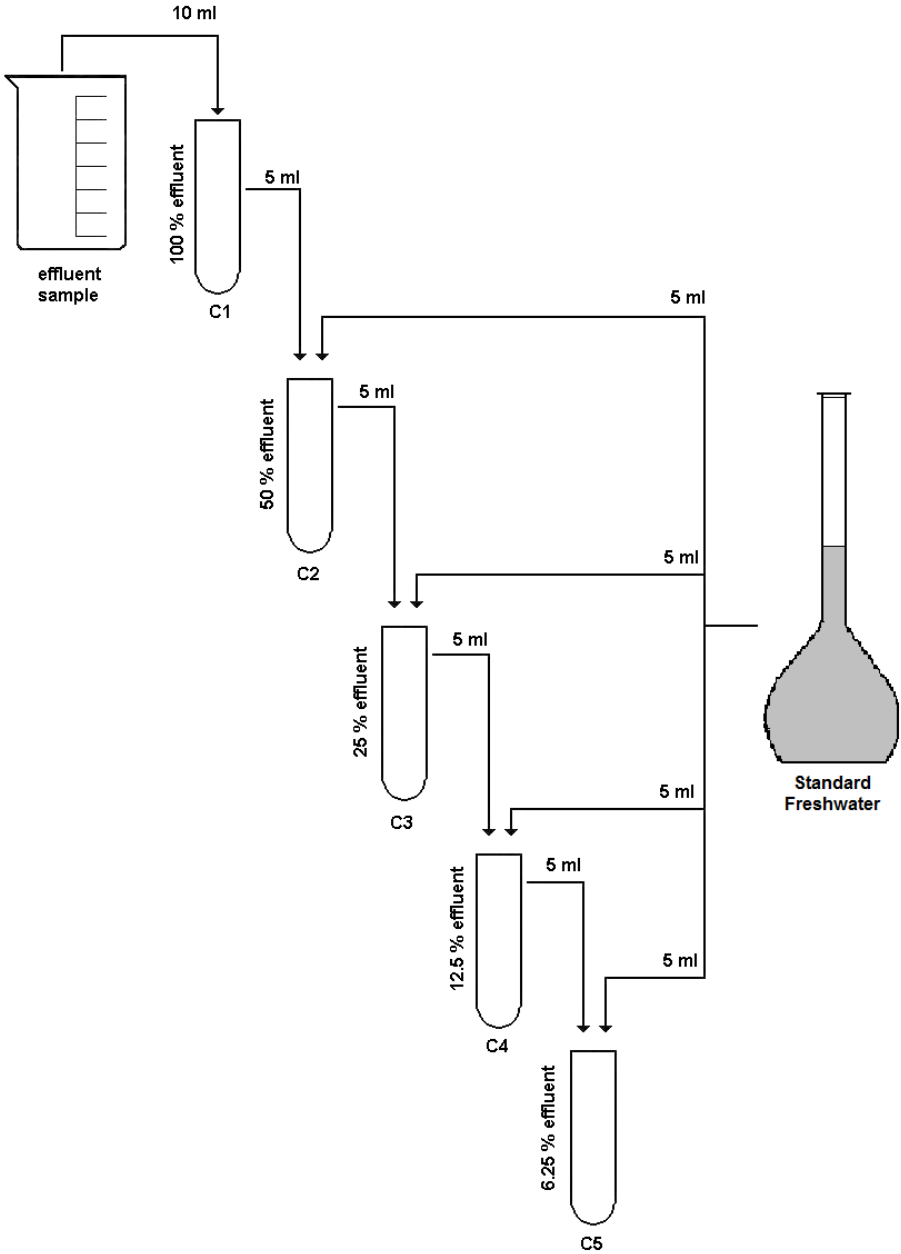
1. Prepare 20 ml of diluted Standard Freshwater by adding 17.5 ml deionized water to 2.5 ml Standard Freshwater.
2. Open a tube with cysts and fill it with diluted hatching medium (approximately 1 ml).
3. Close the tube with the stopper and shake it at regular intervals during a 30 minutes period.

Transfer of the pre-hydrated cysts into the hatching petri dish

1. Empty the contents of the vial with pre-hydrated cysts into one of the two small petri dishes; make sure most of the cysts are transferred by rinsing the tube with diluted Standard Freshwater.
2. Add 10 ml diluted Standard Freshwater medium to the petri dish and swirl gently to distribute the cysts evenly.
3. Cover the hatching petri dish and incubate at 25 °C for **20-22 hours**, under continuous illumination (light source of min. 3000-4000 lux).

PREPARATION OF THE TOXICANT DILUTIONS

A. EFFLUENTS



5. PREPARATION OF THE TOXICANT DILUTION SERIES

The TOXKIT bioassays have been designed primarily for cost-effective acute toxicity screening.

This section of the Standard Operational Procedure describes a simple and rapid way to make toxicant dilution series with the aid of disposable 10 ml plastic tubes and disposable 1 ml and 10 ml plastic graduated pipettes.
Greater precision may be gained by using conventional laboratory glassware.

A. Effluents

A dilution series 100% - 50% - 25% - 12.5% and 6.25% of the effluent sample is prepared by the serial dilution procedure; each dilution is made by diluting the previous concentration by half (cf. US-EPA/600/4-85/013, 1985).

Procedure (see figure)

1. Add 5 ml Standard Freshwater to test tubes 2, 3, 4, and 5.

2. Add 10 ml effluent sample to test tube 1 and rinse the pipette.

3. Using the same pipette, transfer 5 ml from test tube 1 to test tube 2 and rinse the pipet; cap and shake test tube 2.

4. Repeat this procedure (step 3) for the next dilutions (Table 1).

    * 5 ml from test tube 2 to test tube 3
    * 5 ml from test tube 3 to test tube 4
    * 5 ml from test tube 4 to test tube 5

5. Proceed to section 6 : **Filling of the Test Plate.**

PREPARATION OF THE TOXICANT DILUTIONS

B. CHEMICAL COMPOUNDS

Range finding test

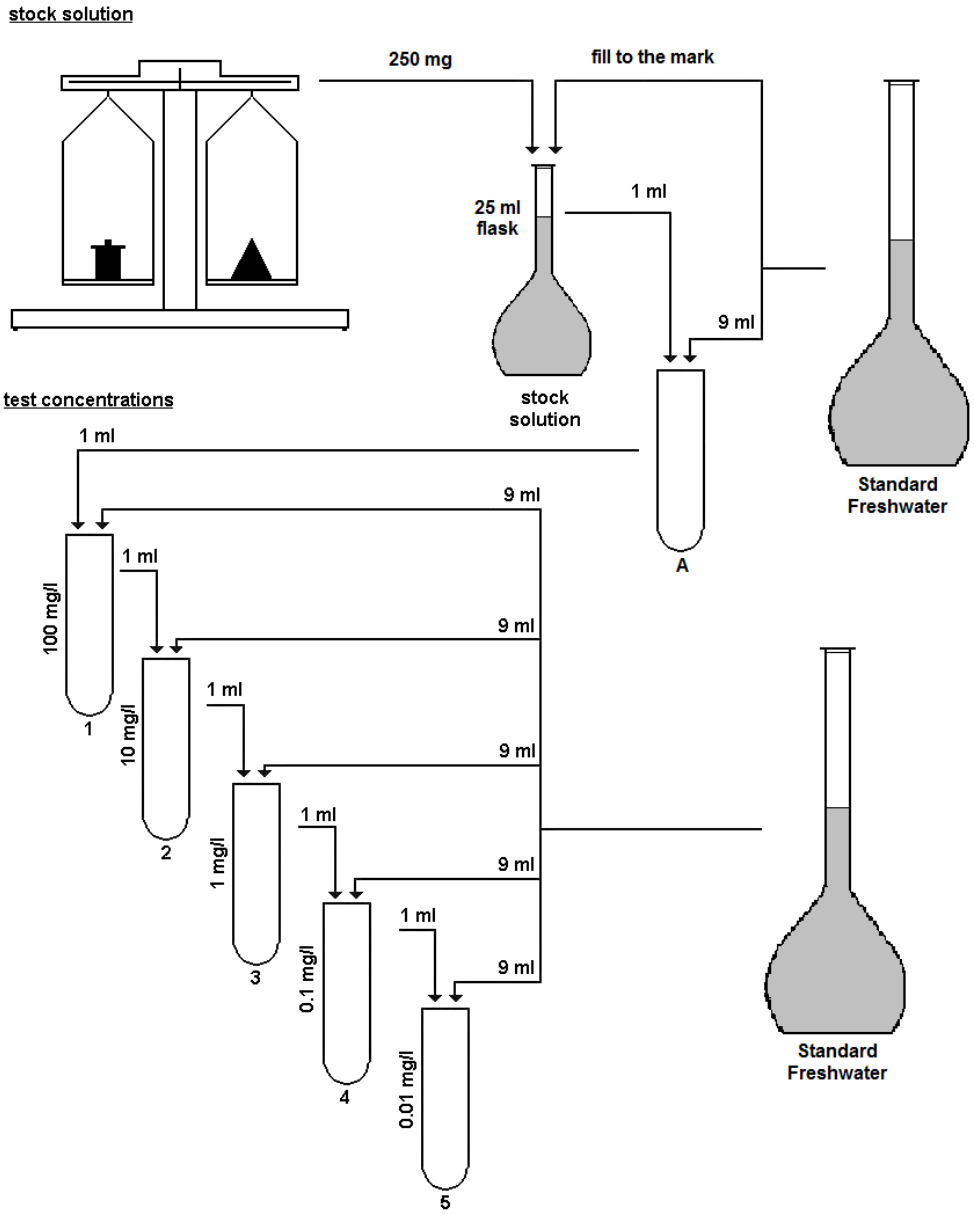


Table 1 : Dilution series of the effluent

Test tube	Effluent concentration (%)
1	100
2	50
3	25
4	12.5
5	6.25

B. Chemical compounds

If the approximate toxicity of the chemical compound is known, one may proceed directly to the **definitive test**.
If the approximate toxicity of the chemical is **not** known, a **range finding** test must be carried out.

Range finding test

The dilution series: 100 mg/l, 10 mg/l, 1 mg/l, 0.1 mg/l, and 0.01 mg/l will be prepared for testing.

Procedure (see figure)

Stock solution

1. Weigh 250 mg of the chemical on an analytical balance and transfer it into a 25 ml volumetric flask.

2. Fill to the mark with Standard Freshwater, cap and shake vigorously.

3. Transfer 1 ml of the stock solution to test tube A and rinse the toxicant pipette.

4. Add 9 ml Standard Freshwater, cap and shake the test tube.

Test concentrations (Table 2)

1. Add 9 ml Standard Freshwater to test tubes 1, 2, 3, 4, and 5.

2. Add 1 ml of test tube A to test tube 1 and rinse the toxicant pipette; cap and shake test tube 1.

3. Add 1 ml of test tube 1 to test tube 2 and rinse the toxicant pipette; cap and shake test tube 2.

4. Repeat this procedure (step 3) for the next dilutions:

    * 1 ml from test tube 2 to test tube 3.
    * 1 ml from test tube 3 to test tube 4.
    * 1 ml from test tube 4 to test tube 5.

5. Proceed to section 6 : **Filling of the Test Plate**.

Table 2 : Dilution series of chemical compound

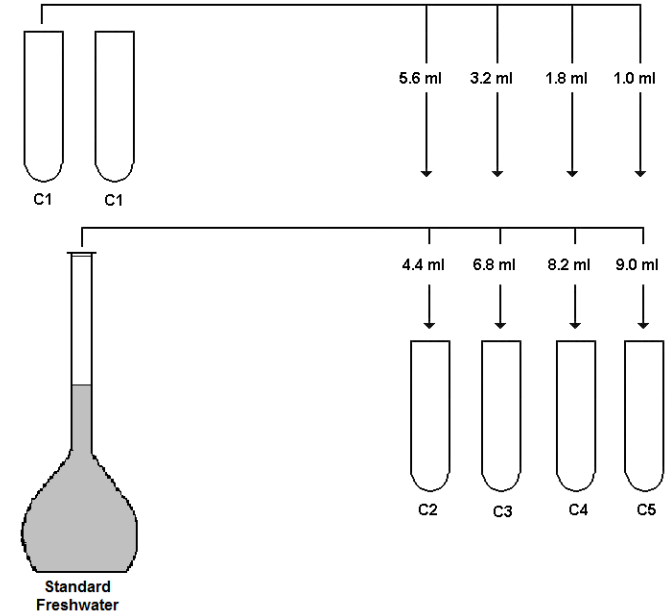
Test tube	Chemical concentration (mg/l)
1	100
2	10
3	1
4	0.1
5	0.01

Definitive test

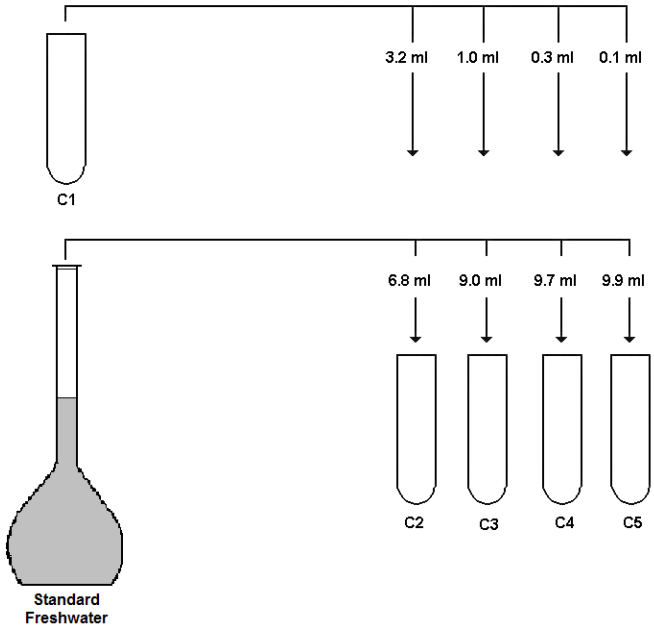
The dilution series to be tested in the definitive test spans the range of the lowest concentration producing 100 % mortality and the highest concentration producing 0 % mortality in the range finding test. This range can span one order of magnitude (case A) or two orders of magnitude (case B) as indicated in Table 3. This concentration range will be called C1 - C5.

Definitive test

A : C1- C5 spans one order of magnitude



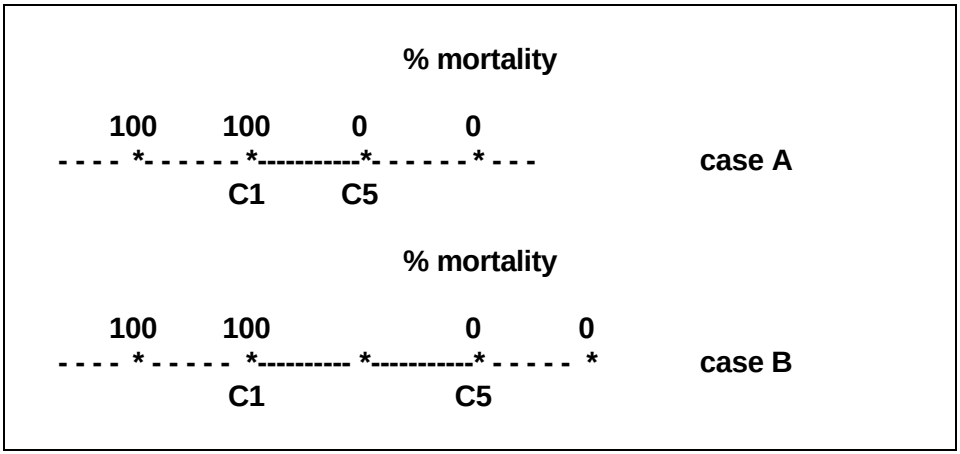
B : C1 - C5 spans two orders of magnitude



Procedure (see figure)

A dilution series ranging from C1 (100 % mortality) to C5 (0 % mortality) is prepared.
C1 is prepared according to the dilution instructions given in Table 3.

Table 3 : Diagrammatic representation of the 100 % and 0 % mortality concentration range, as determined in the range finding test



A. C1 - C5 spans one order of magnitude.

Important remark : in this case concentration C1 must be prepared in duplicate (two test tubes).

1. Add the volumes of Standard Freshwater as indicated in Table 4 to the respective test tubes.

2. Add the volumes of toxicant concentration C1 as indicated in Table 4.

3. Cap and shake the test tubes.

Table 4 : Dilution series C1 - C5

Test tube	Standard Freshwater (ml)	C1 (ml)
C1	0	10
C2	4.4	5.6
C3	6.8	3.2
C4	8.2	1.8
C5	9.0	1.0

4. Calculate the actual concentrations of C1, C2, C3, C4 and C5 (these figures are needed for the LC_{50} estimation).

C1 = ...mg/l

C2 = 0.56 × C1 = ...mg/l

C3 = 0.32 × C1 = ...mg/l

C4 = 0.18 × C1 = ...mg/l

C5 = 0.10 × C1 = ...mg/l

5. Proceed to section 6 : **Filling of the Test Plate.**

B. C1 - C5 spans two orders of magnitude.

Remark : in this case only one test tube of the C1 concentration has to be prepared.

1. Add the volumes of Standard Freshwater as indicated in Table 5 to the respective test tubes.

2. Add the volumes of toxicant concentration C1 as indicated in Table 5.

3. Cap and shake the test tubes.

Table 5 : Dilution series C1 - C5

Test tube	Standard Freshwater (ml)	C1 (ml)
C1	0	10
C2	6.8	3.2
C3	9.0	1.0
C4	9.7	0.3
C5	9.9	0.1

4. Calculate the actual concentrations of C1, C2, C3, C4, and C5 (these figures are needed for the LC_{50} estimation).

C1 = ...mg/l

C2 = 0.32 × C1 = ...mg/l

C3 = 0.10 × C1 = ...mg/l

C4 = 0.03 × C1 = ...mg/l

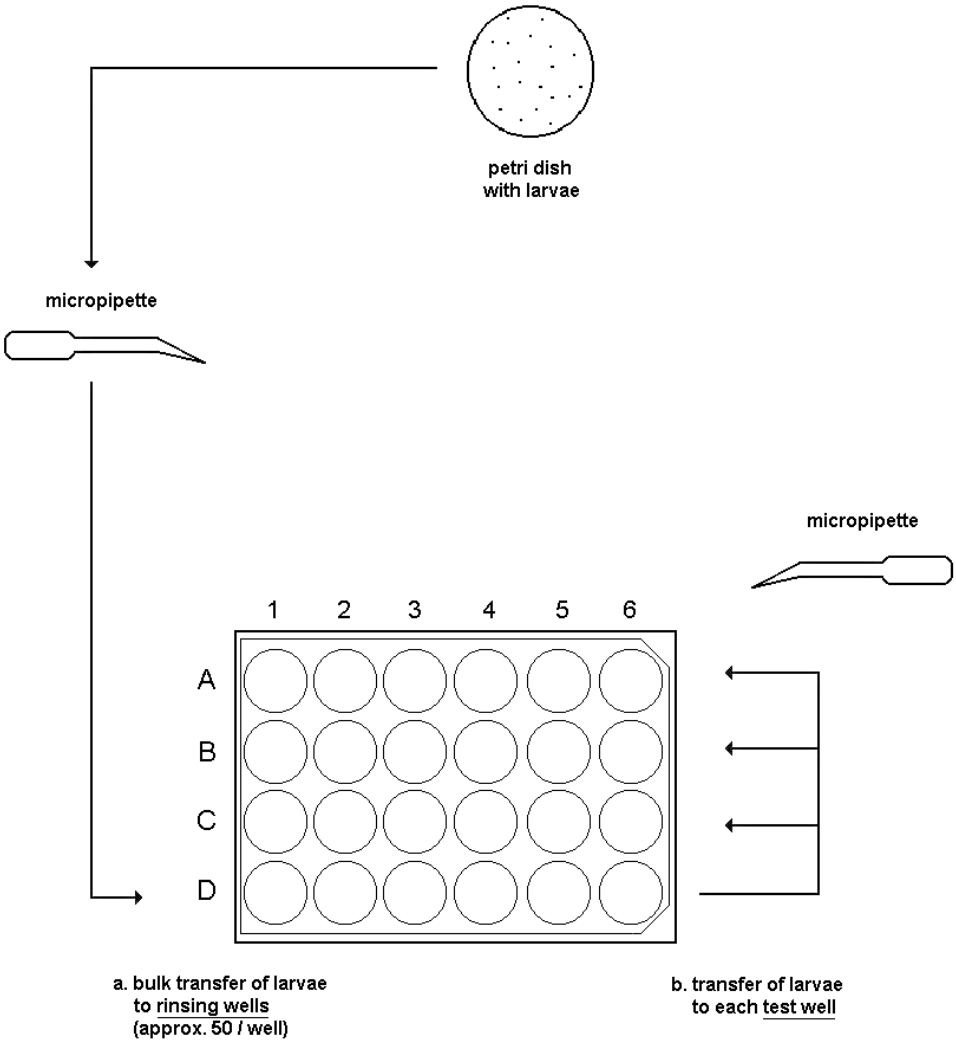
C5 = 0.01 × C1 = ...mg/l

5. Proceed to section 6 : **Filling of the Test Plate.**

6. FILLING OF THE TEST PLATE

Each toxicant dilution has to be transferred into all the wells of one column in the multiwell plate. The wells are labelled from 1 to 6 horizontally and from A to D vertically.
The distribution of the test solutions will always be carried out starting with the control (left, column 1) towards the highest concentration (right, column 6).

TRANSFER OF THE LARVAE INTO THE WELLS



Procedure (see figure)

Controls :

1. Add 1 ml Standard Freshwater to each well of column 1 (wells A1, B1, C1, D1).

Toxicant dilutions :

2. Shake each test tube thoroughly.

3. Transfer 1 ml of test tube 5 to each well of column 2 (wells A2, B2, C2, D2).

4. Repeat this procedure (steps 2 and 3) with test tubes 4, 3, 2 and 1 to fill the wells of columns 3, 4, 5 and 6, respectively.

7. TRANSFER OF THE LARVAE TO THE TEST WELLS

The micropipette should be held like a pencil with the index finger and tumb providing pressure on the bulb. This position usually provides the best control and produces the least fatigue, but any position may be used if it feels more comfortable. The bulb should be ***squeezed gently*** *to provide adequate suction. It will take a little practice to develop a feel for just the right pressure. Micro-pipetting of larvae is an easily acquired skill. After about 15 minutes of practice, most people become sufficiently skilled at collecting, counting, and transferring larvae rapidly to successfully complete the bioassay. Once accustomed to it, micro-pipetting becomes comfortable for most people.*

Transfer of the fairy shrimp larvae to the multiwell plate is accomplished in two steps :

a. transfer of the larvae from the petri dish into the rinsing wells of the multiwell plate (D1 to D6).
b. transfer of the larvae from the rinsing wells to the actual test wells (rows A, B, C).

Remark : The intermediate transfer of the larvae through rinsing wells (row D) minimizes the dilution of the toxicant solutions in the test wells (rows A, B, C).

Procedure (see figure)

1. Take the hatching petri dish out of the incubator and wait approximately five minutes to allow the nauplii to congregate.

The next steps are executed under a dissection microscope at magnification 10-12x.

2. Put the petri dish on the microscope stage and using the micropipette, transfer **approximately 50 larvae** from the petri dish into each rinsing cup (each well of row D), in the following sequence : D1 (control), D2, D3, D4, D5 and D6 (increasing concentrations of toxicant). **Try to carry over as little as possible liquid from the petri dish to the wells during this transfer.**

3. Put the multiwell plate on the stage of the dissection microscope and transfer **10 larvae** from rinsing well D1 into the 3 other wells of column 1 (A1, B1 and C1).
   Count the larvae as they exit the micropipette to confirm transfer of 10 test organisms per well.

4. Repeat this transfer for columns 2, 3, 4, 5 and 6 (in this sequence !).

8. INCUBATION OF THE TEST PLATE

Procedure

1. Put the Parafilm strip on top of the multiwell plate and put the cover on tightly.

2. Put the multiwell plate in the incubator at 25 °C in darkness, for 24 hours.

9. SCORING OF THE RESULTS

Procedure

1. Take the multiwell plate out of the incubator and put it under the dissection microscope.

THAMNOTOXKIT F - RESULTS SHEET

Name of operator : .....

Date of performance of test : .....

Toxicant tested : .....

Type of test : O range finding
O definitive

Dilution series tested : concentration 1 = ...
concentration 2 = ...
concentration 3 = ...
concentration 4 = ...
concentration 5 = ...

	Control	Conc. 5	Conc. 4	Conc. 3	Conc. 2	Conc. 1
A						
B						
C						
Total	/30	/30	/30	/30	/30	/30
% Mortal.						

Mortality scores

2. Check all the wells of row A, B, and C and record the number of dead larvae.

* *The larvae are considered dead if they do not show any movement during 10 seconds of observation.*

3. Score the mortality figures on the RESULTS SHEET.

4. Total the number of dead larvae for each concentration and calculate the % mortality*.

10. ESTIMATION OF THE LC_{50}

There are many procedures for calculating 50% effect thresholds. A data treatment program to calculate the 24h EC_{50} for the Thamnotoxkit microbiotest is available on demand from MicroBioTests Inc.

11. TEST VALIDITY

For the toxicity test to be acceptable, the percentage mortality of the test organisms in the controls should not be higher than 10%

12. REFERENCE TEST

In order to check the correct execution of the test procedure and the good physiological condition of the test animals, it is advisable to perform a reference test from time to time.
Such a quality control test can e.g. be performed with the reference toxicant potassium dichromate ($K_2Cr_2O_7$).

Procedure

1. Weigh 100 mg potassium dichromate on an analytical balance, transfer it to a 100 ml volumetric flask and fill to the mark with deionized water.
2. Make a dilution series of the reference toxicant (e.g. in 10 ml plastic tubes), according to the procedure indicated in section 4 : **Chemical compounds - Definitive test.**

The dilution series for the reference test ranges between 0.032 mg/l and 0.32 mg/l. First a 1 mg/l dilution of the stock solution is prepared, from which the sub-dilutions C1-C3 are made (C3 to be made in duplicate in case 10 ml test tubes are used). Dilutions C4 and C5 are subsequently prepared out of C3. The listing hereunder shows the 5 dilutions to be used for the reference test :

C1 (one test tube) : 0.32 mg/l
C2 (one test tube) : 0.18 mg/l
C3 (two test tubes): 0.10 mg/l
C4 (one test tube) : 0.056 mg/l
C5 (one test tube) : 0.032 mg/l

3. Proceed to section 6. **Filling of the Test Plate.**

From the data obtained in the quality control test, a 24 h LC_{50} has to be calculated, the value of which should be situated within the limits (range) stipulated in the specification sheet.

N.B. : The mean 24h LC50 value for potassium dichromate calculated for the International Interlaboratory Comparison on the Thamnocephalus platyurus test was 0.100 mg/l with 95% confidence limits of 0.052 - 0.148 mg/l.

LIST OF TOXKIT MICROBIOTESTS

Tests for freshwater and soils

PROTOXKIT F : 24h reproduction inhibition test based on the ciliate protozoan *Tetrahymena thermophila*. This assay is under consideration as an OECD Guideline.

ROTOXKIT F : 24h mortality test, based on the rotifer *Brachionus calyciflorus*. This assay adheres to ASTM Standard Guide E1440-91.

ROTOXKIT F chronic : 48h reproduction inhibition test based on the rotifer *Brachionus calyciflorus*. This assay adheres to ISO norm 20666 and AFNOR norm T90-377.

THAMNOTOXKIT F : 24h mortality test, based on the anostracan crustacean *Thamnocephalus platyurus*. This assay adheres to ISO norm 14380.

CERIODAPHTOXKIT F : 24h mortality test, based on the cladoceran crustacean *Ceriodaphnia dubia*. This assay is in current practice in the USA as an EPA Method.

DAPHTOXKIT F: 24h-48h mobility inhibition test, based on the cladoceran crustacean *Daphnia magna*. This assay adheres to ISO norm 6341 and OECD Guideline 202.

OSTRACODTOXKIT F : 6 days chronic mortality and growth inhibition test with the ostracod crustacean *Heterocypris incongruens*. This assay adheres to ISO norm 14370.

RAPIDTOXKIT F Thamno: 30-60 min particle ingestion inhibition test based on the anostracan crustacean *Thamnocephalus platyurus*. This assay adheres to ISO norm 14380.

ALGALTOXKIT F : 72h growth inhibition test, based on the green alga *Selenastrum capricornutum* (presently named *Pseudokirchneriella subcapitata*). This assay adheres to ISO norm 8692 and OECD Guideline 201.

PHYTOTOXKIT solid samples: 3 days germination and root growth inhibition test with seeds of 3 higher plants.

PHYTOTOXKIT liquid samples: A short germination and root/shoot growth inhibition microbiotest for determination of the direct effect of chemicals on higher plants.

DUCKWEED TOXKIT F : 72h growth inhibition test with the duckweed species *Spirodela polyrhiza*.

Tests for estuarine/marine environments

ROTOXKIT M : 24h mortality test based on the rotifer *Brachionus plicatilis*. This assay adheres to ASTM Standard Guide E1440-91.

ARTOXKIT M : 24h mortality test based on the anostracan crustacean *Artemia salina* (renamed *Artemia franciscana*). This assay adheres to ASTM Standard Guide E1440-91.

ALGALTOXKIT M : 72h growth inhibition test based on the marine diatom *Phaeodactylum tricornutum*. This test adheres to ISO norm 10253.

Kleimoer 15
9030 Gent
Belgium
www.microbiotests.com