CERIODAPHTOXKIT F ACUTE

Crustacean Acute Toxicity Screening Test

for Freshwater

STANDARD OPERATIONAL

PROCEDURE

TABLE OF CONTENTS

INTRODUCTION TO THE DAPHTOXKITS

Origin:
The screening microbiotests with the daphnid species *Daphnia magna* and *Ceriodaphnia dubia* have been 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 recently been renamed Laboratory for Environmental Toxicology and Aquatic Ecology (LETAE)*

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

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 activated "on demand", prior to performance of the toxicity test.
This eliminates the need for continuous recruitment and/or stock culturing of test organisms and hence the major practical burden and cost factor.
All Toxkits tests have been "miniaturized" into practical and user friendly microbiotests which can be performed with conventional lab materials and equipment, on little bench space .

Biological characteristics of the Daphtoxkits:
The Daphtoxkits make use of the dormant eggs of the crustaceans *Daphnia magna* or *Ceriodaphnia dubia*, which are used worldwide for toxicity testing. These eggs are protected by a chitinous capsule called ephippium, and can be stored for long periods of time without losing their viability. When the ephippia are placed in specific environmental conditions and triggers, the eggs develop in 3 - 4 days of time into neonates which can then be used immediately for the toxicity tests.

Principle of the Daphtoxkits

Acute and/or chronic bioassays are performed in disposable multiwell test plates departing from neonates, uniform in size and in age, hatched from ephippia.

Test methodology:
Daphtoxkit tests are performed in accordance with test procedures prescribed by national and international organizations (e.g. OECD, ISO, EEC, USEPA, ASTM).

Features:
Each acute Daphtoxkit contains all the (disposable) materials to perform 6 complete bioassays (range finding or definitive tests). The only equipment needed is an incubator or a temperature controlled room at 25 °C, a small light table or a dissection microscope, and conventional laboratory glassware.

Sensitivity:
Comparative research in several laboratories in different countries has shown that the sensitivity of the bioassays performed with Daphtoxkits is similar to that of the conventional toxicity tests carried out with neonates from stock cultures.

Precision:
Since Daphtoxkits contain standard test materials (and biomaterials), the repeatability of these microbiotests is very high.

Shelf life:
The *Ceriodaphnia dubia* ephippia must be stored in darkness, at 5 °C (+/- 2 °C) to keep their viability. The hatching success of the ephippia kept in the former conditions is guaranteed for several months as indicated on the expiry date label on each kit.

Representativity
Like rotifers and copepods, cladocerans are ecologically very important members of freshwater aquatic communities. Daphnia's are the most commonly used crustacean test species for determination of the effects of xenobiotics on primary consumers in freshwater aquatic ecosystems.

CONTENTS OF THE CERIODAPHTOXKIT F ACUTE

Vials with ephippia:
Six 2 ml plastic tubes covered by aluminum foil, containing ephippia of *Ceriodaphnia dubia*, to be stored in a refrigerator at 5 °C (+/- 2 °C) until use. The number of neonates obtained from each vial suffices for one full toxicity test.

Concentrated salt solutions:
Five small glass bottles, each containing a concentrated solution of one salt, to make up one liter of Standard Freshwater (moderately hard US EPA medium) with deionized or distilled water, for preparation of the hatching and toxicant dilution medium.
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)

Petri dishes:
Two polystyrene petri dishes of 5 cm diameter, for the hatching of the ephippia.

Multiwell test plates:
Six polystyrene test plates (9 x 13 cm) with 24 wells (3 ml) which will serve as test containers.

Parafilm strips:
Six Parafilm strips for sealing the multiwell plates to minimize evaporation during the incubation period.

Micropipettes:
Six polyethylene micropipettes for transfer of the test organisms.

Microsieve:
A small sieve with 100 µ mesh, for rinsing of the ephippia

Standard Operational Procedure manual:
A detailed brochure with all instructions for performance of range finding and/or definitive assays on pure chemicals or wastes.

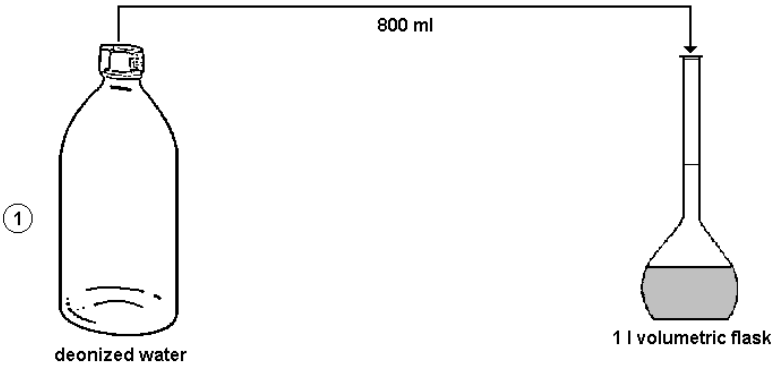
Bench protocol:
An abbreviated version of the Standard Operational Procedure manual.

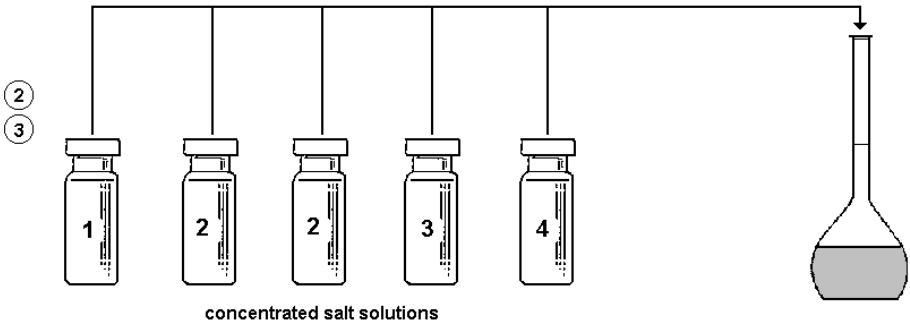
Results sheets:
Six sheets for scoring of the results and calculation of the mean percentage of mortality.

Specification sheet:
A sheet indicating the batch number and shelf life of the ephippia, the batch number of the concentrated salt solutions, the expiry date of the Ceriodaphtoxkit and the 24 LC_{50} value for the reference chemical potassium dichromate.

All the non-biological materials provided in the Ceriodaphtoxkit 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 describes hereunder are prepared with distilled water or deionized water. To avoid repetition, only the wording “distilled water” will be used further on.

*The **Ceriodaphtoxkit F acute** contains a set of 5 vials with concentrated salt solutions to prepare one liter of a "reconstituted" natural freshwater, according to the formula of the US Environmental Protection Agency (US EPA) for “moderately hard water”.*

The Standard Freshwater will be used as hatching medium for the ephippia and as dilution medium for preparation of the toxicant dilution series.

Procedure for the preparation of 1 liter Standard Freshwater :
(see figure)

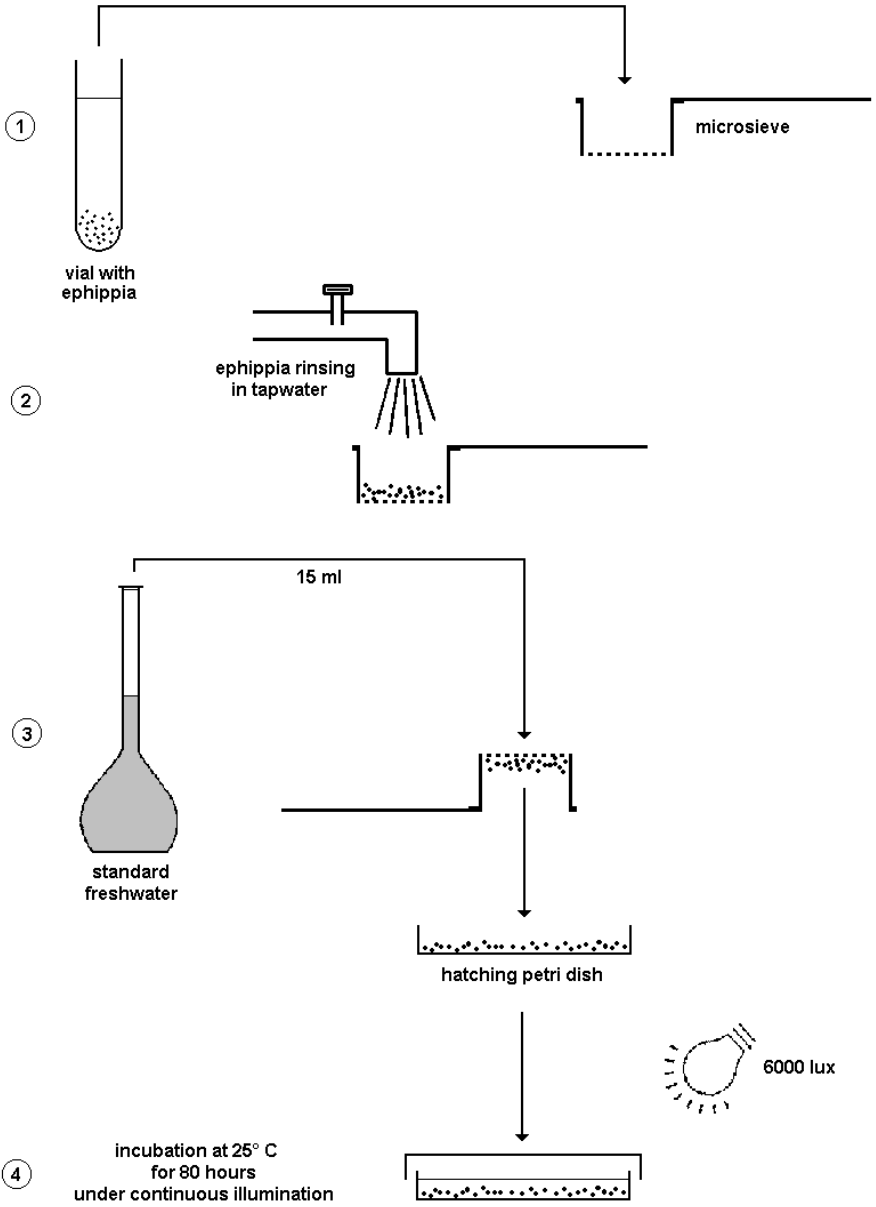
1. Fill a 1000 ml volumetric flask with approximately 800 ml distilled water.
2. Uncap vial labeled number 1 ($NaHCO_3$), and pour the contents in the flask.
3. Repeat step 2 for the other vials with concentrated salt solutions, i.e. two vials number 2 ($CaSO_4.2H_2O$), one vial number 3 ($MgSO_4.7H_2O$)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

2. STORAGE OF THE HATCHING AND DILUTION MEDIUM

One liter Standard Freshwater suffices for performance of 6 complete acute Ceriodaphtoxkit F bioassays.

If all the tests are not carried out within a few days after preparation of the Standard Freshwater, the medium should be stored in the refrigerator in darkness. Take care to bring the cooled medium (gradually) back to room temperature prior to use.

HATCHING OF THE EPHIPPIA



3. 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 ephippia 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.

4. HATCHING OF THE EPHIPPIA

Hatching of the ephippia must be initiated about 80 hours prior to the start of the toxicity test (see box "Important considerations with regard to ephippia hatching").

Procedure (see figure) :

1. Pour the contents of one vial with ephippia into the microsieve; make sure that all the ephippia are transferred.

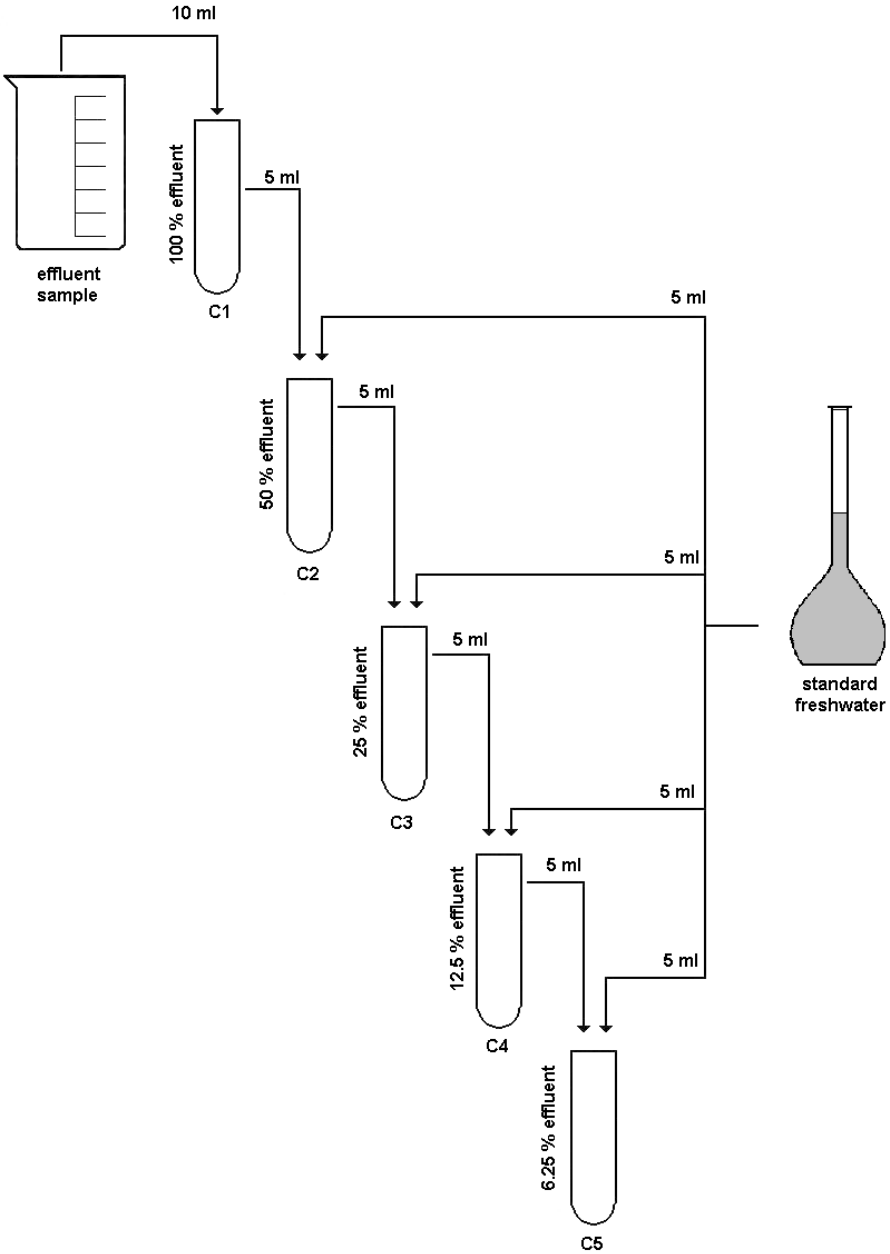
2. Rinse the ephippia thoroughly with tap water to eliminate all traces of the storage medium.

3. Transfer the ephippia into the hatching petri dish in 15 ml pre-aerated Standard Freshwater (one can also use a 10 cm diameter dish with 50 ml pre-aerated Standard Freshwater).

4. Cover the hatching petri dish and incubate **for about 80h, at 25 °C under continuous illumination of min. 6000 lux** (light intensity at the top of the petri dish).

PREPARATION OF THE TOXICANT DILUTIONS

1. EFFLUENTS



IMPORTANT CONSIDERATIONS WITH REGARD TO EPHIPPIA HATCHING

The embryonic development of *Ceriodaphnia dubia* eggs takes about 80 hours in optimal conditions. Under the illumination and temperature indicated above (i.e. 6.000 lux and 25 °C) hatching of the first neonates should start after about 78 - 80 h incubation.

Since the neonates should not be older than 24h at the start of the toxicity test, hatching should be checked after 80h incubation and the test organisms must be collected within 24h after appearance of the first neonates.

5. PREPARATION OF THE TOXICANT DILUTION SERIES

All TOXKIT bioassays have been designed primarily for cost-effective acute toxicity screening ; consequently 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 pipets.

Clearly greater precision may be gained by using conventional laboratory glassware.

5.1. TESTS ON 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. Take five 10 ml test tubes and label them from C1 to C5.

2. Add 5 ml dilution water to test tubes C2, C3, C4, and C5.

3. Add 10 ml effluent sample to test tube C1 and rinse the pipet.

4. Using the same pipet, transfer 5 ml from test tube C1 to test tube C2 and rinse the pipette ; cap and shake test tube C2.

5. Repeat this procedure (step 4) for the next dilutions (Table 1), i.e.

   - 5 ml from test tube C2 to test tube C3
   - 5 ml from test tube C3 to test tube C4
   - 5 ml from test tube C4 to test tube C5

6. Proceed to section 6 : **Filling of the test plate.**

Table 1 : Dilution series of the effluent

Test tube	**Effluent concentration (in %)**
C1	**100**
C2	**50**
C3	**25**
C4	**12.5**
C5	**6.25**

In case the lowest effluent concentration tested out (6.25% produces more than 50% mortality, the test has to be repeated with a lower dilution series. The highest effluent concentration to be selected for preparation of the 1:1 dilution series will be the lowest one that produced 90-100% mortality in the first test.

5.2. TESTS ON CHEMICAL COMPOUNDS

*If the approximate toxicity (order of magnitude) of the chemical compound to Ceriodaphnia dubia is known, a **definitive test** can be performed immediately. If no such information is available, then two consecutive assays must be performed :*

*a) a **range finding** test to determine the 0-100% tolerance range of the Daphnids to the toxicant,*

*b) a **definitive test** to determine the 50% effect concentration* (EC_{50})

A. RANGE FINDING TEST

A dilution series 1:10 must be prepared. An example is given below for a concentration series ranging from 100 mg/l down to 0.01 mg/l.

Procedure (see figure)

Stock solution

1. Take a 25 ml volumetric flask and label it Stock solution A. Take six 10 ml test tubes and label them as follows : Stock solution B, C1, C2, C3, C4 and C5.

2. Weigh 250 mg of the chemical on an analytical balance and transfer it into the volumetric flask labeled Stock solution A.

3. Add dilution water to the mark, cap and shake vigorously.

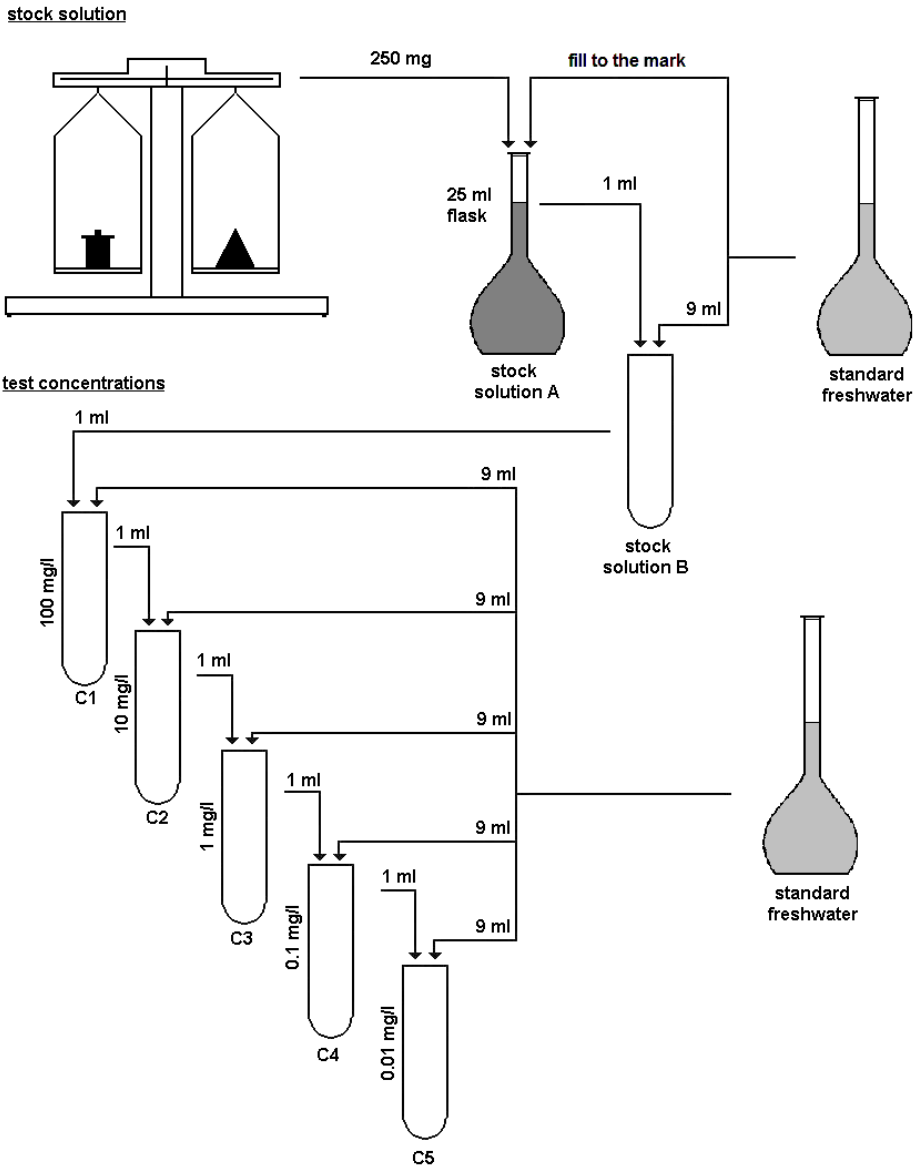
4. Transfer 1 ml from the Stock solution A volumetric flask to the tube labeled Stock solution B.

5. Add 9 ml dilution water, cap and shake the test tube.

PREPARATION OF THE TOXICANT DILUTIONS

2. CHEMICAL COMPOUNDS

A. Range finding test



Test concentrations (Table 2)

1. Add 9 ml dilution water to test tubes C1, C2, C3, C4, and C5.

2. Add 1 ml of tube Stock solution B to tube C1 and rinse the toxicant pipet; cap and shake tube C1.

3. Add 1 ml of tube C1 to tube C2 and rinse the toxicant pipet; cap and shake tube C2.

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

   - 1 ml from tube C2 to test tube C3.
   - 1 ml from tube C3 to test tube C4.
   - 1 ml from tube C4 to test tube C5.

5. Proceed to section 6 : **Filling of the test plate.**

Table 2 : Dilution series of chemical compound

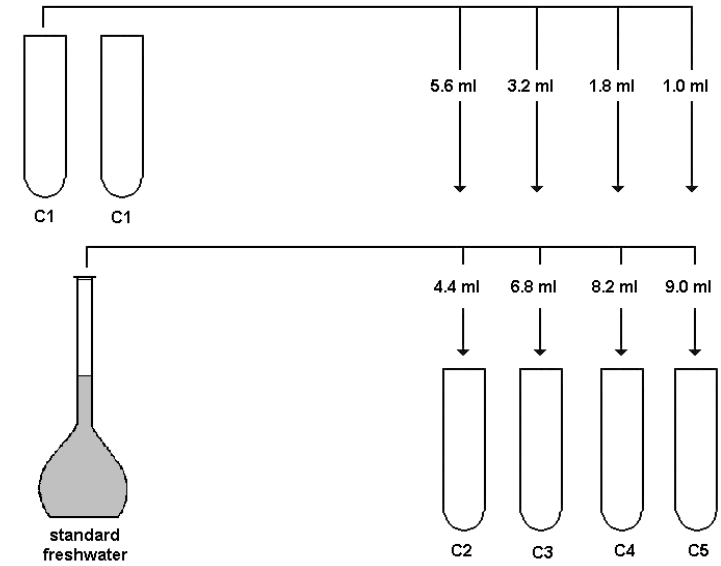
Test tube	Chemical concentration (in mg/l)
1	100
2	10
3	1
4	0.1
5	0.01

B. DEFINITIVE TEST

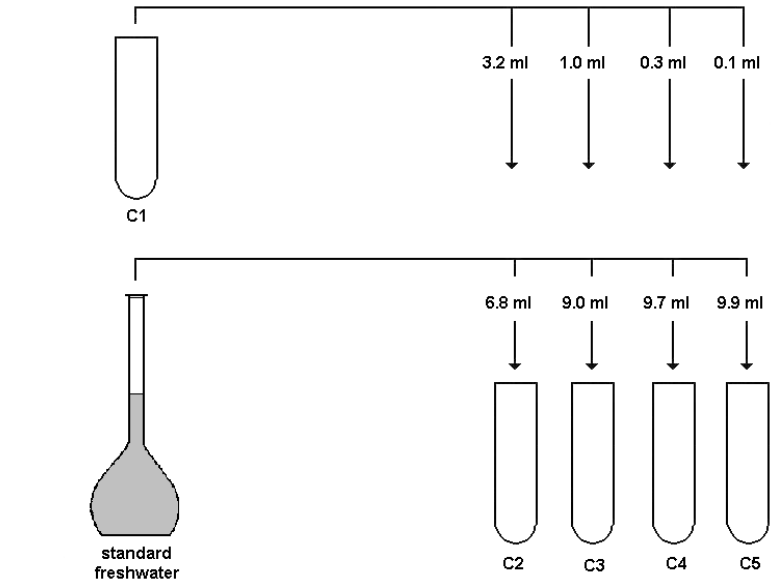
The dilution series to be tested in the definitive test spans the range of the lowest concentration producing 100 % mortality and the highest concentration producing 0 % mortality in the range finding test. As shown in Table 3 this range can span one order of magnitude (case A) or two orders of magnitude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

B. Definitive test

A : C1- C5 spans one order of magnitude



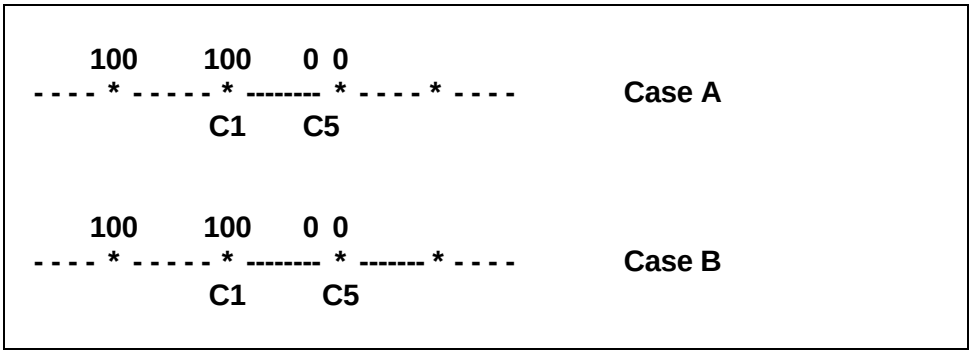
B : C1 - C5 spans two orders of magnitude



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 : Schematic presentation of the 100% - 0% mortality concentration range, determined in the range finding test



Case 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 dilution water 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	dilution water (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 x C1 = ...mg/l

C3 = 0.32 x C1 = ...mg/l

C4 = 0.18 x C1 = ...mg/l

C5 = 0.10 x C1 = ...mg/l

5. Proceed to section 6 : **Filling of the test plate**.

Case B. C1 - C5 spans two orders of magnitude.

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

1. Add the volumes of dilution water 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	dilution water (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 x C1 = ...mg/l

C3 = 0.10 x C1 = ...mg/l

C4 = 0.03 x C1 = ...mg/l

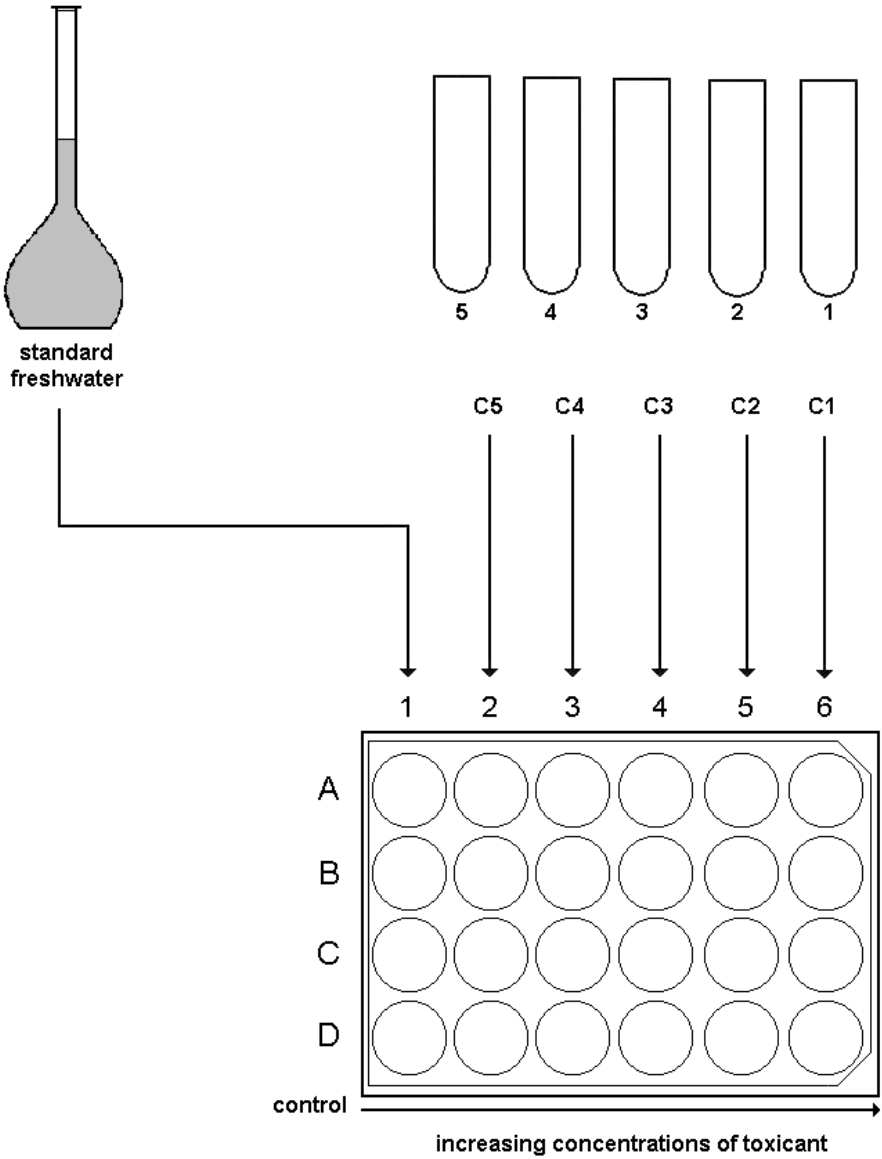
C5 = 0.01 x C1 = ...mg/l

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).

FILLING OF THE TEST PLATE



Procedure (see figure)

Controls :

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

Toxicant dilutions :

2. Shake each test tube thoroughly.

3. Transfer 1 ml of tube C5 to each well of column 2 (wells A2, B2, C2, D2).

4. Repeat this procedure (steps 2 and 3) with test tubes C4, C3, C2 and C1 to fill the wells of columns 3, 4, 5 and 6, respectively.

7. TRANSFER OF THE TEST ORGANISMS TO THE TEST WELLS

The transfer of the Ceriodaphnia neonates into the test wells is performed at the aid of a micropipette.

The transfer of the organisms from the hatching petri dish to the multiwell plate is accomplished in two steps:

a. transfer from the petri dish into the rinsing wells of the multiwell plate (D1 to D6).

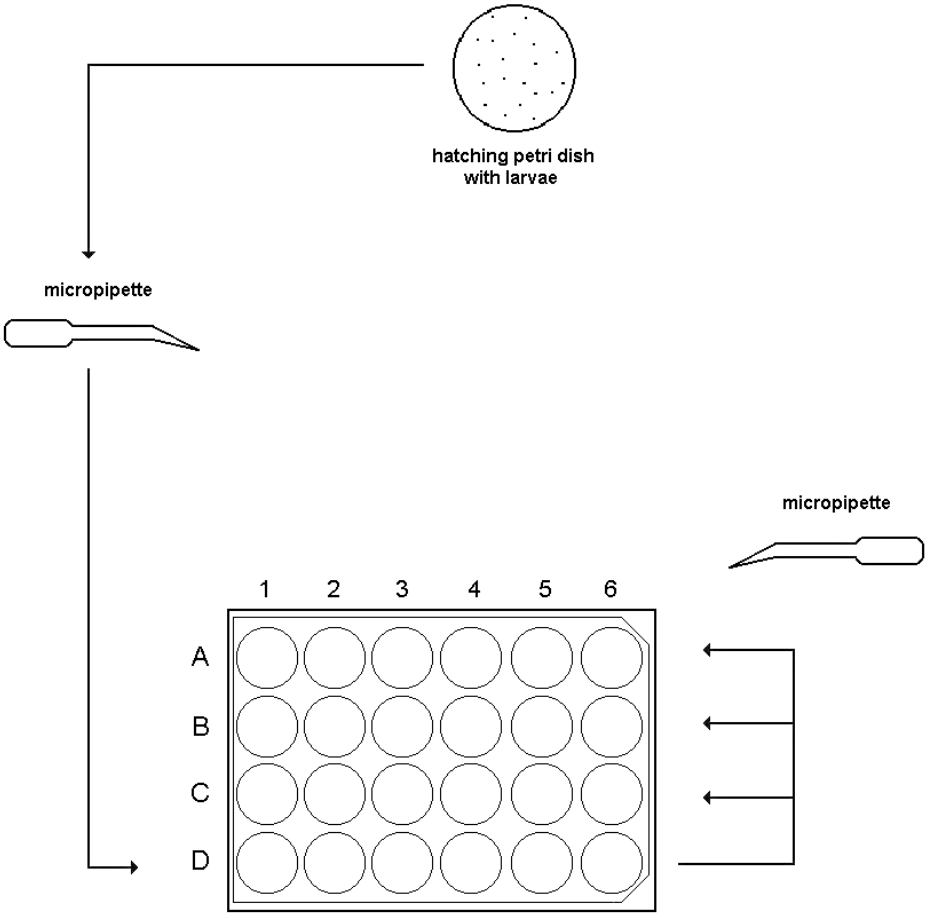
b. transfer of the neonates from the rinsing wells to the actual test wells (rows A, B, C).

Remark : The intermediate transfer of the test organisms 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 petri dish containing the hatched *Ceriodaphnia dubia* out of the incubator.

TRANSFER OF THE TEST ORGANISMS TO THE TEST WELLS



The next steps are executed under a dissection microscope at magnification 10-12x, or alternatively on a light box with a magnifying glass (6-8 x).

2. Put the petri dish on the microscope stage or on the light box, and using the micropipette, transfer **approximately 35-40 organisms** 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 or on the light box and, **after thorough rinsing of the micropipette** transfer **10 neonates** from rinsing well D1 into each of the 3 other wells of column 1 (A1, B1 and C1).
   Count the organisms as they exit the micropipette to ensure 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 or on the light box.

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

* *The organisms 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. Calculate the total number of dead *Ceriodaphnia* for each concentration and calculate the % mortality.

10. VALIDITY OF THE TEST

Next to all other specific validity conditions prescribed in standard Daphnia bioassay protocols (e.g. with regard to pH and oxygen concentration) a major condition is that ***the number of dead organisms should not exceed 10 % in the controls.***

11. ESTIMATION OF THE 24hLC$_{50}$

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

12. REFERENCE TEST

In order to check the correct execution of the test procedure and the sensitivity of the test animals, it is advised to perform from time to time a reference test.
Such a quality control test can e.g. be performed with the reference toxicant potassium dichromate (K_2CrO_7).

Procedure

1. Make a dilution series of the reference toxicant (e.g. in 10 ml plastic tubes), according to the procedure indicated in section 5 : **Chemical compounds - Definitive test**.

CERIODAPHTOXKIT F ACUTE - RESULT 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

The dilution series to be prepared for the reference test with the Ceriodaphtoxkit ranges between 0.1 mg/l and 1 mg/l.

N.B. Two test tubes have to be prepared with the concentration 1mg/l.

The list hereunder shows the 5 dilutions to be used for the reference test :

C1 (two test tubes) : 1 mg/l
C2 (one test tube) : 0.56 mg/l
C3 (one test tube): 0.32 mg/l
C4 (one test tube) : 0.18 mg/l
C5 (one test tube) : 0.1 mg/l

2. Proceed to section 6. **Filling of the test plate.**

From the data obtained in the quality control test, a 24h LC_{50} shall be calculated, the value of which should be situated within the limits stipulated in the specification sheet included in each Ceriodaphtoxkit.

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 short 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.

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

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.

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

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

SPIRODELA DUCKWEED TOXKIT : 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.

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

MANUFACTURED BY :

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