ARTOXKIT M™

Artemia Toxicity Screening Test

for Estuarine and Marine Waters

STANDARD OPERATIONAL

PROCEDURE

TABLE OF CONTENTS

	Page
Introduction to the Artoxkit M	2
Contents of the Artoxkit M	4
1. Preparation of the Standard Seawater	7
2. Storage of the medium	9
3. Hatching of the cysts	9
4. Preparation of the toxicant dilutions	11
A. Effluents	11
B. Chemical compounds	12
- Range finding test	14
- Definitive test	15
5. Filling of the test plate	21
6. Transfer of the larvae to the test wells	21
7. Incubation of the test plate	23
8. Scoring of the results	25
9. Estimation of the LC_{50}	25
10. Reference test	27

INTRODUCTION TO THE ARTOXKIT M

Origin : This screening bioassay was developed by the research team of Prof.Dr.G.Persoone at the State University of Ghent, Belgium.

Scope : The TOXKITS are aquatic toxicity tests in kits containing all materials as well as the test animals to perform simple, rapid, sensitive and cost-effective acute toxicity tests for chemicals or effluents released in freshwater, estuarine or marine environments.

The major advantage and asset of the TOXKITS, in comparison to conventional bioassays, is that each kit contains the test animals in the form of dormant eggs (cysts) from which larvae can be hatched "on demand". This eliminates one of the major bottle-necks in aquatic toxicology : the need for continuous stock culturing of test animals.

Principle : A 24 h LC_{50} bioassay is performed in a multiwell testplate using instar II-III larvae of the brine shrimp *Artemia franciscana* (formerly *Artemia salina*), which are hatched from cysts.

Features : Each Artoxkit M contains the (disposable) materials to perform 6 complete screening toxicity tests (range finding of definitive 24h LC_{50}) or 5 bioassays and one quality control test with a reference toxicant. Using larvae of the brine shrimp *Artemia franciscana* 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 somewhat lower sensitivity for some chemicals (in comparison to other test species) is largely compensated by the many advantages and the very high cost-efficiency of routine screening tests with brine shrimp.

Precision : Since each Artoxkit contains standard test materials, dilution water and reference cysts, the repeatability of the toxicity test is very high.

Cysts viability : Cysts can be stored in dry conditions in darkness in the refrigerator at 5 °C (+/- 2 °C) for at least six months, without losing their viability.

Representativity : Although *Artemia franciscana* is not found in estuaries or in the sea (for ecological reasons it has retracted into waters with high salinity), it is one of the most euryhaline marine species. Brine shrimp indeed thrive very well (for growth and reproduction) in laboratory cultures in brackish as well as in coastal and oceanic waters.

CONTENTS OF THE ARTOXKIT M

Vials with *Artemia franciscana* cysts
Six 1 ml plastic vials containing reference cysts of the brine shrimp *Artemia franciscana*, which can be stored at room temperature. 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.

Concentrated salt solutions
One vial containing NaCl and six 15 ml glass bottles, each containing a concentrated solution of one specific salt, to make up one liter Standard Seawater, for preparation of the hatching and the toxicant dilution media.

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

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

Parafilm strips
Six 10 x 15 cm strips of Parafilm for sealing the multiwell plate to minimize evaporation during the incubation period.

Micropipettes
Six polyethylene micropipettes for transferring the larvae.

Bench protocol
An abbreviated version of the extended Standard Operational Procedure manual, for those who are familiar with aquatic toxicity tests.

Test protocol
The Standard Operational Procedure manual for the *Artemia franciscana*

bioassay, with detailed instructions for performance of range finding tests and/or definitive tests, on pure chemicals or effluents.

Data- and calculation sheets

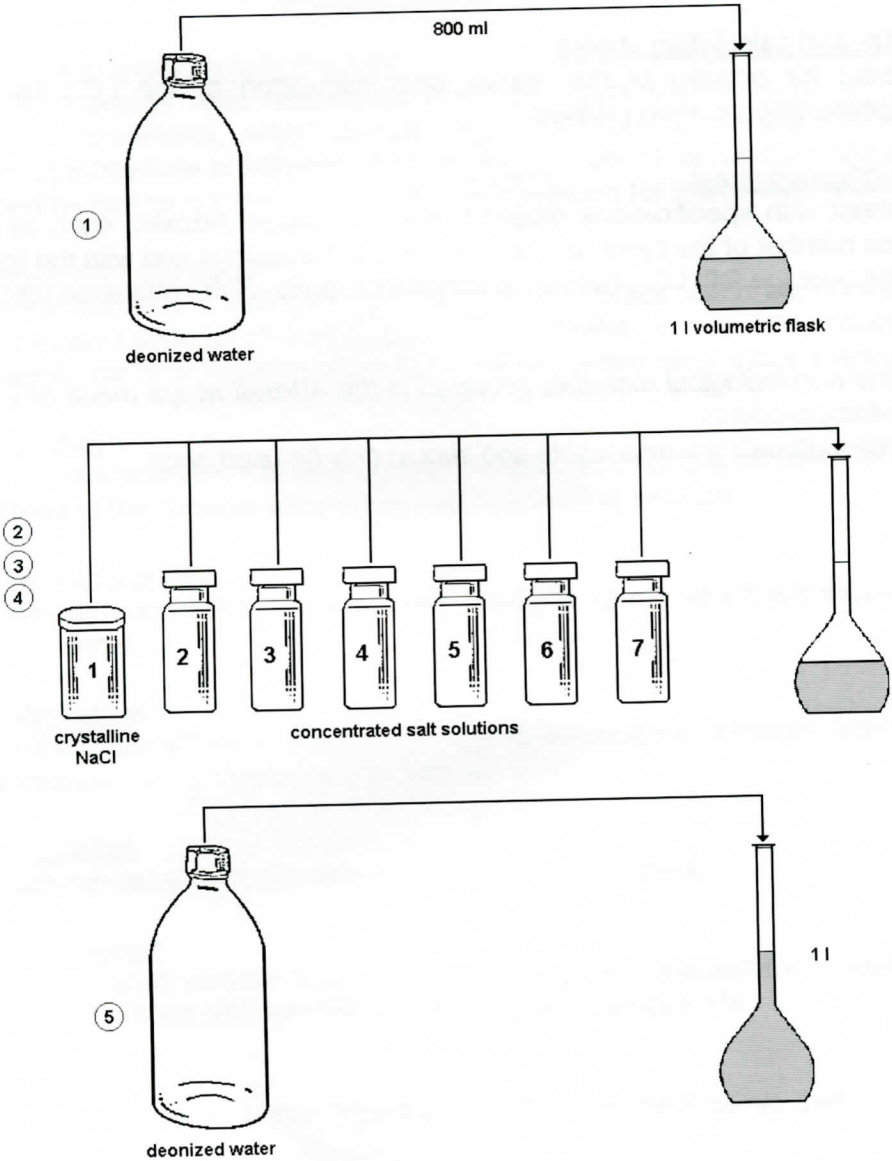
Sheets for scoring of the results and calculation of the LC_{50} by the graphical interpolation method.

Specification sheet

A sheet with specifications proper to each individual Artoxkit, such as the batch number of the cysts, the concentrated salt solutions and with the figure of the average 24h LC_{50} (with 95% confidence limits) of the reference test.

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

PREPARATION OF STANDARD SEAWATER



1. PREPARATION OF THE STANDARD SEAWATER

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 furtheron.

The vials with salt and concentrated salt solutions provided in the kit, are used to prepare one liter standard (artificial) seawater of normal seawater salinity (35 ppt). The Standard Seawater solution is used to prepare the hatching medium for the cysts, and as dilution medium for the toxicant dilution series.

Procedure (see figure)

1. Fill a 1 liter volumetric flask with approximately 800 ml deionized water.
2. Take vial number 1 (NaCl) and pour the contents in the flask. Shake until all the salt is dissolved.
3. Uncap the vial with concentrated salt solution labeled number 2 (KCl), and pour the contents into the volumetric flask.
4. Repeat step 3 for the other vials with concentrated salt solutions i.e. vial number 3 ($CaCl_2$), vial number 4 ($MgCl_2$), vial number 5 ($MgSO_4$), vial number 6 ($NaHCO_3$) and vial number 7 (H_3BO_3), respecting this sequence.
5. Add deionized water up to the 1000 ml mark and shake to homogenize the medium.

Important remark : For estuarine water bioassays, seawater of reduced salinity can be prepared by diluting the Standard Seawater with deionized water. This "brackish" water has to be used (instead of the 35 ppt Standard Seawater) as dilution medium for preparation of the toxicant dilution series.

Table 1 gives the ratios of Standard Seawater to deionized water to prepare 100 ml dilution medium of various salinities.

HATCHING OF THE CYSTS

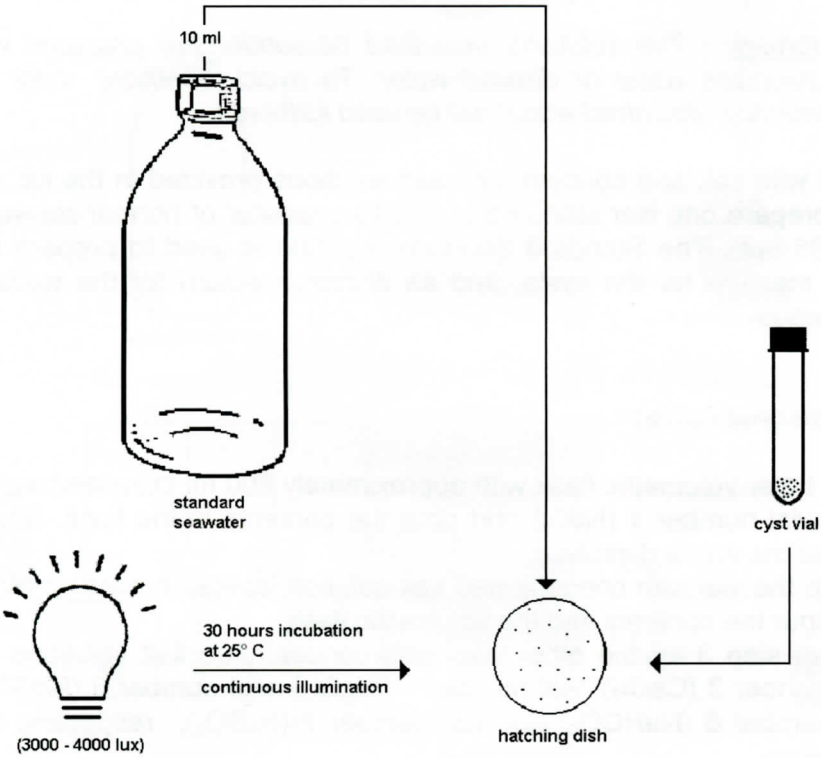


Table 1 : Preparation of seawater of reduced salinity

Salinity (ppt)	Standard Seawater (ml)	Deionized water (ml)
5	14	86
10	29	71
15	43	57
20	57	43
25	71	29
30	86	14

2. STORAGE OF THE MEDIUM

The 1 liter solution of Standard Seawater 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 Seawater in the refrigerator in darkness.
Take care to bring the cooled medium (gradually) back to room temperature prior to use.

3. HATCHING OF THE CYSTS

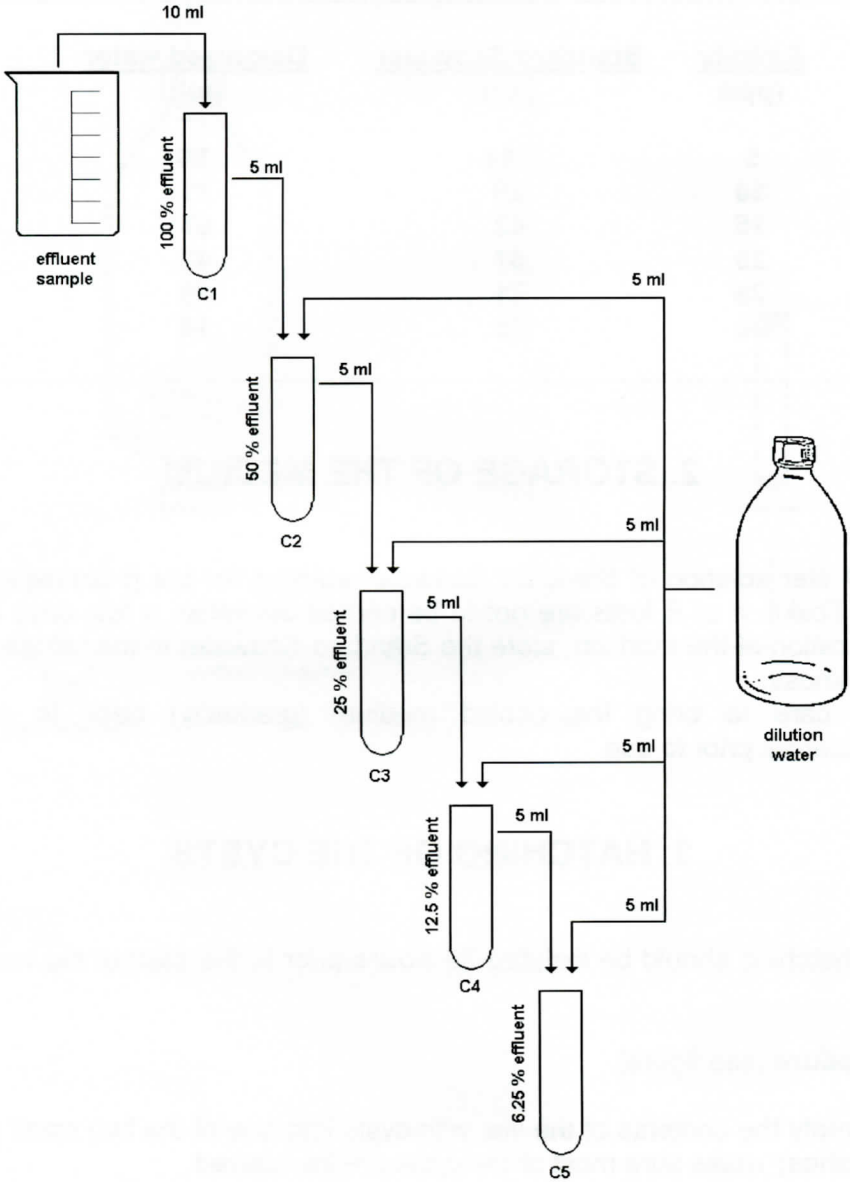
Cyst hatching should be initiated **30 hours** prior to the start of the toxicity test.

Procedure (see figure)

1. Empty the contents of the vial with cysts into one of the two small petri dishes; make sure most of the cysts are transferred.

PREPARATION OF THE TOXICANT DILUTIONS

A. EFFLUENTS



2. Add 10 ml Standard Seawater into the petri dish and swirl gently to distribute the cysts evenly.
3. Cover the hatching petri dish and incubate at 25°C for **30 hours**, under continuous illumination (light source of 3000-4000 lux).

Hatching will start after about 18-20 hours, after 30 hours most of the larvae will have moulted into the instar II-III stage.

4. PREPARATION OF THE TOXICANT DILUTION SERIES

The TOXKIT bioassays have been designed primarily for cost-effective acute toxicity screening; consequently this section of the Standard Operational Procedure proposes 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.

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 dilution water to test tubes 2, 3, 4, and 5.

2. Transfer 10 ml effluent sample to test tube 1 and rinse the pipet.

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 100 mg of the chemical on an analytical balance and transfer it into test tube A.
2. Add 10 ml dilution water; cap and shake vigorously.
3. Transfer 1 ml of test tube A to test tube B and rinse the toxicant pipet.
4. Add 9 ml dilution water, cap and shake the test tube.

Test concentrations (Table 3)

1. Transfer 9 ml dilution water to test tubes 1, 2, 3, 4, and 5.
2. Add 1 ml of test tube B to test tube 1 and rinse the toxicant pipet; cap and shake test tube 1.
3. Add 1 ml of test tube 1 to test tube 2 and rinse the toxicant pipet; 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 3 : Dilution series of chemical compound

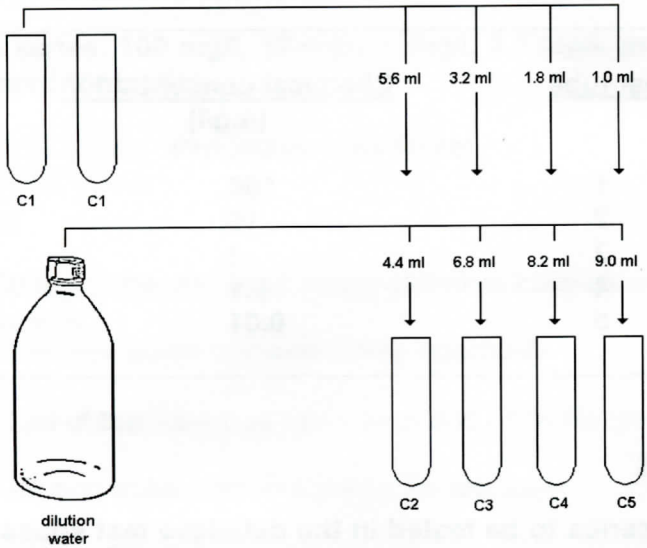
Test tube	Chemical concentration (mg/l)
1	100
2	10
3	1
4	0.1
5	0.01

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 4. 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

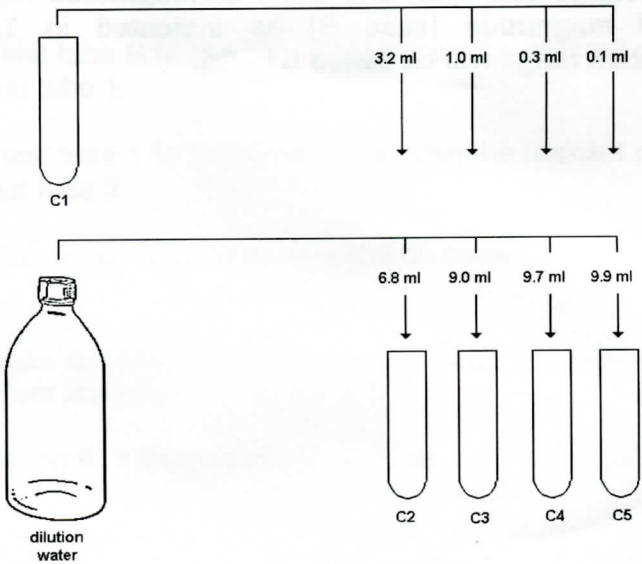


Table 4 : Diagrammatic representation of the 100 % and 0 % mortality concentration range, as determined in the range finding test

```
                    % mortality

   100      100       0        0
---- *------*----------*------*---          case A
            C1        C5

                    % mortality

   100      100                0        0
---- *----- *---------- *----------*----- *      case B
            C1                  C5
```

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.

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

1. Add the volumes of dilution water as indicated in Table 6 to the respective test tubes.

2. Add the volumes of toxicant concentration C1 as indicated in Table 6.

3. Cap and shake the test tubes.

Table 6 : 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 × C1 = ...mg/l

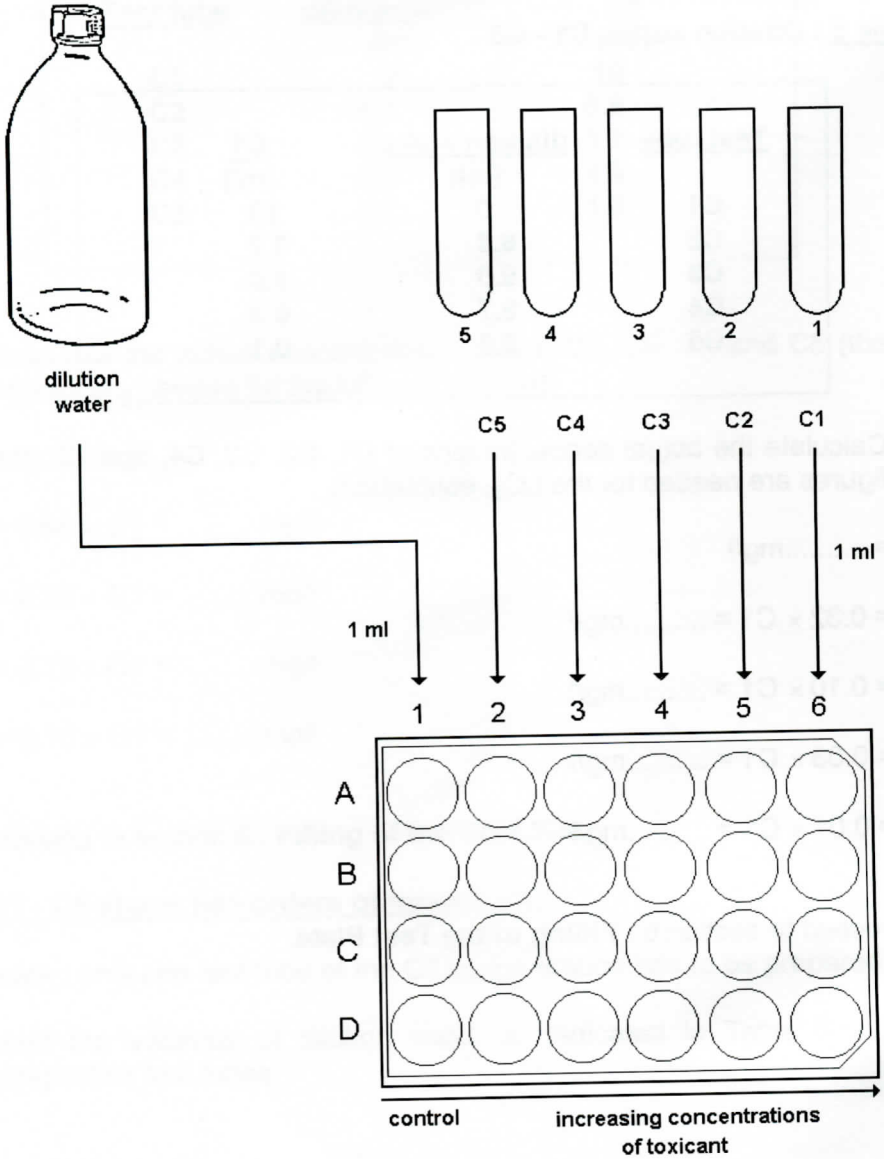
C3 = 0.10 × C1 = ...mg/l

C4 = 0.03 × C1 = ...mg/l

C5 = 0.01 × C1 = ...mg/l

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

FILLING OF THE TEST PLATE



5. FILLING OF THE TEST PLATE

Each toxicant dilution has to be transferred to 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).

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

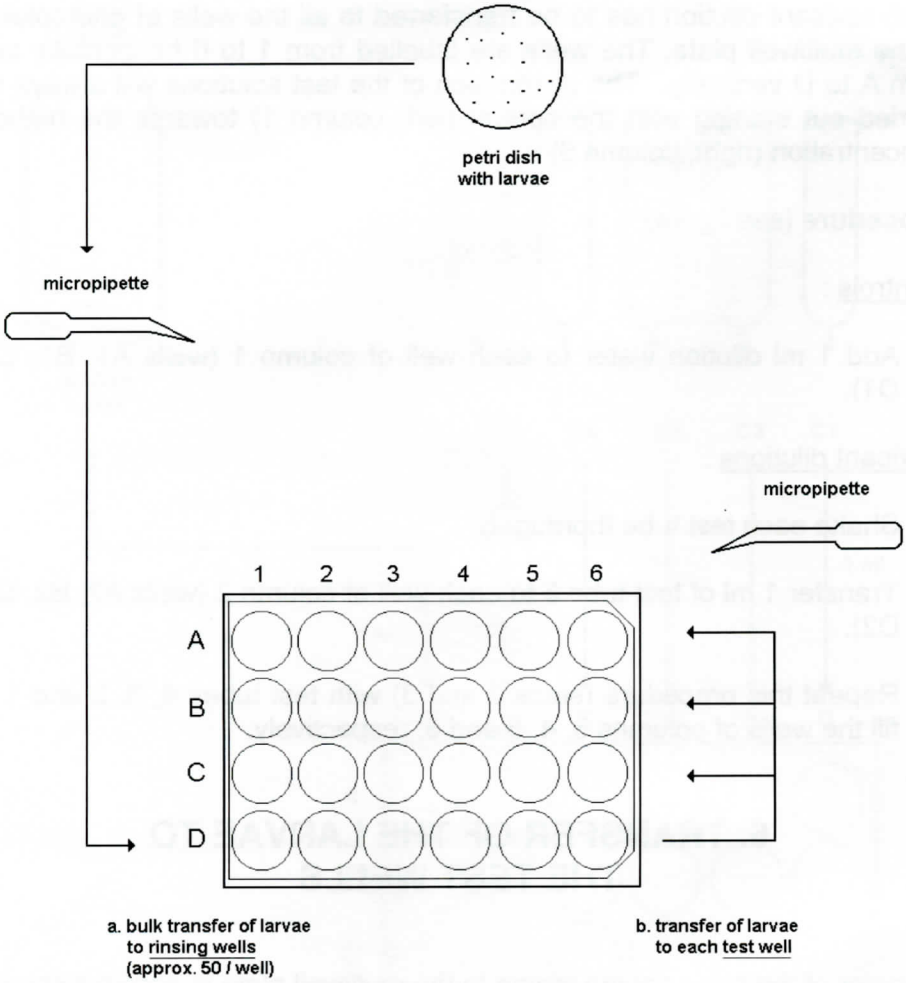
4. Repeat this procedure (steps 2 and 3) with test tubes 4, 3, 2 and 1 to fill the wells of columns 3, 4, 5 and 6, respectively.

6. TRANSFER OF THE LARVAE TO THE TEST WELLS

Transfer of the brine 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).

TRANSFER OF THE LARVAE INTO THE WELLS



Remark : *The intermediate transfer of the brine shrimp 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 transfer 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 micropipet, 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 en C1).
   Count the larvae as they exit het micropipet to confirm transfer of 10 test organisms per well.
4. Repeat this transfer for columns 2, 3, 4, 5 and 6 (in this sequen-ce !).

7. 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.

ARTOXKIT M - RESULTS SHEET

Name of operator : .....

Date of performance of test : .....

Toxicant tested : .....

Type of test : O range finding
O definitive

Salinity of the test medium : ..... ppt

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

8. SCORING OF THE RESULTS

Procedure

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

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

* *If the mortality in the controls exceeds 10 %, the bioassay is considered invalid and the test must be repeated !*

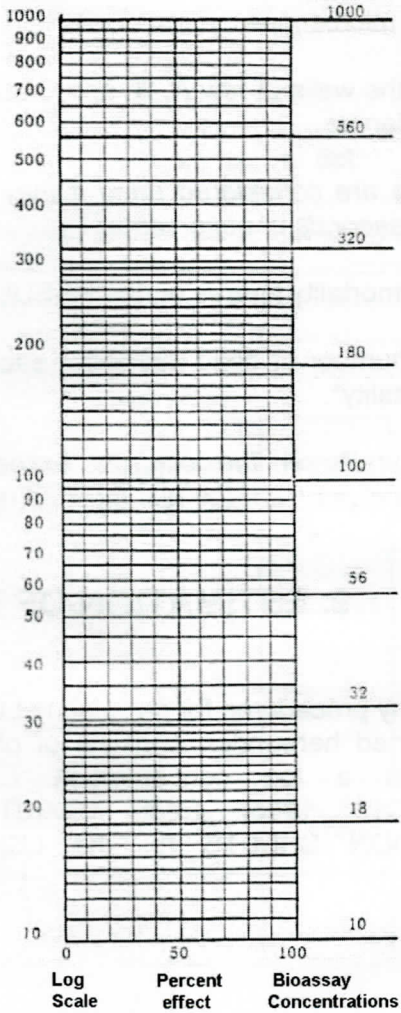
9. ESTIMATION OF THE LC_{50}

There are many procedures for calculating LC_{50}'s. A very simple procedure which is outlined hereunder, consists of plotting the calculated percent mortalities on a log concentration / % mortality sheet (US-EPA/600/485/013, 1985). Each TOXKIT contains 6 GRAPHICAL INTERPOLATION SHEETS for the LC_{50} estimation by "graphical interpolation".

TOXKIT

GRAPHICAL INTERPOLATION SHEET

- **Test species :**
- **Toxicant :**
- **Exposure time :**



O **LC50 :**

O **EC50 :**

Procedure

1. Indicate the concentrations or dilutions used in the dilution series on the Y-axis.
2. Plot the calculated percent mortality on the horizontal line at the height of each concentration or dilution.
3. Connect the plotted mortality points with a straight line.
4. Locate the two most adjacent points on the graph which are separated by the vertical 50 % mortality line and read the LC_{50} at the intersect of the two lines.

10. 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_2CrO_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 Standard Seawater.
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 to be made and used for the test is the following :

C1 (two test tubes) : 100 mg/l
C2 (one test tube) : 56 mg/l
C3 (one test tube) : 32 mg/l
C4 (one test tube) : 18 mg/l
C5 (one test tube) : 10 mg/l

3. Proceed to section 5. : **Filling the Test Plate**.

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

V100603

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 E14490-91.

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

THAMNOTOXKIT F : 24h mortality test, based on the anostracan crustacean *Thamnocephalus platyurus*. This assay is endorsed by several countries for acute toxicity testing.

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.

DAPHTOXKIT F pulex : 24h-48h mobility inhibition test, based on the cladoceran crustacean *Daphnia pulex*. This assay adheres to 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*.

RAPIDTOXKIT F : 30-60 min particle ingestion inhibition test based on the anostracan crustacean *Thamnocephalus platyurus*.

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

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.

MANUFACTURED BY :
MicroBioTests Inc.
Industriezone 'De Prijkels'
Venecoweg 19
9810 Nazareth
Belgium
www.microbiotests.be