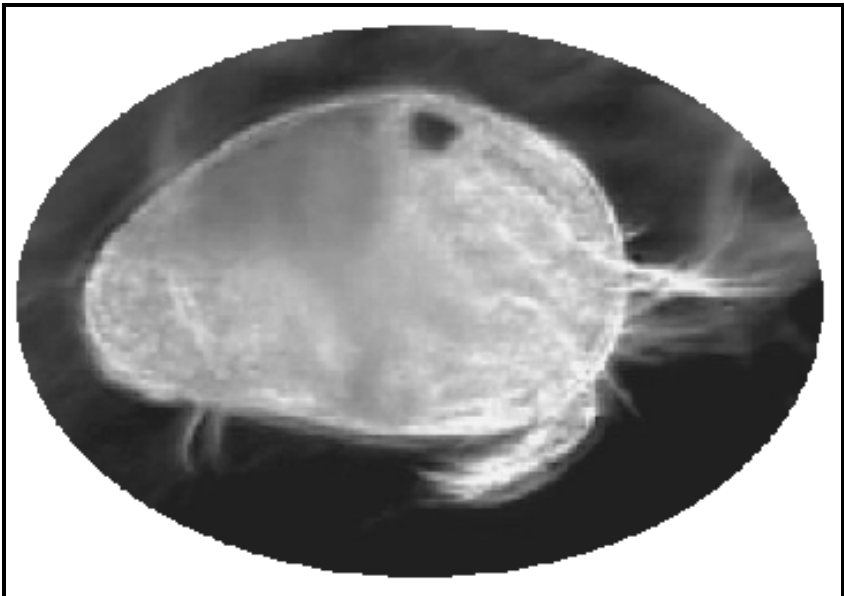


OSTRACODTOXKIT F

“Direct contact” Toxicity Test
for Freshwater Sediments



STANDARD OPERATIONAL
PROCEDURE

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INTRODUCTION TO THE OSTRACODTOXKIT F

A “direct contact” Toxicity Test for Freshwater Sediments

Origin :

The 6 days “direct contact” ostracod toxicity test for freshwater sediments was developed by Prof. Dr. G. Persoone and associates at the Laboratory for Environmental Toxicology and Aquatic Ecology of the Ghent University in Belgium.

Scope :

TOXKITS are microbiotests 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 environments and the **Ostracodtoxkit F** has been developed specifically to detect and quantify the toxicity of freshwater sediments and by extension also soils contaminated by inorganic or organic pollutants.

Principle :

A “direct sediment contact” bioassay is performed in multiwell test plates using neonates of the benthic ostracod crustacean ***Heterocypris incongruens*** hatched from cysts. After 6 days contact with the sediment (or the soil), the percentage mortality and the growth of the crustaceans are determined and compared to the results obtained in a (non-toxic) reference sediment.

N.B. : Since the growth of the ostracods is also determined after 6 days of exposure as a second effect criterion, this assay is in fact a “sub chronic” test.

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 “on demand” prior to performance of the toxicity tests.

Specific advantage of the Ostracodtoxkit F microbiotest

Sediment toxicity is often only carried out on the “pore water” fraction with the aid of test organisms representative for the water column. These assays

only reveal the toxic impact of the contaminants which are “dissolved” in the interstitial waters. Yet, for a full assessment of sediment toxicity “direct contact” tests with “benthic” organisms are also needed to show the impact of toxicants resulting from the contact of the test biota with the contaminated sediments and/or from the ingestion of (toxic) sediment particles. The Ostracodtoxkit F is **the very first “sediment contact” microbiotest with a crustacean test species** for the assessment of the “total” toxicity of sediments, hence including the toxic hazard of both dissolved and not-dissolved pollutants.

Features :

Each Ostracodtoxkit F contains all the disposable materials to perform 3 and even up to 5 bioassays (see Table and Figure). The only equipment needed is an incubator (25 °C (+/- 1 °C)), a dissection microscope (magnification 10-12x) and conventional laboratory glassware.

Sensitivity :

The sensitivity of the 6 days Ostracodtoxkit F with *Heterocypris incongruens* compares favourably with the 10 days contact sediment toxicity test with the amphipod crustacean *Hyalella azteca* and is substantially more sensitive than the sediment contact test with the midge larvae *Chironomus riparius*.

Precision :

Since all Ostracodtoxkits contain the same standard test (bio)materials and test media, the repeatability of this bioassay is very high.

The precision of the *Heterocypris incongruens* test has been determined on a reference chemical in an International Interlaboratory Comparison involving 26 laboratories from 14 countries. The outcome of this ringtest revealed the high intra- and interlaboratory reliability of this microbiotest.

Standardization :

Subsequent to the International Interlaboratory Comparison which had revealed its high degree of standardization, the ostracod microbiotest has been taken into consideration by the ISO (International Organization for Standardization) as a new toxicity test for freshwater sediments under the name “ISO 14371 - Water quality - Determination of freshwater sediment toxicity to *Heterocypris incongruens* (Crustacea, Ostracoda).

Cyst 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 :

Ostracod crustaceans are ecologically important members of the meiofauna of freshwater sediments. *Heterocypris incongruens* has a cosmopolitan distribution and can be found in diverse freshwater benthic habitats in all continents.

GENERAL INFORMATION ON THE TEST DESIGN

The test procedure consists in exposing freshly hatched ostracods for 6 days to a thin layer of sediment, covered by Standard Freshwater, in multiwell cups.

*Two different effects are determined at the end of the exposure period : **mortality** and **growth inhibition**.*

The results are compared to those obtained in a parallel test with a reference sediment.

Extensive investigations led to the selection of the following test materials and parameters :

- polystyrene multiwell plates with 2 x 3 cups (36 mm diameter)
- 1 ml sediment per test cup
- 2 ml Standard Freshwater per test cup
- 2 ml algal suspension per test cup (= food complement)
- 10 organisms per test cup
- a standard sand as the (control) reference sediment
- a sediment/water ratio of 1 ml/4 ml i.e. 1 : 4.

NUMBER OF DUPLICATES AND NUMBER OF TOXICITY TESTS

Since the volume of test sediment (1 ml) put in the replicate test wells is rather small, the replicate samples put in the test cups can - depending of the type of sediment - differ in their "percentual" composition with regard to the size of the particles. This can influence the amount of toxicants in each sample and hence the effects found in the replicate test wells.

In order to obtain a representative overall picture of the toxicity of any type of sediment the Standard Operational Procedure of the Ostracodtoxkit is based on 6 replicates.

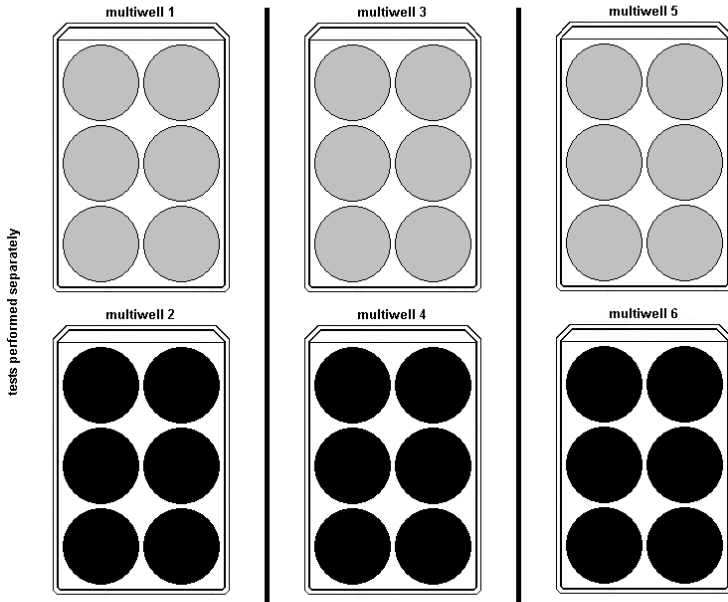
The table and figure hereunder indicate the number of toxicity tests that can be performed with the materials contained in the Ostracodtoxkit, depending on the time of performance of the bioassays (separate or concurrent).

**Number of tests that can be performed
with one Ostracodtoxkit**

	6 replicates for each sediment sample
Tests performed separately	3
All tests performed concurrently	5

The configuration of the wells to be filled with the reference sediment and the test sediment respectively is visualised in the next figure for the cases indicated above.

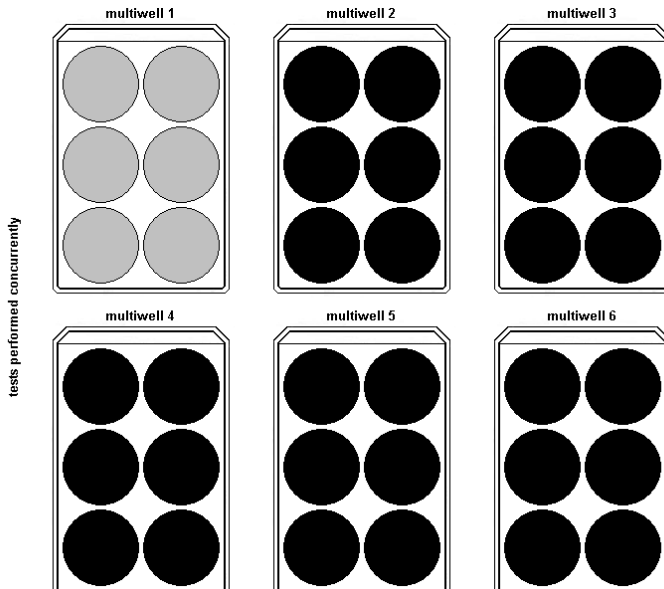
Overview of the 6 test plates with reference sediment and test sediment, for tests performed separately or concurrently, with 6 replicate test wells



reference sediment



test sediment



CONTENTS OF THE OSTRACODTOXKIT F

Ostracod cysts

Three 1 ml tubes containing reference cysts of the ostracod *Heterocypris incongruens*, which should be stored in a refrigerator at 5 °C (+/- 2 °C) until use to maintain maximum hatchability. If the hatching procedure is followed properly, the number of ostracods obtained from each cyst tube will exceed by far the number of test organisms needed for the toxicity test.

Multiwell test plates

Six polystyrene plates with 6 wells (36 mm diameter) which will serve as test containers.

Multiwell plate for “length” measurement

One “thin-bottom” multiwell with 48 small cups, to be used for the length measurement of the ostracods

Parafilm strips

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

Petri dishes

Six polystyrene Petri dishes (5 cm diameter) with cover. Three Petri dishes will be used for the hatching of the ostracod cysts; the others will serve for the transfer and the counting of the number of live ostracods at the end of the test.

Concentrated salt solutions

Five small glass bottles each containing a concentrated solution of one particular salt, to make up 1 liter Standard Freshwater (moderately hard synthetic water, US EPA formula) with deionized water. The Standard Freshwater is used for cyst hatching and preparation of the algal food suspension.

Composition :

Vial 1 : NaHCO_3 (96 mg - dissolved in 1 l. = 96 mg/l)

Vial 2 : $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ (60 mg - dissolved in 1 l. = 60 mg/l)

Vial 3 : $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ (60 mg - dissolved in 1 l. = 60 mg/l)

Vial 4 : $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (123 mg - dissolved in 1 l. = 123 mg/l)

Vial 5 : KCl (4 mg - dissolved in 1 l. = 4 mg/l)

Algal food

Three tubes containing small “algal beads” with green algae (*Scenedesmus spec.*) immobilized in an inert matrix. The tubes with the algal beads must be kept in the refrigerator at 5 °C (+/- 2 °C) in darkness until use.

Matrix dissolving medium

Three vials containing a special medium to dissolve the matrix in which the micro-algae are immobilized. The matrix dissolving medium must be stored in the refrigerator at 5 °C (+/- 2 °C)) prior to use.

Spirulina powder

Three tubes with Spirulina powder for pre-feeding the freshly hatched ostracods prior to the test.

Reference sediment

One plastic pot (with a 500 µl spoon) containing a standard sand to be used as reference (control) sediment in the tests.

Sediment pots

Five plastic pots (with a 500 µl spoon) to be filled with the test sediments.

Spatulas

6 plastic spatulas to strike of the sediment from the 500 µl spoon.

Micropipettes

Six micropipettes in glass, for the transfer of the ostracods.

“Large mouth” micropipettes

Six micropipettes for the transfer of the sediment suspension to the microsieve at the end of the test.

Lugol solution

One small glass bottle with Lugol fixative solution to fix the ostracods for easy length measurement at the end of the test.

Lugol micropipette

One polyethylene micropipette to be used for the transfer of the Lugol fixative.

Micrometer slip

One transparent slip (18 x 18 mm) with perpendicular micrometres of 1 cm length, with subdivisions of 50 µm, to measure the length of the ostracods. The precision of the total length and of the 50 µm subdivisions of the perpendicular micrometer is >95%.

Microsieve

One sieve of 100 µm mesh, to wash out the smallest sediment particles at the end of the test.

Standard Operational Procedure manual

A detailed brochure with all the instructions for the performance of the toxicity tests.

Bench protocol

An abbreviated version of the extended Standard Operational Procedure manual.

Results sheets

Sheets for scoring of the mortality of the test organisms and the length of the surviving ostracods at the end of the test.

Specification sheet

A sheet with specifications proper to each individual Ostracodtoxkit, such as the batch number of the cysts, the concentrated salt solutions, the algal food, etc.

All the non-biological materials provided in the Ostracodtoxkit F are made of inert, non-toxic products.

These materials are disposable and should only be used once.

TEST PROCEDURE

The test procedure described hereafter has been visualised in a "slide show" with photos and text showing step by step the manipulations involved in the performance of the assay.

The Ostracodtoxkit slide show is accessible on website www.microbiotests.be in the section "Technical Info - Slide Shows".

1. PREPARATION OF STANDARD FRESHWATER

The vials with concentrated salt solutions provided in the Ostracodtoxkit are used to prepare 1 liter Standard Freshwater. The synthetic freshwater selected for the Ostracodtoxkit tests is a "moderately hard synthetic water", US EPA formula. The Standard Freshwater is used as hatching medium for the cysts and as the medium for preparation of the algal food suspension.

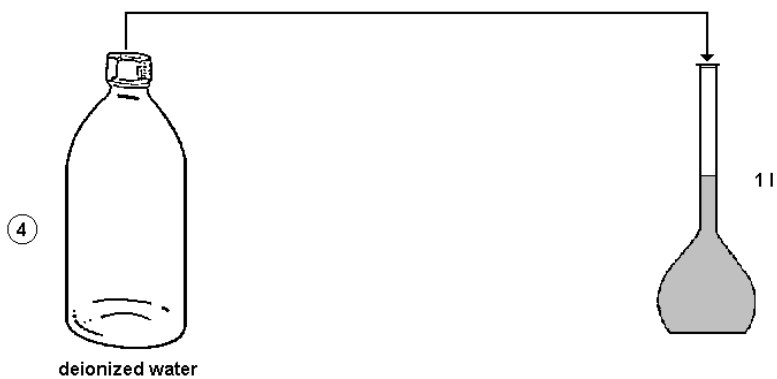
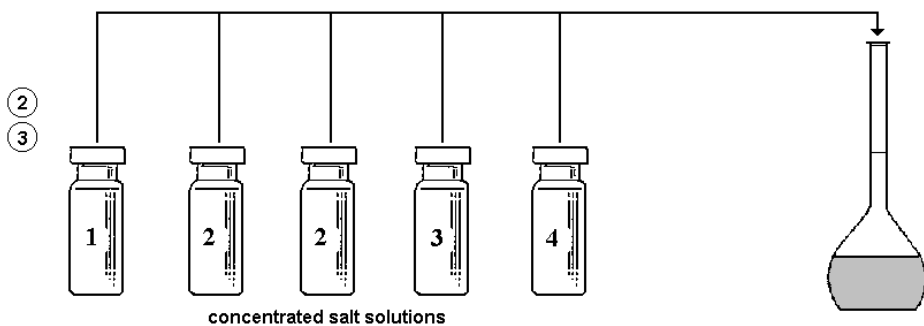
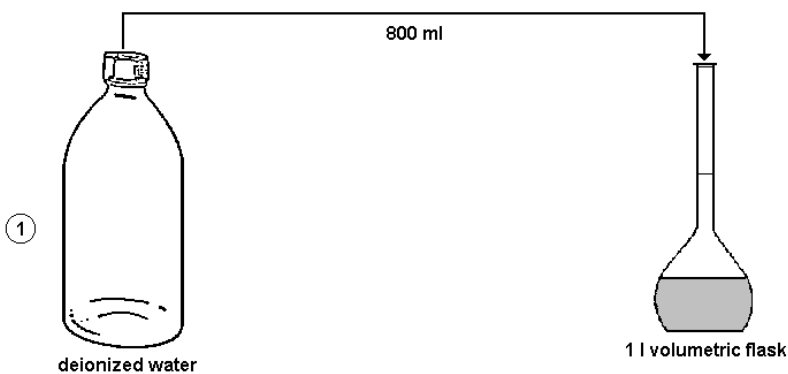
Procedure (see Figure)

1. Fill a 1 liter volumetric flask with approximately 800 ml deionized water.
2. Uncap the vial with concentrated salt solution labelled 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 ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$), one vial number 3 ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and one vial number 4 (KCl), respecting this sequence.
4. Add deionized water up to the 1 liter mark and shake to homogenize the medium.

2. STORAGE OF THE STANDARD FRESHWATER

The 1 liter Standard Freshwater suffices for all the bioassays which can be performed with one Ostracodtoxkit. If all the tests are not 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.

PREPARATION OF STANDARD FRESHWATER



3. PRE-AERATION OF THE STANDARD FRESHWATER

It is strongly advised to aerate the Standard Freshwater for at least 15 minutes prior to using it for the hatching of the cysts and for the preparation of the algal food suspension.

Pre-aeration can be performed very easily by air bubbling through a plastic tube connected to an aquarium air pump.

4. HATCHING OF THE OSTRACOD CYSTS

*The hatching of the ostracod cysts should be initiated **52 hours** prior to the start of the toxicity test.*

Procedure (see Figure)

1. Put 8 ml Standard Freshwater in one of the Petri dishes.
2. Open a vial with cysts and fill it with 1 ml. Standard Freshwater.
3. Close the vial with the stopper and shake it (this step will result in a better hatching success).
4. Empty the contents of the vial into the Petri dish.
5. To secure the complete transfer of the cysts, the vial should be rinsed twice with 1 ml Standard Freshwater.
6. Cover the hatching Petri dish with its lid and incubate **at 25 °C (+/- 1 °C) for 52 hours under continuous illumination** (light source of min. 3000-4000 lux).

5. PRE-FEEDING OF THE FRESHLY HATCHED OSTRACODS

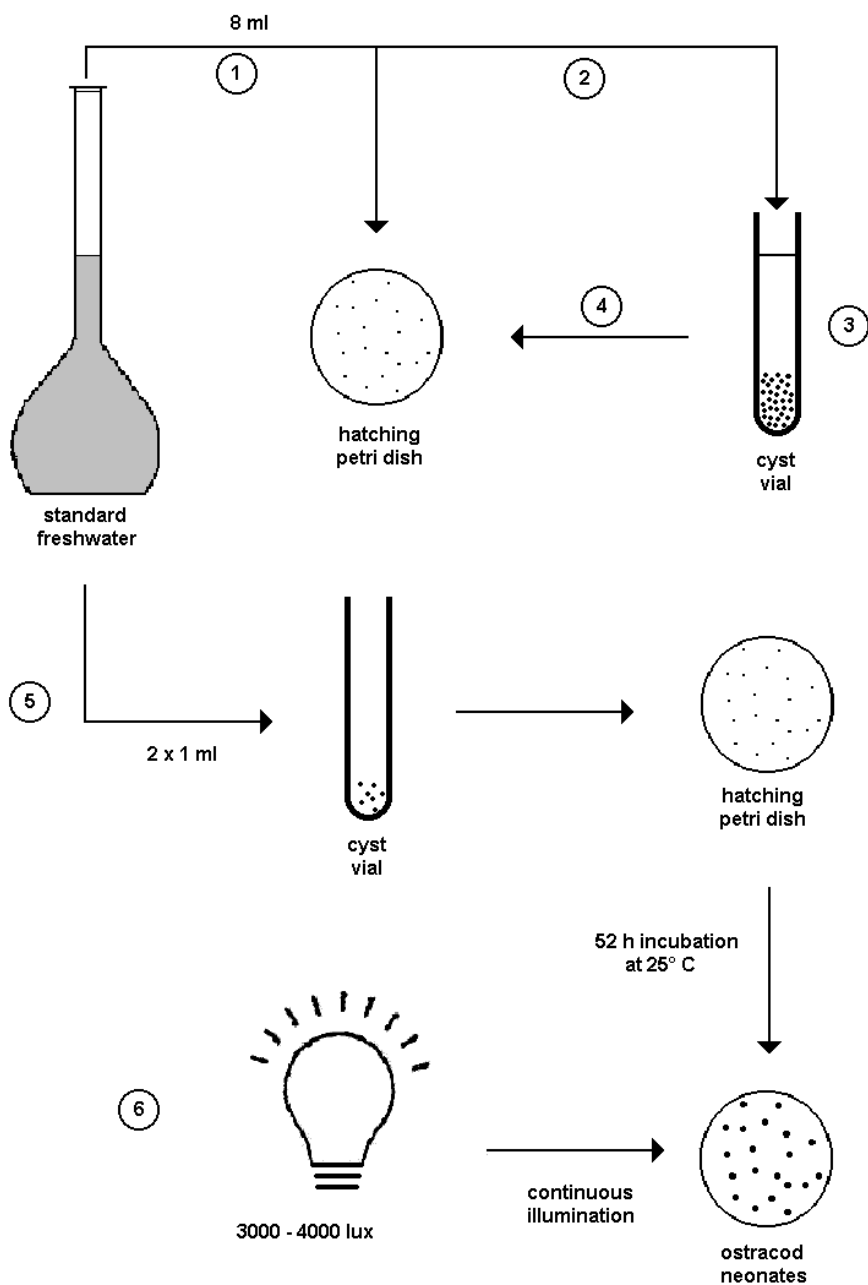
In order to provide the ostracods with food immediately after they have hatched, a short pre-feeding step is carried out after 48h incubation of the cysts.

Procedure

1. Take one of the tubes with Spirulina powder and fill it with Standard Freshwater.
2. Stopper the tube and shake the contents thoroughly to disperse the Spirulina particles.

N.B. Mixing on a Vortex is recommended to obtain a homogenous suspension.

HATCHING OF OSTRACOD CYSTS



3. Pour the Spirulina suspension in the hatching Petri dish 48 h after the start of the incubation of the cysts.
4. Put the hatching Petri dish back in the incubator and continue to incubate for 4 hours.

6. LENGTH MEASUREMENT OF FRESHLY HATCHED OSTRACODS

As soon as possible after hatching (i.e. shortly after the 52 h incubation) the length of the ostracods must be determined. The length measurements can be performed very easily with the aid of the special micrometer.

Procedure (see Figure)

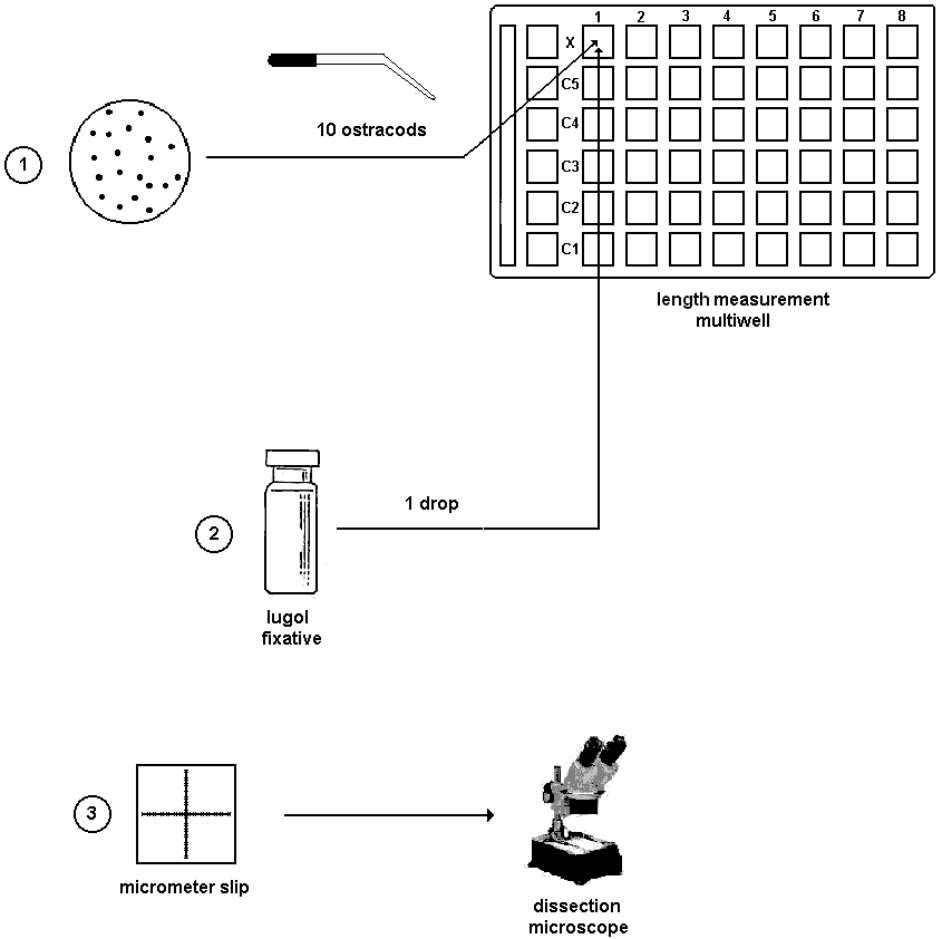
1. Pick up about 10 ostracods from the hatching Petri dish with a **glass micropipette** and transfer them into one cup of the (thin-bottom) multiwell for “length” measurement.

N.B. Ostracods are “thigmotactic”, i.e. they try to resist to water currents by clinging themselves to any possible solid surface. This can create a problem during the transfers of the ostracods because some of them “cling” to the inside walls of “plastic” micropipettes. For this reason special glass micropipettes have been included in the Ostracodto kits for the ostracod transfers. The glass micropipettes have very smooth walls to which the crustaceans cannot attach.

The glass micropipettes are very fragile and must be handled with much care !

2. Add one drop of Lugol fixative solution to the cup with the ostracods and wait for a few minutes till the organisms are completely immobile.
3. Take the micrometer slip and position it “exactly in the middle” of the glass plate on the bottom stage of the dissection microscope. Verify at high magnification that the intersection of the two (1 cm) perpendicular micrometer lines is exactly in the centre of the visual field.
4. Fix both sides of the micrometer slip to the glass plate with transparent tape.
5. Put the “multiwell for length measurement” on the stage of the dissection microscope, and position the cup containing the ostracods on top of the micrometer slip.

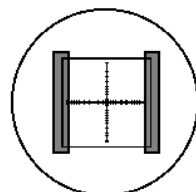
LENGTH MEASUREMENT OF OSTRACOD NEONATES



4

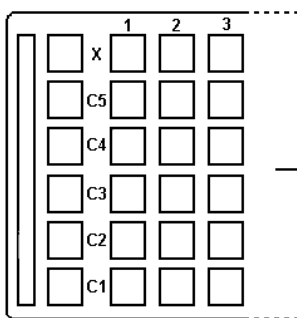


dissection microscope

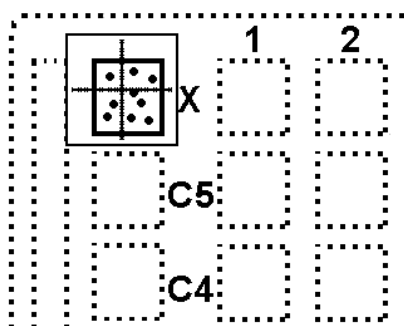


fixation of micrometer slip on the glass plate

5



6



6. Rotate the multiwell to put the ostracods one after the other with their length axis exactly on top of one of the 2 micrometer lines, and measure the length of the organisms.

N.B. The smallest subdivisions of the micrometer lines are 50 μm . Freshly hatched ostracods have a length of about 150-250 μm .

7. Score the length for each ostracod on Results Sheet B - Growth inhibition (in column DAY 0).

7. PREPARATION OF ALGAL FOOD SUSPENSION

Although most sediments contain a certain amount of organic matter, neither the composition of this organic material nor its quantity may be adequate nor sufficient to feed the ostracods during the 6 days exposure period.

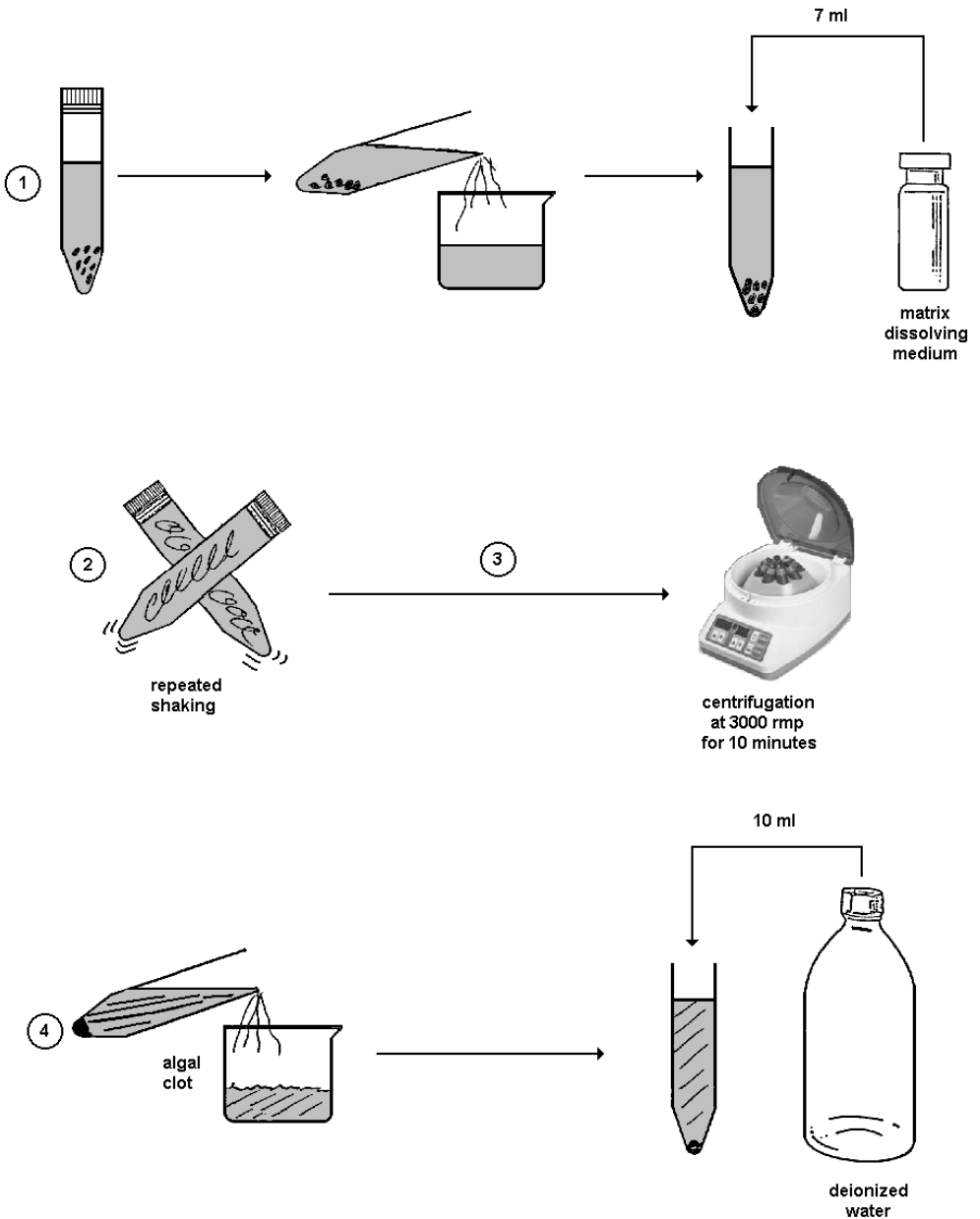
To preclude biased results due to starvation of the test organisms, a standard amount of algal cells is therefore added to the test wells as food complement, at the start of the assay.

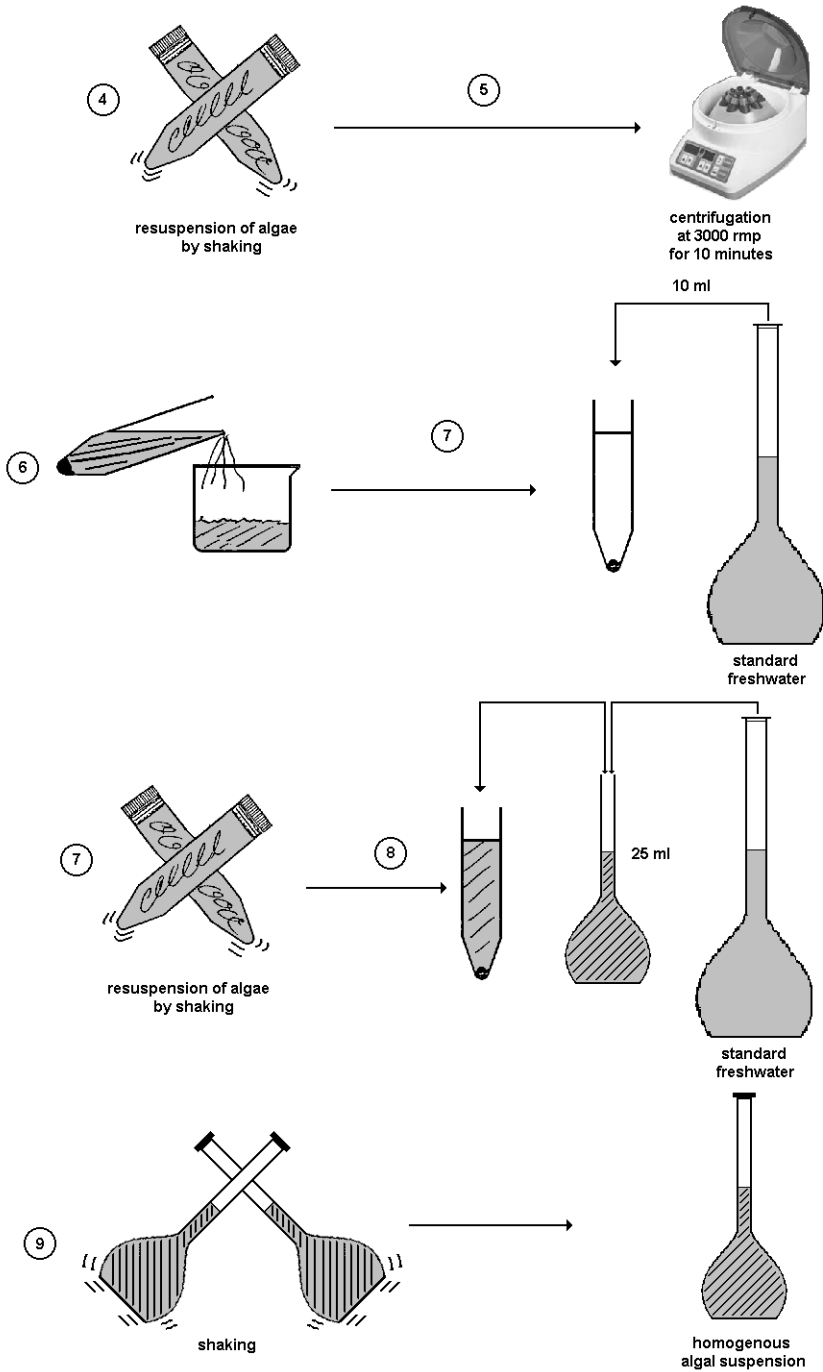
Procedure (see Figure)

1. Take one tube with algal beads, pour out the storage medium and add 7 ml matrix dissolving medium.
2. Cap the tube and shake the contents intermittently (shaking on a Vortex mixer is advised) until the matrix surrounding the algae has fully dissolved and the microalgae are totally set free. This will take approximately 10-15 min.
3. Centrifuge the tube at 3000 rpm for 10 minutes.
4. Pour out the supernatant, add 10 ml distilled water and re-suspend the algae by shaking (shaking on a Vortex mixer is again advised).
5. Centrifuge the tube again at 3000 rpm for 10 minutes.
6. Pour out the rinsing water.
7. Fill the tube with 10 ml Standard Freshwater, cap and shake to re-suspend the algae.
8. Transfer the algal suspension to a 25 ml volumetric flask and add Standard Freshwater to the mark.
9. Cap the flask and shake thoroughly to re-suspend the algae and obtain a homogenous algal suspension.

N.B. The algal concentration in the flask will be around 1.5×10^7 cells/ml.

PREPARATION OF ALGAL FOOD SUSPENSION





8. ADDITION OF STANDARD FRESHWATER, SEDIMENT, ALGAL FOOD AND OSTRACODS TO THE TEST PLATE

The following operations have to be performed with one test plate for the reference sediment, and one test plate for the test sediment.

Procedure (see Figure)

Addition of Standard Freshwater

1. Put 2 ml Standard Freshwater into each well of a multiwell test plate.

Addition of sediment

1. Take one of the “sediment pots” (which are provided with a blue spoon on the lid) and fill the spoon with sediment.
2. Strike of the excess sediment from the spoon with the plastic spatula and transfer the remaining sediment (500 μ l) into the well at the top left side of the multiwell plate. Use the tip of the spatula to empty the spoon completely.
3. Add a second spoon with 500 μ l sediment to the same well.
4. Repeat the former operations to add 1000 μ l sediment to the 5 other wells of the test plate.
5. Keeping the multiwell horizontally, gently shake it to distribute the sediment evenly over the bottom surface of the test cups.
6. Wait for a few minutes to allow the sediment to settle to the bottom of the wells.

Addition of algal food

1. Shake the flask with the algal suspension thoroughly and transfer very gently 2 ml algal suspension into each well of the test plate.

Addition of ostracods

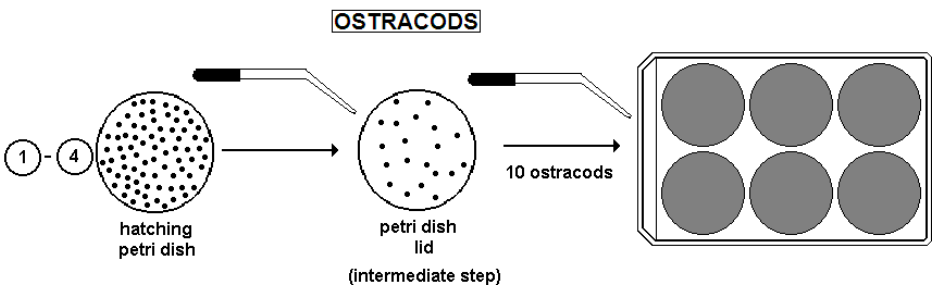
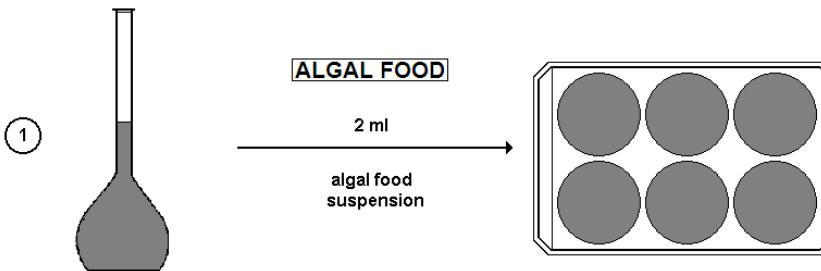
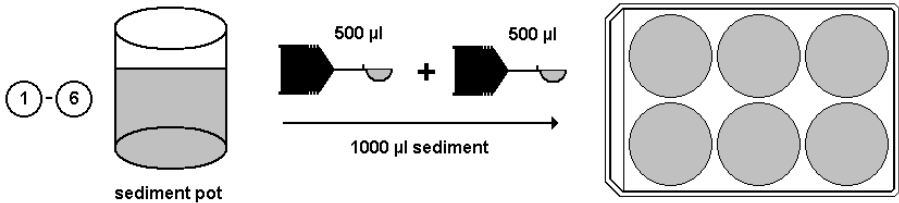
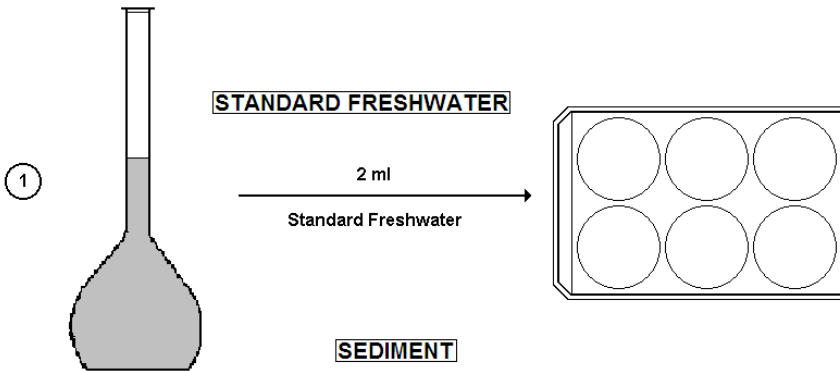
1. Take the lid of the hatching Petri dish and fill it with 5 ml Standard Freshwater.
2. Put the hatching Petri dish on the stage of the dissection microscope and transfer - with the glass micropipette - part of the ostracod neonates into the lid of the hatching Petri dish.

This operation shall be carried out at a magnification of 10-12 x.

N.B. This intermediate transfer step will facilitate the subsequent transfer of the ostracod neonates into the wells of the multiwell test plate.

3. Put the lid of the hatching Petri dish containing the ostracods on the stage of the dissection microscope and with the aid of the glass micropipette transfer **exactly 10 ostracods** into each well of the test plate.

ADDITION OF STANDARD FRESHWATER, SEDIMENT, ALGAL FOOD AND OSTRACODS TO THE TEST WELLS



9. INCUBATION

1. Cover the test plate with a piece of Parafilm and close it with the lid.
2. Put the multiwell plate in the incubator at 25 °C (+/- 1 °C), in darkness, **for 6 days**.

10. RECOVERY OF THE SURVIVING OSTRACODS

At the end of the 6 days exposure the ostracods have to be recovered from the multiwells to determine the percentage mortality and to make the length measurements of the surviving ostracods for calculation of the growth inhibition.

The ostracods can be recovered “directly” from the wells containing reference sediment since the organisms are easily visible under a dissection microscope.

This is, however, not the case for the cups with test sediment if the latter also contains very fine particles. For such sediments a “sieving procedure” has to be applied to recover the ostracods.

10.1. Recovery of the ostracods from the test plate with reference sediment

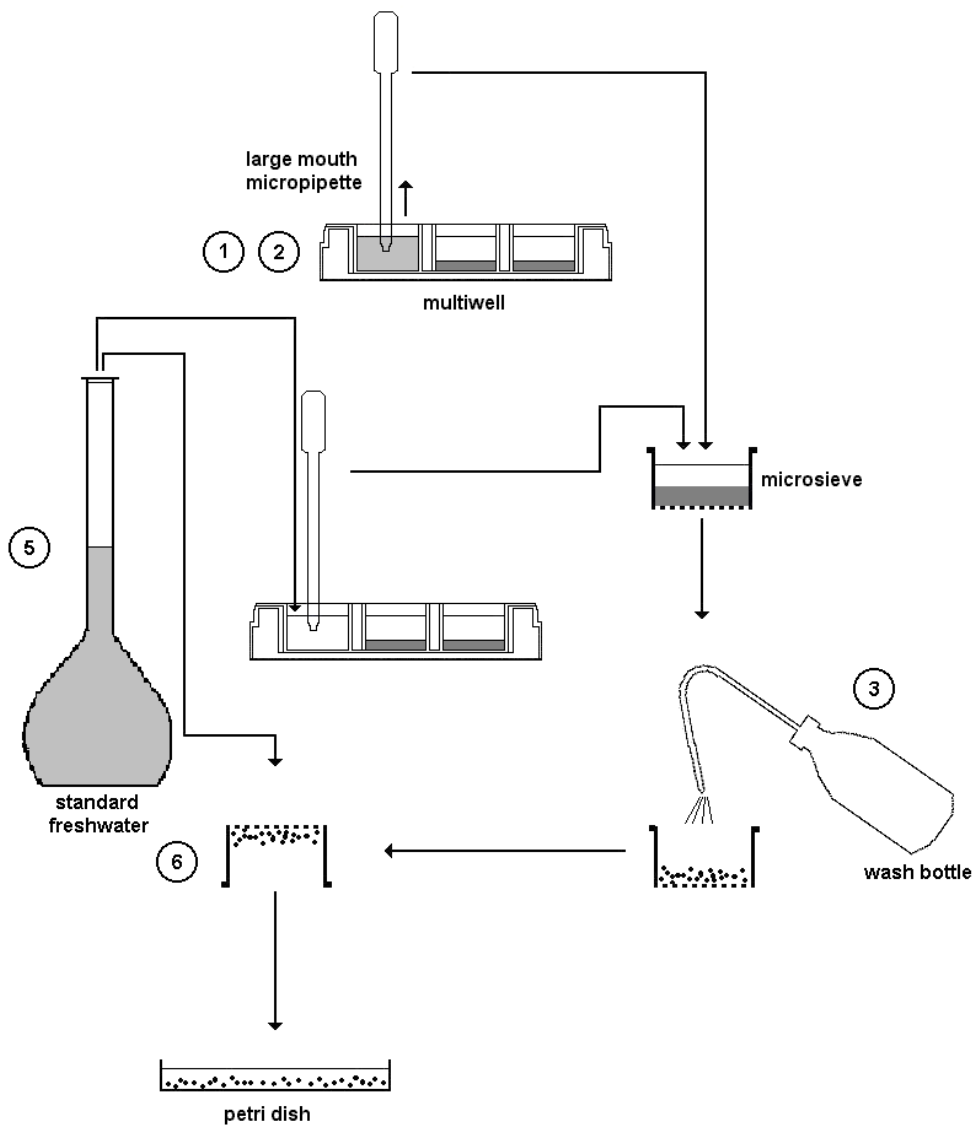
Procedure

1. Place the multiwell on the stage of the dissection microscope and switch on (preferably) both the top and the bottom illumination.
2. With the aid of the glass micropipette pick up all the living ostracods from the first well of the test plate and transfer them into the second cup of the top row of the “length measurement multiwell”.

N.B. It is extremely important to recover “all” the living ostracods from the well, for the correct evaluation of the percentage mortality.

3. Proceed the same way to recover the living ostracods from the 5 other wells of the test plate, and put these organisms into the next 5 cups of the length measurement multiwell.
4. Add one drop of Lugol fixative to each cup of the length measurement multiwell containing live ostracods, to immobilize them.
5. Count the number of ostracods into each cup and score the numbers on the Results Sheet A - Mortality.

RECOVERY OF THE OSTRACODS FROM THE WELLS WITH TEST SEDIMENT



10.2. Recovery of the ostracods from the test plate with test sediment

Procedure (see Figure)

1. Take a “large mouth” micropipette and very gently mix the sediment in the first test cup with the water layer on top of the sediment.
2. Suck up part of the sediment suspension and transfer it into the microsieve.
3. Gently rinse the contents of the microsieve with tap water (put in a wash bottle) till all the fine sediment particles are washed out.
4. Proceed further with the stepwise transfer of the sediment suspension from the well to the microsieve, followed by rinsing with tap water, till most of the sediment has been transferred from the well to the microsieve.
5. Add a few ml Standard Freshwater to the cup, mix it with the remaining sediment and transfer the latter to the microsieve for rinsing. Repeat this operation several times if necessary to make sure that all the sediment and all the ostracods have been transferred.
6. Turn the microsieve upside down on top of a small Petri dish and rinse the contents of the microsieve back into the Petri dish with Standard Freshwater. Make sure that the full contents of the microsieve are transferred into the Petri dish.

N.B. The Petri dish will contain the remaining (large) sediment particles + the living and dead ostracods, which can now easily be seen under a dissection microscope.

7. Put the Petri dish on the stage of the dissection microscope, and with the aid of the glass micropipette transfer all the living ostracods into the second cup of the third row of the length measurements multiwell.
8. Add one drop of Lugol fixative to each cup of the length measurement multiwell containing the living ostracods, to immobilize them.
9. Count the number of ostracods in the cup and score the number on the Results Sheet A - Mortality.
10. Perform the same rinsing and transfer operations for the 5 other cups with test sediment.

11. DETERMINATION OF THE PERCENTAGE MORTALITY

1. With the data filled out in Results sheet A - Mortality, calculate the total number of surviving ostracods in the 6 test cups (= A).
2. Subtract this number (A) from 60 (= the 6 x 10 ostracods inoculated at the start of the assay), to obtain the total number of dead ostracods (B = 60 - A).
3. Determine the percentage mortality in the reference sediment and in the test sediment with the formula :

$$\% \text{ mortality} = B/60 * 100$$

12. DETERMINATION OF THE PERCENTAGE GROWTH INHIBITION

Growth inhibition is the second effect criterion of the Ostracodtoxkit microbiotest which allows to evaluate the “sub lethal” toxicity of sediments.. The growth inhibition is determined by comparing the length of the surviving ostracods in the test sediment with that in the reference sediment at the end of the test.

Determination of the “sub lethal” impact of sediment toxicants clearly only makes sense for test sediments which don’t have a high “lethal” effect, i.e. induce a high mortality of the ostracods.

The growth inhibition should therefore only be determined on test sediments for which less than 30% mortality has been found.

Important remark : *Even if the growth inhibition effect must not be assessed, the length of the ostracods must nevertheless be measured in the cups with the control (reference) sediment at the end of the exposure period. These length measurements of the organisms in the control wells are indeed needed for the calculation of the “growth increment” of the ostracods, which is a validity criterion for the assay (see Section 12).*

Procedure

1. Put and fix the micrometer coverslip in the middle of the glass plate of the dissection microscope.

OSTRACODTOXKIT F

RESULTS SHEET A - MORTALITY

Name of operator :

Date of performance of test :

Test sediment :

Replicate	Control sediment	Test sediment
1		
2		
3		
4		
5		
6		
Total number of living ostracods (A)		
Total number of dead ostracods (B = 60 – A)		
% mortality (B/60*100)		

2. Put the multiwell for length measurements on the bottom stage of the dissection microscope and centre the cup containing the ostracods transferred from the first well of the test plate with reference sediment.
3. Rotate the multiwell to put the ostracods one after the other with their length axis exactly on top of one of the 2 micrometer lines, and measure the length of the organisms.
4. Score the length of each measured ostracod on Results Sheet B - Growth inhibition.
5. Proceed the same for the other 5 cups containing the surviving ostracods from the multiwell with reference sediment, and for the 6 cups with ostracods from the multiwell with test sediment.

Treatment of the data on Results Sheet B - Growth inhibition

1. Calculate the mean length of the ostracods at the start of the test (= A) and score this figure in the corresponding box of the Results Sheet B - Growth inhibition.
2. Calculate and score the mean length of the surviving ostracods at the end of the test for the 6 wells with reference sediment and the 6 wells with test sediment.
3. Calculate and score “the overall mean length” of the ostracods at the end of the test in the reference sediment and in the test sediment (= B = the mean of the 6 replicates).
4. Calculate and score the mean growth of the ostracods at the end of the test in the reference sediment and in the test sediment (= B-A).
5. Calculate and score the percentage growth inhibition in the test sediment with the formula :
% growth inhibition : $100 - [(growth\ in\ test\ sed./growth\ in\ refer.\ sed.) * 100]$

An Excel version of Results Sheet A - Mortality and B - Growth inhibition can be obtained from the company MicroBioTests Inc.

After typing in the number of living ostracods in the boxes of Results Sheet A, and (if appropriate) the length of the surviving ostracods in the boxes of Results Sheet B, the Excel program will automatically calculate the percentage mortality and the percentage growth inhibition.

OSTRACODTOXKIT F

RESULTS SHEET B – GROWTH INHIBITION

To be filled with the length data of the ostracods (in µm)

Organism	Day 0						Day 6 (end of test)					
	Control sediment						Test sediment					
	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Rep. 6	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Rep. 6
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
Mean length at the start (A)												
Mean length at the end for each replicate												
Mean length at the end for the 6 replicates (B)												
Mean growth for the 6 replicates (B-A)												
% growth inhibition (C)												

* % growth inhibition = $100 - [(\text{Growth in toxicant} / \text{Growth in control}) \times 100]$

13. VALIDITY OF THE TEST

For the toxicity test to be acceptable, the following two criteria must be fulfilled :

- a) the percentage mortality of the ostracods in the reference sediment should not be higher than 20%.
- b) the mean length of the ostracods in the reference sediment should have increased by a factor 1.5 to the mean length of the organisms at the start of the test.

14. REFERENCE TEST

In order to check the correct execution of the test procedure and the sensitivity of the test organisms, 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 **copper sulphate (CuSO₄.5H₂O)** in the range 1-10 mg/l.*

The toxicant is spiked in the reference sediment which is used as control sediment in the Ostracodtoxkit assay.

*The solutions of the reference toxicant have to be prepared in Standard Freshwater "**in double strength**" (= two times the concentrations to which the test organisms will be exposed during the test).*

*N.B. The rationale for this is that after addition of the 2 ml toxicant solution to the test wells, a volume of 2 ml algal food suspension is also added to each well, which decreases the toxicant concentration in the wells by half. Since the reference test involves 5 toxicant dilutions+ the negative control, the assay is performed **in 3 replicates** instead of 6.*

N.B. The percentage mortality in the highest test concentration (10 mg/l) should (normally) be above 50%, and as indicated in Section 11 there is hence no need in the quality control test described hereunder to also determine "growth inhibition".

Procedure

Length measurement of the ostracods at the start of the test

Similarly to the procedure for test sediments, the length of 10 ostracods has to be measured at the start of the test, as prescribed in Section 6.

The data of these measurements shall be scored in the column “Day 0” on the specific “Results Sheet - Length of organisms” of the Ostracodtoxkit F - Reference test.

Preparation of the toxicant dilutions

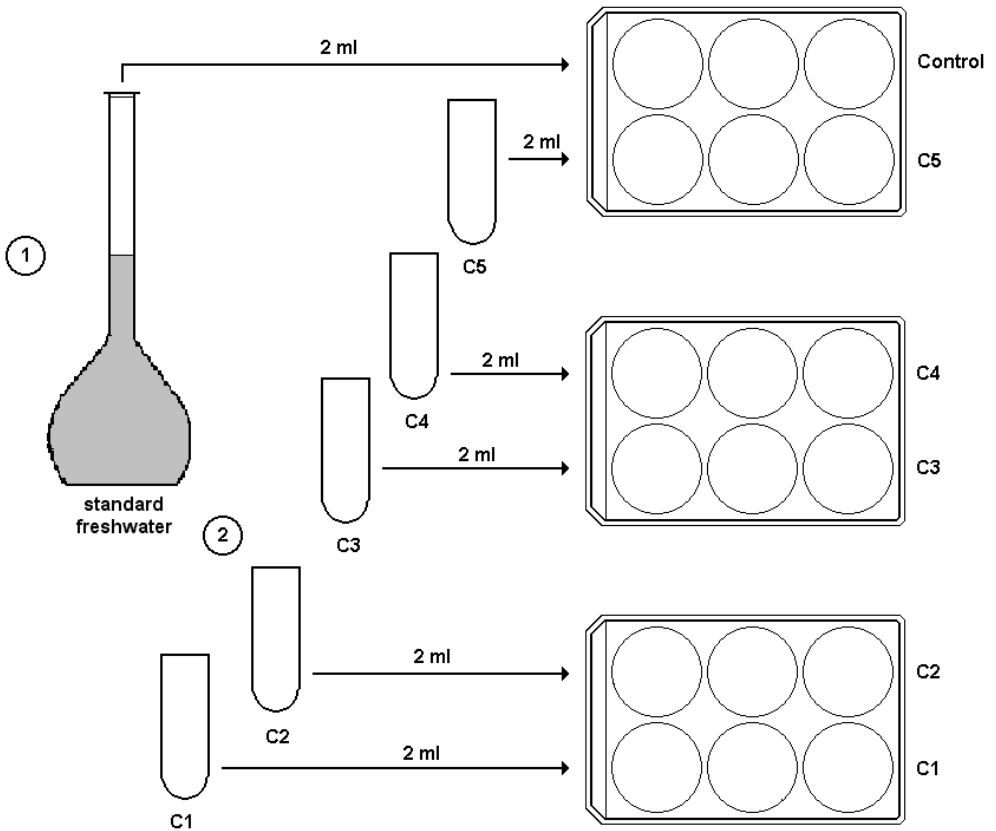
1. Take two 100 ml volumetric flasks and label them as “stock solution” and “sub-stock solution”.
2. Weigh 100 mg copper sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) on an analytical balance.
2. Put the weighted chemical into the “Stock solution” flask and fill this flask to the mark with deionized water.
3. Shake the flask to homogenize the stock solution (= 1000 mg/l $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$).
4. Transfer 2 ml stock solution into the “sub-stock solution” flask and fill this flask to the mark with Standard Freshwater.
5. Shake the flask to homogenize the sub-stock solution (= 20 mg/l $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$).
6. Take 5 test tubes of 15 ml content and label them C1-C2-C3-C4-C5.
7. Put the following volumes of sub-stock solution and Standard Freshwater into the test tubes, and shake the tubes to homogenize the contents :

- **C1** : **20.0 mg/l** = 10.0 ml sub-stock solution
- **C2** : **11.2 mg/l** = 5.6 ml sub-stock solution + 4.4 ml Standard Freshwater
- **C3** : **6.4 mg/l** = 3.2 ml sub-stock solution + 6.8 ml Standard Freshwater
- **C4** : **3.6 mg/l** = 1.8 ml sub-stock solution + 8.2 ml Standard Freshwater
- **C5** : **2.0 mg/l** = 1.0 ml sub-stock solution + 9.0 ml Standard Freshwater

Addition of the toxicant solutions to the test wells (see figure)

1. Put 2 ml Standard Freshwater in the first 3 wells of a test plate, and put 2 ml of the lowest toxicant concentration (C5) in the 3 other wells of this test plate.
2. Proceed similarly with the addition of 2 ml of the other toxicant concentrations to the wells of the second and the third test plate.
3. Add 2 x 500 μl reference sediment, 2 ml algal food suspension and 10 ostracods to each well of the 3 test plates as prescribed in section 8.

Addition of toxicant dilutions, reference sediment, algal food and ostracods to the the test wells



- 3 Addition of 1000 μ l reference sediment, 2 ml algal food suspension and 10 ostracods to each test well (as shown in the Figure in Section 8)

Incubation and recovery of the surviving ostracods

Proceed further as indicated in Sections 9 and 10.1 of the Ostracodtoxkit test procedure.

Scoring of the results and data treatment

1. Score the number of recovered living ostracods from each test well on the “Results Sheet - Mortality” of the Ostracodtoxkit F - Reference test.
2. Calculate the total number of surviving ostracods in the controls and in the 5 toxicant dilutions (= A), and the number of dead ostracods (B = 30-A)
3. Determine the percentage mortality with the formula :
$$\% \text{ mortality} = B/30 \cdot 100$$
4. Calculate the 6d LC₅₀ with the aid of a toxicity data treatment programme.

Determination of the growth of the ostracods in the control wells

1. Transfer the ostracods from the 3 control wells into 3 cups of the “length measurement multiwell” and add one drop of Lugol fixative to immobilize them.
2. Measure the length of the ostracods in each cup and score the data on the Results Sheet - Length or organisms of the Ostracodtoxkit F - Reference test.
3. Calculate the mean length of the ostracods for each replicate and the mean length for the 3 replicates (= B).
4. Determine the mean growth of the ostracods in the control wells by subtracting A from B.

Validity of the reference test

The same two validity criteria indicated in Section 13 with regard to the percentage mortality and the growth of the ostracods in the controls must also be fulfilled for a reference test to be acceptable.

Furthermore, if the reference test has been performed with copper sulphate (CuSO₄·5H₂O), the 6d LC₅₀ shall be compared with the data of the “International Interlaboratory Comparison on the *Heterocypris incongruens* microbiotest”.

According to this ringtest, the mean 6d LC₅₀ for CuSO₄·5H₂O was 5.79 mg/l, and the results of a reference test with this compound should be in the range **2.21 - 9.37 mg/l**.

OSTRACODTOXKIT F - REFERENCE TEST

RESULTS SHEET - MORTALITY

Name of operator :

Date of performance of test :

Toxicant tested : $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

Dilution series tested : concentration 1 : 10 mg/l
concentration 2 : 5.6 mg/l
concentration 3 : 3.2 mg/l
concentration 4 : 1.8 mg/l
concentration 5 : 1 mg/l

Replicate	Control	C5	C4	C3	C2	C1
1						
2						
3						
Total number of living ostracods (A)						
Total number of dead ostracods (B = 30 - A)						
% mortality (B/30*100)						

OSTRACODTOXKIT F – REFERENCE TEST

RESULTS SHEET – LENGTH OF ORGANISMS

Name of operator :

Date of performance of test :

Length of ostracods (in μm) at the start of the test and at the end in the control wells

	Day 0	Day 6		
Organism		Rep.1	Rep.2	Rep.3
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
Mean length at the start (A)		X	X	X
Mean length at the end for each replicate	X			
Mean length at the end for the 3 replicates (B)	X			
Mean growth for the 3 replicates (B-A)	X			

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.

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