RAPIDTOXKIT

Microbiotest for rapid detection

of water contamination

STANDARD OPERATIONAL

PROCEDURE

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INTRODUCTION TO THE RAPIDTOXKIT MICROBIOTEST

Origin :
The development of the **Rapidtoxkit** microbiotest originated because of the demand for "rapid assays" to detect the presence of toxicants in water supplies. The Rapidtoxkit is a "short" version of the 24h Thamnotoxkit microbiotest and measures a (sub-lethal) toxic stress **after 15 minutes, 30 minutes or one hour exposure** of the crustacean larvae to water samples suspected to contain toxicants.

Biological background of the assay :
Zoo-planktonic biota such as the crustaceans take up their food (bacteria, micro-algae, detritus, etc.) by filtration from the water column.
When the organisms are "stressed", either by an environmental factor or by the presence of toxicants, the rate of filtration decreases and even stops in cases of very severe stress.
This biological phenomenon can be used for rapid toxicity tests by exposing the organisms for a short period of time to suspected water samples and observing whether or not there is a significant difference in the uptake of particulate material in comparison with the controls.

Scope : Like all other Toxkit microbiotests, the Rapidtoxkit contains all the materials (including the test organisms) to perform rapid, sensitive and reproducible toxicity tests at low cost. Whereas the other Toxkits are better suited for routine toxicity testing of chemicals and wastes released in aquatic or terrestrial environments, **the Rapidtoxkit has been developed specifically for rapid detection of water contamination**.
This test is particularly apt for **water contamination emergencies** resulting from accidental or deliberate introduction of toxicants in water supplies.
The Rapidtoxkit test can also be applied to "leachates" of solids suspected to contain toxicants.

Advantages of Toxkit microbiotests :
The major advantage and asset of all Toxkit microbiotests, in comparison with 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 tests.

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

Principle :
The Rapidtoxkit assay is performed in disposable test tubes using larval crustaceans (*Thamnocephalus platyurus*) hatched from cysts. The test organisms are exposed for a very short period of time (from 15 minutes up to 1 hour) to the suspected water samples after which time a suspension of red microspheres is added. In the control (a standard freshwater) the organisms ingest the microspheres which colour the digestive tract deep red. Stressed (intoxicated) organisms do not take up the coloured particles anymore or ingest them at a much lower rate. The presence (and the intensity of uptake) or the absence of the coloured particles in the digestive tract of the larval crustaceans is observed after 15 minutes under a stereomicroscope at low magnification.

Features :
Each Rapidtoxkit contains all the (disposable) materials to perform 3 independent series of tests. Each series allows for the analysis of up to 7 water samples if the assays are performed in 2 replicates, or up to 15 water samples if tested without replication. The only equipment needed is an incubator or a temperature controlled room at 25°C for the hatching of the cysts and a stereomicroscope to observe the uptake of the coloured microspheres.

Shelf life :
The tubes with cysts must be stored in darkness at 4°C to keep their viability. The hatching success of the cysts kept in the aforementioned conditions is guaranteed for several months as indicated on the expiry label on each kit.

Detection threshold :
Studies on a variety of inorganic and organic chemicals have shown that the larval crustaceans used in the Rapidtoxkit microbiotest detect the presence of toxicants in water samples at a level substantially below the "Human Lethal Doses" indicated in scientific literature.

Sensitivity :
The Rapidtoxkit microbiotest has been shown to be as sensitive for many compounds as the rapid tests based on bacteria. The Rapidtoxkit was also found to be substantially more sensitive than rapid bacterial assays for metals and in particular for biological toxins and constitutes as such an excellent complement to the latter to cover all categories of toxic compounds and avoid false negatives.

CONTENTS OF THE RAPIDTOXKIT MICROBIOTEST

Vials with cysts
Three 1 ml plastic tubes containing cysts of the anostracan crustacean *Thamnocephalus platyurus*, to be stored in a refrigerator at 4°C until use.

Flask with standard freshwater
One polyethylene flask containing 120 ml standard freshwater (moderately hard EPA medium).

Hatching vessel
Three 25 ml polystyrene cell culture flasks with stoppers, for the hatching of the cysts.

Sub-sampling tubes
Six transparent 10 ml conical base polystyrene tubes provided with plugs, for transfer of 0.5 m larval suspension into the test tubes.

Test tubes
Forty eight transparent 10 ml conical base polystyrene tubes provided with plugs, for the control water and the test waters.

Test tube holders
Six cardboard holders for one sub-sampling tube and 8 test tubes in two rows.

Vial with red beads
A 10 ml glass bottle with a suspension of red coated polystyrene microspheres (5 µm diameter) for ingestion by the test organisms. The red beads must be stored in the refrigerator (at 4°C) until use.

Vial with fixative
A 15 ml glass dropper bottle with a fixative agent (Lugol solution) to fix the test organisms at the end of the exposure period prior to their observation under the stereomicroscope.

Observation plates
Six rectangular (57 x 95 mm) transparent polystyrene plates, with 8 shallow wells (15 mm diameter) for microscopic observation of the test organisms after fixation.

Blue plastic rectangular sheet and Counting grid
A blue plastic rectangular sheet with a transparent counting grid on top, to be placed on the bottom stage of the stereomicroscope for observation of the test organisms.

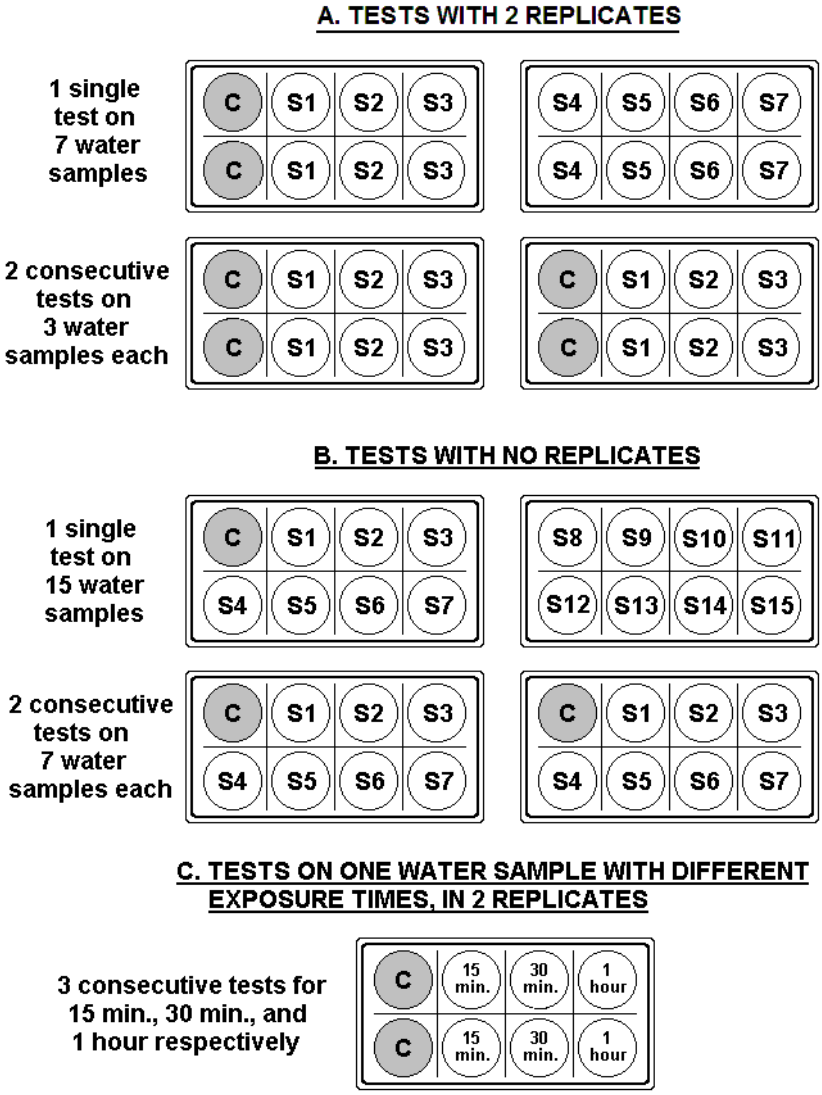
Standard Operational Procedure manual
A detailed brochure with all the instructions for the performance of the rapid tests.

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

Results sheets
Six sheets for the scoring of the results and the calculation of the percentage mean inhibition of the particle uptake.

Specification sheet
A sheet indicating the batch number and shelf-life of the cysts and the batch number of the standard freshwater, the fixative and the red beads suspension.

Figure 1 : **Overview of the number of water samples which can be analysed with the test organisms hatched from 1 tube of cysts**



NUMBER OF WATER SAMPLES

The materials included in the Rapidtoxkit allow to perform 3 independent series of tests, each using one tube of cysts. Each series can be performed on different numbers of water samples, depending on the combinations and the number of replicates.
Figure 1 gives an overview of the different possibilities in this regard.

Option A is for tests performed with 2 replicates, and shows that up to 7 water samples can be analysed concurrently, or two consecutive tests (to be performed the same day) on 3 water samples each.

Option B is for tests performed without replication. In this case up to 15 water samples can be analysed concurrently or two consecutive tests can be made on 7 water samples.

Option C shows that, in case of real "urgency" one can make tests in 2 replicates with a first analysis of the test organisms after only 15 minutes exposure to the suspected water sample, and perform a second and third analysis after 30 minutes and 1 hour exposure respectively.

For reasons of simplicity, the procedure given below describes one series of tests with 2 replicates, on 3 water samples and with an exposure time of 1 hour.

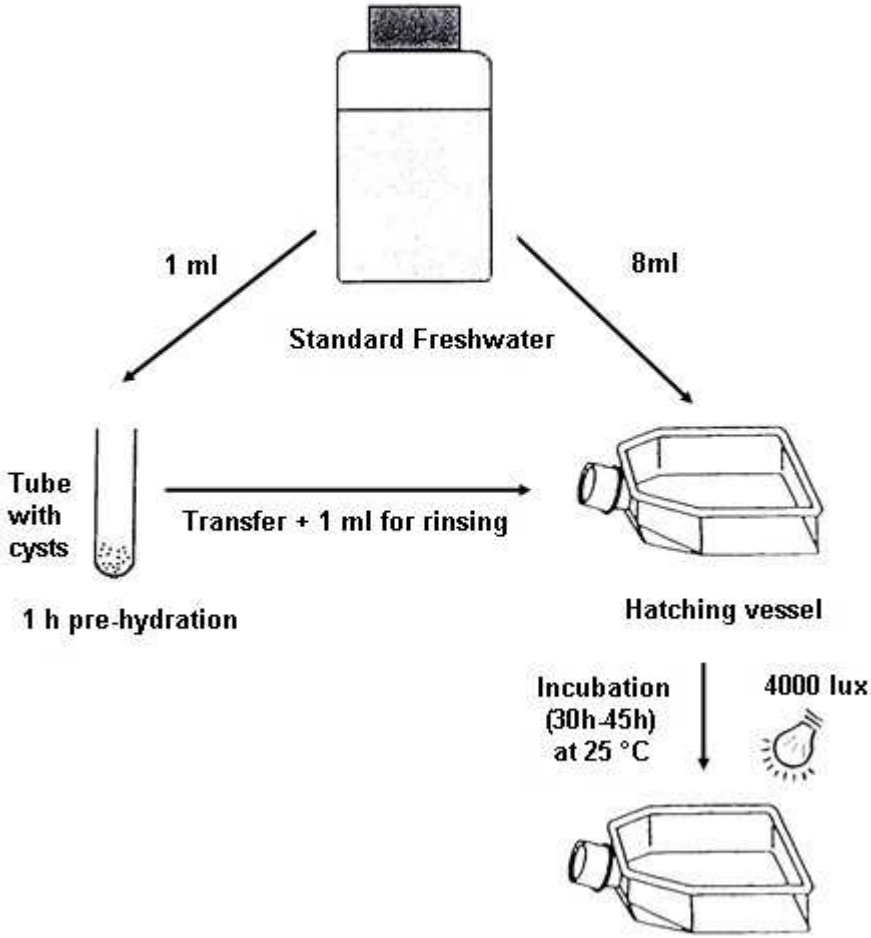
1. HATCHING OF THE CYSTS (see Figure 2)

1.1 Pre-hydration of the cysts

In order to avoid floating during the hatching period and ensure their hydration, the cysts have to be "pre-hydrated" for a short time prior to their incubation leading to the hatching of the larvae.

1. Open a tube with cysts and add 1 ml standard freshwater.
2. Close the tube with the stopper and shake it thoroughly to suspend all the cysts.
3. Let the cysts pre-hydrate for one hour.

Figure 2. HATCHING OF THE CYSTS



1.2. Transfer of the pre-hydrated cysts into the hatching flask

1. Empty the contents of the tube with pre-hydrated cysts into a hatching vessel.
2. Add one ml standard freshwater to the tube and transfer the contents into the hatching tube to make sure that all the cysts are carried over.
3. Add 8 ml standard freshwater to the hatching vessel.
4. Close the hatching vessel, shake it gently in all directions (to avoid cysts adhering to the walls) and place the hatching vessel flat in the incubator.
5. Incubate **for 30h (minimum) to 45h (maximum) at 25°C, under continuous illumination** (light source of min. 3000-4000 lux).

2. FILLING OF THE TEST TUBES WITH CONTROL WATER AND 3 TEST WATERS (see Figure 3)

1. Fill the first 2 tubes adjacent to the sub-sampling tube to the horizontal mark with standard water.
2. Fill the next 2 tubes the same way with test water 1 and repeat this operation for the second and third test waters.

3. TRANSFER OF ORGANISMS TO THE SUB-SAMPLING TUBE AND SAMPLE TUBES (see Figure 4)

1. Take the vessel with the hatched larvae, shake it gently to distribute the organisms evenly and pour the contents in the “sub-sampling tube” located on the left side of the cardboard holder.
2. Set the 1 ml micropipette to 0.5 ml.
3. Lower the tip of the micropipette in the sub-sampling tube till the aperture is in the middle of the water column.
4. Distribute the larvae evenly in the water column by repeated aspiration and dispensation of water + larvae from the micropipette.
5. Take up 0.5 ml larval suspension and transfer it into the first test tube.
6. Repeat steps 3 and 4 for all the test tubes in the holder.
7. Stopper all the tubes.
8. Place the holder with the tubes in an incubator set at 25°C, **for one hour** in darkness.

Figure 3. FILLING OF THE TEST TUBES WITH CONTROL WATER AND TEST WATERS

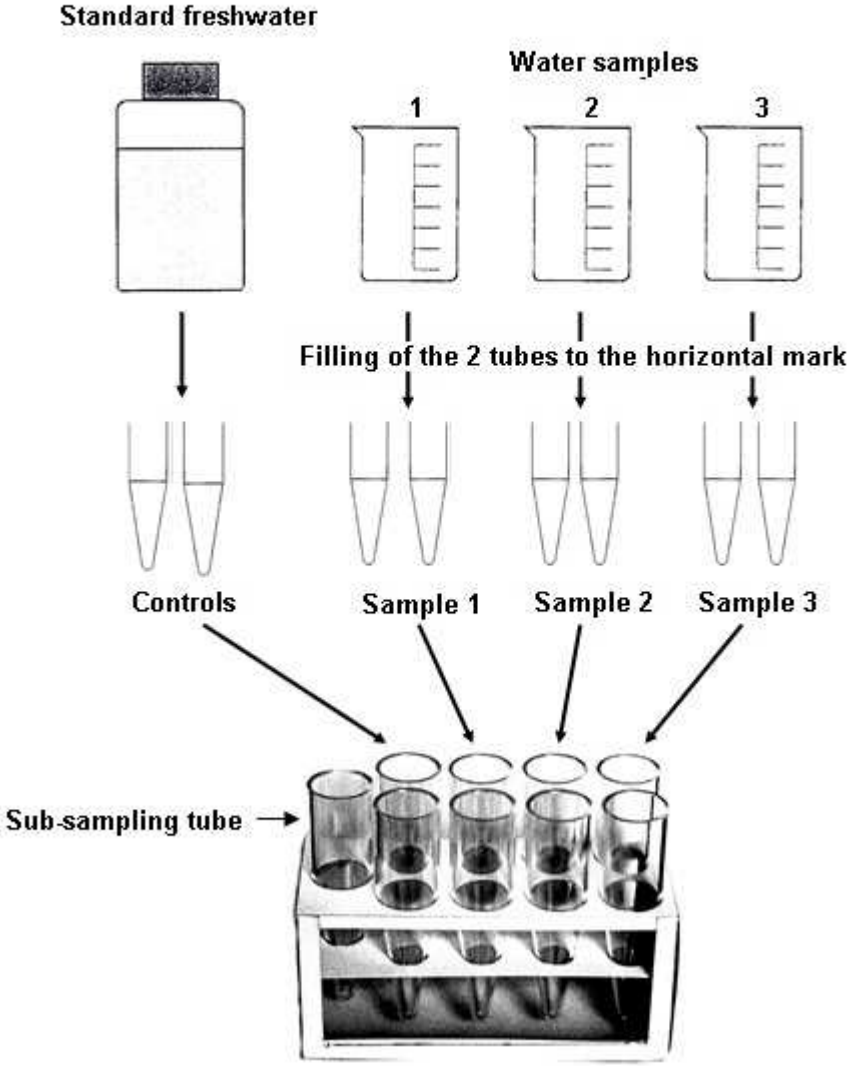


Figure 4. TRANSFER OF ORGANISMS IN THE SUB-SAMPLING TUBE AND TEST TUBES

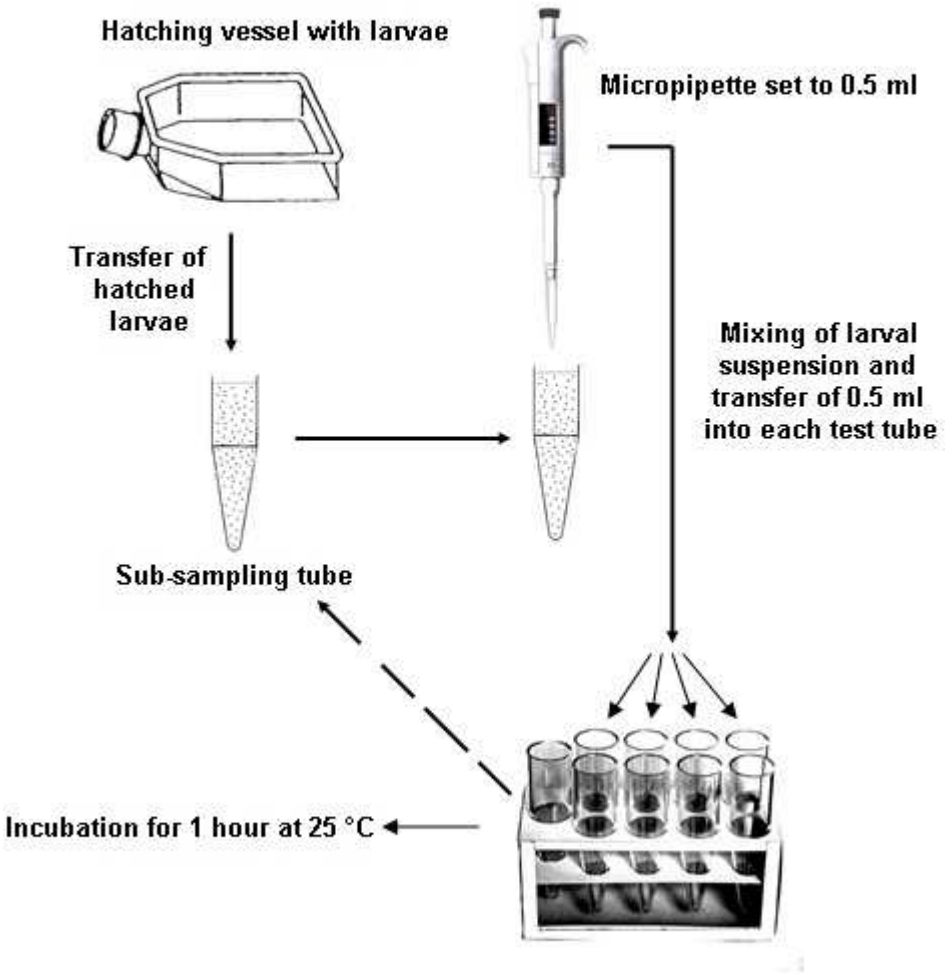


Figure 5. ADDITION OF COLORED MICROSPHERES TO THE TEST TUBES

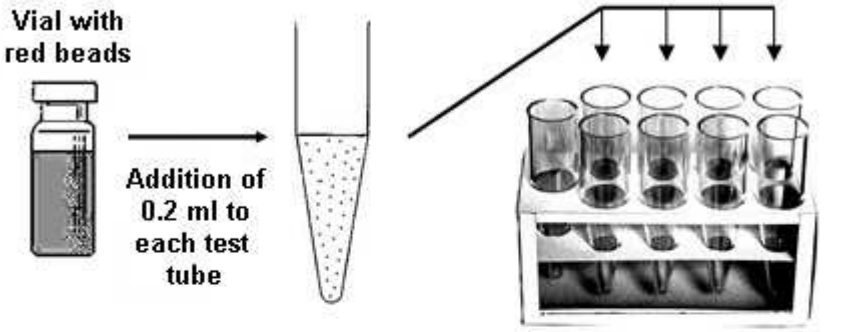
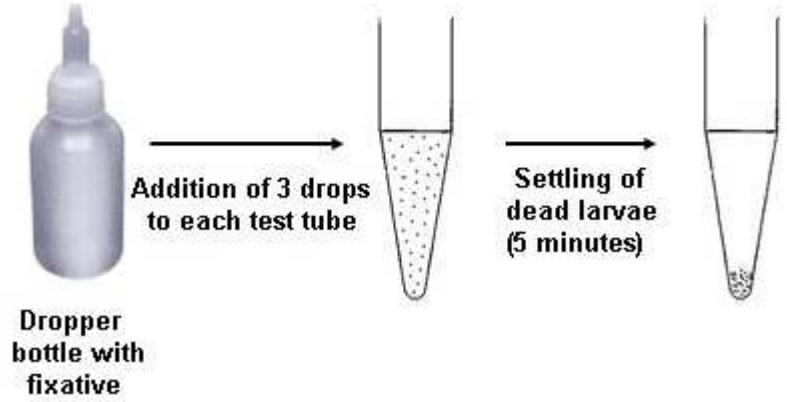


Figure 6. FIXING OF THE TEST ORGANISMS



4. ADDITION OF COLOURED MICROSPHERES TO THE TEST TUBES (see Figure 5)

1. Take the bottle containing the suspension of red microspheres and mix the contents thoroughly (*preferably using a Vortex mixer*) to obtain a homogenous suspension.
2. Remove the stoppers from the test tubes and add 0.2 ml of the bead suspension to the tubes with control water and test waters.
3. Put the stoppers back and shake each tube gently to distribute the microspheres evenly throughout the water column.
4. Put the holder with the test tubes in the incubator and incubate **for 15 to 30 minutes** at 25°C in darkness.

N.B. Although uptake of the colored particles is apparent after 15 minutes, the color of the digestive tract is generally further enhanced after 30 minutes uptake.

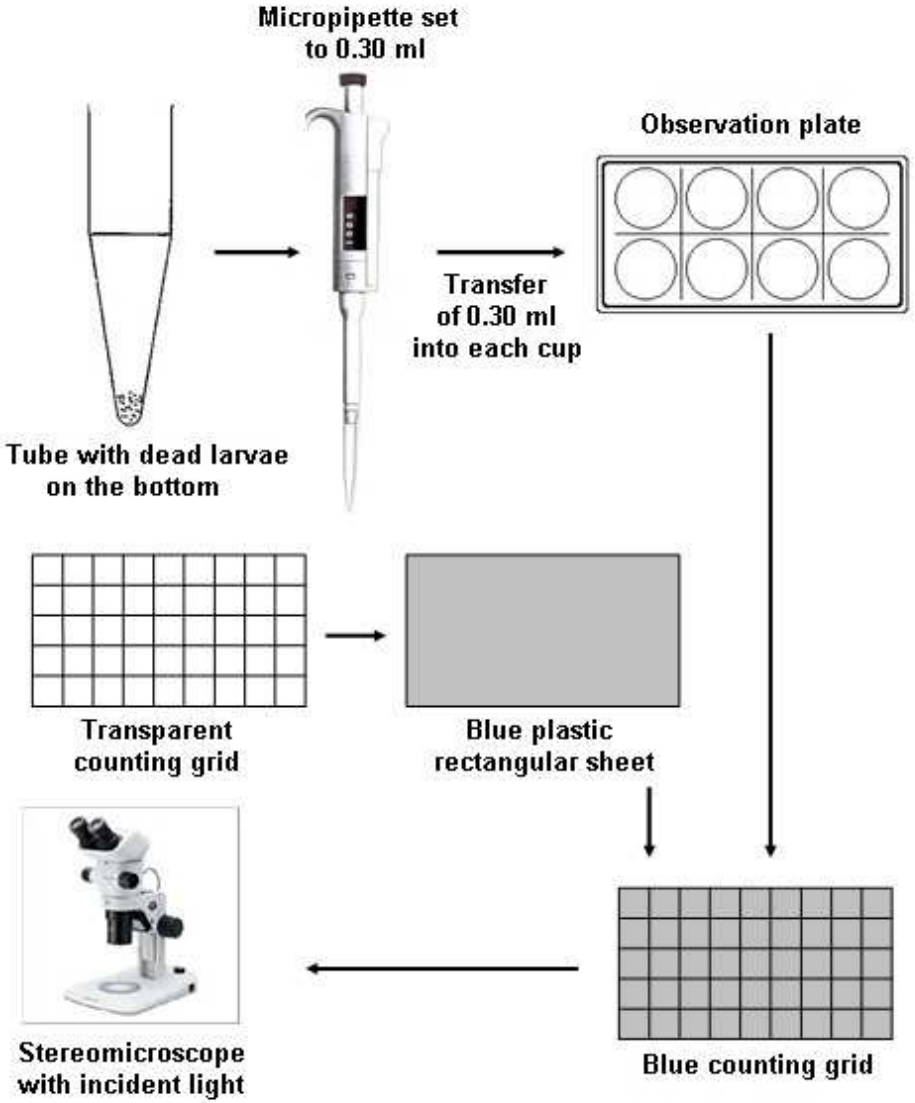
5. FIXING OF THE TEST ORGANISMS (see Figure 6)

1. Remove the stoppers from the test tubes and add 3 drops fixative from the dropper bottle to each tube.
2. Put the stoppers back and shake the tubes gently to distribute the fixative evenly in the water column.
3. Wait for approximately 5 minutes for the dead organisms to settle to the bottom of the conical test tubes.

6. TRANSFER OF THE TEST ORGANISMS INTO THE WELLS OF THE OBSERVATION PLATE (see Figure 7)

1. Set the volume of the micropipette to 0.30 ml.
2. Insert the micropipette into the first “control” tube and lower the tip until its aperture is close to the test organisms lying at the bottom of the tube.
3. Collect (in one rapid movement) all the dead larvae.
4. Empty the contents of the micropipette into the first well of the observation plate.
5. Repeat this operation for the second control tube and all the tubes with the water samples, in the sequence of their position in the holder.

Figure 7. TRANSFER OF THE TEST ORGANISM IN THE WELLS OF THE OBSERVATION PLATE



7. ANALYSIS OF THE TEST ORGANISMS UNDER THE STEREOMICRO-SCOPE AND SCORING OF THE RESULTS

Prerequisites :
The microscopic observations have to be carried out with "incident" illumination.
In order to obtain a good contrast between the coloured digestive tract of the test organisms and the rest of their body the observation plate must be placed under the stereomicroscope on top of the blue rectangular sheet with its transparent counting grid provided in each Rapidtoxkit.

Strong illumination is needed for the microscopic analyses. The use of either fibre optic cold light with a flexible arm (with or without coupling to a central ring) or a ring light illumination with LEDs is strongly recommended.

1. Put the transparent counting grid on top of the blue plastic rectangular sheet on the bottom stage of the stereomicroscope.
2. Put the observation plate on top of the counting grid on the bottom stage of the stereomicroscope.
3. Centre the first well and select the magnification which allows to view the entire surface of the well containing the dead test organisms.
4. With the aid of a needle, spread the organisms over the entire surface of the well, for more easy observation and counting.
5. Count the total number of larvae in the well and the number of larvae with clearly coloured digestive tracts.

N.B. The blue rectangular sheet gives a very good contrast for observation and counting of the larvae with or without uptake of red particles.

6. Score the numbers in the respective columns and rows on the Result Sheet.
7. Repeat this operation for all the wells.
8. Calculate the mean percentage particle uptake for the "control" tubes and for the "test water" tubes.

RAPIDTOXKIT

RESULTS SHEET

Date : .....

Name of operator : .....

Age of test organisms : .....

Type of water samples :

Sample 1 : .....

Sample 2 : .....

Sample 3 : .....

	Replicate	Total number of organisms	Number of coloured organisms	% coloured organisms
Control	1			
	2			
	Total			A =
Test water 1	1			
	2			
	Total			B =
Test water 2	1			
	2			
	Total			B =
Test water 3	1			
	2			
	Total			B =
Test water 4	1			
	2			
	Total			B =
Test water 5	1			
	2			
	Total			B =
Test water 6	1			
	2			
	Total			B =
Test water 7	1			
	2			
	Total			B =

% inhibition of particle uptake =

$$\frac{A - B}{A} \times 100$$

- Test water 1 : ..... %
- Test water 2 : ..... %
- Test water 3 : ..... %
- Test water 4 : ..... %
- Test water 5 : ..... %
- Test water 6 : ..... %
- Test water 7 : ..... %

9. Calculate the mean percentage inhibition of particle uptake using the following formula :

$$\text{Mean percentage inhibition of particle uptake} = \frac{A - B}{A} \times 100$$

With A = mean percentage particle uptake in the control
and B = mean percentage particle uptake in the water sample

IMPORTANT REMARKS FOR CORRECT APPLICATION OF THE RAPID TEST :

Various factors have to be taken into consideration for the scoring of the results and the interpretation of the findings such as larval stage, vitality, intraspecific differences and intensity of particle uptake.

***1. Larval stage** :*

A. *The first larval stages of anostracan crustaceans (such as Thamnocephalus platyurus) survive on their yolk reserves and as yet do not have a fully developed digestive tract. Consequently, the first instars cannot take up food nor any other particles (see Photo 1).*
Only after metamorphosis into the next instar stages does the digestive tract become "operational" and the organisms start to take up food and/or other particulate material such as the coloured microspheres employed here (see Photo 2).

REMARK 1.
The test organisms should only be collected "at the earliest" after 30h from the start of the incubation of the cysts.

B. *Hatching of the cysts is, however, not totally synchronous. This means that at the time of collection some larvae may still be in an instar stage which cannot yet take up particles.*
The first instars can be distinguished very easily from the older larval stages because they are smaller, orange in colour (because of the yolk) and not transparent.

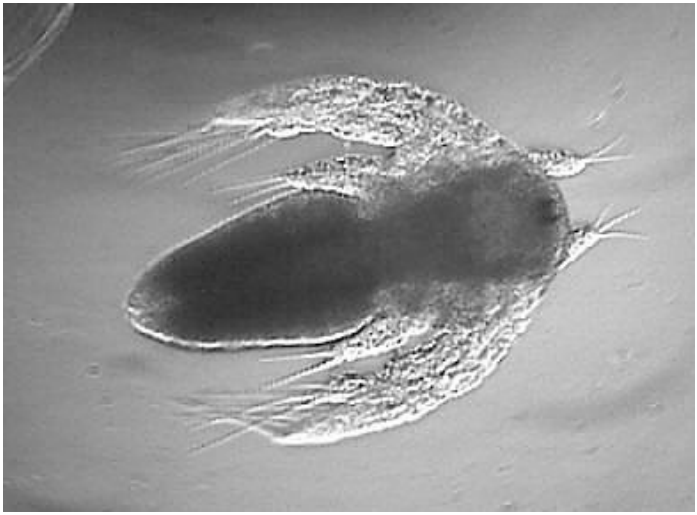


Photo 1 : First larval stage (300 µm) of the crustacean test organism, not capable of ingesting particulate material

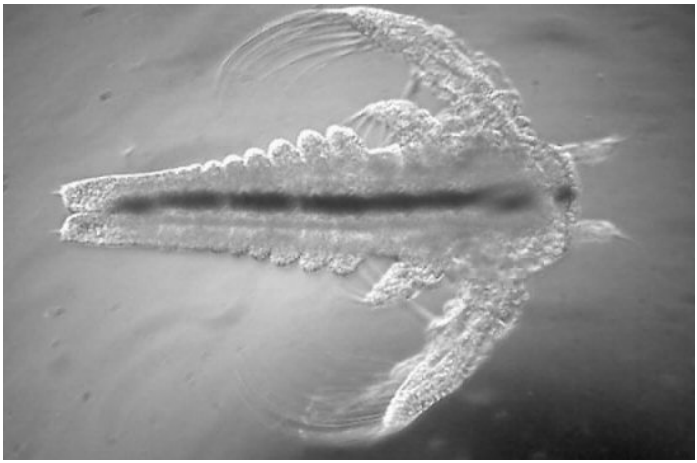


Photo 2 : Older larval stage (400-500 µm) of the crustacean test organism, with digestive tract full of ingested particles

REMARK 2.
The opaque (orange) instars should be excluded for the purpose of scoring*.*

2. "Vitality" of the test organisms *:*
It has been found experimentally that the particle uptake is very low or even nil when the larvae become very weak (which is the case when they remain for long in the hatching medium owing to the lack of food).

REMARK 3.
The organisms have to be collected and used for the assays in the time span of 30h to 45h from the start of the incubation of the cysts*.*

3. Intraspecific differences
In every population of biota there is always a mixture of strong and weak individuals. In addition, since the hatching of the cysts is not totally synchronous, the test population will also consist of a mixture of larvae "of different ages" and hence different vitality.
Therefore, it will be observed that in some larvae the whole digestive tract is filled with coloured microspheres, whereas in others only the gut and/or part of the intestine is coloured and in some larvae there will be complete lack of any uptake of coloured particles.

REMARK 4.
Even in the controls the percentage of "coloured" organisms will never be 100% but usually between 60 and 90%.

This does, however, not matter for the interpretation of the results! The evaluation is actually based on the percentage of "coloured" organisms in the test water(s) in comparison with the percentage coloured larvae in the controls (which does not need to be 100 %).

4. Intensity of uptake of coloured microspheres
It will also observed from time to time that in the (toxic) water samples, the "intensity" of the particle uptake and/or the total amount of coloured microspheres in the digestive tract of the larvae is significantly lower than that noted for the organisms in the control water.

For reasons of simplicity and practicality the test protocol of the Rapidtoxkit is based on the sole calculation of the number of organisms "with or without uptake of coloured particles without any distinction for the "intensity" of the particle uptake.

REMARK 5.
A "weaker" particle uptake by the test organisms in the test waters compared with those in the control water is an additional sign of (toxic) stress and should also be taken into consideration in the final interpretation !

8. INTERPRETATION OF THE RESULTS AND EVALUATION OF THE TOXIC HAZARD OF THE WATER SAMPLE

The Rapidtoxkit is a "pass-fail" microbiotest using non-diluted test waters with a very simple interpretation of the observations, namely the level of uptake of coloured particles considered to be "significantly different" from that in the controls.

As a guideline, **30% inhibition of particle uptake** is suggested as "a threshold signal" for the presence of "unwanted" compounds in the water sample, which should trigger further attention and action.

9. INTERFERENCES

The Rapidtoxkit microbiotest can be employed without any difficulty on "coloured" waters.

Turbid water samples must, however, be filtered through a membrane filter (0.2 µ porosity) prior to performance of the assays since particulate material will interfere with the uptake of the coloured microspheres.

10. TEST VALIDITY

Tests in which less than 50% of the test organisms in the control water exhibit ingestion of coloured microspheres, should be disregarded.
Too low uptake in the controls indeed points to a too low vitality of the test population. This may be due to various factors such as inadequate hatching or inappropriate test conditions.

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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