

PHYTOTESTKIT

for determination of the direct effects of chemicals
on seed germination and early growth of plants

LIMIT TEST WITH 3 PLANT SPECIES



STANDARD OPERATIONAL

PROCEDURE

TABLE OF CONTENTS

	<u>Page</u>
Introduction to the Phytotestkit microbiotest	2
Assets of the Phytotestkit microbiotest	3
Contents of the Phytotestkit limit test	6
Test Procedure of the Phytotestkit limit test	8
1. Filling of the test plates with the foam pad, the Parafilm sheet and the white filter paper and spiking with the test compound	8
2. Placing of the black filter paper and the seeds, and closing of the test plate	8
3. Incubation of the test plates	10
4. Image recording at the end of the exposure period	10
5. Analysis and measurements of the germinated seeds in the stored files	12
6. Calculation of the effect of the spiked chemical on seed germination and root and shoot growth	17

INTRODUCTION TO THE PHYTOTESTKIT MICROBIOTEST

ORIGIN

The **Phytotestkit** is a “variant” of the **Phytotoxkit** microbiotest, which was developed as a simple and practical alternative to conventional phytotoxicity tests which are performed “in pots” and hence quite space demanding and time consuming.

Both the **Phytotoxkit** and the **Phytotestkit** microbiotests allow for direct length measurements of roots and shoots in special transparent test containers, with the aid of image analysis, which eliminates the multiple manipulations inherent to conventional phytotoxicity assays.

The Phytotoxkit test measures the decrease (or the absence) of seed germination and early growth of plants “in contaminated soils” in comparison to germination and growth in a “reference” soil. This microbiotest can also be used to determine the impact of chemicals on the plants by “spiking” of the reference soil with a chemical or a water sample.

SCOPE

Contrary to the Phytotoxkit, the **Phytotestkit** microbiotest measures the “intrinsic” (direct) effect of chemical compounds on plants, without incorporation of the latter in a soil.

Besides studies of the impact of “toxic” chemicals on seed germination and early growth of plants this bioassay is also suited for the analysis of (positive or negative) effects of e.g. soil fertilisers, agricultural chemicals and biocides, on germination and early plant growth.

This microbiotest can furthermore also be used for the analysis of the dynamics of early plant growth in function of the concentration of the spiked compound(s). The transparent test plates indeed allow to take pictures of the growing plants at any moment of time during the entire incubation period.

PRINCIPLE

The Phytotestkit makes use of the same unique flat and shallow transparent test plates as the Phytotoxkit.

In the Phytotestkit test procedure however the lower part of the test plate is not filled with soil, but with a thick white filter paper placed on top of a Parafilm sheet and a foam pad. The white filter paper is subsequently spiked with a solution of the chemical under analysis.

Seeds of the test plant(s) are positioned at equal distance near the middle ridge of the test plate, on a black filter paper placed on top of the spiked white filter paper.

The test plates are then closed with their transparent cover and placed vertically in a holder for incubation at 25 °C (+/- 1 °C) for 3 days.

At the end of the incubation period a “digital” picture is taken of the test plates in which the germinated plants can clearly be seen underneath the transparent cover. The pictures are taken either with a digital camera, a webcam camera or a flatbed paper scanner, and stored in a computer file.

The visual analyses of the germination success and the length measurements of the roots and the shoots can be made immediately or postponed since the pictures of the test plates are continuously available.

ASSETS OF THE PHYTOTESTKIT

The Phytotestkit has multiple advantages over conventional assays with higher plants :

- the technology allows to determine the “intrinsic” (direct effects of chemicals on germination and early growth of plants without interference of a soil substrate
- the test plates are small and require little bench space and incubation space which allows to perform multiple concurrent tests
- the transparent test plates allow for direct observation of the germinated seeds and the growing plants
- the pictures of the test plates with the germinated seeds are stored in computer files which allows to postpone the measurements
- the length measurements of the roots and shoots is rapid and automatic by the use of image analysis techniques
- this microbiotest also allows to evaluate the early growth dynamics of the plants

FEATURES

The Phytotestkit microbiotest analyses two kinds of effects :

1. the effect of the chemical compound on seed germination, in comparison to the not spiked control
2. the effect of the chemical on the early growth of the plant, in comparison to the not spiked control

The Phytotestkit limit test is provided with seeds of 3 plant species selected on the basis of their rapid germination and the rapid growth of the roots and shoots, which allows to complete the assays after only 3 days of incubation :

- 1) the monocotyl Sorgho (*Sorghum saccharatum*)
- 2) the dicotyl garden cress (*Lepidium sativum*)
- 3) the dicotyl mustard (*Sinapis alba*).

N.B. The Phytotestkit microbiotest can also be performed with other types of seeds but the exposure time will then have to be selected in function of their specific speed of germination and root and shoot growth.

The Phytotestkit microbiotest is available in 2 distinct versions :

1. A “PHYTOTESTKIT LIMIT TEST WITH 3 PLANT SPECIES” which allows to perform a bioassay on each of the 3 plant test species mentioned above, at one (selected) concentration of a chemical (in comparison to the control). Each assay is performed in 3 replicates.

2. A “PHYTOTESTKIT COMPLETE TEST WITH ONE PLANT SPECIES” which allows to perform a “complete test” with 5 dilutions of a chemical (in comparison to the control) on one of the 3 plant species mentioned above and with 3 replicates with calculation of the EC50.

N.B. The Standard Operational Procedure described hereunder deals specifically with the Phytotestkit limit test.

Equipment

The only equipment needed for performance of the tests is an incubator or a temperature controlled room and a digital camera (or a webcam camera or a flatbed paper scanner) to shoot pictures of the test plates with the germinated seeds, for subsequent storage in a computer file.

The length measurements of the roots and the shoots of the germinated seeds is made with an image analysis programme, such as e.g. "ImageJ", which can be downloaded directly from the Internet, or which can be obtained at no charge from the company MicroBioTests.

SHELF LIFE

The shelf life of the Phytotestkit limit test is function of the germination potential and the growth strength (vigour) of the 3 types of seeds provided in the kit. If the seeds are stored properly, their shelf life is at least half a year.

CONTENTS OF THE PHYTOTESTKIT LIMIT TEST

Test plates

18 transparent test plates (21 x 15.5 x 0.8 cm) in polyvinylchloride (PVC). The test plates are composed of a bottom part separated by a middle ridge into two compartments, and a flat cover. The two parts of the test plates are provided laterally with small rectangular “cavities and bulges” for closing the plates tightly by a click system.

Foam pads

18 rectangular white polyether foam pads (8.7 x 12.5 cm, thickness 0.6 cm) with rounded edges, to be placed in the lower part of the bottom of the test plates.

Parafilm sheets

18 sheets of Parafilm, which are slightly larger than the foam pads, to be placed on top of the former to preclude that liquid of the (saturated) thick white filter paper passes into the foam pad.

Thick filter papers

18 thick white cellulose filter papers (8.7 x 12.5 cm, thickness 1.7 mm) with rounded edges, to be placed on top of the Parafilm sheets for subsequent spiking with the chemical compound under investigation.

Envelope with black filter papers and labels

An envelope containing :

- a) 18 rectangular high purity seed testing black filter papers (8.7 x 12.5 cm, thickness 0.54 mm) with rounded edges, to be placed on top of the spiked white filter paper, in the bottom compartment of the test plates
- b) 18 labels for identification of the individual test plates.

Test plate holders

3 cardboard holders (17 x 6 x 10.5 cm), each for 6 test plates.

Tubes with seeds

3 tubes with quality controlled seeds of 3 selected plant species : the monocotyl *Sorghum saccharatum* (Sorgho) and the dicotyls *Lepidium sativum* (garden cress) and *Sinapis alba* (mustard).

For optimal germination and longest shelf life the tubes with seeds should be stored in the refrigerator (5 °C (\pm 2 °C)).

Results sheets

3 sheets for the scoring of the seed germination data and the mean root lengths and shoot lengths for the 3 plants, and for calculation of the percentage effect of the spiked chemical on seed germination and early plant growth.

Standard Operational Procedure manual

A brochure with detailed instructions for the performance of the Phytotestkit limit test.

Bench protocol

An abbreviated version of the Standard Operational Procedure manual.

Specification sheet

A sheet indicating the batch number and the shelf life of the seeds.

TEST PROCEDURE OF THE PHYTOTESTKIT LIMIT TEST

The procedure described hereunder outlines the handlings on one test plate, with spiking of the white filter paper with one selected concentration of a chemical compound, or with deionized or distilled water for the control test plate.

1. FILLING OF THE TEST PLATE WITH THE FOAM PAD, THE PARAFILM SHEET AND THE WHITE FILTER PAPER AND SPIKING WITH THE TEST COMPOUND

1. Put one foam pad in the bottom compartment of a test plate, place one Parafilm sheet on top and put one thick white filter paper on top of the Parafilm sheet (*Figure 1 and Figure 2*)
2. Fill a syringe with 20 ml of the prepared concentration of the chemical (or with 20 ml deionized or distilled water for the control test plates) and slowly spread it over the entire surface of the thick white filter paper to hydrate the latter completely (*Figure 3*).

2. PLACING OF THE BLACK FILTER PAPER AND THE SEEDS AND CLOSING OF THE TEST PLATES

1. Put one thin black filter paper on top of the hydrated thick white filter paper (*Figure 4*) and wait until the black filter is completely wet

N.B. If air bubbles are trapped under the black filter paper, lift the paper up and put it back slowly.

2. Place 10 seeds of one of the 3 plants on top of the black filter paper, in one row and at equal distance of each other. The seeds shall be placed near the top of the filter paper, at about 1 cm of the middle ridge of the test plate (*Figure 5*).

*N.B. The correct placing of the 10 seeds is facilitated by the 10 marks at equidistant intervals on the middle ridge, and the mark on the two lateral sides of the test plate (see *Figure 6*).*

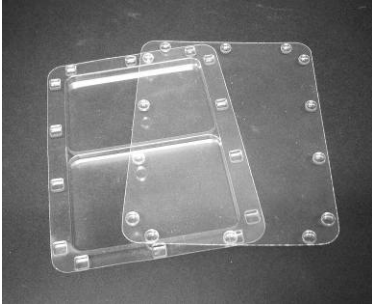


Figure 1



Figure 2

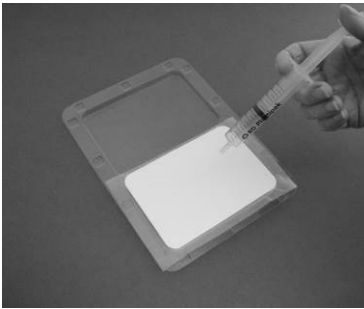


Figure 3



Figure 4



Figure 5

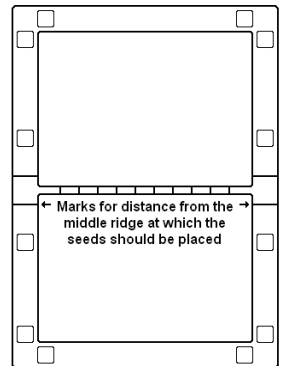
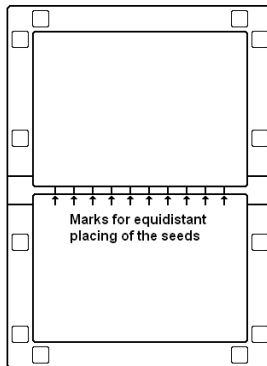


Figure 6

- Carefully place the cover on the bottom part of the test plate and click the lateral bulges on the cover into the corresponding cavities of the bottom part, to close the test plate tightly.

N.B. This “closing” operation shall be started in the middle of the test plate to avoid that the position of the seeds changes during the closing operation.

- Write the specifics of the test plate (name of the plant species, concentration of the spiked chemical, number of the replicate) on one of the small labels, and stick this label laterally on the bottom compartment of the test plate, in between two rectangular cavities.
- Turn the closed test plate upside down for a few hours to facilitate the hydration of the seeds (Figure 7).

N.B. Since the Phytotoxkit limit test is performed on 3 different plant species and with 3 replicates for each of them, the manipulations described above have to be repeated for each plant species with 3 replicate test plates for the control and 3 replicate test plates for the spiked chemical.

3. INCUBATION OF THE TEST PLATES

- Put the test plates in a vertical position in the cardboard holders, with 6 test plates per holder (Figure 8).
- Put the cardboard holders with the test plates in the incubator and incubate for 3 days at 25 °C (+/- 1 °C).

N.B. Light does not seem to play a significant role for the germination of the seeds nor the growth of the roots and shoots during the short incubation time of only a few days.

It is therefore advised not to provide illumination during the test period (unless one wants to specifically study the influence of light, in case of longer exposure times).

4. IMAGE RECORDING AT THE END OF THE EXPOSURE PERIOD

A picture of the test plates is taken at the end of the exposure period either with a webcam camera, a digital camera or a flatbed paper scanner.

4.1. Image recording with a webcam camera

Follow the specific instructions of the webcam camera to take a computer photo of the test plate, which then should be saved as a JPEG file.

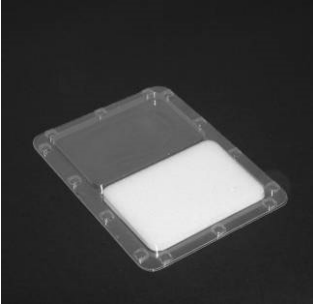


Figure 7



Figure 8

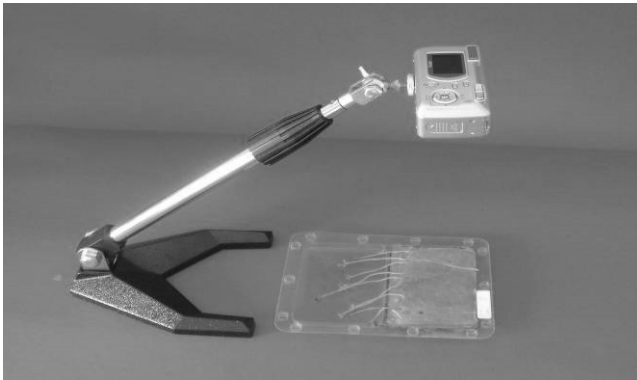


Figure 9

4.2. Image recording with a digital camera

The digital camera can be mounted on a vertical stand or a stand with a telescopic arm. (*Figure 9*).

Depending on the type of stand, the test plates shall be placed “horizontally” or “vertically” to take the photo.

N.B. In both cases the distance between the camera and the test plate must be selected so that the total surface of the bottom compartment of the test plate fills the screen of the LCD monitor (or the viewfinder), including the label on the left side.

After shooting the pictures, the frames shall be transferred to the computer and stored as JPEG files in the selected Directory.

4.3. Image recording with a flatbed paper scanner

1. Put the test plate “surface down” on the paper scanner.
2. Record the image of the test plate with the appropriate programme.
3. Give the file a name (with a JPEG extension) and store it in the selected Directory.

N.B. It happens that condensation occurs on the inside of the lid of the test plates which interferes with the visibility of the roots. In such case, the lid shall be lifted off (very carefully) from the bottom part of the test plate prior to shooting the picture.

During this operation, care has to be taken that the germinated plants don't stick to the cover.

The condensation has then to be wiped off from the lid and the latter placed back to shoot the picture. In case the picture is made with a webcam camera or a digital camera and the test plate is placed horizontally, it is not even necessary to put the lid back on the test plate.

5. ANALYSIS AND MEASUREMENTS OF THE GERMINATED SEEDS IN THE SAVED PHOTOS OF THE TEST PLATES

A major asset of the Phytotestkit test procedure is that contrary to most conventional phytotoxicity assays the analyses and length measurements don't need to be made immediately at the end of the exposure period, but can be postponed to any appropriate timing. The computer file with the saved images indeed remains permanently available for further analyses and measurements.

5.1. Counting of the number of germinated seeds

1. Open the file with the saved Image of a test plate with one of the plant species for the control, and count the number of germinated seeds.

2. Record the figure in the Results Sheet in Section “1. Seed germination” - Replicate 1.
3. Proceed similarly for the second and the third test plate of the same plant species and record the data in Replicate 2 and Replicate 3 in the Results Sheet.
4. Count the number of germinated seeds in the 3 test plates for the 2 other plant species and record the data in the Result Sheet.

N.B. : For reasons of uniformity only the seeds which have developed a root of at least 1 mm length shall be considered as being germinated.

5.2. Measurement of the root and shoot lengths of the germinated seeds

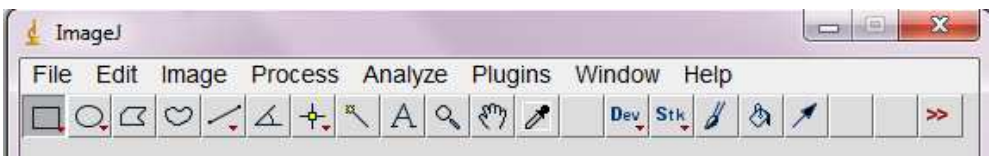
These measurements will be performed with the aid of an “Image Analysis program”, such as e.g. the Image J program which can be downloaded free of charge from the Internet (or obtained at no charge from the company MicroBioTests).

The procedure for the root and shoot measurements with the Image J program is given in detail hereafter.

IMAGE J

Access to the picture of the test plate and increase of the picture on the screen to the size of the bottom compartment of the test plate

1. Open the Image J program - A horizontal bar appears with a number of icons and words.



2. Go to Analyze and click Set Measurements. Make sure that none of the checkboxes are ticked (untick them if they have been ticked). Then click OK (to save the new settings).

3. Go to File and click Open image.
4. Select the Directory and click on the file with the saved photo of one of the test plates.
5. Enlarge the picture of the test plate by clicking the + key several times.
6. Move the picture on the screen by pressing the space bar (and keeping it pressed) and moving “the mouse” of the computer in the desired direction.

N.B. : when keeping the space bar pressed, the + sign changes to a “small hand”.

7. Enlarge the picture on the screen till the bottom compartment of the test plate almost totally fills up the computer screen (if too large, the size can be decreased with the - key).

Calibration of the measurements

1. Click Image, then Type and 8 bit color - Click OK.
2. Click the icon / (the icon on the right of the icon showing a heart).

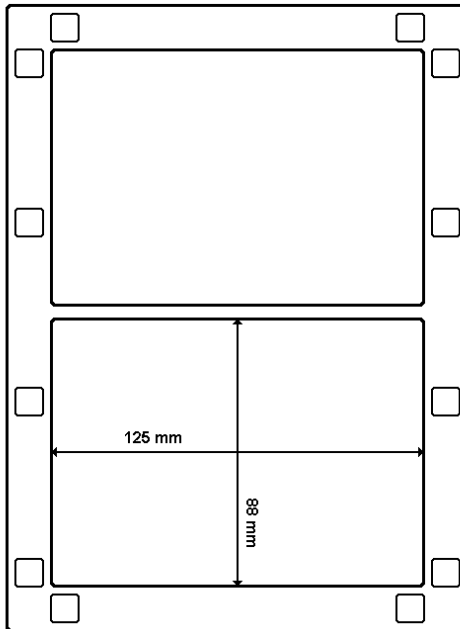
N.B. : if this icon is in another view (e.g. showing an indented line instead of a full line), click right on the mouse and select “straight line”.

3. Position the cursor on the left border of the bottom compartment of the test plate and draw a straight line from the left border to the right border (= the width of the bottom compartment of the test plate) (see *Figure 10*).

N.B. : Alternatively a straight “vertical” line can also be drawn, from the top border to the bottom border.

4. Click Analyze and Set scale.
5. Fill out the box Known distance with the figure **125** (in case a horizontal line has been drawn) or **88** (in case a vertical line has been drawn).
6. Type “mm” in the box Unit of length.

FIGURE 10 : CALIBRATION OF LENGTH MEASUREMENTS (in millimeter)



7. Click OK (this will subsequently automatically make the calculation of the root or shoot lengths in mm).

Measurement of the root and the shoot lengths

1. Click on the icon / and click right on the mouse to select "free hand line" (the icon will now show an indented line).
2. Move the + cursor on the screen to the place where the root or the shoot of the first germinated seed protrudes from the seed and then move the cursor along the total length of the root or the shoot.

N.B. : this operation has to be made "in one continuous movement". In case of a problem during this measurement, stop drawing the line and start the measurement again.

NOTE : For the monocotyl species Sorghum saccharatum, the root starts exactly at the point where it protrudes from the seed.

For the dicotyl species Lepidium sativum and Sinapis alba, the boundary between the root and the shoot is situated exactly at the point where the tiny “secondary” roots start to develop laterally on the (primary) root. This boundary is furthermore also relatively easy to find, since the shoot is somewhat thicker than the root (see Photo below).

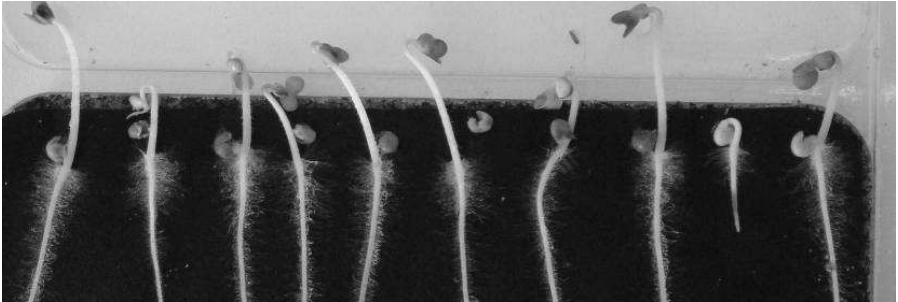


Photo of part of a test plate with germinated seeds of Sinapis alba, showing the lateral secondary roots on the (primary) roots

IMPORTANT REMARK

Similarly to what is mentioned in Section 5.1. with regard to seed germination, measurements of root and shoot lengths shall only be made on the seeds which have developed a root of at least 1 mm length.

3. Simultaneously click Ctrl and the letter M on the keyboard. This will open the Result box of the Image J program and show the first length measurement.
4. Shift the + cursor to the second root or shoot and proceed similarly to perform the length measurement of this root or shoot.
5. Save the result of this measurement by clicking Ctrl and M.

N.B. : as long as “Ctrl and M” are not clicked the measurement values will not be saved in the Result box !

6. Proceed further similarly for all the other roots or shoots of the test plate.

Saving of the root and shoot length measurements

1. Go to the Results box of the Image J program (which contains the length values of all the germinated seeds in the test plate) and click on all the values (which will then show up on a black background).
2. Click right on the mouse and click Copy.
3. Open Excel and click Paste to transfer the root or shoot length data to an Excel sheet.
4. Save the Excel file and give it a name (e.g. “root (or shoot) lengths germinated seeds first test plate – plant species X - Control”).
5. Close the Image J program for the analyzed test plate.
6. Open the Image J program again and proceed similarly as indicated above for the root and shoot length measurements of the germinated seeds in the other test plates for the 3 plant species.
7. Save each time the data for each test plate in an Excel file with a specific name.

Data treatment

1. Open the Excel sheets with the saved root and shoot length data and calculate the mean root and shoot length for each test plate for the control (= A) and for the tested chemical (= B).
 2. Transfer these data for each test plant to the corresponding Results Sheet in the sections “Length of roots” and “Length of shoots”.
- 6. CALCULATION OF THE PERCENTAGE EFFECT OF THE SPIKED CHEMICAL ON SEED GERMINATION AND ROOT AND SHOOT GROWTH**
1. Calculate in the Results Sheets for the 3 plant species the average number of germinated seeds for the 3 replicates in the control and in the test chemical.

2. Calculate the percentage effect of the chemical compound on seed germination and on root and shoot growth with the formula :

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

with A = average number of germinated seeds and average root and shoot length in the control

and B = average number of germinated seeds and average root and shoot length in the concentration of the tested chemical.

NOTE : An Excel program for the Data Treatment of the “Phytotestkit limit test” has been worked out by the company MicroBioTests Inc.

This program allows to transfer the root and shoot length data measured for the 3 plant species with the Image J program in specific Excel Sheets which automatically calculate the percentage root and shoot growth inhibition.

This Excel program can be obtained free of charge from the company MicroBioTests Inc.

PHYTOTESTKIT LIMIT TEST - RESULTS SHEET

Date :

Name of operator :

Plant species :

Chemical compound :

Test concentration :

1. SEED GERMINATION

		Number of germinated seeds
Control	Replicate 1	
	Replicate 2	
	Replicate 3	
	Average (= A)	
Test chemical	Replicate 1	
	Replicate 2	
	Replicate 3	
	Average (= B)	

2. LENGTH OF ROOTS

		Number of germinated seeds
Control	Replicate 1	
	Replicate 2	
	Replicate 3	
	Average (= A)	
Test chemical	Replicate 1	
	Replicate 2	
	Replicate 3	
	Average (= B)	

2. LENGTH OF SHOOTS

		Number of germinated seeds
Control	Replicate 1	
	Replicate 2	
	Replicate 3	
	Average (= A)	
Test chemical	Replicate 1	
	Replicate 2	
	Replicate 3	
	Average (= B)	

$$\text{Percentage effect} = \frac{A - B}{A} \times 100 = \quad \%$$

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 tricorutum*. This test adheres to ISO norm 10253.

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