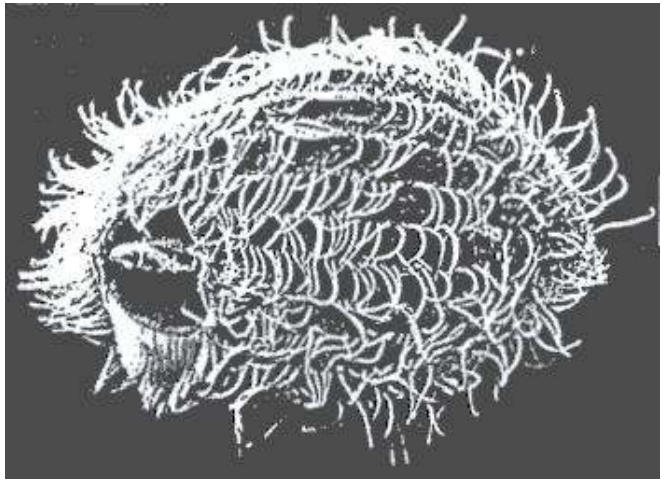
PROTOXKIT F

Freshwater Toxicity Test

with a Ciliate Protozoan



STANDARD OPERATIONAL

PROCEDURE

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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 the AFNOR norm T90-377.

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

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

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

CERIODAPHTOXKIT F : 24h mortality test, based on the cladoceran crustacean *Ceriodaphnia dubia*. This assay is in current practice in the USA as an EPA Method.

OSTRACODTOXKIT F : 6 days chronic mortality and growth inhibition test with the ostracod crustacean *Heterocypris incongruens*.

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

ALGALTOXKIT F : 72h growth inhibition test, based on the green alga *Selenastrum capricornutum* (presently named *Pseudokirchneriella subcapitata*). This assay adheres to ISO norm 8692 and OECD Guideline 201.

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

Tests for estuarine/marine environments

ROTOXKIT M : 24h mortality test based on the rotifer *Brachionus plicatilis*. This assay adheres to ASTM Standard Guide E1440-91.

ARTOXKIT M : 24h mortality test based on the anostracan crustacean *Artemia salina* (renamed *Artemia franciscana*)
This assay adheres to ASTM Standard Guide E1440-91.

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

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INTRODUCTION TO THE PROTOXKIT

Origin :
The protozoan growth inhibition bioassay originates from the research team of Dr. W. Pauli at the Institute of Biochemistry and Molecular Biology, Free University of Berlin, Germany. The test was adapted to the TOXKIT concept in cooperation with the research group of Prof. Dr. G. Persoone at the Laboratory for Biological Reseearch in Aquatic Pollution (LABRAP) at the University of Ghent, Belgium.

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

Advantages of TOXKIT tests :
The major advantage of TOXKITS, in comparison to ”conventional” bioassays, is that the test organisms are incorporated in the kits in a ”dormant” or ”immobilized” form, from which they can be activated ”on demand” prior to performance of the toxicity test.
This eliminates the need for continuous recruitment and /or stock culturing of test organisms, and hence the major cost factor.
Furthermore, all TOXKITS have been ”miniaturized” into low cost microbiotests which can be performed with conventional lab materials and equipment, on little benchspace.

Advantages of the PROTOXKIT test :
- No stock-culturing
The PROTOXKIT makes use of ciliates which are maintained alive at room temperature for several months, in a very specific medium, without any prejudice to their good physiological condition.

A major additional assett of the Tetrahymena microbiotest in comparison to the Toxkits with invertebrate test species, is that the bioassay can be started immediately with the ciliates contained in the stock culture vials, without any intermediate time loss for hatching of the test biota.

REFERENCE TEST

In order to check the correct performance of the test and the sensitivity of the test organisms, it is advised to regularly run a quality control test with the reference chemical potassium dichromate ($K_2Cr_2O_7$). Despite the low toxicity of this negatively charged metal complex to *Tetrahymena* (in contrast to cationic - positively charged - heavy metals), potassium dichromate has been chosen for performance of the control test, because of its wide acceptance as reference chemical in ecotoxicological testing.

The toxicant dilution series to be prepared ranges from 100 mg/l down to 10 mg/l.

Procedure

1. Make a stock solution of 100 mg/l potassium dichromate by weighing 25 mg of the compound and dissolving it in distilled water in a 250 ml calibrated flask.
2. Follow the procedure given for TESTING OF CHEMICALS - DEFINITIVE TEST - Case A : C1-C5 spans one order of magnitude.

The 5 dilutions to be used for the reference test are the following:
C1 (56 mg/l); C2 (32 mg/l); C3 (18 mg/l); C4 (10 mg/l); C5 (5.6 mg/l).

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

VALIDITY OF THE TEST

For the toxicity test to be acceptable, the OD of the controls after 24h incubation must show a decrease of the T0 value by at least 60% ; in other words : the OD at T24 shall be 40% or less of the OD at T0.

IMPORTANT REMARK

If the 60% OD decrease in the controls is not reached after 24h incubation, a second OD scoring of all the test cells can be attempted 2-4h later. If the new OD readings of the controls fulfill the 60% decrease criterion, this points to a slightly slower growth of the ciliates (this, however, will not affect the test results). Consequently the bioassay can in this case (and on the basis of the second set of OD readings) still be considered as being valid.

DATA TREATMENT

1. Calculate the mean for the two parallels for each toxicant dilution and the control.
2. Calculate the difference between the mean OD at T0 and T24 for each toxicant dilution (ΔODC1-C5) and for the control (ΔODC0).
3. Calculate the % inhibition for each toxicant dilution by the following equation :

$$\%\, inhibition_{(C1\text{-}C5)} = \left(1 - \frac{\Delta OD_{(C1\text{-}C5)}}{\Delta OD_C 0}\right) x\, 100$$

4. Calculate the 24h EC_{50} using the specific Protoxkit computer programme that can be obtained - free of charge - from all Toxkit distributors.

- Short term assessment of chronic toxicity

The Protoxkit assay is a multigeneration growth test which includes 5-6 generations, and is completed in 24 hours.

- Rapid scoring

The test is based on optical density measurements.The PROTOXKIT uses disposable polystyrol spectrophotometric cells of 1 cm path length as test containers. The cells can be used with any type of spectrophotometer.

Principle of the PROTOXKIT test :

A 24h growth inhibition test is performed with the ciliate *Tetrahymena thermophila*. The OECD has given the development of a Test Guideline with Tetrahymena a priority. The test is based on the turnover of substrate into ciliate biomass. While normal proliferating cell cultures clear the substrate suspension in 24h, inhibited culture growth is reflected by remaining turbidity. Optical density measurements of the turbidity quantify the degree of inhibition.

Features :

Each PROTOXKIT contains all the (disposable) materials to perform 6 complete 24h assays (one control and 5 concentrations, each in duplicate). Besides conventional laboratory glassware and a suitable piston/automatic laboratory pipette for 40 µl volumes, the only equipment needed is:

a) an incubator or a temperature controlled room, at 30°C

b) a spectro-photometer (440 nm).

Sensitivity :

To minimize “toxic buffering” of e.g. heavy metals and/or reactive and highly lipophilic substances (by binding to organic components in the medium which may interfere with the bioavailability of the toxicants) the PROTOXIT medium has an organic content more than a factor 100 lower than that of the proteose-peptone media, used in "conventional" *Tetrahymena* tests.

Precision :

Comparative studies with conventional *Tetrahymena* tests revealed that the precision of the PROTOXKIT assays is at least equal to and often better than that of the conventional tests with this ciliate.

Shelf Life :
The stock-cultures containing the *Tetrahymena* can be stored at ambient temperatures (18-22°C) for a few months without loss of viability.
Consequently PROTOXKIT assays can be carried out within a period of several months as indicated by the expiry date label on each kit.

2. Add the volumes of distilled water and toxicant concentration C1 indicated in Table 5 to each tube.

Table 5 : Dilution series C1 - C5

Test tube	**distilled water** (ml)	**C1** (ml)
C1	0	10
C2	6.8	3.2
C3	9.0	1.0
C4	9.7	0.3
C5	9.9	0.1

TESTING OF COLOURED SAMPLES

Coloured samples will interfere with the OD measurements and hence bias the results.

Colour interferences can be taken care off as follows :

1. After the preparation of the toxicant dilution series in the (glass) tubes, but prior to the addition of the ciliates and the food substrate, fill one test cell of each toxicant dilution with 2 ml of the corresponding dilution.

2. Keep the 5 test cells with the (coloured) dilutions separately. Prior to the measurement (at 0h and 24h) of the OD of the test cells containing the toxicant dilutions with the ciliates and the food substrate, zero-calibrate the spectrophotometer with the test cells containing the corresponding coloured toxicant dilutions.

The test cells containing the coloured toxicant dilutions shall preferably also be incubated in the same light and temperature conditions as the test cells in the cell holders. This way, changes in colour during the 24h exposure will automatically be taken into account by the zero-calibration with the corresponding test cells.

In case of interference of OD readings by turbidity this method can also be used.

2. Preparation of toxicant dilution series

A. C1-C5 spans one order of magnitude

1. Take six 15 ml tubes (preferably in glass), label two tubes C1 and the others C2,C3,C4 and C5.
2. Transfer 10 ml of the stock toxicant solution into each C1 tube.
3. Add the volumes of distilled water indicated in Table 4 to the respective test tubes.

Table 4 : Dilution series C1 - C5

Test tube	**distilled water** (ml)	**C1** (ml)
C1	0	10
C2	4.4	5.6
C3	6.8	3.2
C4	8.2	1.8
C5	9.0	1.0

4. Add the volumes of toxicant concentration C1 indicated in Table 4 to each tube.
5. Cap and shake the test tubes.
6. Proceed further as indicated in the **Test Procedure for Effluents : Sections 2, 3, 4 and 5.**

N.B. The actual concentrations of C1 up to C5 are needed for the EC_{50} estimation and shall be calculated as follows :

C1 = ...mg/l
C2 = 0.56 x C1 = ...mg/l
C3 = 0.32 x C1 = ...mg/l
C4 = 0.18 x C1 = ...mg/l
C5 = 0.10 x C1 = ...mg/l

B. C1-C5 spans two orders of magnitude

Proceed exactly as in case A, except for the following :

1. Only one test tube C1 is needed.

CONTENTS OF THE PROTOXKIT

Stock-culture
One glass vial containing 3 ml of live Tetrahymena suspension, to be stored at room temperature until use. Samples for performing the individual tests can be removed aseptically by a sterile syringe through the vial septum.

Syringes
Sterile, disposable syringes for removal of small aliquots of the stock-culture.

Substrate vials
Six small tubes with food substrate, to prepare the food suspension for the ciliates. Substrate vials must be stored below 0°C (preferably at -20°C) until use.

Reconstitution solution vials
Six small tubes with reconstitution solution for preparation of the food suspensions, also to be stored preferably at - 20°C until use.

Stock culture cells
Six disposable 1 cm polystyrol spectrophotometric cells of 1.5 ml contents, with lid, for determination of the ciliate density in the stock-cultures.

Ciliate inoculum tubes
Six 5 ml disposable tubes with stopper, for dilution of the stock-culture to the inoculation ciliate density.

Test cells
Eighty disposable 1 cm polystyrol spectrophotometric cells with lids, to be used as test containers, allowing the direct reading of the optical density in a spectrophotometer.

Cell holders
Two cell holders in cardboard, for the 12 cells used in each Protoxkit bioassay.

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

Bench Protocol
An abbreviated version of the detailed Standard Operational Procedure manual.

Results sheets
Sheets for data scoring of the optical density readings and calculation of the effect percentages.

Specification sheet
A sheet indicating the batch number of the stock-culture and the substrate, the expiry date of the TOXKIT and the 24h EC_{50} values for the reference chemical potassium dichromate.

5. Repeat the 1 ml transfer from tube C2 to C3, and subsequently from C3 to C4 and from C4 to C5.
6. Proceed further as indicated in the **Test Procedure for Effluents : Sections 2, 3, 4 and 5.**

DEFINITIVE TEST

The dilution series to be prepared must span the range of the lowest concentration producing 80-100% effect and the highest concentration producing less than 20% effect in the range-finding test.

As shown in Table 3, this range can span one order of magnitude (case A) or two orders of magnitude (case B).

The new concentration range to be tested will again be called C1-C5.

Table 3: Schematic presentation of the 100-0% effect range determined in the range-finding test.

Case A	**% growth inhibition** **100 100 0 0** **- - - * - - - * ---------- * - - - - * - - -** **C1 C5**
Case B	**% growth inhibition** **100 100 0 0** **- - - * - - - * -------- * -------- * - - - -*** **C1 C5**

1. Preparation of chemical stock solution

Prepare **20 ml** of the C1 toxicant concentration (this can be done by serial dilution 1:10, following e.g. Table 2).
C1 is the lowest concentration which, in the range finding test produced 80-100 % growth inhibition.

RANGE FINDING TEST

An example is given below for a concentration series, ranging from 100 mg/l down to 0.01 mg/l.

1. Preparation of chemical stock solution

Prepare a 100 mg/l stock solution by weighing (on an analytical balance) 25 mg of the substance to be tested, and dissolving it in distilled water in a 250 ml calibrated flask.
Poorly soluble compounds should first be dissolved in an organic solvent (e.g. DMSO, acetone, ethanol or methanol).

N.B. In order to avoid (toxic) interference, the concentration of the solvent in the highest test solution should not exceed 0.2 % (v/v).

Preparation of toxicant dilution series

A 1:10 dilution series with distilled water will be prepared, starting with the stock solution (100 mg/l) as the highest test concentration. For poorly soluble compounds, the highest soluble concentration containing no more than 0.2 % solvent shall be used as the highest test concentration.

1. Take five 15 ml test tubes (preferably in glass) and label them from C1 to C5. C1 is the highest and C5 the lowest concentration of the compound to be tested (see Table 2).

Table 2: Dilution series of the chemical compound

Test tube	chemical concentration (mg/l)
C1	100
C2	10
C3	1
C4	0.1
C5	0.01

2. Transfer 10 ml of the stock solution (100 mg/l) into tube C1.
3. Put 9 ml distilled water into all the other test tubes.
4. Transfer 1 ml from tube C1 into C2 and mix.

STANDARD OPERATIONAL PROCEDURE

General remark : The preparation of the toxicant dilution series is performed for pure chemicals with distilled or deionized water.
From a physiological point of view, this may seem strange, since ciliates (like all other biota) cannot survive in a medium which does not contain particular ions or salts.
These vital constituents are, however, present in sufficient amounts in the reconstituted substrate suspension, which is added to all test cells.

In order to avoid unnecessary continuous repetitions in the following text, the use of "distilled water" stands for both "distilled or deionized water".

TESTING OF EFFLUENTS

General remark : The procedure described below for the testing of effluents (or liquid wastes) can also be applied to any other type of contaminated liquids (e.g. surface waters, groundwaters, sediment pore waters, soil or waste leachates, etc.)

SAMPLE PREPARATION

In order to avoid interference by particulate matter and/or biota which may be present in particular effluent samples, it is recommended to clean the samples prior to testing.
This can be performed by filtering the samples over a 45 µm membrane filter or by removing the particulate matter and/or biota by centrifuging.

TEST PROCEDURE

1. Preparation of toxicant dilution series

A dilution series (100% - 50% - 25% - 12.5% and 6.25%) of the centrifuged (or filtered) effluent sample is prepared by serial 1:2 dilution with distilled water.

1. Take five 15 ml test tubes (preferably in glass) and label them from C1 to C5. C1 will contain the undiluted effluent, C5 the highest dilution (see Table 1).

Table 1: Dilution series of the effluent

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

2. Put 10 ml of the undiluted (treated) sample in tube C1 (= 100%).
3. Fill C2 to C5 with 5 ml distilled water each.
4. Transfer 5 ml from C1 to C2 and mix (= 50%).
5. Repeat this operation for C2 to C3 (= 25%), C3 to C4 (= 12.5%) and C4 to C5 (= 6.25%).

2. Preparation of ciliate inoculum

(see Figure : *Preparation of ciliate inoculum*)

1. Take the stock culture vial and shake it gently to homogenize the contents.
2. Take 500 µl from the ciliate stock culture with a sterile syringe.
3. Transfer the 500 µl stock suspension into a 1.5 ml stock-culture cell and add 1 ml distilled water.
4. Cover the stock-culture cell with its lid, shake gently and measure the optical density (OD) at 440 nm.
5. Calculate the dilution factor needed to arrive at a 'theoretical' OD value of 0.040 with the formulas :

   F = ODvalue / 0.040
   V = 0.5 x (F-1)

   (e.g. if the original OD is 0.090, the 1.5 ml ciliate suspension should be diluted by a factor 0.090/0.040 = 2.25 times).
6. Transfer 500 µl of the diluted ciliate stock into the ciliate inoculum tube and add Vml distilled water.
7. Close the tube with the stopper and mix gently.

PROTOXKIT F

RESULTS SHEET

Name of operator : .....

Date of performance of test : .....

Toxicant tested : .....

Type of test : O range finding test
O definitive test

Dilution series tested : concentration 1 = ...
concentration 2 = ...
concentration 3 = ...
concentration 4 = ...
concentration 5 = ...

OPTICAL DENSITY MEASURED AT 440 nm							
Exposure time	Replicate	Control	C5	C4	C3	C2	C1
t_0	1						
	2						
	Mean						
t_{24}	1						
	2						
	Mean						

$24hEC_{50}$ = ...

3. Record the T0 data on the Result Sheet.
4. Put all the cells back in their holding tray and put the tray in an incubator at 30°C for 24 hours.

IMPORTANT REMARK

In some cases (which are batch dependent) it is advised to extend the incubation period to 28 hours in order to obtain enough OD decrease in the controls (see also section Validity of the test - Important Remark on page 18).

N.B. Since the growth of the ciliates is very temperature dependent, the outcome of the bioassays and their repeatability will also be highly dependent of the temperature precision and stability of the incubator ...

5. After 24h incubation, recalibrate the measuring equipment with a test cell containing 2 ml distilled water. Gently shake each cell and determine again the OD at 440 nm (= time T24 scoring).
6. Record the T24 data on the Result Sheet.

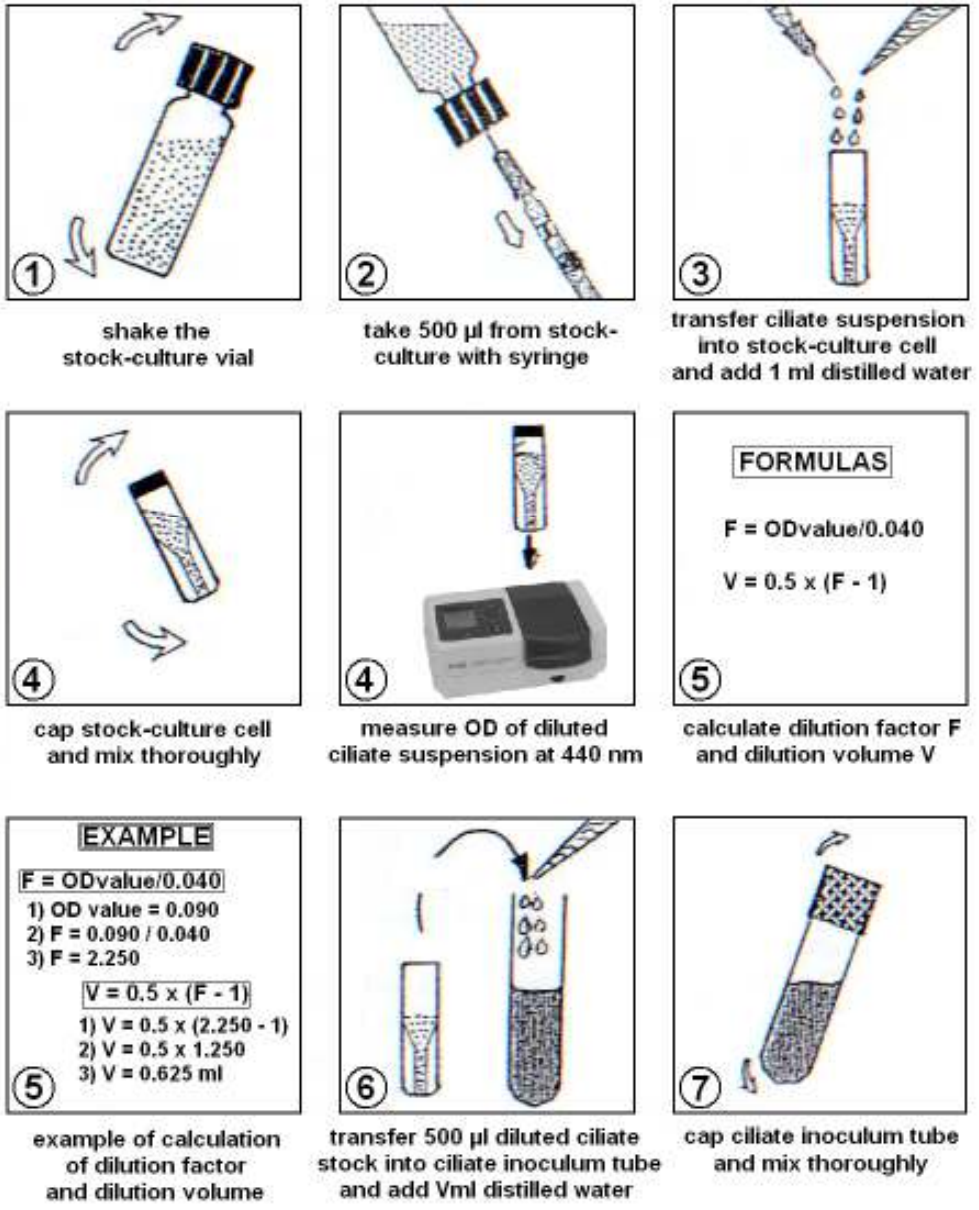
N.B. If after 24h incubation the lowest effluent concentration (6.25%) inhibits the protozoan growth (relative to the control) substantially (i.e. by close to or over 50%), a second test has to be performed with a new (higher) dilution series. The highest effluent concentration to be selected for this second dilution series is the lowest one that produced 80-100% inhibition in the first test.

TESTING OF CHEMICAL COMPOUNDS

If the approximate toxicity of the chemical compound to protozoans is known, a **definitive test** can be performed immediately. However, if no data are available on the toxicity of the chemical to unicellulars, two consecutive assays must be performed:

a) a **range finding test** to determine the 0-100% tolerance range of Tetrahymena to the toxicant,
b) a **definitive test** to determine the 50% inhibition threshold with more precision.

PREPARATION OF CILIATE INOCULUM



3. Preparation of the food suspension

(see Figure: *Preparing the Substrate)*

1. Take one vial of reconstitution medium and one vial of food substrate out of the deepfreezer and defrost the contents slowly.
2. Transfer (by micropipeting) the full contents of the vial with reconstitution medium into the food substrate tube.
3. Close the food substrate tube and mix thoroughly.

4. Inoculation of the test cells

(see Figure: *Test Procedure*)

1. Take 12 test cells and label them in pairs C0 to C5 (two cells for each dilution).
2. Add 2 ml distilled water to the two C0 cells (controls).
3. Add 2 ml from dilution tubes C1 to C5 to the respective C1 to C5 test cells (two cells for each dilution).
5. Take the food substrate tube and mix the contents thoroughly. Add 40 µl food suspension to each of the 12 test cells.
6. Take the ciliate inoculum tube and homogenize the contents by gentle shaking. Transfer 40 µl into each of the 12 test cells. Close all the cells with their lids.

N.B. The transfer of 40 µl ciliate inoculum into the test cells will result in a start concentration of (close to) 100 protozoans per ml in each of the cells.

5. Optical density measurements and incubation of test cells

1. Zero-calibrate the spectrophotometer at 440 nm, with a test cell containing 2 ml distilled water.
2. Subsequently, and after gentle shaking (by inverting the cells a few times), measure the optical density (OD) of each test cell at 440 nm (= time T0 scorings).

N.B. Make sure to position the cells in exactly the same way in the measuring equipment during the T0 scorings (look at the mark on each cuvette). The test cells must indeed all be positioned in the same way for the scoring after 24h incubation.