8. SPIROTOX TEST – *SPIROSTOMUM AMBIGUUM* ACUTE TOXICITY TEST

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1. Objective and scope of the test method

Protozoa play an important role in the environment as primary consumers. With bacteria they are major organisms in water self-purification systems. They are attractive in ecotoxicology due to their short life cycle, ease of culturing and high susceptibility to toxicants. The Spirotox test can be used as a screening tool for toxicity assessment of various kinds of environmental samples such as freshwaters and drinking waters. It can also be used for monitoring the toxicity of effluents before and after purification steps, leachates and sediment pore water. In a battery of toxicity tests it can be used for evaluating the toxicity of pure chemicals including volatile compounds.

The Spirotox test is carried out in 24-well disposable microplate. The technique is very simple. It can be performed with conventional laboratory equipment on little bench space and at low cost. As *Spirostomum ambiguum* has impressive dimensions, the observed effects can be seen even without a microscope.

2. Summary of test procedure

Spirotox is a 24-hour microplate test undertaken with a very large ciliated protozoan *Spirostomum ambiguum*. The test is carried out in 24-well microplate (6 x 4 wells). In a single microplate 5 samples can be tested in a screening assay or one sample with 5 dilutions in a definitive assay. Two endpoints can be observed with the use of a dissection microscope: sublethal effects such as deformations, shortening and immobilisation of the cell of the protozoan and lethality. The Spirotox test is usually conducted for 24 hours, however in some cases prolongation of the test to 48 hours may significantly increase its sensitivity. For some samples (organic compounds), however, a short 2-hour test may be sufficient, as 2h-EC50 values can match those of 24h-LC50's.

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Table 1. Rapid summary of the test procedure.

Test organism	Ciliated protozoan *Spirostomum ambiguum*
Type of test	acute, static
Test format	24-well disposable microplate (6 x 4 wells)
Volume contents of wells	1 ml of test solution
Test organism numbers	10 organisms per well
Incubation time	24 hours (optionally also 2 and 48 h may be used)
Incubation temperature	25 ± 2°C in darkness
Experimental configuration	a) screening test: 5 samples + control, each with 3 replicates b) definitive test: 5 dilutions of a sample + control, each with 3 replicates
Endpoints	a) sublethal deformations: EC50 b) lethality: LC50
Reference toxicants	Cd^{2+} as $Cd(NO_3)_2$; Zn^{2+} as $ZnSO_4$; SDS (sodium dodecyl sulphate)

3. Overview of applications reported with the toxicity test method

Spirostomum ambiguum has been used as a test organism in Municipal Waterworks in Warsaw (Poland) for routine monitoring of the Vistula river since 1988. The test was first carried out in glass bakers, at room temperature and a light:dark photoperiod of 16:8 h. Since 1990 intensive studies on this protozoan have been undertaken in the Department of Environmental Health Sciences, Medical University of Warsaw. A simple microplate bioassay technique with the protozoan *Spirostomum ambiguum* was first presented during the 6th International Symposium on Toxicity Assessment and On-line Monitoring in Berlin (Germany) in 1993. Since that time the methodology has been improved and the test has been applied in several fields. Initially, growth and maintenance requirements were estimated. *S. ambiguum* was found to survive more than 48 hours in a wide range of pH and total hardness. One very important finding was that the protozoa were able to live in media containing non-detectable levels of dissolved oxygen. Additionally, *S. ambiguum* can be stored for several weeks at a wide range of temperatures from 5 to 28°C.

The evaluation of the sensitivity of *S. ambiguum* began with inorganic compounds. Spirotox uses an inorganic medium as a diluent and it does not require any food during the test. In comparison with other tests Spirotox was the most sensitive to heavy metals with 24h-EC50 as low as 4, 8 and 20 µg/L for copper,

silver and mercury, respectively (Nałęcz-Jawecki and Sawicki, 1998). Additionally, 1h-EC50 results were comparable to 24h-LC50's (Nałęcz-Jawecki et al., 1995). In contrast to cations, *S. ambiguum* in a short test was insensitive to inorganic anions (Nałęcz-Jawecki and Sawicki, 1998). However, extending the test from 24 h to 96 h increased the toxicity of potassium dichromate, for example, more than 10 times.

Volatile compounds are an important class of environmental pollutants. Estimation of their toxicity in standard, multi-well test plates is not simple, due to some loss of the compounds during exposure and cross contamination of wells. The special Spirotox-volatile procedure was developed for evaluating the toxicity of organic compounds (Nałęcz-Jawecki and Sawicki, 1999). A short time after introducing the protozoa into the wells of the microplate, each well was impregnated with silicone fat, and the microplate was tightly closed with a polyethylene film. Since 2003 silicone fat and polyethylene film were replaced by an adhesive film. Following intensive studies on the toxicity of organics towards Spirotox, a database comprising more than 150 compounds was created (Nałęcz-Jawecki and Sawicki, 1999; 2002a; 2002b). The results of the Spirotox test were compared to 4 bioassays: Microtox®, *Tetrahymena pyriformis, Daphnia magna* and *Pimephales promelas*. The sensitivity of the ciliated protozoa *S. ambiguum* and *T. pyriformis* was similar for most of the tested compounds. For non-polar narcotics good correlation was found between the tests and 48h-EC50 values generated with the Spirotox test were generally 3-4 times higher than those of Microtox®, *D. magna* and *P. promelas*. In contrast, no correlation was found between the Spirotox assay and other tests for polar narcotics, electrophiles and weak acid respiratory uncouplers. In these groups some compounds proved to be much more toxic to the protozoan than to the other test organisms.

S. ambiguum can survive in a broad range of pH from 5 to 8. In this sense, it can be a valuable tool in evaluating toxicity/pH relationships. Our first paper in this field studied the relationship for nitrophenols (Nałęcz-Jawecki and Sawicki, 2003a). Such evaluations are helpful to estimate more adequate QSAR equations.

Apart from simple organics the Spirotox toxicity database contains the results for selected pesticides (Nałęcz-Jawecki et al., 2002a), drugs (Nałęcz-Jawecki and Sawicki, 2003b) and cationic surfactants (Nałęcz-Jawecki et al., 2003). In the group of 24 pesticides tested *S. ambiguum* was extremely sensitive to fungicides with 24h-EC50's of 2, 4 and 6 μg/L for dichlorofluanid, captan and thiram, respectively (Nałęcz-Jawecki et al., 2002a). The first report on the toxicity of pharmaceuticals towards freshwater protozoa showed that drugs used in the treatment of the human nervous system caused toxic effects towards *S. ambiguum* at concentrations lower than 1 mg/L (Nałęcz-Jawecki and Sawicki, 2003b). The Spirotox test was used as one element of a battery of four bioassays to investigate the biological activity of a new group of cationic surfactants (Nałęcz-Jawecki et al., 2003). The toxicity of tested compounds varied from 0.2 to 1 mg/L. Structure analysis studies showed that the presence of a long hydrophobic chain lowered the toxicity for the Microtox® test, but did not alter that in the protozoan test.

Traditionally, pulp and paper processing activities have been considered as a serious source of environmental pollution. Resin acids and phenolic compounds are

two main classes of toxic compounds that were identified in effluents of this industrial sector. A battery of bioassays comprising Spirotox, Microtox® and Thamnotoxkit F™ was used for evaluating the biological activity of effluents from pulp and paper mills (Michniewicz et al., 2000; Nałęcz-Jawecki et al., 2000). The Toxicity Equivalency Unit (TEU) approach was applied for estimating the potential toxicity of individual compounds towards aquatic organisms. Spirotox was the most sensitive to resin acids, with the exception of 12,14-dichlorodehydroabietic acid, which was most toxic to Thamnotoxkit F™. Microtox® was much more sensitive than the crustacean and protozoan to phenols and chlorophenols. TEU's for 7 effluents were calculated based on the concentrations of the 17 major toxicants and the EC(LC)50 values for individual compounds. The effluents were toxic in all bioassays conducted, especially Microtox®. However, real toxicity expressed in toxicity units (TU) was much higher than the predicted TEU values suggesting that part of the toxicants remained undetected by chemical analysis.

Cyanobacteria can produce a wide range of toxins (Namikoschi and Rinehart, 1996). Toxic and non-toxic strains can be found together. There is no simple method to distinguish toxic cyanobacterial blooms from non-toxic ones. Historically, the mouse bioassay was used to evaluate the biological activity of cyanobacteria. A battery of six bioassays was used to examine the toxicity of cyanobacterial blooms from Central Poland (Nałęcz-Jawecki et al., 2002b; Tarczyńska et al., 2000; 2001). Spirotox and Thamnotoxkit F™ were the most sensitive bioassays and their toxicity results were correlated with the microcystin LR concentration. However, following a 2-year monitoring study (Tarczyńska et al., 2001), this correlation was not confirmed, though these bioassays were also the most sensitive.

Apart from environmental applications Spirotox was used for quality control of medical devices (Nałęcz-Jawecki et al., 1997). Protozoa are unique in that they are both eucaryotic cells as well as complete, unicellular, self-sufficient organisms (Ricci, 1990). Our preliminary results (data not published) showed that *S. ambiguum* were comparably sensitive to medical device extracts as the legal tissue culture tests with mammalian lymphocytes (method performed according to the Polish Pharmacopeia). There were no false positive samples for Spirotox, while 10% of samples proved toxic in the Microtox® test and non-toxic to mouse lymphocytes used in the legal test. Clearly, the protozoan test is not meant to replace legal tests, but it can be used as a screening tool for monitoring the production of medical devices.

4. Advantages of conducting the Spirotox test

The Spirotox test is a very simple acute bioassay conducted with a ciliated protozoan. Protozoa play an important role in natural and artificial ecosystems as primary consumers and are main components of water self-purification systems. Thus, they should be incorporated in a battery of bioassays. In the Spirotox test a simple mineral medium is used as a diluent and no food is added during the test. It minimises the influence of complexation and sorption of toxicant(s) to the components of medium and food particles. *Spirostomum ambiguum* is a very large protozoan 2-3 mm long, hence, scoring of test results is very simple even with the

naked eye. Additionally, due to this " convenient size" of the protozoan, manipulation during the test is simple and transferring organisms to test vessels takes only a few minutes. The Spirotox test is carried out in disposable, standard multiwells. Dilutions of the sample are performed directly in the multiwell plate. In addition to a potentially short exposure time, testing in multiwells reduces the "consumption" of glassware and minimizes sample contamination and/or sorption to laboratory materials. Its short and simple procedure enables the initiation of more than 5 tests per hour.

S. ambiguum has simple environmental requirements (Tab. 2). It is not an anaerobic organism, but it can survive at a very low level of oxygen. Hence, different kinds of samples can be investigated including leachates and effluents with high TOC.

Table 2. Environmental requirements of S. ambiguum.

Parameters	
pH	5.0 – 8.0
Dissolved oxygen (% saturation at 25°C)	0 – 100
Total hardness ($CaCO_3$ mg/L)	0.3 – 250
Salinity (NaCl mg/L)	3 – 1100
Temperature (°C)	5 – 28

5. Test species

Spirostomum ambiguum is a very large ciliated protozoan, 2 –3 mm long, easily seen with the naked eye (Fig. 1). It has been used as a test organism for nearly one hundred years (Czerniewski et al., 1935; Seyd, 1936). Due to its size it has been a very useful organism in studies on regeneration (Seyd, 1936) and cytological and microscopic observations (Finley et al., 1964). Due to its high sensitivity to chemical, mechanical or electrical stimulation, it was a valuable tool in physiological studies (Rostkowska and Moskwa, 1968; Ettienne, 1970; Applewhite, 1972; Jones, 1966). *S. ambiguum* can be easily cultured in laboratory at low cost and with modest bench space.

Figure 1. Spirostomum ambiguum.

5.1 TAXONOMY

Phylum	Protozoa
Class	Ciliata
Order	*Spirotricha Bütschli*
Suborder	*Heterotricha Stein.*
Species	*Spirostomum ambiguum Ehrbg.*

S. ambiguum lives in small forest ponds. It has been observed in Kampinos National Park in Central Poland and in Central France (Grolière and Njine, 1973). The strain described in this paper has been cultured in Municipal Waterworks in Warsaw for more than 25 years and in our department since 1990. The strain can be obtained from the Department of Environmental Health Sciences, Medical University of Warsaw, Banacha 1, str., 02-097 Warsaw, Poland. It can survive without food up to 6 weeks within a 5 – 28°C range.

6. Culture / maintenance of organisms in the laboratory

All culturing, maintenance and toxicity testing areas should be free of potential toxicant input. Culture of the protozoa must be separated from the toxicity testing area. *Spirostomum ambiguum* is very sensitive to heavy metals especially silver and copper. Special attention should be given to water systems in terms of tap water filters and tap water installation. Do not use silver filters! If the tap water installation is made of copper, carefully rinse all materials and equipment with glass distilled or de-ionized water.

Spirostomum ambiguum can be cultured in a 5 L aquarium containing 4 L of natural, unpolluted water. Cultures should be maintained at room temperature (15 – 25°C) in darkness or in a dim light.

6.1 MATERIALS

Materials required for culturing and testing *S. ambiguum* are listed below.

Box 1. Materials required for the culturing and testing of S. ambiguum.

Glass aquaria or beakers: 5 to 10 L capacity.
Food: Flaked oats + dried alder leaves (50:1). Alder leaves are used to prevent the development of fungi. Other leaves with a high level of tannin could be used.
Graduated glass pipettes: 1 and 10 mL.
Adjustable automatic pipette (1 mL).
Automatic pipette (1 mL).
Polyethylene or glass micropipettes (0.5 mL) for transferring the test organisms.
Polystyrene or glass Petri dishes (10 x 50 mm and 15 x 100 mm).
Disposable, rigid, polystyrene microplates 24-well (6 x 4). Capacity of well ~3.5 mL.
Adhesive sealing film for multiwell plates should be used only for testing volatile compounds.
Beakers: 100, 250 and 1000 mL capacity.
Graduated cylinders: 25 and 100 mL capacity.
Volumetric flasks: 100, 200 and 1000 mL capacity.

6.2 EQUIPMENT

All equipment should be adequately maintained and regularly calibrated. Any equipment in contact with protozoa, reagents, test samples, etc. must be made of chemically inert material: glass, stainless steel and plastic, and of course, clean and free of substances which could interfere with testing.

Box 2. Equipment required for the culturing and testing of S. ambiguum.

Laboratory incubator set at 25 ± 2.0°C. Do not use incubators made of copper.
Refrigerator.
Dissection microscope with a magnification of 8 x.
pH-meter.
Millipore Super – Q™ water purification system or equivalent (glass distilled).
Analytical balance for weighing chemicals.
Magnetic stirrer.
Vortex.
Thermometer.

6.3 WASHING OF GLASSWARE

All reusable glassware must be cleaned carefully. Any glassware used in culture/ maintenance of the organism should be washed without cationic detergents. In our department an acid detergent is used as cleaning medium. Then, glassware is rinsed twice with tap water (from a non copper installation!) and rinsed with de-ionized water. Finally, the glassware is oven dried at 105°C. New glassware should be rinsed with acid (5% HNO_3) and then treated as above.

Avoid copper and silver! Use only glass or stainless steel materials. Do not use water filters made of silver. If the tap water installation in your lab is made of copper, use distilled water to rinse the glassware.

For final rinsing, de-ionized water should be used. It may be replaced with glass distilled water, but not with distilled or double distilled water, where metal containers are used.

6.4 PREPARATION OF REAGENTS AND CULTURE MEDIA

6.4.1 Culture medium

The protozoa are cultured in natural water. Different sources of water were evaluated, but no good artificial water has been found so far. Each laboratory should therefore choose a suitable water source. The chemical composition of water used in our laboratory is shown in Table 3.

Protozoa are grazed on bacteria that are fed with a mixture of flaked oats and dried alder leaves. Flaked oats can be bought in health food markets (use oats with no preservatives!). Alder leaves are added in order to prevent the development of fungi. Other leaves with a high level of tannin could be used. In our laboratory alder

leaves are collected in natural forests in spring. Then, they are dried at 60°C and comminuted. The food is prepared by mixing 1 g of leaves with 50 g of flaked oats.

Table 3. Physico-chemical composition of water sources used for culturing S. ambiguum.

Parameters	***Source 1: 230 m deep***	***Source 2: 330 m deep***
pH	7.0	6.7
Hardness ($CaCO_3$ mg/L)	104	89
Conductivity (mS)	0.76	0.66
Cl^- (mg/L)	102	94

6.4.2. Diluent

All chemicals must be of analytical grade quality. Millipore Super Q™ or glass-distilled water must be used for preparation of reagents.

As a diluent, a diluted Tyrod solution is used. Per L, it comprises: 125 mg NaCl, 3.13 mg KCl, 3.13 mg $CaCl_2$, 1.56 mg $MgCl_2$, 15.63 mg $NaHCO_3$ and 0.78 mg NaH_2PO_4. Two 100-fold stock solutions are prepared with the reagents listed below (all salts anhydrous). To start off, label two 200 mL volumetric flasks: Tyrod 1 and Tyrod 2, then add 150 mL of water to each.

Weigh each chemical and add individually to each solution. Ensure that each chemical is dissolved prior to adding the next chemical. Then, adjust the volume of solutions to 200 mL with water. Stock solutions can be stored at 4°C up to 2 months.

Box 3. Ingredients of stock solution Tyrod 1.

Sodium chloride	NaCl	2.50 g
Potassium chloride	KCl	62.6 mg
Calcium chloride	$CaCl_2$	62.6 mg
Magnesium chloride	$MgCl_2$	31.2 mg

Box 4. Ingredients of stock solution Tyrod 2.

Sodium bicarbonate	$NaHCO_3$	312.6 mg
Sodium phosphate	NaH_2PO_4	15.6 mg

6.4.3 Diluent – Tyrod solution (Tyrod)

Normal strength Tyrod solution is prepared by adding 10 mL of each stock Tyrod solutions (1 and 2) to a 1 L beaker filled with about 900 mL of water. Mix well between each addition. Use moderate mixing with the magnetic stirrer and adjust the final pH to 7.4 ± 0.2 with 1*N* HCl or 1*N* NaOH. Then the volume of the solution should be adjusted to 1 L in a volumetric flask.

6.4.4 Procedure of culturing Spirostomum ambiguum

Spirostomum ambiguum is not cultured under axenic conditions. Hence, no sterilisation of test containers and media is required. Culture should be carried out at room temperature (15-25°C), in darkness or in a dim light.

New cultures of *S. ambiguum* should be started in small, 250 mL beakers, then transferred to 1 L beakers and finally to 5 L aquaria.

- Pour 200 mL of culture water into a 250 mL beaker and add 200 mg of food.
- Cover the beaker with Petri dish and leave it at room temperature for 2 days.
- Inoculate the culture with the protozoa. The organisms are sent to the laboratory in ampoules in culturing medium, which also contain an inoculum of bacteria. Whole contents of the ampoule should be poured into the beaker.
- Once a week add 100 mg of food to the beaker.

After 4 weeks transfer the culture to a 1 L beaker, prepared as follows:

- Pour 600 mL of culture water into the beaker and add 400 mg of food.
- Cover the beaker and leave it at room temperature for 2 days.
- Inoculate the protozoa by pouring the contents of the small beaker to the 1 L beaker.
- Once a week add 100 mg of food to the beaker.

After 4 weeks transfer the culture to a 5 L aquarium, prepared as follows:

- Pour 3.5 L of culture water into a 5 L aquarium and add 1 g of food.
- Cover the aquarium and leave for 2 days.
- Inoculate the protozoa by pouring ½ of the culture from the 1 L beaker into the aquarium.

6.4.5 Maintenance of the culture

A minimum of three aquaria should be operating at the same time. Every working day the culture should be observed carefully. The culture is healthy if the protozoa are swimming in the whole volume of medium or if they are ”grazing” on the flaked oats. Twice a week the protozoa should be observed under the dissection microscope and pH of the medium should be measured. The change of pH must not be greater than 0.5. Every week ½ of the water should be replaced with a fresh supply and 0.5 g of food should be added. Every month a new aquarium should be prepared and inoculated with 500 mL of the old culture.

7. Preparation of protozoa for testing

Before testing the protozoa should be separated from the culture medium. Using a 10 mL glass pipette transfer the dense culture of protozoa from the bottom of the aquarium to a 25 mL capacity graduated cylinder. Fill the cylinder with Tyrod solution. Wait a few minutes until the protozoa drop to the bottom of the cylinder. (Do not wait too long! After an additional few minutes the protozoa will start swimming in the whole medium and you will have to start from the beginning). Then carefully pour out as much medium as you can. Fill the cylinder with the Tyrod solution and repeat the rinsing three times. Finally transfer the suspension of cells in the Tyrod solution to a small Petri dish (50 mm of diameter).

8. Testing procedure

8.1 INFORMATION/GUIDANCE REGARDING TEST SAMPLES PRIOR TO CONDUCTING BIOASSAYS

8.1.1 Chemicals

For the health and safety of laboratory personnel, physical, chemical and toxicological (if available) properties of the substance(s) to be tested should be obtained. Stock solutions of each substance should be prepared in MilliQ water. Stock solutions of chemicals not readily soluble in water may be prepared by using organic solvents, *e.g.,* methanol, acetone and DMSO (dimethylsulphoxide). If solvent is used to dissolve a chemical in preparation for testing, an additional solvent control must be incorporated into the experiment at the highest concentration used.

If the pH of a stock solution is outside the 5-8 pH range, it must be adjusted to the nearest border (*i.e*., samples with an initial pH lower than 5 should be adjusted to 5 and samples with an initial pH exceeding 8 should be adjusted to 8).

For volatile compounds a special procedure should be performed.

8.1.2 Environmental samples

Environmental samples should be collected according to standard procedures. Samples are usually placed in clean, labelled containers of inert material, filled to the brim (minimal headspace) and transported in the dark on ice. Twenty mL of sample are sufficient for conducting the Spirotox test from the range-finding to the definitive test, although it is recommended to collect 0.5-1 L of sample if physico-chemical measurements are also to be made. Environmental samples should be tested as soon as possible but no longer than 3 days after collection. Prior to testing, samples should be stored in a refrigerator at 4°C.

As the Spirotox test is based on visual observations of protozoa under the dissection microscope, suspended solids and coloured samples are not sources of interference. Hence, there is no necessity to filter the sample. If the pH of a sample lies outside a 5-8 pH range, it must be adjusted to the nearest border (*i.e*., samples with an initial pH lower than 5 should be adjusted to 5 and samples with an initial pH exceeding 8 should be adjusted to 8).

8.2 SELECTING A TESTING PROCEDURE

The experimental procedure depends on the type of sample and objective of the assay. Two main procedures can be performed utilizing a screening assay and one requiring dilutions. A screening test (see Section 8.4) helps to identify toxic samples from a large number of (possibly non toxic) samples for further definitive assays. For example, a screening test should be carried out for evaluating the toxicity of drinking water sources and/or low-contamination freshwaters. If the toxicity of a sample is unknown and unpredictable, a range-finding dilution assay (see Section 8.5) should be performed with 1 L dilutions ranging from 100 to 0.1%. If the approximate toxicity of a sample is known, a definitive dilution test (see Section 8.6) can be carried out and EC(LC)50 values estimated.

8.3 SELECTING TEST CONCENTRATIONS

If the approximate toxicity of a sample to *S. ambiguum* is known after a screening test, test concentrations are prepared that will encompass a range of responses from 0% to 100%.

For chemicals, whose toxicity is unknown, stock solutions should be prepared at the following concentrations.

- For water-soluble compounds the highest tested concentration is usually 100 mg/L. Only for special purposes (*e.g*, solvents tests) will concentrations exceed 1000 mg/L.
- For compounds of low solubility in water the concentrations should be close to their solubility limit.
- If an organic solvent is used, the stock solution should be at least 50 times more concentrated that the highest test concentrations. The concentration of the solvent in the test should not be higher than the NOEC (no observed effect concentration). The NOEC for methanol, ethanol and acetone for Spirotox is 2%.

In the range-finding test, numerous concentrations are assayed (*e.g.,*: 100; 50; 25; 12.5; 6.25; 3.12; 1.56; 0.78; 0.39; 0.20; 0.10%). From the results of the range-finding test, the concentrations for the definitive test should be chosen. Under ideal conditions the test should include at least one concentration that will have no effect and at least one concentration that will kill all the protozoa. However, EC(LC)50 values can be estimated if at least one concentration causes a toxic effect below 50% and at least one above 50%.

8.4 SCREENING TEST

The test design incorporates one concentration of the undiluted sample in 3 replicates. Five samples (and control) can be assayed in one multiplate (Fig. 2). A rinsing row serves to prevent dilution of the toxicant during the transfer of test organisms from a Petri dish to the test wells.

	1	**2**	**3**	**4**	**5**	**6**
A	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Control
B	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Control
C	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Control
D	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Control

R I N S I N G R O W

Figure 2. Configuration of a microplate in the screening test.

The screening test procedure is explained in Box 5.

Box 5. Screening test.

Prepare a sufficient number of multiwells (one per 5 samples).

Dispense 1 mL of each tested sample into all 4 wells of one column.

Dispense 1 mL of Tyrod solution into the wells of column No 6 in each multiplate.

Transfer the protozoa into the wells with a micropipette. A glass or a plastic micropipette may be used. Transfer is usually carried out under a dissection microscope at a magnification of ~8 x. It is also possible to use a magnifying lens.

Place the Petri dish with the rinsed protozoa (see Section 7) under the dissection microscope. While looking at the end of the micropipette, catch approximately 40 protozoa. Dispense this number into each well of rinsing row (D). Do not touch the sample with the pipette to avoid contamination! If that happens change or rinse the pipette.

Transfer exactly 10 protozoa from each rinsing well to the corresponding wells in each column of the microplate. Change micropipette or rinse it with Tyrod after each sample.

8.5 RANGE-FINDING TEST

The range-finding test is a preliminary assay designed to establish the approximate toxicity of an unknown sample. Test design incorporates a control and eleven 2-fold dilutions of tested sample in two replicates. Figure 3 illustrates the experimental disposition of a microplate.

	1	2	3	4	5	6
A	Sample 100 %	Sample 50%	Sample 25%	Sample 12.5%	Sample 6.25%	Sample 3.12%
B	**Control**	Sample 0.10%	Sample 0.20%	Sample 0.39%	Sample 0.78%	Sample 1.56%
C	Sample 100 %	Sample 50%	Sample 25%	Sample 12.5%	Sample 6.25%	Sample 3.12%
D	**Control**	Sample 0.10%	Sample 0.20%	Sample 0.39%	Sample 0.78%	Sample 1.56%

Figure 3. Configuration of microplate in the range-finding test.

The insertion of the control between the highest concentration of sample is meant to check for the presence of volatile compounds. If this occurs, control mortality is observed. In this case a special Spirotox-volatile procedure should be performed.

The range-finding test procedure is explained in Box 6 and schematised in Figure 4. Sample dilutions are prepared directly in the multiplate.

Box 6. Range-finding test.

Dispense 1 mL of Tyrod into all wells of the microplate with the exception of A1 and C1.

Dispense 1 mL of a tested sample into A1, A2, C1 and C2.

Using the same pipette and pipette tip mix the contents of A2 by withdrawing and dispensing the sample 5 consecutive times.

Transfer 1 mL from A2 to A3 and mix contents with the pipette. Continue this process until A6, then transfer 1 mL from A6 to B6, and continue this process until B2. Discard 1 mL of sample from B2 to a waste container. B1 is the control well.

Repeat this dilution procedure in rows C and D.

Transfer protozoa into the wells with a micropipette. A glass or a plastic micropipette may be used. The transfer is usually carried out under a dissection microscope at a magnification of ~8 x. It is also possible to use a magnifying lens.

Place the Petri dish with the rinsed protozoa (see Section 7) under the dissection microscope. While looking at the end of the micropipette, catch exactly 10 protozoa. Drop them into each well starting from the controls. Do not touch the sample with the pipette to avoid contamination. If that happens change or rinse the pipette.

Verify that 10 organisms are actually in each well of the microplate.

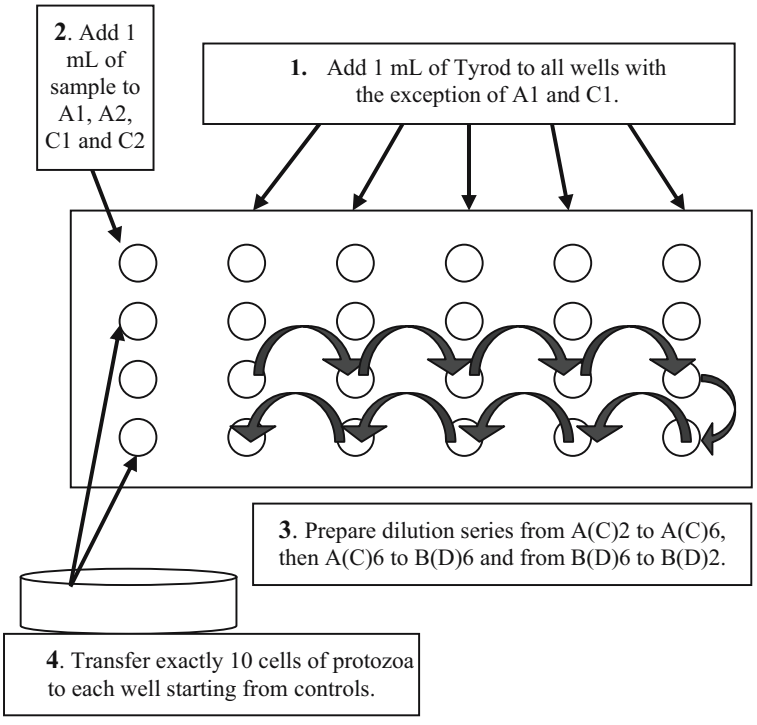

Figure 4. Range-finding test.

8.6 DEFINITIVE TEST

The definitive test is an assay designed to establish the precise toxicity of a sample. Test design incorporates a control and five 2-fold dilutions of tested sample with three replicates. Figure 5 displays the experimental disposition of a microplate.

	1	2	3	4	5	6
A	Sample 100 %	Sample 50%	Sample 25%	Sample 12.5%	Sample 6.25%	Control
B	Sample 100 %	Sample 50%	Sample 25%	Sample 12.5%	Sample 6.25%	Control
C	Sample 100 %	Sample 50%	Sample 25%	Sample 12.5%	Sample 6.25%	Control
D	Sample 100 %	Sample 50%	Sample 25%	Sample 12.5%	Sample 6.25%	Control

R I N S I N G R O W

Figure 5. Configuration of microplate in the definitive test.

A rinsing row serves to prevent dilution of the toxicant during the transfer of the test organisms from a Petri dish to the test wells. The procedure of the definitive test is explained in Box 7 and schematised in Figure 6. Sample dilutions are prepared directly in the multiplate.

Box 7. Definitive test.

Dispense 1 mL of Tyrod into all wells of the microplate with the exception of column 1.

Dispense 1 mL of a tested sample into wells in the columns 1 and 2.

Using the same pipette and pipette tip, mix the contents of A2 by withdrawing and dispensing sample 5 consecutive times.

Transfer 1 mL from A2 to A3 and mix contents with the pipette. Continue this process until A5. Discard 1 mL of sample from A5 to a waste container. A6 is the control well.

Repeat this dilution procedure in rows B, C and D.

Transfer protozoa into the wells with a micropipette. A glass or a plastic micropipette may be used. The transfer is usually carried out under a dissection microscope at a magnification of ~8 x. Yet it is also possible to use a magnifier.

Place the Petri dish with the rinsed protozoa (see Section 7) under the dissection microscope. Looking at the end of the micropipette, catch approximately 40 protozoans. Dispense them into each well in the rinsing row (D). Do not touch the sample with the pipette to avoid contamination! If that happens change or rinse the pipette.

Transfer exactly 10 protozoa from the rinsing wells to the corresponding wells in the column of the multiwell starting from the control.

8.7 EXPOSURE CONDITIONS

Experimental microplates are placed in an incubator set at 25 ± 2°C, without illumination. Total exposure time is 48 h.

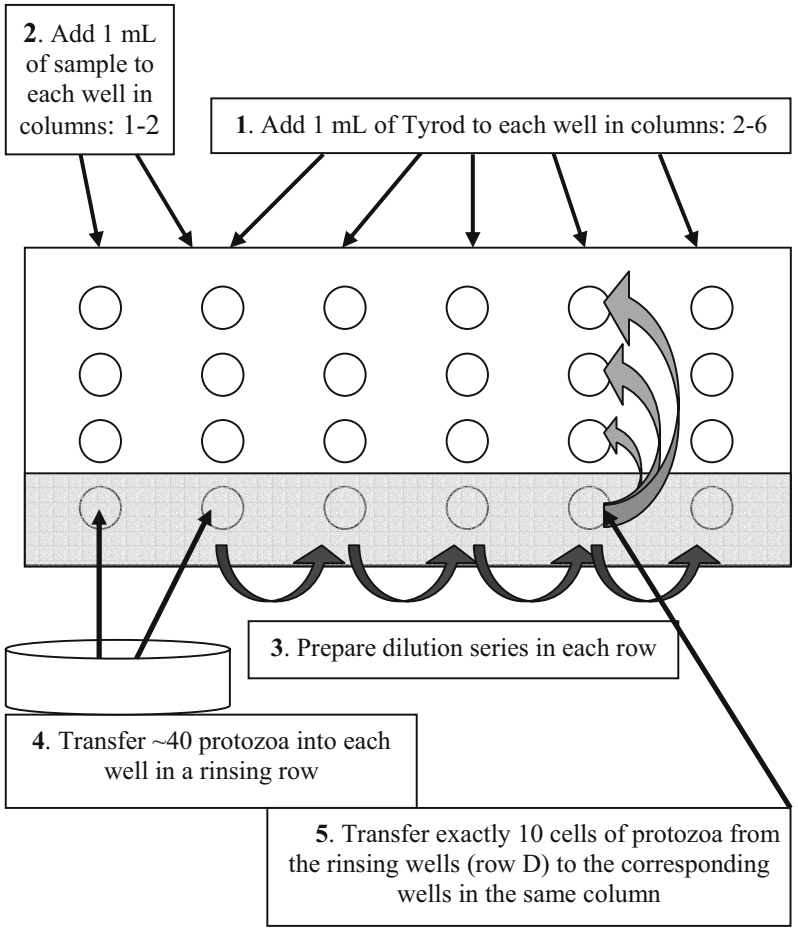

Figure 6. Definitive test.

9. Post-exposure observations/measurements and endpoint determinations

9.1 ENDPOINTS

Two toxicity effects can be observed with the use of a dissection microscope (magnification of 8 x).

- Sublethal responses: such as bending, shortening of the cell and immobilisation of the protozoan. Some deformations of the protozoan are presented in Figure 7.
- Lethal response: spherical deformation and autolysis. After autolysis the protozoa disappear, so one must be sure that they were added into the well of the microplate! A dead *S. ambiguum* is shown in Figure 8.

Figure 7. Sublethal deformations of Spirostomum ambiguum.

Figure 8. Autolysis of Spirostomum ambiguum.

9.2 SCORING THE RESULTS

- Place the multiwell under the dissection microscope.
- Check all the wells of row A, B and C and record the number of living normal and deformed (def) protozoa in each well. Subtract the number of living (normal and deformed) cells from 10 = the number of dead organisms (let). Report the number of dead and deformed protozoa on the data sheet. Keep in

mind that if the protozoan is scored as dead, it was first deformed and should also be considered as deformed (see example below).

– Calculate the % of sublethal responses (% def) and lethal responses (% let) in each column of the microplate.

Table 4. Scoring of the results – an example.

Row	***Normal protozoa***	***Deformed protozoa (def)***	***Dead protozoa (let)***
A	3	3	10 – (3+3) = 4
B	4	2	10 – (4+2) = 4
C	3	4	10 – (3+4) = 3
Effects (Σ)	-	def = 9 + 11	let = 11
Effects (%)	-	% def = (9+11)/30 x 100 = 67%	% let = 11/30 x 100 = 37%

Table 5. Spirotox data sheet.

24 h	***100%***		***50%***		***25%***		***12.5%***		***6.25%***		***Control***	
	def	***let***	***def***	***let***	***def***	***let***	***def***	***let***	***def***	***let***	***def***	***let***
A	-	10	5	5	2	1	1	-	-	-	-	-
B	-	10	2	8	5	2	-	1	-	-	-	1
C	-	10	7	3	3	-	1	-	2	-	1	-
Effects (Σ)	0+30	30	14+16	16	10+3	3	2+1	1	2+0	0	1+1	1
Effects (%)	100	100	**100**	**53**	**43**	**10**	10	3	7	0	7	3

9.3 ENDPOINT DETERMINATION

The endpoint reported depends on the type of test. In the screening test percent of effects (% let and % def) caused by the sample are presented. In the definitive test EC50 and LC50 values are typically calculated. An EC50 is a sample concentration causing 50% of sublethal effects. An LC50 is a sample concentration causing 50% mortality of exposed organisms.

9.3.1 Screening test

– If the % def value is lower than 20% the sample is considered non toxic.
– If the % def value is between 20 and 50% the sample is considered somewhat toxic.
– If the % def value is greater than 50% the sample is considered toxic and a

range-finding and/or definitive test should be performed to estimate the EC(LC)50 value.

9.3.2 Definitive test

Calculate the % def and % let values and report results on the data sheet.

There are several procedures for calculating EC(LC)50 values. Methods used to estimate the EC(LC)50 from multi-concentration tests depend on the number of partial deformities (sublethal effects) and mortalities (lethal effects) observed. *S. ambiguum* rarely gives partial mortality in more than one concentration. A simple, graphical procedure described below is sufficient in most cases. It is based on the U.S. EPA method (Weber, 1993). The procedure is described below in Box 8 and in Figure 9. If the results are scored not only after 24 h but also after 48 h and/or 2 h, the calculation should be made for each time period.

Box 8. Graphical procedure for estimating EC(LC)50 values.

Choose two % def values: one lower than 50% and the other greater than 50%. See bolded values in Table 5.

Indicate the concentrations on the Y-axis and corresponding % def values on the X-axis.

Connect the plotted points with a straight line.

Read the EC50 value at the intersection of the plotted line and the vertical 50% effect line.

Estimate the LC50 value in the same way.

EC50 and LC50 values from data presented in Table 5 are 27% and 48%, respectively.

This graphical procedure can be carried out with any computer programme that allows calculation of log values (*e.g.*, MS Excel). The macro can be obtained from the author of this chapter (grzes@farm.amwaw.edu.pl).

9.4 CONDITIONS FOR VALIDITY AND BUILT-IN QUALITY CONTROL

9.4.1 Conditions for test validity

The test is valid if toxic effects (both deformations and lethal effects) observed in control wells do not exceed 10%.

9.4.2 Built in quality control

Deviation from normalcy (in the case of a test result with a reference toxicant) may indicate a change in laboratory performance (health of test organisms, culture, contamination, faulty diluent, improper washing of glass or procedural error). Three reference toxicants were chosen for the Spirotox test:

Cd^{2+} as $Cd(NO_3)_2$; Zn^{2+} as $ZnSO_4$; SDS (sodium dodecyl sulphate)

The reference tests should be performed with the same batch of protozoan culture and the same batch of Tyrod stock solutions.

Reference toxicant data should be within ±2 standard deviations of values obtained in previous tests. Data based on 50 experiments performed by the Department of Environmental Health Sciences, Medical University in Warsaw, Poland, yielded the average values shown in Table 6.

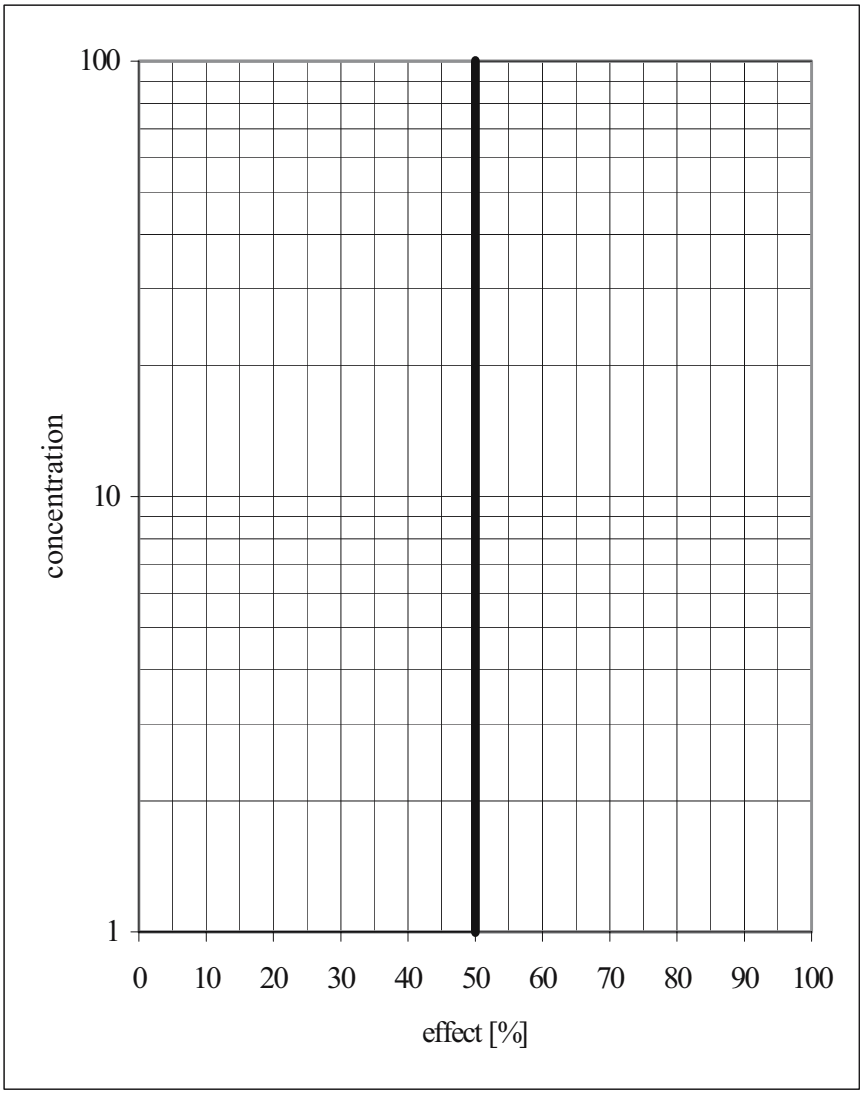

Figure 9. Graphical interpolation sheet.

Table 6. Reference toxicant data for 24h LC50's.

Reference toxicant	*Mean (mg/L)*	*± SD*	*Range (± 2 SD)*
Zn^{2+}	0.472	0.101	0.270 – 0.674
Cd^{2+}	0.224	0.050	0.124 – 0.324
SDS	6.92	1.83	3.26 – 10.58

10. Factors capable of influencing performance of test organism and testing results

Toxicity tests, regardless of their degree of standardisation, can be subject to various factors linked to procedure that will limit their applications if they are not properly addressed. Some such factors may be excluded by application of special procedures (volatile compounds). Factors having the potential to adversely influence testing results are briefly recalled and commented below.

10.1 ADHERENCE OF CHEMICALS TO WELLS

Because small 1 mL wells are used, high area to volume ratio can cause great toxicant adhesion on the test containers. The problem is most crucial, when very low concentrations of toxicants are tested. Some researchers do not use plastic microplates due to potential toxicant adhesion on wells. Similarly, toxicants can adhere to glass beakers (*e.g.*, metals), especially when beakers are used repeatedly. Since affinity for plastic or glass is chemical-dependent, no material is always the most convenient.

10.2 VOLATILE COMPOUNDS

For testing volatile compounds the special Spirotox-volatile procedure should be applied, in which each well is tightly closed with a plastic film.

10.3 LOW WATER-SOLUBLE COMPOUNDS

In testing low water-soluble compounds some precipitation of the tested substance can occur. This can lead to two specific problems: increased toxicity due to suspension uptake by protozoa or lower toxicity due to a decreasing concentration of the substance. If precipitation is linked to evaporation of an applied organic solvent, the Spirotox-volatile procedure can be employed. If no obvious reason can be accounted for, any noted precipitation effect should be reported.

10.4 MISCELLANEOUS FACTORS

All types and brands of 24-well polystyrene microplates may not be adequate for the test. Some microplates may be toxic to the protozoa. It is highly recommended to

thoroughly investigate new brands/types of microplates with reference toxicants before using them in toxicity tests.

11. Application examples (case studies) with the protozoan toxicity test

An application of the Spirotox test described in this chapter was first presented during the 7th Meeting of the Central and Eastern European Regional Section of SECOTOX (Society of Ecotoxicology and Environmental Safety) in Brno in the Czech Republic (Nałęcz-Jawecki et al. 2002c).

During the 1960's and 1970's unwanted pesticides were deposited in several hundreds of tombs all over Poland. After a few years the tombs started to leach out chemicals into ground water. During liquidation and remediation works 30 ground water samples were collected in the vicinity of 8 tombs. Their toxicity was evaluated with the following battery of bioassays: Microtox®, Spirotox, Protoxkit F™, Thamnotoxkit F™ and Daphnia test. First, screening tests were performed (Tab. 7) followed by definitive tests with the toxic samples (Fig. 10). EC(LC)50 results were then transformed into toxic units [TU = 100%/EC(LC)50].

Fifty seven percent of samples were not toxic according to the Spirotox test. Four samples (13%) caused toxicity effects between 20 and 50%, and were considered somewhat toxic. Nine samples (30%) were toxic in the Spirotox test and a definitive test was then performed (Fig. 10).

Table 7. Evaluation of ground water sample toxicity in the Spirotox screening test.

Sample	***% def***	***Sample***	***% def***	***Sample***	***% def***
M1	10	R1	13	S1	**100 (T)**
M2	**100 (T)**	R2	0	S2	**100 (T)**
M3	13	R3	3	S3	**100 (T)**
B1	**100 (T)**	P1	0	W1	**100 (T)**
B2	7	P2	7	W2	0
B3	10	P3	0	W3	10
B4	17	P4	**100 (T)**	K1	**100 (T)**
B5	**90 (T)**	D1	30 (ST)	K2	45 (ST)
B6	0	D2	16	K3	30 (ST)
B7	3	D3	13	K4	33 (ST)

T – toxic sample
ST – somewhat toxic sample
def - deformities

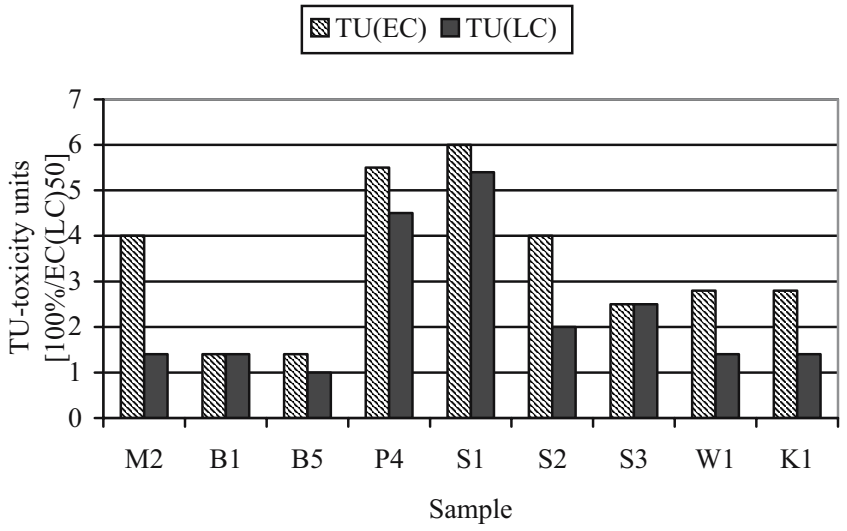

Figure 10. Toxicity of ground water samples in the definitive Spirotox test.

12. Accessory/miscellaneous test information

Laboratory personnel need not be specifically educated in biological/microbiological techniques, but must be trained in analytical techniques such as weighing, pipetting etc. Considerations for safety must be in place prior to carrying out tests with toxic substances and effluents, which may be not only toxic, but also infectious.

In terms of applicability, the Spirotox test can be carried out on any liquid medium, including coloured samples, suspensions and samples with low dissolved oxygen levels. Its use was reported for effluents, ground and surface waters, solid waste leachates and different extracts. At present, most of the published data concern the sensitivity of the test towards inorganic and organic compounds.

An experienced operator can easily initiate 5 microplate tests per hour. Twenty samples can be diluted and microplates filled in one-half of a work day. Post exposure counting is simple and takes only a few minutes per microplate, so scoring results and data reduction can be done during another one-half day. Hence, a batch of 20 microplates could be processed daily, depending on the time necessary for sample preparation (*e.g.*, adjusting of pH).

Costs to process 20 samples depend on the purchase price of microplates (the most expensive material) and wages of laboratory personnel.

The Spirotox test is currently performed in four scientific and university laboratories in Poland and in Municipal Waterworks in Warsaw (Poland).

13. Conclusions/prospects

The Spirotox test was introduced as a simple and low-cost toxicity test with ciliated protozoans. Protozoa play an important role in the environment as primary consumers. With bacteria they are major organisms in water self-purification systems. Hence, they should be incorporated into a battery of bioassays.

In order to explore new fields of application for this test, a sediment/soil direct contact procedure is now under investigation. By way of this direct contact test, cells are also exposed to particle-bound substances of low water solubility. Direct contact tests are of interest because they have higher ecological relevance than tests performed on pore waters or solvent extracts.

New endpoints allowing shorter exposure times (1-2 hours) are also being explored. They include physiological observations of food uptake and biochemical techniques with fluorescent dyes. Assuming that such tests are found to be sufficiently sensitive, they would then have useful applications as screening tools in the assessment of waterworks.

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Abbreviations

def	deformed
% def	% of sublethal deformity responses
EC50	sample concentration causing a 50% sublethal effect
DMSO	dimethylsulfoxide
LC50	sample concentration killing 50% of exposed organisms
let	lethal
% let	% of lethal response
NOEC	no observed effect concentration
QSAR	Quantitative structure-activity relationship
SDS	sodium dodecyl sulfate
ST	somewhat toxic sample
T	toxic (sample)
TEU	Toxicity Equivalency Unit
TOC	Total Organic Carbon
TU	Toxicity Units.