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Protocols belonging to the report 'Toxicity measurements in concentrated water samples'

(RIVM Report 607013010/2010)

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Abstract

Protocols belonging to the report 'Toxicity measurements in concentrated water samples'

This report contains protocols for bioassays, i.e. technical descriptions of how bioassays should be carried out. Using this information, researchers can perform these methods in exactly the same way as the Dutch RIVM and Centre for Water Management.

Bioassays use living organisms to gain insight in water quality, in contrast with chemical methods. An earlier report (RIVM-report 607013010 and Centre for Water Management report 2009.003) summarised the information about bioassays, but the protocols were still missing. The current report fills this gap.

Key words: bioassays, protocols

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Rapport in het kort

Protocollen behorend bij het rapport 'Metingen van toxiciteit in geconcentreerde watermonsters. Evaluatie en validatie'

Dit rapport bevat protocollen voor bioassays, oftewel de technische beschrijvingen hoe zij moeten worden uitgevoerd. Met deze informatie kunnen onderzoekers de methoden op exact dezelfde manier uitvoeren als het RIVM en de Waterdienst.

Bioassays zijn methoden die in tegenstelling tot chemische methoden, met behulp van levende organismen inzicht geven in de waterkwaliteit. In een eerder rapport (RIVM-rapport 607013010 en Waterdienst-rapport 2009.003) is de bestaande informatie over bioassays samengevat, maar de protocollen ontbraken nog. Dit rapport voorziet in deze lacune.

Trefwoorden: bioassays, protocollen

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Summary

A previous RIVM/Centre for Water Management-report discussed the evaluation and validation of bioassays. In an annex, it was mentioned that the protocols, necessary to perform the bioassays in exactly the same way as RIVM and Centre for Water Management do, would be published later. In the current report, all the protocols are included.

1 Introduction

In RIVM-report 607013010, entitled ‘Toxicity measurements in concentrated water samples. Evaluation and validation’, is referred to protocols for the execution of bioassays in appendix I at page 107 (RIVM, 2009). Publication of the protocols is useful to complete the extended documentation given in that report. However, not all users will be interested in the level of detail given in the protocols. Therefore, this separate report containing the protocols is published only digitally, allowing any one who is interested to read how the bioassays are performed. In the next chapters, protocols will be given for sampling, sample treatment, and seven bioassays (the protocol for Daphnia immobility test was not found).
The intention is that the protocols are readable independently. For that reason, some information is repeated in many chapters, e.g. in footnotes. For the same reason, references are given on a per-chapter basis.
RIVM often applies the bioassays mentioned in this report in combination with the sampling and pretreatment procedures as described in chapter 2 and 3. However, the bioassays could be applied with other sampling or pretreatment procedures. Therefore, the description of the bioassays applies to all samples, no matter the pretreatment.

Chapter 2 through 9 were originally drafted by AquaSense, chapter 10 is based on Pieters et al. (2008).

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2 Sampling and preservation of surface water for use in toxicity tests

Keywords: monitoring, surface water, sampling, preservation

2.1 Subject

This protocol describes the methods by which surface water samples should be taken for use in toxicity tests. It also describes the procedures for preserving the samples. The methods described apply to both manual and automated sampling (random samples in both cases). Surface water samples will generally undergo pretreatment before being used in toxicity tests. For a description of this procedure, see chapter 3, 'Sample pretreatment for use in toxicity tests'.

2.2 Area of application

The protocol described here is primarily intended for sampling in surface water, though it can in principle be used for any water system.

2.3 Principle

Sampling and preservation are vital elements of toxicity tests on surface water samples or extracts thereof. Many factors can influence the toxicity of a sample, including homogeneity, stratification, contamination, adsorption, evaporation, photodegradation, biodegradation, physical-chemical transformations and hydrolysis. To minimise these negative impacts, samples must be taken and preserved in the correct way.

2.4 Terms and definitions

Random sample:	sample taken at a random time at a random sampling point
PE, PP, PC:	polyethylene, polypropylene, polycarbonate
Stratification:	layered structure

2.5 Reagents and additives

No additives and/or preservatives are used to preserve samples for use in toxicity tests.

2.6 Equipment

2.6.1 Sampling (manual)

- Buckets (**PE, PP or PC**) with spout and cord (10 litres)
- Funnel (plastic)
- Jug (1 litre, plastic)
- 25-litre sealable sampling vessels (**stainless steel**)
- Writing materials
- Sampling form
- Labels
- Personal protection equipment
- Refrigerated storage (4 °C)

2.6.2 Sampling (automatic)

- Pumping system (peristaltic, centrifugal or submersible pump), minimum pumping speed 0.5 m/s
- Pump hoses (*silicon only for use with peristaltic pumps*)
- Clamps
- Pulley extension cable 220 V
- Water hoses
- Connectors
- Generator (220 V), if no electricity supply is present
- 25-litre sealable sampling vessels (**stainless steel**)
- Writing materials
- Sampling form
- Labels
- Personal protection equipment
- Refrigerated storage (4 °C)

2.7 Sampling method

Implementation (manual)

- Check you have all the material needed, including the correct number of (clean) sampling vessels.
- Check the location details and sampling points. Take particular note of any possible currents and stratification.
- Ensure a clean working environment at the sampling point, free of dust, exhaust fumes, solvents and fuels.
- Fill the bucket with the water for sampling by lowering it gently below the surface to a depth of approximately 10 to 15 cm.
- Ensure that the bucket does not scrape the side of the boat and/or bank as you raise it.
- Rinse the funnel and jug using the sample.
- Rinse the sampling vessels at least twice using the sample.
- Fill the sampling vessels with the sampled water, regularly stirring the sample in the bucket to guarantee the homogeneity of the sample (suspended particulate matter).
- Ensure that the sampling vessels are filled to the brim, with no air bubbles above the liquid.
- Seal the sampling vessels immediately.

- Ensure that the sample is fully and correctly coded. Note at least the following on the sampling vessel: type of sample, date and time of sampling, location details, client/sender, code (if desired).
- Complete the sampling form in full.
- Preserve the samples according to instructions.

Implementation (automatic)

- Check you have all the material needed, including the correct number of (clean) sampling vessels.
- Check the location details and sampling points. Take particular note of any possible currents and stratification.
- Ensure a clean working environment at the sampling point, free of dust, exhaust fumes, solvents and fuels.
- Submerge the suction point of the pump below the water surface to a depth of 50-100 cm, with the inlet towards the direction of flow. The pump must be at least 50 cm above the bed. When sampling from a boat, the suction point must be at least 1.5 m from the hull, and far enough upstream to guarantee the free flow of surface water.
- Set the pump to the required flow rate (see section 6.2).
- Allow the system to flush through for 5-10 minutes before commencing sampling.
- Ensure that no air bubbles enter the pump system.
- Prevent coarse particles from entering the sampling by fitting a filter (wide mesh) or gauze (plastic) in front of the suction point.
- Rinse the sampling vessels at least twice using the sample.
- Fill the sampling vessels with the sampled water.
- Ensure that the sampling vessels are filled to the brim, with no air bubbles above the liquid.
- Seal the sampling vessels immediately.
- Ensure that the sample is fully and correctly coded. Note at least the following on the sampling vessel: type of sample, date and time of sampling, location details, client/sender, code (if desired).
- Complete the sampling form in full.
- Preserve the samples according to instructions.

2.8 Preservation of surface water samples

- After sampling the samples should be stored as soon as possible in a cool (4 °C) dark place.
- Transport the samples to the test laboratory immediately (within 24 hours). Ensure that the samples are kept in cool (4 °C) dark conditions during transportation.
- If the samples cannot be processed immediately (within 24 hours) at the test laboratory, they should be frozen (-20 °C).
- Small portions of no more than 1 litre should be frozen for use in toxicity tests. The portions should be divided into PE sample pots. The pots should be filled to no more than 80% of their total volume and sealed airtight. Before the pots are filled, they should be rinsed with the sample. Stir the sample regularly when distributing it among the pots to ensure it is homogeneous.
- If several sampling vessels have been used at each location (sampling point), their contents should first be mixed to form a single combined sample before it is divided among the sample pots.
- If the surface water sample is to be used for sample conversion (XAD), at least 20% should be removed from the 10-litre sampling vessel before it is placed in the freezer. If several vessels have been used at each location (sampling point), they need not be combined to form a single sample. This will take place after XAD extraction.

2.9 Reporting

Sampling should be reported on the specially designed form, which can be found in the section 'Sampling form' later in this chapter.

2.10 Quality assurance

Quality assurance focuses on the sampling procedures and the conduct of staff. The following factors are particularly important:

- flow patterns in the sampling area;
- possible stratification at the sampling point;
- homogeneity of the surface water at the sampling point;
- use of correct, clean sampling material;
- accurate coding of the sampling vessels.

2.11 Safety

- Wear appropriate protective clothing during sampling (safety goggles and gloves).
- Ensure that the sampling vessels are secured during transportation.
- Be aware of danger at sampling locations (slippery surfaces etc.).

2.12 Maintenance

The specific equipment used for automatic sampling does not require regular maintenance or calibration.

2.13 References and additional information

More information can be found in:

RIZA/RIKZ (1996) GMP. Rijkswaterstaat voorschrift nr. 913.00.W001. Monsterneming van oppervlaktewater met behulp van emmer.

RIZA/RIKZ (1996). GMP. Rijkswaterstaat voorschrift nr. 913.00.W002. Monsterneming van oppervlaktewater met behulp van pompsysteem.

Maagd, G-J. (2000). Monstername en monstervoorbehandeling in Totaal-Effluentbeoordeling. RIZA, januari 2000.

ISO 5667-6 (1990). Water quality, Sampling, Part 6: Guidance on sampling of rivers and streams.

ISO 5667-4 (1987). Water quality, Sampling, Part 4: Guidance on sampling from lakes, natural and man-made.

2.14 Sampling form

Date	Time	Code	Sample type	Location	Preservation	Remarks

3 Sample pretreatment for use in toxicity tests

Keywords: XAD, extracts, concentrate, eluate, microcontaminants, monitoring, surface water

3.1 Subject

This protocol describes the methods by which surface water samples should be pretreated for use in toxicity tests. The conversion process consists of three elements, which eventually produce a concentrated water sample for use in toxicity tests. The protocol is based on methods developed by the National Institute for Public Health and the Environment's Ecotoxicology Laboratory (presently called Laboratory for Ecological Risk Assessessment) in Bilthoven.

3.2 Area of application

The protocol described here is primarily intended for surface water, though it can in principle be used for any water sample.

3.3 Terms and definitions

XAD:	polymeric adsorbent resin (Amberlite)
XAD-4:	polystyrene divinyl benzene copolymer resin
XAD-8:	polymethyl methacrylate resin
Solid phase extraction:	extraction of liquids using a solid adsorbent
Eluate:	organic fraction collected after elution of XAD
Water concentrate:	aqueous sample containing microcontaminants in concentrated form
Concentration factor:	value denoting the degree of concentration of the original sample

3.4 Principle of water sample conversion

The conversion of water samples begins with concentration using XAD resins. The sample is mixed with the XAD resins, isolating apolar substances and substances with weak polarity. The resins are then eluted using an organic solvent (acetone), and then converted to a concentrated sample. The sample is distilled so that what eventually remains is a highly concentrated aqueous sample which can be diluted for use in toxicity tests.

3.5 Reagents and additives

- Sodium hydroxide (4% v/v)
- Perchloric acid ($HClO_4$)
- Methanol p.a.
- Acetone p.a.
- Milli-Q water

- XAD-4 resin (Rohm & Haas, Antwerp, Belgium)
- XAD-8 resin (Supelite DAX-8, Supelco, Alrich, Zwijndrecht, the Netherlands)
- EPA medium (US EPA, 1985)
- Nitrogen
- Cyclohexane p.a.
- Ethanol

3.6 Equipment

- Borosilicate glass bottles (e.g. 10-litre) with Teflon-lined stopper
- Glass beakers
- Volumetric cylinders
- Funnels
- Elution columns, Ø 1.05 cm, 30 cm long, glass with ground-glass joints at top and in bottom of glass filter
- Dosing pipette, glass with broad tip
- Sample vials (30 and 60 ml) with crimp cap (Chrompack, Bergen op Zoom, the Netherlands)
- Sieve, 50 µm, stainless steel
- Deep freeze
- Roller apparatus
- Glass petri dishes
- Analytical balance
- Crimping pliers
- Water bath
- Kuderna Dänish evaporation glassware (mini version) (receiving $vessel_{64723}$ (2 ml), $flask_{64729}$ (250 ml), $condensor_{64839}$, Supelco, Aldrich, Zwijndrecht, the Netherlands)
- Thermometer (min. 100 °C)
- Zeolites
- Aluminium foil
- Digital flow meter
- Soxhlet extractor

3.7 Pretreatment of XAD resins

Before the XAD resins can be used they must first be purified. First, they are washed in sodium hydroxide (repeat 10 x) and then with perchloric acid 4% (repeat 10 x). They are then washed with milli-Q (repeat 10 x) and then with methanol (repeat 2 x). The washing procedure is followed by Soxhlet extraction using methanol (24 hours) and washing in ethanol (repeat 3 x). The resins are then subjected to Soxhlet extraction once again, using a mix of ethanol and cyclohexane (30.5/69.5 V/V%). Finally, the resins are rinsed with methanol (repeat 5 x). They are then stored in methanol p.a. in the dark at room temperature until use.

Purified XAD resin (XAD-4 and XAD-8) can also be obtained from KWR, Nieuwegein, the Netherlands (formerly called KIWA Water Research).

3.8 Method

The method has been divided into three elements: XAD extraction, elution with acetone and the conversion of acetone extract to water concentrate.

3.8.1 XAD extraction

- Take 7.5 ml XAD-4 and 7.5 ml XAD-8 for every 60 litres of water sample.
- Pour the XAD into an elution column using a glass funnel, ensuring that the XAD remains under the methanol. Begin with the XAD-4.
- Rinse the XAD with 10 × bed volume water (= 90ml).
- Transfer the XAD into the water sample (60 litres) using water and a clean glass beaker. Measure out 2.5 to 3 ml XAD-4/8 for each 10-litre sample bottle.
- Place the bottle on a roller apparatus and mix the sample with the resin at room temperature in the dark for at least 48 hours (adsorption).
- After at least 48 hours, sieve the XAD through a 50 µm sieve. Dry the XAD by pressing tissues against the bottom of the sieve.
- Transfer the XAD to a petri dish (Ø 10cm) whose empty weight has been determined, spreading it over the entire surface of the petri dish as far as possible. Shake the petri dish a number of times during drying.
- Dry the XAD in a functioning fume cupboard for at least 24 hours.
- The XAD (15 to 18 ml on methanol base) is dry enough once it weighs <5.4 grammes.

3.8.2 XAD elution using acetone

- Rinse the column with acetone (p.a. quality) and dry with pressured air.
- Transfer the dry XAD to a clean elution column (L = 30 cm, Ø 1.05 cm). See Equipment.
- Add enough acetone to ensure that all XAD is submerged.
- Remove any air bubbles by gently shaking the column a number of times.
- Elute using 25 ml acetone. Elute slowly to ensure that the process runs smoothly (approximately 30 minutes).
- Collect the eluate in a 60 ml sample vial. Note the volume on the sample form (section 3.15).
- Seal the sample vial and store it in the deep freeze at -18 °C.

3.8.3 Conversion of acetone eluate to water concentrate (KD distillation)

- Set up a Kuderna-Dänish (KD) concentrator apparatus in a fume cupboard. The receiving vessel should be suspended in the water bath. The flask is coupled to this, and connected to the condenser (see Figure 1).
- Heat the water bath to 65-70 °C and switch on the cooling water.
- Quantitatively transfer the acetone eluate to the KD apparatus.
- Add a zeolite and 0.7 ml milli-Q.
- Commence distillation by placing the apparatus in the water bath. Wrap the flask in aluminium foil.
- Cease distillation as soon as the residue stops boiling, approximately. 0.2 ml (ensure distillation does not continue for too long).
- Remove and seal receiving vessel immediately. Note the colour of the residue on the sample form (section 3.15).
- Quantitatively transfer the residue, using EPA-medium to a 60 ml sample vial and fill it to 60 grammes.
- Seal the vial and store in the refrigerator until use.

- Note data on sample form (section 3.15).

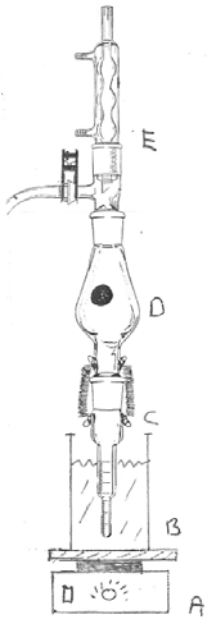

Figure 1. KD apparatus: a) heating plate, b) water bath, c) receiving vessel, d) KD flask, e) condenser.

3.9 Calculating concentration factor

The concentration factor for a sample is calculated as follows:

$$\text{concentration factor} = \frac{\text{original sample volume}}{\text{water concentrate volume}}$$

The volumes should be expressed in the same unit.

The concentration factor in the procedure described is 1000.

3.10 Reporting

The results of the conversion procedures should be reported using the specially designed form which can be found in section 3.15 of this chapter.

3.11 Quality assurance

To guarantee the quality of the conversion technique, the recovery and reproducibility of the technique must be known. Recovery with standard deviation is given below for two types of substances (Collombon et al., 1997):

XAD concentration (1997 method, elute 3 times with acetone)
- 2,4 dichloraniline (in mixture of 12 narcoticising substances): 108 ± 7% recovery (n=6)
- diuron (in mixture of 6 pesticides): 99 ± 6% recovery (n=6)

Water concentrate (1997 method, without N_2 purging)
- 2,4 dichloraniline (in mixture of 12 narcoticising substances): 61 ± 7% recovery (n=3)
- diuron (in mixture of 6 pesticides): 55 ± 3% recovery (n=3)

3.12 Safety

- Always wear suitable protective clothing (lab coat, safety goggles and gloves) when performing the tests.
- Regard the contaminated samples as chemical waste and dispose of accordingly.
- Work in a fume cupboard, as organic solvents are used in this procedure.
- Read the toxicity data and safety instructions for the solvents before use.

3.13 Maintenance

The specific equipment used for the conversion of water samples does not require regular maintenance or calibration.

3.14 References and additional information

M.Collombom, R. van de Kamp, J. Struijs (1997). Procedures for extracting organic micro-pollutants from water samples to monitor toxicological stress. RIVM rapport nr. 607042008.

U.S. EPA, (1985). Methods for measuring the acute toxicity of effluents to freshwater and marine organisms. EPA 600/4-85-013 Envirn. Research Laboratory, Duluth.

More information can be found in:

RIVM (1999) Document ECO/303/01. Rijksinstituut voor Volksgezondheid en Milieu, Laboratorium voor Ecotoxicologie, februari 1999. (In Dutch.)

Beveren, J. van (1989) Voorschrift voor de XAD isolatie in watermonsters van 50 tot 300 liter, deel 2: de opwerking; KIWA Nieuwegein, juni 1989. (In Dutch)

Penders, E.J.M., Hoogeboezem, W. (2000) Biotesten, een bruikbaar instrument voor de kwaliteitsbewaking van oppervlaktewater. RIWA. (In Dutch)

3.15 Sample form for sample pretreatment

Data	Sample code	Sample volume (l)	Distillation residue colour	Water concentrate volume (ml)	Storage	Remarks

4 Toxicity test using luminescent bacteria

Keywords: bacterium *Vibrio fischeri*, quality test, monitoring, surface water, bioluminescence

4.1 Subject

This protocol describes a toxicity test in which the bioluminescent bacterium *Vibrio fischeri* (formerly *Photobacterium phosphoreum*) is exposed to a sample for analysis. The method is derived from ISO 11348-3 (1998) and the NVN 6516 (1993) standard. The reduction in bioluminescence in *Vibrio fischeri* is measured after 5, 15 and 30 minutes. The inhibition of light emission correlates with the degree of toxicity. The protocol also includes a description of a quality test for determining the sensitivity of batches of bacteria. Phenol is used as the reference substance in the quality test.

4.2 Area of application

The protocol described here has been drawn up for tests on surface water and extracts of surface water. Since *Vibrio fischeri* are used, the protocol is applicable to both freshwater and saltwater samples.

4.3 Terms and definitions

Bioluminescence:	light emission by bacteria under the influence of metabolic reactions
Lyophilised:	freeze-dried in a vacuum
EC_{20}/EC_{50}:	the toxicity of a sample expressed as the concentration at which 20% or 50% reduction in luminescence occurs relative to the blank
Reference toxicant:	a substance (phenol in this case) used to establish the sensitivity of a batch of bacteria

4.4 Principle of the test

Vibrio fischeri is a luminescent bacterium, which has the property of emitting part of the energy released in the metabolic reaction (ascorbic acid cycle) as light. Any disruption in this metabolic reaction as a result of the presence of toxic substances will result in a change in the amount of light emitted. This reduction can be quantitatively determined using a luminescence meter (Microtox™ or LUMIStox™). The toxicity of a sample is expressed as the concentration at which a 20% or 50% reduction in luminescence occurs relative to the blank (EC_{20} or EC_{50}).

4.5 Reagents and additives

General

- Test kits/reagents for measuring ammonium and nitrite (e.g. cuvette test developed by Dr. Lange, Tiel or test strips from Merck, Amsterdam)

- Ultrapure water (e.g. Millipore, Etten-Leur or Salm & Kipp, Breukelen)
- Phenol (p.a.)

Microtox™
- Microtox™ reconstitution solution (shelf life: see packaging), Azur Environmental Ltd., Berkshire, UK
- Microtox™ diluent (sodium chloride solution, shelf life: see packaging)
- Microtox™ reagent (lyophilised *Vibrio fischeri*, shelf life: see packaging)
- MOAS (saline solution, shelf life: see packaging)

LUMIStox™
- LUMIStox™ luminescent bacteria (freeze-dried *Vibrio fischeri*, shelf life: at least 9 months at -18 °C), Dr. Lange, Tiel.
- Reactivation solution (sodium chloride, potassium chloride, magnesium chloride solution)
- NaCl (solid)
- Saline solution (2% NaCl)

4.6 Equipment

General
- pH meter
- Oxygen meter
- Conductivity meter
- Balance
- Table centrifuge
- Repeating pipette (10 – 500 µl)
- Automatic pipettes (1 and 5 ml)
- Volumetric flasks and cylinders
- Stopwatch/clock
- Calibration thermometer (max. deviation 0.2 ºC)

Microtox™
- Microtox™ analyzer (M500) connected to a PC with MicrotoxOmni software version 1.15 for Windows
- Microtox™ cuvettes (glass)
- Spectrophotometer (wavelength 490 nm)
- Cuvettes (1 cm)

LUMIStox™
- LUMIStox™ 300 connected to a PC (LUMISsoft™ III software for Windows) with LFD 2000 printer
- LUMIStherm™ (15.0 ± 0.2 °C)
- LUMIStox™ cuvettes (glass)
- Test tube (15 ml)

4.7 Reference substance

Phenol (p.a.) is used as the reference substance for the quality test, measured from a stock solution of 40 mg/l, which must be made fresh prior to each quality test.

4.8 Preparing for the test

4.8.1 Preparing for the test (*environmental samples*)

Surface water/concentrate

- The sample should be free of particles, as they can disrupt the light signal during the test. It should preferably be tested with as little pretreatment as possible. Any particles should be removed initially by means of settling and decanting, thereafter by centrifugation, repeated if necessary[1].

Extracts of surface water

- If extracts are used in a solvent whose toxicity is unknown, its toxicity to *Vibrio fischeri* must first be established. To this end, a test is performed with the solvent. Before testing, the solvent is diluted five times, to produce the following series of concentrations: 10 – 5 – 2.5 – 1.25 vol%. The results of the test allow a sample dilution to be determined. The sample should be diluted in such a way that the solvent causes less than 10% luminescence inhibition in the bacteria (see section 4.13).
- Given the possible effects of physical and chemical parameters, measure the pH, oxygen content, conductivity, and the nitrite and ammonium contents of the sample[2] (surface water or extract).
  - pH (using a pH meter);
  - oxygen content (using an oxygen electrode);
  - nitrite content (using a test kit);
  - ammonium content (using a test kit);
  - conductivity (using a conductivity meter).

4.8.2 Preparing for the test (*quality test*)

Prepare a stock solution by dissolving 0.2 g of phenol in 50 ml milli-Q. Dilute the solution 100 times to produce the stock solution of 40 mg/l (initial test concentration 18 mg/l).

4.9 Microtox™ method

In the description below the methods for testing environmental samples and the quality test using phenol have been combined.

4.9.1 Preparing the test organisms

- Switch on the Microtox™ equipment at least half an hour before use.
- Place 1 ml of reconstitution solution in a cuvette and place it in the appropriate reagent well.
- Once the reconstitution solution has reached the correct temperature in the incubator block, the bacteria can be reconstituted. Add 1 ml of the reconstitution solution (from the reagent well) to a

[1] If the sample is still turbid, it can be filtered over glass fibre or eventually over a 0.45 µm filter.

[2] Assuming sufficient sample volume is available.

pot of freeze-dried bacteria. Gently shake the pot to produce a homogeneous suspension, and transfer this to the cuvette which held the reconstitution solution.

- Place the cuvette with the bacterial suspension in the incubator block and mix it by sucking up 10 × 500 µl with a 1 ml pipette and expelling it back into the cuvette.
- The bacterial suspension is now ready and can be used for the next 4 hours. If the suspension is kept for longer than 4 hours a quality test must be performed simultaneously with the test to check the quality of the bacteria.

4.9.2 Equipment settings

- Turn on the PC and start up Windows (menu controlled by mouse) with password.
- Click on 'Run test'
- Choose the 'Basic test' protocol from the list, NEXT>
- Check the settings in the 'Test parameters' screen. The following standard settings should be shown for the standard test:

Test protocol	Basic Test or Quality Test
Test description	Description of sample
Test toxicant	Surface water, Extract, Solvent or Phenol
Number of controls	1
Control replicates	2
Number of samples	1 or 2
Sample replicates	2
Number of dilutions	4
Initial concentration	45 (freshwater sample), 50 (saltwater sample), 10 (solvent) or 18 (quality test)
Dilution Factor	2
Concentration Units	% or mg/l
Test times	5, 15 and 30 minutes
Time units	minutes

- If desired, note the fact that a solvent control is also being performed, which concentration of solvent is being used, and the number of replicas.
- Click on 'Additional data' and enter the batch number stated on the pot in which the freeze-dried bacteria are stored. NEXT>
- Enter the sample number or name as 'sample name'.
- Follow the 'Wizard', which will guide you through the test (see section 4.9.3)

4.9.3 Test implementation

- Place the test cuvettes in the Microtox incubator block..
- Add 1500 µl of diluent to row A of cuvettes 1 to 4. Add 250 µl MOAS to cuvette 5 row A (do not add MOAS if conductivity > 2564 µS/mm).
- Add 500 µl of diluent to rows B and C of cuvettes 1 to 5. Add 500 µl to a further two cuvettes and place them temporarily in another part of the incubator block.
- Add 10 µl of bacterial suspension to cuvettes B1 to 5, C 1 to 5 and the two extra cuvettes, using an Eppendorf repeating pipette (homogenise the bacterial suspension before use).
- Carefully mix the cuvettes (gently shake by hand).
- The cuvettes must then stand (in the incubator block) for 15 to 20 minutes before the test can commence.
- Now prepare the dilution series. Add 2500 µl of sample to test cuvette A5 (if no MOAS is used, add 3000 µl). Mix well using a pipette. Make a 1:1 dilution by transferring 1500 µl of test solution

from A5 to A4, from A4 to A3 and from A3 to A2. Mix well each time solution is transferred. No sample is added to A1 (= blank).

- Before commencing the test, check the initial values.
  For this purpose, the tubes are measured once, 15 minutes after the bacterial suspension has been added. Place the first cuvette in the READ chamber. Before measuring a series of cuvettes, press the SET button (when measuring the first sample). Then set the initial value using the READ button on the Microtox™. Check the value of all cuvettes, which must differ by no more than 15 units. Should some differ by more than 15 units, check whether the two extra cuvettes fall within the range, and if necessary exchange them for cuvettes that do not. If there is still too large a range, the test will be invalid, and new cuvettes containing diluent and bacterial suspension will have to be placed in rows B and C, after which the initial values will have to be checked again. If the initial values are correct, wait 5 minutes before taking the actual measurements.
- Place cuvette B1 in the READ chamber. Press the SET button on the Microtox™ (reset).
- After the cuvette has been measured, press the space bar and then the READ button. The value will appear on the computer screen and on the Microtox™ display. Then measure the initial values of all cuvettes in the following order: C1, B2, C2, B3 … etc.
- Immediately after taking the reading from C5 pipette 500 µl of the dilution series to rows B and C as follows: 500 µl from A1 to B1, from A1 to C1, from A2 to B2, from A2 to C2 etc. After each dilution in rows B and C mix the A row by sucking up and expelling the dilution three times.
- Immediately after mixing in cuvette C5, press the space bar. The test duration will appear on the screen.
- After 5 minutes the computer will indicate that the cuvettes need to be measured. Begin with cuvette B1. At fixed times (15 and 30 minutes) the screen will indicate that the flashing cuvette needs to be measured. The order of measuring is B1, C1, B2, C2, B3, C3, B4, C4, B5 and C5.
- After the final measurement, enter the desired file name for the results.
- After the final measurement, set the calculation options by choosing: ‘OPTIONS’ ? ‘CALCULATION OPTIONS’ set EC value (20 or 50).
- Print a report by choosing: ‘DATA’ ? ‘GENERATE REPORT FROM CURRENT DATA’ ? ‘PRINT’.

4.10 Microtox™ colour correction

Perform a colour correction if the liquid in the cuvette is clearly discoloured in the concentration where EC_{20} or EC_{50} occurs (visual observation).

- Prepare the solutions of the sample which will be used to calculate the EC_{20} or EC_{50} value.
- Set the spectrophotometer to wavelength 490 nm.
- Zero the spectrophotometer with diluent and measure the transmission (%) of all desired concentrations (1 cm cuvette, glass or quartz).
- Enter the values measured in the MicrotoxOmni worksheet by going to the ‘TEST’ menu and choosing ‘ENTER COLOUR CORRECTION DATA’. An extra column (ABSx) will appear, in which the spectrophotometer results can be entered.

4.11 LUMIStox™ method

A brief description of the LUMIStox™ method is given below. If a reference substance (e.g. phenol) is used, this can be regarded as a sample, and the settings of the LUMIStox™ adjusted accordingly.

4.11.1 Preparing the test organisms

- If necessary, defrost the reactivation solution to 4 °C (in refrigerator).
- Pipette 12 ml of reactivation solution into the test tube.
- Shake vigorously and place the solution in the refrigerator (3-6 °C) to bring it to temperature.
- Pipette 0.5 ml of reactivation solution from the test tube into a tube of freeze-dried bacteria.
- Place the bacterial suspension in the refrigerator (3-6 °C) for 15 minutes, and the remaining reactivation solution in the LUMIStherm at 15 °C.
- The bacterial suspension is now ready and can be used for the next four hours. If the suspension is kept for longer than 4 hours a quality test must be performed simultaneously with the test to check the quality of the bacteria.

4.11.2 Equipment settings

- Start up the LUMISstox 300.
- Select measuring method 'EC' and test '492'.
- Change the settings. The following standard settings should be shown for the standard test:

Number of dilutions:	3 to 9
Preliminary dilution:	2, 4, 8, etc
Dilution type:	DIN or geometric
Unit sample component:	% or mg/l
Conc. sample comp.:	if unknown, enter 100%
Incubation period:	5, 15 and 30

4.11.3 Test implementation (DIN with 9 dilutions)

- Place the test cuvettes in the LUMIStherm.
- Pipette the bacterial suspension (0.5 ml) into the remaining reactivation solution in the test tube and mix thoroughly.
- Pipette 0.5 ml of this solution into cuvettes B1 to B10 and C1 to C10 in the LUMIStherm.
- The cuvettes must then stand in the incubator block for 15 minutes at 15 °C before the test can commence.
- Add to the sample 2% of solid NaCl by, for example, dissolving 0.3 grammes of NaCl in 15 ml of sample (do not add salt if the salinity of the sample >20‰).
- Now prepare the dilution series. Add 1.5 ml of saline solution to test cuvettes A1 to A8, and 1 ml to A9. Add 1.5 ml of sample to cuvettes A8 and A10, and 2 ml to A9. Mix thoroughly using a cuvette. Make a 1:1 dilution by transferring 1.5 ml of test solution from A9 to A7, from A7 to A5 and from A5 to A3. Then make a second dilution series by transferring 1.5 ml from A8 to A6, from A6 to A4 and from A4 to A2. Mix well each time solution is transferred. No sample is added to A1 (= blank).
- I0 measurement: place cuvette B1 in the measuring cell of the LUMIStox and measure it. Remove the cuvette from the LUMIStox and return it to B1 in the LUMIStherm. Pipette 0.5 ml from cuvette A1 to B1 (= test start time). Place cuvette C1 in the measuring cell and measure it. Return the cuvette to C1 and pipette 0.5 ml from cuvette A1 to C1. Repeat with all cuvettes from rows B and C by pipetting from the cuvette in row A with the same number.
- After the incubation periods measure all cuvettes from rows B and C in the following order: B1, C1, B2, C2 … C10.
- After the final cuvette has been measured the EC_{20}, EC_{50} and all measured values will be printed out.

4.12 Parameters

Parameters have been drawn up for the testing of *environmental samples* (Postma et al., 2002). All parameter criteria relate to the values in the actual test concentrations, except for the pH (original sample) The following parameters apply to *Vibrio fischeri*:

- pH: 6-8.5;
- oxygen content: > 30% of saturation value;
- temperature: 15 ± 2 °C;
- ammonium content: < 1000 mg/l (at pH=8.0, 15 °C, 32‰);
- nitrite content: < 70 mg/l;
- conductivity: 25640-46000 µS/cm.

4.13 Validity criteria

The following validity criteria[3] apply to the toxicity test using the *Vibrio fischeri* bacterium:

- the loss of bioluminescence in the blank must be between 0.6 and 1.8;
- the solvent used should cause less than 10% luminescence inhibition relative to the blank;
- the quality test must be valid.

4.14 Statistical processing

In the Microtox™ test an EC_{20} or EC_{50} (Effective Concentration) value is determined using the MicrotoxOmni software (version 1.15). The LUMNIStox™ uses LUMISsoft III software. The EC_{20} or EC_{50} value is defined as the concentration of test medium at which a 20% or 50% loss of bioluminescence can be observed relative to the blank (= dilution medium) after a certain exposure time. The lowest of the EC_{20} or EC_{50} values determined at three points in time is used to indicate the toxicity of the sample. The effects in the test are determined by linear regression analysis of the dose-effect relationship. To obtain a linear correlation between test concentration and effect, the loss of bioluminescence at each exposure concentration is expressed as a gamma value (γ):

$$\gamma = (I_0/I_t)-1$$

where:
γ = gamma value;
I_0 = bioluminescence of blank;
I_t = bioluminescence of a sample after exposure time t.

The concentration-effect relationship for the exposure time is calculated using linear regression analysis:

$$\ln C = b[\ln \gamma] + a$$

where:
γ = gamma value
C = concentration

[3] According to ISO standards, duplicate measurements should not deviate more than 3% from the average value. This is often not feasible and therefore not included here.

b = slope
a = intercept

4.15 Reference substance toxicity

After a quality test has been performed, it will be necessary to check whether all the validity criteria of the test have been fulfilled (see section 4.13). Then, the EC_{20} will have to be determined on the basis of the results and this value entered in the Shewart chart for this quality test. If the EC_{20} does not fall within the range defined, the batch of bacteria must be rejected. The quality test should be carried out once every three months, and at any rate for each batch of bacteria. The EC_{20} value must comply with the following limits for phenol[4]: EC_{20}, 5 min: 3.0 – 10.0 mg/l.

4.16 Reporting

The report of the test will need to include at least the following:
- pretreatment of sample;
- sample data;
- sample parameter measurements;
- calculated toxicity data for the sample (EC_{20} and/or EC_{50});
- calculated toxicity data for the reference substance ($EC_{20, 5\ min}$).

4.17 Quality assurance

The methods drawn up by the RIKZ and laid down in standard instruction no. i013.90 (Validation of analysis methods and control/evaluation of performance characteristics) and i020.90 (Drawing up analysis characteristics) are used to calculate the (preliminary) quality criteria. The following criteria are important:

Repeatability:	unknown
Reproducibility:	29%
Rounding interval	1 decimal place (mg/l)

4.18 Safety

- Always wear suitable protective clothing (lab coat and gloves) when performing the tests.
- Regard the contaminated samples as chemical waste and dispose of accordingly.
- Handle the reference toxicant with due care. Read the toxicity data and safety instructions before use.

[4] Besides phenol, other substances can be used: 3,5-dichlorophenol, zinc sulfate heptahydrate and potassium dichromate. Compliance criteria: after 30 minutes of exposure, 20 to 80% of inhibition should occur for 3.4 mg/l of 3,5-dichlorophenol, 2.2 mg/l of Zn^{2+} and 18.7 mg/l of Cr^{6+}.

4.19 Maintenance

The temperature of the incubator block of the Microtox analyzer (M500) or the LUMIStherm must be checked once a year using a calibrated thermometer. Once every two years the analyzer must be serviced by the supplier.

4.20 References and additional information

ISO 11348-3 (1998) Water quality- Determination of inhibitory effect of water samples on the light emission of *Vibrio fischeri*, Part 3 method with freeze dried bacteria.

NVN 6516 (1993). Water - Bepaling van de acute toxiciteit met behulp van *Photobacterium phosphoreum*. Nederlands Normalisatie Instituut, Delft, August 1993.

Postma JF, de Valk S., Dubbeldam M., Maas. J.L., Tonkes, M., Schipper CA, Kater B.J. (2002). Confounding factors in bioassays with freshwater and marine organisms. Ecotox. Environ. Saf. 53: 226-237.

More information can be found in:

Dr. Lange (1999). Luminescent bacteria test DIN 38412 L34, L341, NEN 6516 and AFNOR T90-320. Luminescent bacteria test LCK 492.

Microbics (1992). Microtox, Update manual, Carlsbad, CA, USA.

rivm

4.21 *Vibrio fischeri* sample data and parameters form

Project:	:	
Sample	:	
Storage conditions	:	
Date of receipt	:	
Concentration series	:	1)…… 2)…… 3)……. 4)…… 5)…….. controls)……..
Sample type	:	surface water; extract; solvent; reference substance; other:
Sample frozen?		yes/no
Extract dilution	:	
Date of test	:	

O_2 adjustment ☐ yes, always to approximately 100% (sample with high O_2 consumption)
☐ yes, if < ..... % raise to at least ..... %

Sample	**Treatment[5]**							**Test organism**		**O_2 (%)**		**pH**		**Temp. (ºC)**	**Conductivity (µS/mm)**	**NO_2^- (mg/l)**	**$NH_4^+ + NH_3$ (mg/l) (ºC,pH,sal)[6]**	
	No treatment	Settle, decant	Centrifuge	Fibre-glass filterl	Filter 0.45 µm	Aerate	adjust pH											
								Vibrio fischeri		>30		6 – 8.5		-	2564-4600[7]	<70	<1000 (15, 8, 32)	
								Time	Conc.	before	after[8]	before	after					remarks

[5] Surface water and effluent/influent should preferably be tested in an untreated condition. If it contains too many particles, allow them to settle and decant the sample. Then use a centrifuge and/or filtration through fibre-glass filter and, finally, through a filtration filter.

[6] salinity (‰) = conductivity (µS/mm) × 0.0078

[7] if < 2564 µS/mm add saline solution (MOAS)

[8] value before and after adjustment shown in table

5 PAM test: acute effects on photosynthesis in algae

Keywords: green alga, *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák, monitoring, photosynthesis, surface water

5.1 Subject

The University of Amsterdam's Department of Aquatic Ecology and Ecotoxicology has developed an alternative algae test in collaboration with the National Institute for Public Health and the Environment and Wageningen University's Laboratory for Plant Physiology. In this test, the green alga *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák is exposed to a concentration series of a sample for 4.5 hours. The inhibition (EC_{50}) of photosynthetic efficiency is then determined using a pulse-amplitude modulation (PAM) fluorometer.

5.2 Area of application

The protocol described here has been drawn up for tests on surface water and extracts of surface water. Since the green alga *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák (formerly *Raphidocelis subcapitata* and *Selenastrum capricornutum*) is used, the protocol is applicable only to freshwater samples.

5.3 Terms and definitions

EC_{50}:	The toxicity of a sample expressed as the concentration at which 50% inhibition of photosynthetic efficiency occurs.
Internal standard:	A toxic substance (atrazine in this case) which is used to establish the sensitivity of the test system.

5.4 Principle of the test

The green alga *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák is exposed to a concentration series of the sample for 4.5 hours. The inhibition of photosynthetic efficiency is then measured using a PAM fluorometer connected to an autosampler and a computer. The determination of photosynthetic activity is based on the following principle. Light energy is absorbed by antenna pigments and transmitted to the alga's photosystems I and II. The energy is then transformed into photoproducts and heat. Light is also emitted (fluorescence). Blocking of the photosynthesis system by toxicants, for example, results in changes to the level of fluorescence. The fluorescence signal generally increases as the photochemical and/or heat production reduces. The PAM fluorometer records changes in the fluorescence signal as a saturating light pulse is applied. Photosynthetic efficiency can be derived from the signal recorded. The effects of the toxicant on photosynthetic efficiency are then quantified (EC_{50}).

5.5 Reagents and additives

- *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák green alga suspension
- Dilution water (Dutch Standard Water, consisting of 1.36 mM $CaCl_2 \cdot 2\ H_2O$ 0.50 mM $MgSO_4 \cdot 7\ H_2O$; 1.14 mM $NaHCO_3$; 0.20 mM $KHCO_3$, pH = 8.2 ± 0.2)
- Test kits/reagents for measuring ammonium and nitrite (e.g. cuvette test developed by Dr. Lange, Tiel or test strips from Merck, Amsterdam)
- Ultrapure water or demi-water (e.g. Millipore, Etten-Leur or Salm & Kipp, Breukelen)
- Internal standard (atrazine p.a., Riedel-de Haën, 0.1311 mM dissolved in 98% ethanol)
- Ethanol

5.6 Equipment

- Test vials: glass, 3 ml
- Incubator (continuous light: 100 µE $m^{-2}s^{-1}$, 650 nm and continuous agitation: 100 rpm, Infors AG)
- Fluorometer (Walz; measuring head WATER-PAM/F) connected to an autosampler, both controlled by a PC with WinControl v.1.01.04, Excel and Graphpad software
- Rinsing vial
- Parafilm
- Chrompack decapper
- Light meter (LI-COR, Lincoln, Nebraska, USA, LI-1000) connected to a test vial
- pH-meter
- Conductivity meter
- Glass beakers (250 and 1000 ml)
- Volumetric flask (200 ml)
- Automatic pipettes (100, 1000 and 5000 µl)
- Repeating pipettes (25 and 50 ml)
- Cover plate
- Magnetic stirrer + flea
- Waste receptacle

5.7 PAM fluorometer

A fluorometer is used to measure fluorescence signals. The PAM fluorometer differs from other fluorometers in terms of its measuring principle, which uses pulsed excitation light. Short weak light pulses (pulse length 3 µsec, 650 nm) from a LED (light emitting diode) are conducted to the algal suspension via a glass fibre ('measuring light'). These light pulses induce a fluorescence signal. Given the brief half-life of fluorescence (nsec.) the induced fluorescence pulses will coincide with the excitation light pulses. Chlorophyll-*a* fluorescence has a wavelength between 660 and 760 nm. The fluorescence pulses are conducted to a detector via a glass fibre.
Filtering the measuring light with a short pass filter (which allows wavelengths < 695 nm through) and shielding the detector with a long pass filter (which allows wavelengths > 700 nm through) prevents the pulsed measuring light from reaching the detector. Only the pulsed fluorescence signal is then amplified by a photomultiplier. The selective pulse amplification allows a fluorescence signal to be measured in unfiltered base lighting.

5.8 Internal standard

To verify the sensitivity of the test system, during each test the inhibition of photosynthetic efficiency in an algal suspension is determined using a standard concentration of 0.437 µM of atrazine (herbicide). To this end, 30 µl 0.1311 mM atrazine in 98% ethanol is added to two vials of an identical composition to the controls.

5.9 Method

The method for testing extracts of surface water (XAD) is described below.

5.9.1 Starting up equipment (beginning of test day)

- Switch off the fluorescent lighting in the lab.
- Switch on the incubation table's transformer.
- Place an incubator block on the incubator and set the light intensity of the incubator's reference position to 72 ± 1 µE $m^{-2}s^{-1}$ using the test vial connected to the light meter.
- Switch on the robot (on the back) and transformer in the measuring array.
  NB: first switch on the robot, then the computer!!
- Switch on the computer and start up WinControl.
- Activate batch-file AAN.TXT (FILE, EXECUTE BATCH FILE, OPEN BATCH FILE, AAN, OK).
- Place an incubator block on the measuring array and set the light intensity to 72 ± 1 µE $m^{-2}s^{-1}$ using the test vial connected to the light meter (test array reference position).

5.9.2 Making the algal suspension

The test is performed using the green alga *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák from continuous culture. The alga must be in the exponential phase. For information on the algal culture, the medium used, maintaining the continuous culture etc., see the University of Amsterdam's PAM test guidelines (Beusekom et al., 1999).

- Enter the date and other details on the test form (section 5.19).
- Fill a glass beaker (800 ml) with DSW.
- Calculate the amount of algal culture needed.
  The formula below is used to calculate the amount of algal culture needed for 200 ml of algal suspension, which is enough to perform seven tests.

$$\text{number of ml of algae} = \frac{0.3 \times 10^7}{\text{concentration of culture (cells/ml)}} \times 200$$

- Note the number of ml of algal culture needed on the test form.
- Pipette the calculated amount into a 200 ml volumetric flask and make up to 200 ml with DSW.
- Pour the contents of the volumetric flask into a glass beaker (250 ml), add a flea (bar magnet) and place the beaker on the magnetic stirrer.
- Start up the magnetic stirrer (100 rpm).

5.9.3 Sample pretreatment

Surface water/concentrate
The sample should be free of particles (XAD concentrate generally contains no particles). It should preferably be tested with as little pretreatment as possible. Any particles should be removed initially by means of settling and decanting, thereafter by centrifugation, repeated if necessary.

Extracts of surface water
If extracts are used in an unknown solvent, its toxicity to the alga must first be established. To this end, a test is performed with the solvent. Before testing, the solvent is diluted ten times and then 1:1, to produce the following series of concentrations: 10 – 5 – 2.5 – 1.25 vol%. The results of the test allow a sample dilution to be determined. The sample should be diluted in such a way that the solvent causes less than 10% fluorescence inhibition in the alga relative to the blank (see section 5.11).
Given the possible effects of physical and chemical parameters, measure the pH, oxygen content, conductivity, and the nitrite and ammonium contents of the control (DSW) and the highest concentration. If the parameter criteria are exceeded in the highest concentration, measure the parameters in the other dilutions[9].

- pH (using a pH meter);
- nitrite content (using a test kit);
- ammonium content (using a test kit);
- conductivity (using a conductivity meter).

5.9.4 Making the concentration series

The concentration series should be produced in duplicate. The description is based on a standard concentration series for XAD concentrates with nominal concentration factors (C.F.) of 500×, 150×, 50×, 15×, 5×, 1.5×, 0.5× and 0× (=control), with DSW as the dilution medium. The concentration series may be adjusted if necessary (due to a shortage of concentrate, testing of other toxicants).

As well as a double concentration series (Figure 2: positions 1 to 16), the entire sample series includes a number of extra samples. These extra samples can be used for setting the fluorometer, correcting the test results, checking the condition of the algae and verifying the implementation of the test.

Internal standard (atrazine; 0.437 µM) (positions 17 and 18)
To verify the sensitivity of the test system, during each test the inhibition of photosynthetic efficiency in an algal suspension is determined using a standard concentration of 0.437 µM of atrazine (herbicide). To this end, 30 µl 0.1311 mM atrazine in 98% ethanol is added to two vials of identical composition as the controls.

Fluorescence of the sample (position 19)
The measured fluorescence signals must be corrected for the fluorescent properties of the sample. For this purpose, put the same volume as used for the highest test concentration into one vial. Treat in the same way as the highest testconcentration but add 1 ml of DSW instead of 1 ml of algae.

Photosynthetic efficiency after adaptation to the dark (positions 20 and 21)
These two vials have exactly the same composition as the controls. During the 4.5 hours of incubation these vials are exposed to light. After transfer to the measuring array they receive no light for

[9] It is important to note exceedances. During processing of the data, this information can be used to decide whether all toxicity data can be used.

approximately 20 minutes. Then, after the algae have adapted to the dark, their optimum photosynthetic efficiency is determined.

F-zero (position 24)
To correct for background fluorescence and the fluorescence of DSW, before measurement commences the signal measured by the fluorometer must be set to zero. 3 ml of DSW is pipetted into this vial. F-zero is set just once a day, and therefore need only be placed in the first incubator block.

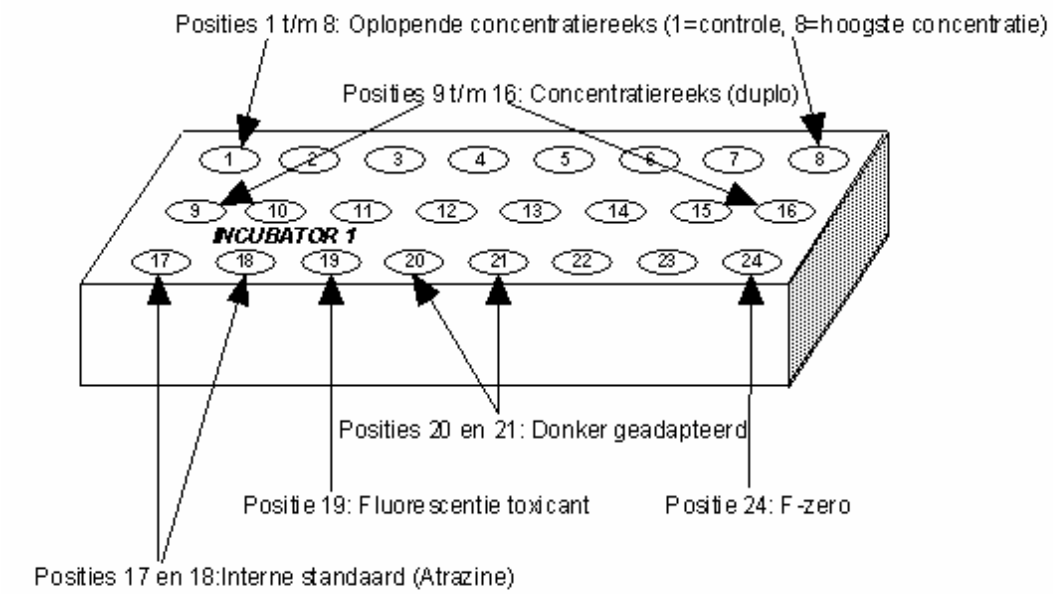

Figure 2. Layout of the incubator block. Positions 1 to 8: Increasing concentration series (1=control, 8=highest concentration). Positions 9 to 16: Concentration series (in dupl.). Positions 17 and 18: Internal standard (atrazine). Position 19: Toxicant fluorescence. Positions 20 and 21: Dark-adapted. Position 24: F-zero

A description of the production of a single concentration series is given below.

- Place 10 vials on the lab table (20 for double series) as shown in Figure 3.
- Add 2 ml DSW to all vials except A_1 and B_1 (repeating pipette; 50 ml tip, setting 2).
- Add 555 µl DSW to vial A_1 (1000 µl pipette).
- Add 1722 µl DSW to vial B_1.
- Note details of concentrate (test form, appendix 2).
- Shake and open the Chrompack vial containing XAD concentrate (using Chrompack decapper).
- Add 1667 µl XAD concentrate to vial A_1.
- Add 500 µl XAD concentrate to vial B_1.
- Set the pipette to 222 µl and mix the content of vial A_1 by sucking up the contents and expelling them into the vial three times.

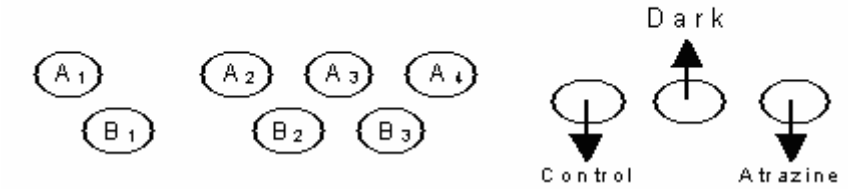

Figure 3. Layout of concentration series, where vial A_1 = highest concentration in the series, vial B_1 = second highest concentration in the series, Control = contains no toxicant, Dark = identical to control, atrazine = internal standard.

- (Use same pipette tip for transferrals from A_1 to A_4 inclusive.)
- Then transfer 222 µl from vial A_1 to vial A_2.
- Mix the content of vial A_2 and transfer 222 µl to A_3.
- Mix the content of vial A_3 and transfer 222 µl to A_4.
- Mix the content of vial A_4 and transfer 222 µl to the waste receptacle.
- Do the same for vials B_1 to B_3.
- Add 30 µl atrazine solution (internal standard) to the 'atrazine' vial (see Figure 1).
- (200 µl pipette; suck up the atrazine solution several times in view of vapour pressure caused by ethanol (rinse), insert pipette until resistance is felt).
- Interlock series A and B to produce a declining concentration series.
- Place the vials in an incubator block in the correct order (see Figure 1).
- Add 555 µl of DSW to vial '19', add 1667 µl of sample, mix well, transfer 222 µl to the waste receptacle, add 1 ml of DSW instead of algla suspension and place this vial in the incubator block (position 19). Note the concentration on the test form (section 5.19).
- Add 1 ml of algal suspension (repeating pipette; 25 ml tip, setting 2) to all vials except vial 19 and note the time on the test form (appendix 2) (= start of incubation).
- In the first test of the day, add 3 ml DSW to a vial and place it in the incubator block (position 24: F-zero).

5.9.5 Incubation

- Place the incubator block in the incubator.
- Check the light intensity of the incubator (reference position 23).
- Switch on the agitation table (if necessary, reset to 100 rpm).
- Check the light intensity in the incubator (reference position 23) every hour during incubation.
- Activate the batch-file 2 hours before performing the first measuring procedure
- OPWARMEN.TXT ('FILE' → 'EXECUTE BATCH FILE' → 'OPEN BATCH FILE' → 'OPWARMEN' →'OK').

5.9.6 Measurements

- Fill rinsing vial with DSW.
- Clasp a sheet of parafilm onto the cover plate.
- After 4 hours and 20 minutes of incubation, remove the incubator block from the incubator and mix the content of the vials by firmly pressing down the cover plate onto the incubator block and gently shaking.
- Place the incubator block on the measuring array.

- Check the light intensity of the measuring array in the reference position.
- During the first test of the day, zero the fluorometer as follows:
  - Activate batch-file F-ZERO.TXT ('FILE' → 'EXECUTE BATCH FILE' → 'OPEN BATCH FILE' → 'F-ZERO' → 'OK')
  - Check the current fluorescence signal (F* in the WinControl Report window). If it does not equal zero or is fluctuating sharply within five seconds of setting the F-zero value, the batch-file must be activated again.
- Start the measuring procedure 4.5 hours after adding the algae to the sample series:
  - Activate batch-file PAM.TXT ('FILE' → 'EXECUTE BATCH FILE' → 'OPEN BATCH FILE' → 'PAM'→ 'OK')
  - Name the data-file (output) and click 'OK'. A layout of the incubator block will appear.
  - Click 'OK' and the measuring procedure will commence.

5.9.7 Calculating EC_{10} and EC_{50} values

- The measuring procedure takes approximately 20 minutes. The data will be stored in a data-file under the given name in the C:\wincont\resultaten directory.
- Transfer the data-file (copy to disk) to a computer on which Excel and Graphpad have been installed.
- Open Excel file EC50-PAM.XLS and save under a new name (referred to below as NIEUW.XLS).
- Enter the test data in the light blue cells.
- Open the data-file in Excel:
  - On the first page of the Text Import Wizard choose 'DELIMITED' and click on NEXT>.
  - Tick 'TAB' and 'COMMA' and click on 'FINISH'.
- Copy block F5 to H122 from the data-file to cell B2 (cursor position) of file NIEUW.XLS.
- Copy blocks V18 to X25 from the xls-file to GraphPad Prism®.
- Start up Graphpad Prism® and open the file PAM/PZM.
- Click on the 'DATA' tab.
- Place the cursor at position X1 and paste the copied block.
- Click on the 'RESULTS' tab.
- Copy blocks X2 to Y23.
- Return to Excel and paste the results block in position AA9 of NIEUW.XLS.
- Save and print NIEUW.XLS.

5.10 Parameters

No criteria have been drawn up for most of the parameters in the PAM toxicity test using the green alga *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák:

- pH: unknown;
- temperature: 20 ± 2 °C;
- ammonium content: unknown;
- nitrite content: unknown;
- conductivity: unknown.

5.11 Validity criteria

The following validity criteria apply to the toxicity test using *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák:

- The solvent used should cause less than 10% inhibition relative to the blank.
- The quality test must be valid.

5.12 Statistical processing

A dose-response relationship is obtained by expressing the photosynthetic efficiency of the concentration series as percentages of the controls (without toxicant). To determine the EC_{10} and EC_{50} values a dose-response curve is fitted using a non-linear least squares method according to a logistic response model (Haanstra et al., 1985). The EC_{10} and EC_{50} values can be calculated quite simply by copying the data-file to a standard Excel file and using the Graphpad Prism® software package.

5.13 Reference substance toxicity

To verify the condition of the test algae, a simultaneous internal quality test is performed using atrazine. After the quality test has been performed, it will be necessary to check whether all the validity criteria (acceptance limits) of the test have been fulfilled. The results will be entered in the Shewart chart for this quality test. If the EC_{50} does not fall within the range defined, the test must be rejected.

(Preliminary) acceptance limits for quality test: EC_{50}: 55-80 µg/l atrazine

5.14 Reporting

The report of the test must include at least the following:

- pretreatment of sample;
- sample data;
- raw data;
- measured sample parameters.;
- calculated toxicity data of the sample (EC_{50}) and quality test.

5.15 Quality assurance

The methods drawn up by the RIKZ and laid down in standard instruction no. i013.90 (Validation of analysis methods and control/evaluation of performance characteristics) and i020.90 (Drafting analysis characteristics) are used to calculate the (preliminary) quality criteria. The following criteria are important:

- Repeatability: unknown
- Reproducibility: 17% (Beusekom et al., 1999)
- Rounding interval: 0 decimal places (µg/l)

5.16 Safety

- Always wear suitable protective clothing (lab coat and gloves) when performing the tests.
- Regard the contaminated samples as chemical waste and dispose of accordingly.
- Handle the herbicide atrazine with due care. Read the toxicity data and safety instructions before use.

5.17 References and additional information

Beusekom, van S.A.M., Admiraal, W., Sterkenburg, A. en Zwart, de D. (1999). Handleiding PAM-Test. ECO notitie 98/09. RIVM, Bilthoven.

Haanstra , L, Doelman, P., Oude Voshaar, J.H. (1985). The use of sigmoidal dose response curves in soil ecotoxicological research. Plant and Soil 84:293-297.

Postma JF, de Valk S., Dubbeldam M., Maas. J.L., Tonkes, M., Schipper CA, Kater B.J. (2002). Confounding factors in bioassays with freshwater and marine organisms. Ecotox. Environ. Saf. 53: 226-237.

More information can be found in:

NPR 6503 (1980). Benodigdheden, werkwijze en medium voor het kweken van *Daphnia magna* en van de hiervoor als voedsel benodigde algen.

5.18 Parameters form *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák

Project: :
Sample: :
Storage conditions :
Date of receipt :
Concentration series : 1) ……. 2) ……. 3) ……. 4) ……. 5) ……. 6) …….
Sample type : surface water; extract; solvent; reference substance; other:
Sample frozen? : yes/no
Dilution of extract :
Date of test :

O_2 adjustment ☐ yes, always to approximately 100% (sample with high O_2 consumption)
☐ yes, if < ..... % raise to at least ..... %

Sample	**Treatment**[10]							**Test organism**		**O_2 (%)**		**pH**		**Temp. (ºC)**	**Conductivity**[11]	**NO_2^- (mg/l)**	**NH_4^+ + NH_3**	
	No treatment	Settle, decant n	Centrifuge	Fibre-glass filter	Filter 0.45 µm	Aerate	Adjust pH	*Pseudokirchneriella subcapitata* (Korshikov) F. Hindák		n.d.		n.d.		20 ± 2	n.d.	n.d.	n.d.	
								Time	Conc.	before	after[12]	before	after	remarks				

[10] surface water and effluent/influent should preferably be tested in an untreated condition. If it contains too many particles, allow them to settle and decant the sample. Then use a centrifuge and/or filtration through fibre-glass filter and, finally, through a filtration filter.
[11] salinity (‰) = conductivity (µS/mm) x 0.0078
[12] value before and after adjustment shown in table

5.19 Test form *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák

Date of test:	
Performed by:	
Project :	
Sample	
Solvent	
Sample data	
Date of algal culture:	
Algal culture data:	
Calculated/required amount of algal culture (ml):	
Algal suspension added at:	
Concentration of known toxicant/DSW mixture (position 19):	
Remarks:	

6 Algae plate test to measure toxicity to *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák

Keywords: green alga, *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák, green alga, fluorescence, monitoring, surface water

6.1 Subject

This protocol is a variation on the ISO 8692 protocol for assessing the toxicity of freshwater samples. The test organism used is *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák, a green alga. Exposure occurs in multiwell test plates, which gives the advantage of allowing the tests to be performed with a small volume of sample, and of considerably reducing the time needed.
Cell concentration is measured during the test by means of in vivo fluorescence, using a plate reader. The endpoint of the test is the concentration at which 50% growth inhibition is found to occur. This EC_{50} value is calculated from the test results using the Toxcalc software.

6.2 Area of application

The protocol described can be used to test effluents, surface water and extracts thereof.

6.3 Terms and definitions

EC_{50}: The toxicity of a sample or substance expressed as the concentration at which 50% growth inhibition occurs.

6.4 Principle of the test

The green alga *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák is exposed to a concentration series of the sample for 72 hours. The test plate is measured using a fluorescence plate reader at the beginning and the end of the test. The cell concentration in each well is calculated on the basis of the fluorescence signal. The increase in cell concentration allows the growth rate in each test concentration to be calculated. The effects of the sample on growth rate are then expressed as an EC_{50}.

6.5 Reagents and additives

- Suspension of *Pseudokirchneriella subcapitata* (Korshikov) F. Hindák
- Milli-Q water
- Ammonium chloride (NH_4Cl)
- Magnesium chloride hexahydrate ($MgCl_2 \cdot 6\ H_2O$)
- Calcium chloride dihydrate ($CaCl_2 \cdot 2\ H_2O$)

- Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7\ H_2O$)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Iron chloride hexahydrate ($FeCl_3 \cdot 6\ H_2O$)
- EDTA ($H_2Na_2EDTA \cdot 2\ H_2O$)
- Hydrogen borate (H_3BO_3)
- Manganese chloride tetrahydrate ($MnCl_2 \cdot 4\ H_2O$)
- Zinc chloride ($ZnCl_2$)
- Cobalt chloride hexahydrate ($CoCl_2 \cdot 6\ H_2O$)
- Copper chloride dihydrate ($CuCl_2 \cdot 2\ H_2O$)
- Sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2\ H_2O$)
- Sodium bicarbonate ($NaHCO_3$)

All chemicals must be reagent grade.

6.6 Equipment

- Multiwell test plates (Greiner 96-well)
- Temperature-controlled incubator with agitator and lighting. INFORS AGCH-4103. Agitation occurs at RPM 140. Temperature setting 23 °C ± 1.
- Bio-tek FL600 plate reader
- Filters: excitation 440/30 nm, emission 680/30 nm
- Toxcalc
- Cling film
- pH meter
- Conductivity meter
- Glass beakers (250 and 1000 ml)
- Volumetric flasks (100 and 1000 ml)
- Sterile conical flasks (250 ml) with filter stopper
- Pipettes (200, 1000 and 5000 µl)
- Multi-channel pipettes

6.7 Making the test medium

The test medium is prepared by diluting the stock solutions as given in Table 1.

Stock solutions 1 to 3 must be sterilised (autoclave 121 °C, 15 min.). Stock solution 4 must be made sterile by means of membrane filtration (0.2 µm).

Table 1. Stock solutions.

Stock solution 1 (Macronutrients)	Concentration in stock		Concentration in medium	
NH_4Cl	1.5	g/l	15	mg/l
$MgCl_2 \cdot 6\ H_2O$	1.2	g/l	12	mg/l
$CaCl_2 \cdot 2\ H_2O$	1.8	g/l	18	mg/l
$MgSO_4 \cdot 7\ H_2O$	1.5	g/l	15	mg/l
KH_2PO_4	0.16	g/l	1.6	mg/l

Stock solution 2 (Fe-EDTA)	Concentration in stock		Concentration in medium	
$FeCl_3 \cdot 6\ H_2O$	64	mg/l	64	µg/l
EDTA ($H_2Na_2EDTA \cdot 2\ H_2O$)	100	mg/l	100	µg/l

Stock solution 3 (Trace elements)	Concentration in stock		Concentration in medium	
H_3BO_3	185	mg/l	185	µg/l
$MnCl_2 \cdot 4\ H_2O$	415	mg/l	415	µg/l
$ZnCl_2$	3	mg/l	3	µg/l
$CoCl_2 \cdot 6\ H_2O$	1.5	mg/l	1.5	µg/l
$CuCl_2 \cdot 2\ H_2O$	0.01	mg/l	0.01	µg/l
$Na_2MoO_4 \cdot 2\ H_2O$	7	mg/l	7	µg/l

Stock solution 4 $NaHCO_3$	Concentration in stock		Concentration in medium	
$NaHCO_3$	50	g/l	50	mg/l

Stock solutions 1 to 3 must be sterilised (autoclave 121 °C, 15 min.). Stock solution 4 must be made sterile by means of membrane filtration (0.2 µm).

6.8 Diluting stock solutions

Place approximately 500 ml of milli-Q water in a 1000 ml volumetric flask. Then add the following amount of stock solution:

- 10 ml stock solution 1;
- 1 ml stock solution 2;
- 1 ml stock solution 3;
- 1 ml stock solution 4.

Fill the volumetric flask to 1000 ml with Milli-Q water. Bring the test medium into equilibrium with the ambient air by bubbling compressed air (passed through a membrane filter) through it for 30 minutes. Measure the pH of the medium and adjust if necessary to 8.1 ± 0.2, using an HCl or NaOH solution (1 mol/l).

6.9 Concentrated test medium (10x)

Place approximately 50 ml milli-Q water in a 100 ml volumetric flask. Then add the following amount of stock solution:

- 10 ml stock solution 1;
- 1 ml stock solution 2;
- 1 ml stock solution 3;
- 1 ml stock solution 4.

6.10 Preparing preculture

A preculture must be started 2 to 4 days before the start of the test. Place 100 ml of test medium in a sterile conical flask. The medium is then injected with a low concentration of algae ($<10^4$ cells/ml). The preculture is cultured in the conditions identical to those used for the test.

6.11 Diluting preculture

Determine the density of algae in the preculture using a Coulter counter (Multisizer II). The preculture will have to be diluted, depending on the density, to ensure that the correct algae concentration (<10000 cells/ml) is achieved in the test plates for each 70 µl of solution. A sample calculation is shown below:

Preculture with 6.6×10^6 cells/ml.

$6.6 \times 10^6 / 3.8 \times 10^4 \approx 173$ (or calculate $3.8 \times 10^4 \times 100 / 6.6 \times 10^6 = 0.580$ ml)

Add 580 µl of preculture to a volumetric flask (100 ml) and fill to 100 ml (≈ 173× dilution). 70 µl of this diluted preculture must be added to each well to achieve a starting concentration of approximately 10000 cells/ml.

6.12 Test solutions

The test concentrations are diluted in the 96-well test plates (Greiner 96 no.) according to the pipetting diagram below.

Pipette 180 µl milli-Q water into the wells as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A			180	180	180	180	180	180	180	180	180	180
B			180	180	180	180	180	180	180	180	180	180
C			180	180	180	180	180	180	180	180	180	180
D			180	180	180	180	180	180	180	180	180	180
E			180	180	180	180	180	180	180	180	180	180
F			180	180	180	180	180	180	180	180	180	180
G			180	180	180	180	180	180	180	180	180	180
H			180	180	180	180	180	180	180	180	180	180

Pipette 270 µl milli-Q water into the wells as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A	270											
B	270											
C	270											
D	270											
E	270											
F	270											
G	270											
H	270											

Pipette 180 µl sample water into the wells as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A		180	180									
B		180	180									
C		180	180									
D		180	180									
E		180	180									
F		180	180									
G		180	180									
H		180	180									

Pipette 180 µl of solution from row three into row 4. Repeat to row 10, sucking up and expelling liquid from the pipette in between. Then **remove** 180 µl of liquid from row 10.

	1	2	3	4	5	6	7	8	9	10	11	12
A			180	▸								
B			180		▸							
C			180			▸						
D			180				▸					
E			180					▸				
F			180						▸			
G			180									
H			180									

remove 180 µl!

Pipette 20 µl concentrated test medium (10x) into the wells indicated below.

	1	2	3	4	5	6	7	8	9	10	11	12
A		20	20	20	20	20	20	20	20	20	20	20
B		20	20	20	20	20	20	20	20	20	20	20
C		20	20	20	20	20	20	20	20	20	20	20
D		20	20	20	20	20	20	20	20	20	20	20
E		20	20	20	20	20	20	20	20	20	20	20
F		20	20	20	20	20	20	20	20	20	20	20
G		20	20	20	20	20	20	20	20	20	20	20
H		20	20	20	20	20	20	20	20	20	20	20

Pipette 70 µl medium into the wells indicated below.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C		70	70	70	70	70	70	70	70	70	70	70
D												
E												
F												
G		70	70	70	70	70	70	70	70	70	70	70
H												

Pipette 70 µl diluted preculture into the wells indicated below. The algae concentration must be <10000 cells per ml at the beginning of the test.

	1	2	3	4	5	6	7	8	9	10	11	12
A		70	70	70	70	70	70	70	70	70	70	70
B		70	70	70	70	70	70	70	70	70	70	70
C												
D		70	70	70	70	70	70	70	70	70	70	70
E		70	70	70	70	70	70	70	70	70	70	70
F		70	70	70	70	70	70	70	70	70	70	70
G												
H		70	70	70	70	70	70	70	70	70	70	70

6.13 Determining the algae concentration in the test plates

Immediately after the start and at the end of the 72-hour incubation the test plates are measured using a fluorescence plate reader. After the plate reader (Bio-tek FL600) has been switched on it must be left to warm up and stabilise before any measurements can be taken. Start up the program that controls the plate reader (KC4). A KC protocol is available for selection of the correct settings. This protocol ensures that the test plate is always measured with similar settings (excitation 440 nm and emission 680 nm, Greiner 96 plate). The results are stored in digital form and exported to a text file.

6.14 Incubation

The test plates are wrapped in cling film to reduce evaporation. Ensure that the film on the top of the plate is as smooth as possible and that it is not doubled over (uneven illumination!!). The test plates are then incubated at 23 ± 1 ºC, under continuous light and agitation (in INFORS AGCH-4103). Place a layer of aluminium foil in the bottom of the agitator to stand the plates on.

6.15 Calculating cell concentration

The cell concentration is calculated before and after incubation on the basis of the fluorescence signal. The values are first corrected for the background signal. The signal is then converted to a cell density, using the following equation:

Cell/ml= 0.25 x (value at ex440/em680)- 56

6.16 Calculating EC_{50} values

Effect values (EC_{10} and EC_{50}) are calculated by means of the Maximum Likelihood Probit method in TOXCALC version 5.0 (Tidepool Scientific Software, 1995). The Maximum Likelihood Probit method has been compared with other methods (Spearman-Karber and linear interpolation) in a number of tests. However, the differences in EC_{50} values were small, and the reliability of the last two methods was lower for some datasets.

6.17 Validity criteria

The test is valid if it meets the following criteria:

- The cell density in the blank must be at least a factor 16 higher after 72 hours of incubation.
- The variation coefficient of the control growth rates may be no greater than 5%.
- The pH in the blank must not deviate by more than 1.5 units from the original medium.

6.18 Reference substance toxicity

To verify the condition of the test algae used, an internal quality test must be performed simultaneously using potassium dichromate. After a quality test has been performed, it will be necessary to check whether all the validity criteria (acceptance limits) of the test have been fulfilled, and the results entered in the Shewart chart for this quality test. If the EC_{50} does not fall within the range defined, the test must be rejected.

Acceptance limits for quality test: EC_{50} 0.3-0.9 mg/l

These are preliminary limit values. More data are needed for more accurate determination of the values!

6.19 Reporting

The report of the test must include at least the following:

- pretreatment of sample;
- sample data;
- raw data;
- calculated toxicity data of the sample (EC_{50}) and quality test.

7 Daphnia IQ toxicity test

Keywords: *Daphnia magna*, *Daphnia pulex*, enzyme activity, quality test, monitoring, surface water

7.1 Subject

Enzyme activity can be an indicator of health in the water flea *Daphnia magna* or *Daphnia pulex*. Daphnia juveniles exposed to toxic substances can have reduced capacity to absorb and enzymatically split a fluorogene-labelled sugar substrate. This test allows the change in enzyme activity caused by stress (toxic substances) in the environment to be determined within two hours.

7.2 Area of application

The protocol described here has been drawn up for tests on surface water and extracts of surface water. Since *Daphnia magna* or *pulex* are used, the protocol is applicable only to freshwater samples. Alternatively, use test organism *Artemia salina* (a saltwater organism) in combination with ASPM (Guillard, 1983) as dilution medium.

7.3 Terms and definitions

Ephippia:	Winter eggs of the water flea
Hatching:	The emergence and development of the ephippia
EC_{50}:	The toxicity of a sample or substance expressed as the concentration at which 50% fluorescence inhibition occurs in test organisms relative to the blank.
NOEC:	No Observed Effect Concentration = the highest concentration of a toxin at which no observable negative effects are found.
Reference toxicant:	A toxic substance (potassium dichromate in this case; DO take notice of safety aspects as mentioned in section 7.15) used to establish the sensitivity of a batch of ephippia.

7.4 Principle of the test

Healthy Daphnia juveniles can absorb the non-fluorescent substrate 4-methylumbelliferyl-B-D-galactoside and split it enzymatically using the galactosidase enzyme. This reaction releases fluorescent umbelliferon, which displays strong fluorescence under UV-light. If one or more steps in the enzymatic process are hampered by stress (toxicant), the fluorescence signal will be weaker, or even absent. The degree of fluorescence is therefore a measure of the toxicity. Fluorescence is measured relative to a control without toxicant.

7.5 Reagents and additives

- Ephippia of the water flea *Daphnia magna* or *Daphnia pulex* (Aqua Survey Inc. New Jersey, USA)
- Dilution water (DSW[13] or Elendt[14])
- MUG or MUF (4-methylumbelliferyl-ß-D-galactopyranoside). Cas. no: 6160-78-7
- Test kits/reagents for measuring ammonium and nitrite (e.g. cuvette test developed by Dr. Lange, Tiel or test strips from Merck, Amsterdam)
- Ultrapure water (e.g. Millipore, Etten-Leur or Salm & Kipp, Breukelen)
- Reference toxicant (potassium dichromate $K_2Cr_2O_7$ p.a.)

7.6 Equipment

- Microsieve (100 µm)
- Climate-controlled test space (20 °C ± 1 °C)
- Longwave ultraviolet light source, wavelength 365 nm (black light)
- pH-meter
- Oxygen meter
- Conductivity meter
- Petri dishes (glass)
- Automatic pipettes (1 and 5 ml)
- Pasteur pipettes
- Suction pipe (Ø <0.5 cm)
- Volumetric flasks, conical flasks and volumetric cylinders

7.7 Reference substance

Potassium dichromate ($K_2Cr_2O_7$ p.a.) is used as the reference substance for the quality test, measured from a stock solution of 1000 mg/l. It can be kept refrigerated for at least a week after preparation (probably much longer; not tested here).

7.8 Method

In the description below the methods for testing environmental samples and for the quality test have been combined.

7.8.1 Preparing the test organisms[15]

- Aerate the dilution water for 4 hours before using it to hatch the winter eggs.
- Pour the winter eggs (ephippia) from the vial into a microsieve and rinse away the remains of the medium in which the eggs were stored with cold tapwater.
- Transfer the eggs to a petri dish containing 50 ml of dilution water.

[13] Dutch Standard Water (DSW) is demineralised water to which some salts were added.

[14] Elendt-medium M4 (Elendt, 1990).

[15] Alternatively, juveniles can be used from a Daphnia culture (age > 24 hours but less than 5 days).

- Cover the petri dish with a lid and place it in an incubator at 20 °C and in continuous light (approximately 90 µE/m^2/sec.= µmol/m^2/sec). Place a dish containing a layer of water in the bottom of the incubator.

The embryonal development of the water flea *Daphnia magna* takes at least 3 days, the development of *Daphnia pulex* at least 5. Use only juveniles that hatch within 24 hours of the first eggs in the toxicity test.

The test organism *Artemia salina* may also be used. Since this is a saltwater organism, ASPM (Guillard, 1983) should then be used as the dilution medium.

7.8.2 Preparing for the test (environmental samples)

7.8.2.1 Surface water/concentrate

The sample should be free of particles, as they can hamper the scoring of the test. It should preferably be tested with as little pretreatment as possible. Any particles should be removed initially by means of settling and decanting, thereafter by centrifugation, repeated if necessary.[16]

7.8.2.2 Extracts of surface water

If extracts are used in a solvent whose toxicity is unknown, its toxicity to the test organism must first be established. To this end, a test is performed with the solvent. Before testing, the solvent is diluted ten times and then 1:1, to produce the following series of concentrations: 10 – 5 – 2.5 – 1.25 vol%. The results of the test allow a sample dilution to be determined. The sample should be diluted in such as way that the solvent causes less than 10% fluorescence inhibition in the test organisms relative to the blank (see section 10).

7.8.2.3 Preparation

Produce from the sample a geometric series of dilutions (dilution factor 2) in test tubes. If desired, a solvent control may be produced.
Given the possible effects of physical and chemical parameters, measure the pH, oxygen content, conductivity, and the nitrite and ammonium contents of the control (DSW) and the highest concentration.[17] If the parameter criteria are exceeded in the highest concentration, measure the parameters in the other dilutions[18].

- pH (using a pH meter);
- oxygen content (using an oxygen electrode);
- nitrite content (using a test kit);
- ammonium content (using a test kit);
- conductivity (using a conductivity meter).

Fill the petri dishes with 50 ml of the sample, using each concentration in turn ([0] is the blank (EPA), [5] is the highest test concentration).[19].

[16] If the sample is still turbid, filtration over glass fibre or eventually 0.45 µm can be applied.

[17] Provided sufficient sample is available.

[18] It is important to note exceedances. During processing of the data, this information can be used to decide whether all toxicity data can be used.

[19] Alternatively, use 5 ml of liquid in 10 ml test tubes.

7.8.3 Test preparation (quality test)

- Make a stock solution by weighing out 1.50 g $K_2Cr_2O_7$ on an analytical balance and dissolving it in 100 ml milli-Q. Dilute this stock solution 10 times using DSW medium to give the stock solution of 1500 mg $K_2Cr_2O_7$/l.
- Use the stock solution (1500 mg $K_2Cr_2O_7$/l) to make a series of dilutions (geometric) in conical flasks, in accordance with Table 2.
- Measure the parameters (oxygen, pH, ammonium, nitrite, conductivity and temperature) in the control (DSW or Elendt) and the highest concentration.
- Fill the petri dishes with 50 ml of each concentration ([0] is the blank (DSW or Elendt), [5] is the highest test concentration).

Table 2. Preparation of dilutions.

Conical flask	Concentration (mg/l)	ml stock	ml DSW
[0]	0	0	150
[1]	10	1.0	149
[2]	17	1.7	148.3
[3]	32	3.2	146.8
[4]	56	5.6	144.4
[5]	100	10	140
	Total needed:	21.5	878.5

7.8.4 Test implementation (quality test + environmental samples)

- Use a glass tube (Ø < 0.5 cm) to transfer 6 juveniles to each petri dish. Try to use as little liquid as possible, in order not to dilute the sample.
- Make a stock solution of 250 mg MUG/l.
- Incubate the petri dishes containing the juveniles for 1 hour at room temperature.
- Pipette 1 ml[20] of the fluorometric biomarker MUG into each petri dish. Incubate for 15 minutes at room temperature.
- Illuminate each petri dish with a longwave UV lamp in a darkened room. Note the intensity of fluorescence in the water fleas (visual assessment).

7.9 Parameters

A number of parameters have been drawn up for the toxicity test using Daphnia. The following parameters apply to this test:

- pH: 5.5-9.5;
- oxygen content: > 20% of saturation value;
- temperature: 20 ± 2 °C;
- ammonium content: < 60 mg/l (at pH=8.0, 20 °C)
  < 15 mg/l (at pH=8.5, 20 °C);
- nitrite content: < 10 mg/l;
- conductivity: < 6500 µS/cm.

[20] If 5 ml of liquid is used, add 100 µl MUG.

7.10 Validity criteria

The following validity criteria apply to the Daphnia IQ toxicity test:

- At least 85% of the juveniles in the control must show strong fluorescence after exposure to a UV lamp.
- The solvent used must cause less than 10% fluorescence inhibition relative to the blank.
- The quality test must be valid.

7.11 Statistical processing

The significance of differences in the average percentage fluorescence per concentration relative to the blank is assessed by means of a hypothesis test (e.g. Dunnett's or William's test). This is used to determine the values of the $NOEC_{fluorescence}$. A parametric (e.g. Maximum Likelihood Probit) or non-parametric (e.g. Spearman-Karber) method is also used to determine the concentration that produces 50% inhibition (EC_{50}) among the test organisms after exposure.

7.12 Reference substance toxicity

After a quality test has been performed, it will be necessary to check whether all the validity criteria of the test have been fulfilled (see section 10). Then, the EC_{50} fluorescence will have to be determined on the basis of the results, and this value entered in the Shewart chart for this quality test. If the EC50 does not fall within the range defined, the batch of juveniles must be rejected.

Preliminary acceptance limits[21] for quality test with cultured juveniles:
EC_{50}: 30 – 50 mg/l potassium dichromate

7.13 Reporting

The report of the test will need to include at least the following:

- pretreatment of sample;
- sample data;
- sample parameter measurements;
- calculated toxicity data for the sample (EC_{50});
- calculated toxicity data for the reference substance (EC_{50});
- shewart chart for the reference substance.

7.14 Quality assurance

To guarantee the quality of the test for application on surface water samples, the test must meet the following quality criteria:

[21] Limits for DCA: 420 – 720 mg/l

- Repeatability: unknown
- Rounding interval: unknown
- Reproducibility: unknown

7.15 Safety

- Always wear suitable protective clothing (lab coat and gloves) when performing the tests.
- Regard the contaminated samples as chemical waste and dispose of accordingly.
- Handle the reference toxicant potassium dichromate with due care. Read the toxicity data and safety instructions before use.

7.16 Reference and additional information

Guillard, R.R.L. (1983). Culture of phytoplankton for feeding marine invertebrates, 108-132. Culture of Marine Invertebrates, Bert C.J.Jr (Editor).

More information can be found in:

Aqua Survey Inc. (1997). DAPHNIA IQ TOXICITY TEST KIT™. Daphnia toxicity screening test for freshwater, instructions.

Elendt, B.P. (1990). Selenium deficiency in Crustacea. An ultrastructural approach to antennal damage in *Daphnia magna* Straus. Protoplasma 154: 25-33.

NPR 6503 (1980). Benodigdheden, werkwijze en medium voor het kweken van Daphnia magna en van de hiervoor als voedsel benodigde algen.

Postma JF, de Valk S., Dubbeldam M., Maas. J.L., Tonkes, M., Schipper CA, Kater B.J. (2002). Confounding factors in bioassays with freshwater and marine organisms. Ecotox. Environ. Saf. 53: 226-237.

7.17 *Daphnia magna* or *pulex* parameter form

Project: :
Sample: :
Storage conditions :
Date of receipt :
Concentration series : 1)…… 2)…… 3)…….4)……5)…….controls)……...
Sample type: : surface water, extract, solvent, reference substance, other:
Sample frozen? : Yes/No
Extract dilution :
Date of test :

O_2 adjustment ☐ yes, always to approximately 100% (sample with high O_2 consumption)
☐ yes, if < ..... % raise to at least ..... %

Sample	**Treatment** [22]							**Test organism**		**O_2 (%)**		**pH**		**Temp. (ºC)**	**Conductivity (µS/mm)** [23]	**NO_2^- (mg/l)**	**NH_4^+ + NH_3**	
	No treatment	Settle, decant	Centrifuge	Fibre-glass filter	Filter 0.45 µm	Aerate	Adjust pH	Daphnia magna/pulex		>20		5 - 11		-	<120	<2	<30 (25, 8.0)	
										before	after[24]	before	after					remarks

[22] surface water and effluent/influent should preferably be tested in an untreated condition. If it contains too many particles, allow them to settle and decant the sample. Then use a centrifuge and/or filtration through fibre-glass filter and, finally, through a filtration filter.
[23] salinity (‰) = conductivity (µS/mm) × 0.0078
[24] value before and after adjustment shown in table

7.18 Score list for acute toxicity test using water flea Daphnia

Performed by: .....
Project number: .....
Sample: .....
Start date: .....
Test started at: ..... **am/pm**
Age of organisms: **juvenile (< 24 hours)**
☐ **24-hour score . Score date: ..... Test scored at: ..... am/pm**

Code	Replica	No. fluorescing at start	No. fluorescing after 24 hrs. per replica
Blank [0] (DSW)	A	6	
	B	6	
	C	6	
	total	18	
conc. [1]	A	6	
	B	6	
	C	6	
	total	18	
conc. [2]	A	6	
	B	6	
	C	6	
	total	18	
conc. [3]	A	6	
	B	6	
	C	6	
	total	18	
conc. [4]	A	6	
	B	6	
	C	6	
	total	18	
conc. [5]	A	6	
	B	6	
	C	6	
	total	18	

8 Toxicity test using *Thamnocephalus platyurus* (THAMNOTOXkit-F)

Keywords: crustacean, *Thamnocephalus platyurus*, quality test, monitoring, surface water

8.1 Subject

This protocol describes a toxicity test using the crustacean *Thamnocephalus platyurus* whereby mortality in juveniles is assessed after 24 hours of exposure. The method is derived from the CREASEL V140195 (Creasel, 1995) guidelines. Mortality is studied as the effect parameter. A quality test for establishing the sensitivity of batches of cysts is also described. The quality test uses potassium dichromate as reference substance.

8.2 Area of application

The protocol described here has been drawn up for tests on surface water and extracts of surface water. Since *Thamnocephalus platyurus* are used, the protocol is applicable only to freshwater samples.

8.3 Terms and definitions

Cysts:	'Dormant eggs' of the crustacean *Thamnocephalus platyurus*.
Hatching:	The emergence and development of the cysts.
LC_{50}:	The toxicity of a sample or substance expressed as the concentration at which 50% of the test organisms die.
NOEC:	No Observed Effect Concentration – the highest concentration of a toxin at which no observable negative effects are found.
Reference toxicant:	A toxic substance (potassium dichromate in this case; DO take notice of safety aspects as mentioned in section 8.15) used to establish the sensitivity of a batch of cysts.

8.4 Principle of the test

During the toxicity test juveniles of the crustacean *Thamnocephalus platyurus* are exposed to a sample for 24 hours. The test is conducted with a control and five concentrations, so that the LC_{50} and NOEC-$_{(mortality)}$ can be determined.

8.5 Reagents and additives

- Cysts of the crustacean *Thamnocephalus platyurus* (Creasel, Deinze, Belgium)
- EPA medium (Freeman, 1953 and US EPA, 1985)
- Test kits/reagents for measuring ammonium and nitrite (e.g. cuvette test developed by Dr. Lange, Tiel or test strips from Merck, Amsterdam)

- Ultrapure water (e.g. Millipore, Etten-Leur or Salm & Kipp, Breukelen)
- Reference toxicant (potassium dichromate $K_2Cr_2O_7$ p.a.)

8.6 Equipment

- Multiwell test plates, plastic (24 wells) (Creasel, Deinze, Belgium)[25]
- Climate-controlled test space (25 °C ± 1 °C)
- Parafilm
- Stereomicroscope (10-30x magnification)
- pH-meter
- Oxygen meter
- Conductivity meter
- Test tubes (10 ml)
- Automatic pipettes (1 and 5 ml)
- Pasteur pipettes (with the tip heated and bent slightly)
- Volumetric flasks and cylinders
- Petri dish

8.7 Reference substance

Potassium dichromate ($K_2Cr_2O_7$ p.a.) is used as the reference substance for the quality test, measured from a stock solution of 1000 mg/l. It can be kept refrigerated for up to a week after preparation.

8.8 Method

In the description below the methods for testing environmental samples and for the quality test have been combined.

8.8.1 Preparing the test organisms

- Use one cup of cysts for each test plate.
- Dilute the EPA medium eight times using milli-Q.
- Add 1 ml of diluted EPA medium to one cup of cysts and shake thoroughly.
- Empty the cup completely into a petri dish. Rinse the cup using a Pasteur pipette if necessary. Add approximately 50 ml of diluted EPA medium per cup to the petri dish.
- Cover the petri dish with a lid.
- Place the petri dish in the incubator for 14-18 hours at 25 °C and in continuous light (approximately 30 $\mu E/m^2/sec. = \mu mol/m^2/sec$). Place a dish containing a layer of water in the bottom of the incubator.

[25] If the test is performed with organic substances or if it is expected that an environmental sample contains organic substances, glass cuvettes instead of plastic plates should be used (Vaal *et al.*, 1998).

8.8.2 Preparing for the test (environmental samples)

8.8.2.1 Surface water/concentrate

The sample should be free of particles, as they can hamper the scoring of the test. It should preferably be tested with as little pretreatment as possible. Any particles should be removed initially by means of settling and decanting, thereafter by centrifugation, repeated if necessary.[26]

8.8.2.2 Extracts of surface water

If extracts are used in a solvent whose toxicity is unknown, its toxicity to *Thamnocephalus platyurus* must first be established. To this end, a test is performed with the solvent. Before testing, the solvent is diluted ten times and then 1:1, to produce the following series of concentrations:
10 – 5 – 2.5 – 1.25 vol%. The results of the test allow a sample dilution to be determined. The sample should be diluted in such as way that the solvent causes less than 10% mortality in the test organisms (see section 8.10).

8.8.2.3 Preparation

- Produce from the sample a geometric series of dilutions (dilution factor 2) in test tubes. If desired, a solvent control may be produced, replacing one concentration in the series, due to lack of space on the test plate.
- Given the possible effects of physical and chemical parameters, measure the pH, oxygen content, conductivity, and the nitrite and ammonium contents of the control (EPA) and the highest concentration.[27] If the parameter criteria are exceeded in the highest concentration, measure the parameters in the other dilutions[28].
    - pH (using a pH meter);
    - oxygen content (using an oxygen electrode);
    - nitrite content (using a test kit);
    - ammonium content (using a test kit);
    - conductivity (using a conductivity meter).

[26] If the sample is still turbid, filtration over glass fibre or eventually 0.45 µm can be applied.

[27] Provided sufficient sample is available.

[28] It is important to note exceedances. During processing of the data, this information can be used to decide whether all toxicity data can be used.

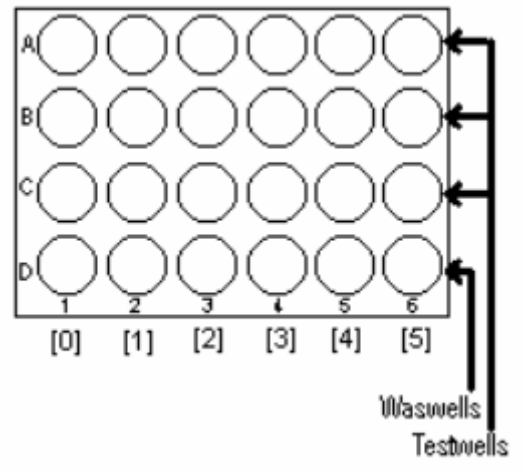

Figure 4. Picture showing the rinsing wells (waswells) and test wells.

- Fill the test wells and rinsing wells of the multiwell test plate with the sample from the test tubes, using each concentration in turn ([0] is the blank (EPA), [5] is the highest test concentration). Place 1.0 ml in each test well and rinsing well.

8.8.3 Test preparation (quality test)

- Make a stock solution by weighing out 0.100 g $K_2Cr_2O_7$ on an analytical balance and dissolving it in 100 ml milli-Q. Dilute this stock solution 1000 times using EPA medium to give the stock solution of 1mg $K_2Cr_2O_7$/l.
- Use the stock solution (1 mg $K_2Cr_2O_7$/l) to make a series of dilutions (geometric) in test tubes, in accordance with Table 3.

Table 3. Preparation of dilutions.

Test tube	Concentration (mg/l)	ml stock	ml EPA
1	0	0	5.0
2	0.032	0.2	4.8
3	0.056	0.3	4.7
4	0.1	0.5	4.5
5	0.18	0.9	4.1
6	0.32	1.6	3.4
	Total needed:	3.4	26.6

- Measure the parameters (oxygen, pH and temperature) in the control (EPA) and the highest concentration.
- Fill the test wells and rinsing wells of the multiwell test plate with the sample from the test tubes, using each concentration in turn ([0] is the blank (EPA), [5] is the highest test concentration). Place 1.0 ml in each test well and rinsing well.

8.8.4 Test implementation (quality test + environmental samples)

- Check whether enough cysts have hatched for the test using a stereomicroscope (10-30x). If necessary, apply a 10-minute 30 °C heat shock, followed by rapid cooling to 25 °C to accelerate hatching.
- Using a Pasteur pipette, place at least 30 specimens from the petri dish in each rinsing well, without allowing the tip of the pipette to come into contact with the liquid in the rinsing well.
- Pipette (using a clean pipette) 10 specimens from the rinsing well into each test well.

- Cover the multiwell test plate with parafilm and lid, and return it to a darkened incubator at 25 °C.
- After 24 hours check the number of mobile specimens using the stereomicroscope (10-30x). Immobile = dead or no swimming activity within 15 seconds of slight agitation of the test plate, though antennae still moving.[29]

8.9 Parameters

A number of parameters have been drawn up for the toxicity test using *Thamnocephalus platyurus* (Postma et al., 2002). The following parameters apply to this test:

- pH: 5-11;
- oxygen content: > 10% of saturation value;
- temperature: 25 ± 2 °C;
- ammonium content: < 30 mg/l (at pH=8.0, 25 °C);
- nitrite content: < 2 mg/l;
- conductivity: < 1200 µS/cm.

8.10 Validity criteria

The following validity criteria apply to the toxicity test using *Thamnocephalus platyurus*:

- Mortality among the animals in the control must be no more than 10% within 24 hours of commencing the test.
- The solvent used should cause less than 10% mortality relative to the blank.
- The quality test must be valid.

8.11 Statistical processing

The significance of differences in the average percentage mortality per concentration relative to the blank is assessed by means of a hypothesis test (e.g. Dunnett's or William's test). This is used to determine the values of the $NOEC_{mortality}$. A parametric (e.g. Maximum Likelihood Probit) or non-parametric (e.g. Spearman-Karber) method is also used to determine the concentration that produces 50% mortality (LC_{50}) among the test organisms after 24 hours of exposure.

8.12 Reference substance toxicity

After a quality test has been performed, it will be necessary to check whether all the validity criteria of the test have been fulfilled (see section 9). Then, the LC_{50} *mortality* will have to be determined on the basis of the results, and the value entered in the Shewart chart for this quality test. If the LC_{50} does not fall within the range defined, the batch of cysts must be rejected.

Acceptance limits for potassium dichromate: LC_{50} mortality: 0.10 – 0.20 mg/l $K_2Cr_2O_7$

[29] Strictly, one should speak about EC_{50} rather than LC_{50}.

8.13 Reporting

The report of the test will need to include at least the following:

- pretreatment of sample;
- sample data;
- sample parameter measurements;
- calculated toxicity data for the sample (LC_{50} and $NOEC_{mortality}$);
- calculated toxicity data for the reference substance (LC_{50}).

8.14 Quality assurance

The methods drawn up by the RIKZ and laid down in standard instruction no. i013.90 (*Validation of analysis methods and control/evaluation of performance characteristics*) and i020.90 (*Drafting analysis characteristics*) are used to calculate the (preliminary) quality criteria. The following criteria are important:

- repeatability: *unknown;*
- reproducibility: 72%;
- rounding interval: 10 decimal places (µg/l).

8.15 Safety

- Always wear suitable protective clothing (lab coat and gloves) when performing the tests.
- Regard the contaminated samples as chemical waste and dispose of accordingly.
- Handle the reference toxicant with due care. Read the toxicity data and safety instructions before use.

8.16 References

Creasel (1995). THAMNOTOXKIT F . Crustacean toxicity screening test for freshwater, standard operational procedure. V140195.

Freeman, W. (1953). A standardized method for determining toxicity of pure compounds to fish. Sewage and industrial wastes, 25:845-848.

Postma JF, de Valk S., Dubbeldam M., Maas. J.L., Tonkes, M., Schipper CA, Kater B.J. (2002). Confounding factors in bioassays with freshwater and marine organisms. Ecotox. Environ. Saf. 53: 226-237.

U.S. EPA (1985). Methods for measuring the acute toxicity of effluents to freshwater and marine organisms. EPA 600/4-85-013 Envirn. Research Laboratory, Duluth.

Vaal, MA, AJ Folkerts (1998). Sensitivity of microscale ecotoxicity tests and their suitability to measure toxicity of environmental samples. RIVM, report no. 607042009.

8.17 *Thamnocephalus platyurus* parameters form

Project: **:**
Sample **:**
Storage conditions **:**
Date of receipt **:**
Concentration series **: 1)…… 2)…… 3)…….4)……5)…….**
Sample type **: surface water, extract, solvent, reference substance, other:**
Sample frozen? **: Yes/No**
Extract dilution **:**
Date of test **:**

O_2 adjustment ☐ yes, always to approximately 100% (sample with high O_2 consumption)
☐ yes, if < ..... % raise to at least ..... %

Sample	**Treatment**[30]							**Test organism**		**O_2 (%)**		**pH**		**Temp. (ºC)**	**Conductivity** [31]**(µS/mm)**	**NO_2^- (mg/l)**	**$NH_4^+ + NH_3$ (mg/l) (ºC, pH)**	
	No treatment	Settle, decant	Centrifuge	Fibre-glass filter	Filtrer 0.45 µm	Aerate	Adjust pH	*Thamnocephalus platyurus*		>20		5 - 11		-	<120	<2	<30 (25, 8.0)	
								Time	Conc.	Before	After[32]	Before	After					Remarks

[30] surface water and effluent/influent should preferably be tested in an untreated condition. If it contains too many particles, allow them to settle and decant the sample. Then use a centrifuge and/or filtration through fibre-glass filter and, finally, through a filtration filter.
[31] salinity (‰) = conductivity (µS/mm) x 0.0078
[32] value before and after adjustment shown in table

8.18 Score list for acute toxicity test using crustacea *Thamnocephalus platyurus*

Performed by: .....
Project number: .....
Sample: .....
Start date: .....
Test started at: ..... **am/pm**
Age of organisms: **juvenile (instar II – III naupli)**
☐ **24-hour score . Score date: ..... Test scored at: ..... am/pm**

Code	Replica	No. mobile at start	No. mobile after 24 hours per replica
Blank [0] (EPA)	A	10	
	B	10	
	C	10	
	total	30	
conc. [1]	A	10	
	B	10	
	C	10	
	total	30	
conc. [2]	A	10	
	B	10	
	C	10	
	total	30	
conc. [3]	A	10	
	B	10	
	C	10	
	total	30	
conc. [4]	A	10	
	B	10	
	C	10	
	total	30	
conc. [5]	A	10	
	B	10	
	C	10	
	Total	30	

9 Toxicity test using *Brachionus calyciflorus* (ROTOXkit-F)

Keywords: rotifer, *Brachionus calyciflorus*, quality test, monitoring, surface water

9.1 Subject

This protocol describes a toxicity test using the rotifer *Brachionus calyciflorus* whereby the effects in juvenile rotifers are assessed after 24 hours of exposure. The method is derived from the Creasel V240195 (1992) guidelines. Mortality is studied as the effect parameter. A quality test for establishing the sensitivity of batches of cysts is also described. The quality test uses potassium dichromate as reference substance.

9.2 Area of application

The protocol described here has been drawn up for tests on surface water and extracts of surface water. Since *Brachionus calyciflorus* are used, the protocol is applicable only to freshwater samples.

9.3 Terms and definitions

Cysts:	'Dormant eggs' of the rotifer *Brachionus calyciflorus*.
Hatching:	The emergence and development of the cysts.
LC_{50}:	The toxicity of a sample or substance expressed as the concentration at which 50% of the test organisms die.
NOEC:	No Observed Effect Concentration – the highest concentration of a toxin at which no observable negative effects are found.
Reference toxicant:	A toxic substance (potassium dichromate in this case; DO take notice of safety aspects as mentioned in section 9.15) used to establish the sensitivity of a batch of cysts.

9.4 Principle of the test

During the toxicity test juveniles of the rotifer *Brachionus calyciflorus* are exposed to a sample for 24 hours. The test is conducted with a control and five concentrations, so that the LC_{50} and NOEC-$_{(mortality)}$ can be determined.

9.5 Reagents and additives

- Cysts of the rotifer Brachionus calyciflorus (Creasel)
- EPA medium (Freeman, 1953 and US EPA, 1985)
- Test kits/reagents for measuring ammonium and nitrite (e.g. cuvette test developed by Dr. Lange, Tiel or test strips from Merck, Amsterdam)

- Ultrapure water (e.g. Millipore, Etten-Leur or Salm & Kipp, Breukelen)
- Reference toxicant (potassium dichromate $K_2Cr_2O_7$ p.a.)

9.6 Equipment

- Multiwell test plates, plastic (36 wells + 6 rinsing troughs + 1 hatching trough) (Creasel, Deinze, Belgium)[33]
- Climate-controlled test space (25 °C ± 1 °C)
- Parafilm
- Stereomicroscope (10-30x magnification)
- pH-meter
- Oxygen meter
- Conductivity meter
- Test tubes (10 ml)
- Automatic pipettes (1 and 5 ml)
- Micropipettes (polyethylene, 1 ml)
- Volumetric flasks and cylinders

9.7 Reference substance

Potassium dichromate ($K_2Cr_2O_7$ p.a.) is used as the reference substance for the quality test, measured from a stock solution of 1000 mg/l. It can be kept refrigerated for up to a week after preparation.

9.8 Method

In the description below the methods for testing environmental samples and for the quality test have been combined.

9.8.1 Preparing the test organisms

- Add 1 ml of EPA medium to 1 cup of cysts and shake thoroughly.
- Empty the cup completely into the hatching trough on the multiwell test plate. If necessary, rinse the cup out using a Pasteur pipette. Add a further 1.5 ml of EPA medium to the hatching trough.
- Cover the multiwell test plate with parafilm and a lid.
- Place the test plate in the incubator for 14-18 hours at 25 °C and in continuous light (approximately 30 $\mu E/m^2/sec$). Place a dish containing a layer of water in the bottom of the incubator.

9.8.2 Preparing for the test (environmental samples)

9.8.2.1 Surface water

The sample should be free of particles, as they can hamper the scoring of the test. It should preferably be tested with as little pretreatment as possible. Any particles should be removed initially by means of settling and decanting, thereafter by centrifugation, repeated if necessary.

[33] If the test is performed with organic substances or if it is expected that an environmental sample contains organic substances, glass cuvettes instead of plastic plates should be used (Vaal en Folkerts, 1998).

9.8.2.2 Extracts of surface water

If extracts are used in an unknown solvent, its toxicity to *Brachionus calyciflorus* must first be established. To this end, a test is performed with the solvent. Before testing, the solvent is diluted ten times and then 1:1, to produce the following series of concentrations: 10 – 5 – 2.5 – 1.25 vol%. The results of the test allow a sample dilution to be determined. The sample should be diluted in such as way that the solvent causes less than 10% mortality in the test organisms (see section 9.10).

9.8.2.3 Preparation

- Produce from the sample a geometric series of dilutions (dilution factor 2) in test tubes. If desired, a solvent control may be produced, replacing one concentration in the series, due to lack of space on the test plate.
- Given the possible effects of physical and chemical parameters, measure the pH, oxygen content, conductivity, and the nitrite and ammonium contents of the control (EPA) and the highest concentration. If the parameter criteria are exceeded in the highest concentration, measure the parameters in the other dilutions[34].
    - pH (using a pH meter);
    - oxygen content (using an oxygen electrode);
    - nitrite content (using a test kit);
    - ammonium content (using a test kit);
    - conductivity (using a conductivity meter).
- Fill the test wells and rinsing troughs of the multiwell test plate with the sample from the test tubes, using each concentration in turn ([0] is the blank (EPA), [5] is the highest test concentration). Place 0.3 ml in each test well and 0.6 ml in the rinsing troughs.

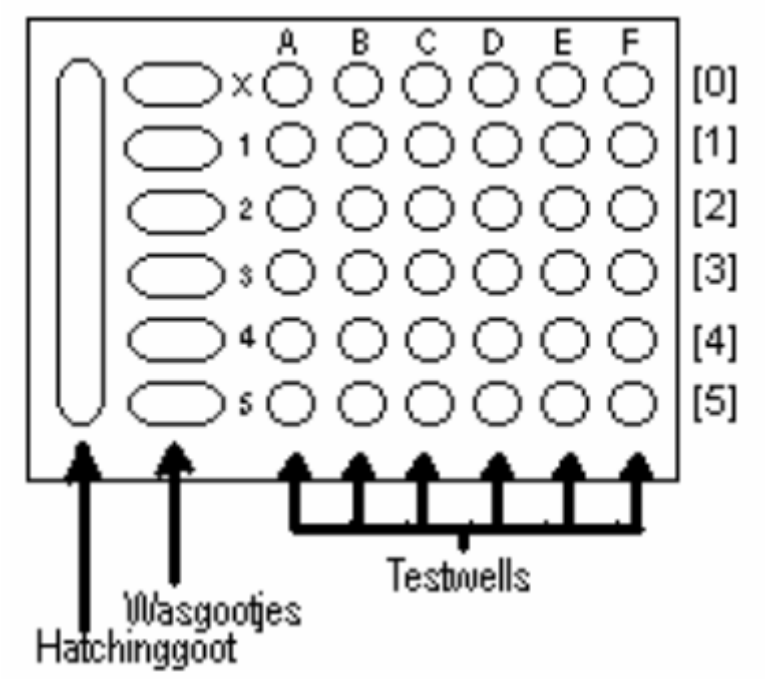

Figure 5. Picture showing the hatching trough (Hatchinggoot), rinsing throughs (Wasgootjes) and test wells.

[34] It is important to note exceedances. During processing of the data, this information can be used to decide whether all toxicity data can be used.

9.8.3 Test preparation (quality test)

- Make a stock solution by weighing out 10 g $K_2Cr_2O_7$ on an analytical balance and dissolving it in 100 ml milli-Q. Dilute this stock solution 1000 times using EPA medium to give the stock solution of 100 mg $K_2Cr_2O_7$/l.
- Use the stock solution (100 mg $K_2Cr_2O_7$/l) to make a series of dilutions (geometric) in test tubes, in accordance with Table 4.

Table 4. Preparation of dilutions.

Test tube	Concentration (mg/l)	ml stock	ml EPA
1	0	0	5.0
2	4	0.2	4.8
3	6	0.3	4.7
4	10	0.5	4.5
5	18	0.9	4.1
6	32	1.6	3.4
	Total needed:	3.4	26.6

- Measure the parameters (oxygen, pH and temperature) in the control (EPA) and the highest concentration.
- Fill the test wells and rinsing troughs of the multiwell test plate with the sample from the test tubes, using each concentration in turn ([0] is the blank (EPA), [5] is the highest test concentration). Place 0.3 ml in each test well and 0.6 ml in each rinsing trough.

9.8.4 Test implementation (quality test + environmental samples)

- Check whether enough cysts have hatched for the test using a binocular microscope (10-30x). If necessary, give a 10-minute 30 °C heat shock, followed by rapid cooling to 25 °C to accelerate hatching.
- Using a micropipette, place at least 30 rotifers from the hatching trough in each rinsing trough, without allowing the tip of the pipette to come into contact with the rinsing trough.
- Pipette (using a clean pipette) 5 rotifers from the rinsing troughs into each test well.
- Cover the multiwell test plate with parafilm and a lid, and return it to a darkened incubator at 25 °C.
- After 24 hours check the number of mobile specimens using the stereomicroscope (10-30x). Immobile = dead or no swimming activity within 15 seconds of slight agitation of the test plate.[35]

9.9 Parameters

A number of parameters have been drawn up for the toxicity test using *Brachionus calyciflorus* (Postma et al., 2002). The following parameters apply to this test:

- pH: 5-9;
- oxygen content: > 15% of saturation value;
- temperature: 25 ± 2 °C;
- ammonium content: < 70 mg/l (at pH=8.0, 25 °C);

[35] Strictly, one should speak about EC_{50} rather than LC_{50}.

- nitrite content: < 50 mg/l;
- conductivity: < 3200 µS/cm.

9.10 Validity criteria

The following validity criteria apply to the toxicity test using *Brachionus calyciflorus*:
- Mortality among the rotifers in the control must be no more than 10% within 24 hours of commencing the test.
- The solvent used should cause less than 10% mortality relative to the blank.
- The quality test must be valid.

9.11 Statistical processing

The significance of differences in the average percentage mortality per concentration relative to the blank is assessed by means of a hypothesis test (e.g. Dunnett's or William's test). This is used to determine the values of the $NOEC_{mortality}$. A parametric (e.g. Maximum Likelihood Probit) or non-parametric (e.g. Spearman-Karber) method is also used to determine the concentration that produces 50% mortality (LC_{50}) among the test organisms after 24 hours of exposure.

9.12 Reference substance toxicity

After a quality test has been performed, it will be necessary to check whether all the validity criteria of the test have been fulfilled (see section 10). Then, the LC_{50} *mortality* will have to be determined on the basis of the results and the value entered in the Shewart chart for this quality test. If the LC_{50} does not fall within the range defined, the batch of cysts must be rejected.

Acceptance limits for potassium dichromate: LC_{50} mortality: 9.60 – 17.80 mg/l $K_2Cr_2O_7$.

9.13 Reporting

The report of the test will need to include at least the following:
- pretreatment of sample;
- sample data;
- sample parameter measurements;
- calculated toxicity data for the sample (LC_{50} and $NOEC_{mortality}$);
- calculated toxicity data for the reference substance (LC_{50});
- shewart chart for the reference substance.

9.14 Quality assurance

The methods drawn up by the RIKZ and laid down in standard instruction no. i013.90 (*Validation of analysis methods and control/evaluation of performance characteristics*) and i020.90 (*Drafting*

analysis characteristics) are used to calculate the (preliminary) quality criteria. The following criteria are important:
Repeatability: *unknown*
Reproducibility: 78%
Rounding interval: 10 decimal places (µg/l)

9.15 Safety

- Always wear suitable protective clothing (lab coat and gloves) when performing the tests.
- Regard the contaminated samples as chemical waste and dispose of accordingly.
- Handle the reference toxicant potassium dichromate with due care. Read the toxicity data and safety instructions before use.

9.16 References

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9.17 *Brachionus calyciflorus* parameter form

Project: :
Sample :
Storage conditions :
Date of receipt :
Concentration series : 1)…… 2)…… 3)……. 4)…… 5)……..
Sample type: surface water; extract; solvent; reference substance; other:
Sample frozen? yes/no
Date of test :

Adjust O_2 ☐ yes, always to approximately 100% (sample with high O_2 consumption)
☐ yes, if < …. % raise to at least ….%

Sample	Treatment[36]							Test organism		O_2 (%)		pH		Temp. (ºC)	Conductivity[37] (µS/mm)	NO_2^- (mg/l)	$NH_4^+ + NH_3$ (mg/l) (ºC,pH)	
	No treatment	Settle, decant	Centrifuge	Fibre-glass filter	Filter 0.45 µm	Aerate	Adjust pH	*Brachionus calyciflorus*		>20		5 - 11		-	<120	<2	<30 (25, 8.0)	
								Time	Conc.	Before	After[38]	Before	After					Remarks

[36] surface water and effluent/influent should preferably be tested in an untreated condition. If it contains too many particles, allow them to settle and decant the sample. Then use a centrifuge and/or filtration through fibre-glass filter and, finally, through a filtration filter.

[37] salinity (‰) = conductivity (µS/mm) x 0.0078

[38] value before and after adjustment shown in table

9.18 Score list for acute toxicity test using rotifer *Brachionus calyciflorus*

Performed by: .....
Project number: .....
Sample: .....
Start date: .....
Test started at: ..... **am/pm**
Age of organisms: **juvenile (instar II – III naupli)**
☐ **24-hour score . Score date: ..... Test scored at: ..... am/pm**

Code	Replica	No. mobile at start	No. mobile after 24 hours per replica
Blank [0] (EPA)	A	10	
	B	10	
	C	10	
	total	30	
conc. [1]	A	10	
	B	10	
	C	10	
	total	30	
conc. [2]	A	10	
	B	10	
	C	10	
	total	30	
conc. [3]	A	10	
	B	10	
	C	10	
	total	30	
conc. [4]	A	10	
	B	10	
	C	10	
	total	30	
conc. [5]	A	10	
	B	10	
	C	10	
	Total	30	

10 Chydotox toxicity test

Keywords: *Chydorus sphaericus*, quality test, monitoring, surface water

10.1 Subject

Chydorus sphaericus is one of the most common cladocerans in The Netherlands, which makes it a more representative test species than *Daphnia magna* for the Dutch situation. The Chydotox-test is a promising alternative for the existing Daphnia sp. acute immobilisation test (OECD, 1984). Working with smaller volumes, materials and being less time consuming than the traditional Daphnia magna test regarding the culture and experimental design, the 'Chydotox-test' shows a comparable sensitivity.

The Chydotox test for surface water is developed from the Chydorid test for sediments (Dekker et al., 2002; Dekker et al., 2006).

10.2 Area of application

The protocol described here has been drawn up for tests on surface water and extracts of surface water. Since *Chydorus sphaericus* is used, the protocol is applicable only to freshwater samples.

10.3 Terms and definitions

Parthenogenetic:	reproduction without males, population consists of females producing diploid eggs which don't need fertilization to develop to young cladocerans. Female cladocerans developed from these eggs are genetically identical to their mothers (clones).
Neonates:	first developmental stadium (before first moulting).
Ephippia:	winter eggs, which is a result of sexual reproduction and indicates stress conditions in the culture.
Hatching:	the emergence and development of the ephippia.
LC_{50}:	the toxicity of a sample or substance expressed as the concentration at which 50% mortality occurs in test organisms relative to the blank.
NOEC:	No Observed Effect Concentration = the highest concentration of a toxicant at which no observable negative effects are found.
Reference toxicant:	a toxic substance (potassium dichromate in this case; DO take notice of safety aspects as mentioned in section 10.15) used to establish the sensitivity of a batch of ephippia.

10.4 Principle of the test

During the toxicity test juveniles of the cladocean *Chydorus sphaericus* are exposed to a sample for 48 hours. The test is conducted with a control and a minimum of five concentration steps, so that the LC_{50} can be determined.

10.5 Reagents and additives

- Neonates (<24 h) of *Chydorus sphaericus* born from adult females containing parthogenetic eggs (clone from one gravid female from lake Drontermeer, collected by University from Amsterdam, summer 1998).
- Dilution water (DSW).
- Nitzschia perminuta, batch-cultured in modified WC medium (Van der Grinten et al. 2005).
- Test kits/reagents for measuring ammonium and nitrite (e.g. cuvette test developed by Dr. Lange, Tiel or test strips from Merck, Amsterdam).
- Ultrapure water (e.g. Millipore, Etten-Leur or Salm & Kipp, Breukelen).
- Reference toxicant (potassium dichromate $K_2Cr_2O_7$ (CAS: 7778-50-9).

10.6 Equipment

- Mesh filter with a diameter of 250 µm (stainless steel)
- 200 ml glass jars with a lid
- 250 µl plastic pipette (flexibility of the pipette is important to transfer the neonates to the vial with as little medium as possible)
- Pasteur pipettes
- Medium DSW
- Reverse dissecting microscope
- 2 ml HPLC vials with crimp cap (borosilicate glass)
- Climate-controlled test space (20 °C ± 1 °C)
- pH-meter
- Oxygen meter
- Conductivity meter
- Petri dishes (glass)
- Automatic pipettes (1 and 5 ml) (Biohit)
- Plastic Pasteur pipettes
- Volumetric flasks, conical flasks and volumetric cylinders

10.7 Reference substance

Potassium dichromate (CAS: 7778-50-9) was chosen as reference toxicant because it is recommended as a reference compound by the OECD for the Daphnia sp. acute immobilization test (OECD, 1984) and consequently, a large set of toxicity data is available for this compound.

10.8 Method

In the description below the methods for testing environmental samples and for the quality test have been combined.

10.8.1 Preparing the test organisms

One day before the start of the experiment, collect adult chydorids containing parthenogenetic eggs from the culture by a mesh sieve with a diameter of 250 µm. Transfer adults into glass jars containing

200 ml of DSW medium. Rinse the mess sieve up and down to separate adults from juveniles. Transfer the adults to another 250 ml beaker glass with 200 ml of DSW. Cover the jars with the lid.
Place the jars overnight in a climate room at 20 °C and a light:dark regime of 16:8 h (culture conditions). The next day, collect juveniles by removing adults with the 250 µm sieve. Concentrate juveniles in a small volume (not more than 50 ml) with the 100 µm sieve. Place 5 juveniles in a droplet (diameter 3 mm) and transfer them into a HPLC-vial. Check in the vial (under a lightbeam) if there are five to (at most) seven (preferably five) chydorids present.

10.8.2 Preparing for the test (environmental samples)

10.8.2.1 Surface water/concentrate

- Rinse a scintillation vial with aceton and blow dry with air.
- Add 1000 µl concentrate (1000×) to the scintillation vial, transfer from this vial 500 µl concentratie to a new vial with a pipette and add 500 µl of DSW medium. Stir the vial on a stirring machine.
- Repeat this procedure for the following dilution series. This way, the concentrate can be diluted to the following dilution range: 1000× - 500× - 250× - 125× - 62.5× - 31× - 16× - 8× - 4× - 2× - 1× - 0.5×.
- Transfer 250 µl from the scintillation vial to the testvial. Repeat for duplicate.
- Close the test vials with a crimp cap lid and place the jars overnight in a climate room at 20 ± 1 °C and incubate for 48 h at a light:dark regime of 16:8 h.
- Every dilution is tested in duplicate, the control is performed in DSW medium.

10.8.2.2 Extracts of surface water

If extracts are used in a solvent whose toxicity is unknown, its toxicity to the test organism must first be established. To this end, a test is performed with the solvent. Before testing, the solvent is diluted ten times and then 1:1, to produce the following series of concentrations: 10 – 5 – 2.5 – 1.25 vol%. The results of the test allow a sample dilution to be determined. The sample should be diluted in such as way that the solvent causes less than 10% immobility in the test organisms relative to the blank (see section 10.10).

10.8.2.3 Preparation

- Produce from the sample a geometric series of dilutions (dilution factor 2) in test tubes. If applicable, a solvent control may be produced.
- Given the possible effects of physical and chemical parameters, measure the pH, oxygen content, conductivity, and the nitrite and ammonium contents of the control (DSW) and the highest concentration. If the parameter criteria are exceeded in the highest concentration, measure the parameters in the other dilutions[39].
    - pH (using a pH meter);
    - oxygen content (using an oxygen electrode);
    - nitrite content (using a test kit);
    - ammonium content (using a test kit);
    - conductivity (using a conductivity meter).
- Fill the petri dishes with 50 ml of the sample, using each concentration in turn ([0] is the blank (DSW), [5] is the highest test concentration).

[39] It is important to note exceedances. During processing of the data, this information can be used to decide whether all toxicity data can be used.

10.8.3 Test preparation (quality test)

Make a stock solution by weighing out 150 mg $K_2Cr_2O_7$ on an analytical balance and dissolving it in 100 ml milli-Q. Dilute this stock solution 10 times using DSW medium to give the stock solution of 150 mg $K_2Cr_2O_7$/l.
Use the stock solution (150 mg $K_2Cr_2O_7$/l) to make a series of dilutions (geometric) in volumetric flasks, in accordance with Table 5.

Table 5. Preparation of dilutions.

Volumetric flask	Concentration (mg/l)	ml stock	ml DSW
[0]	0.032	0.032	149.97
[1]	0.1	0.1	149.9
[2]	0.32	0.32	149.68
[3]	0.56	0.56	149.44
[4]	1.8	1.8	148.2
[5]	3.2	3.2	146.8
[6]	10	10	140
	Total needed:	16	1034

Measure the parameters (oxygen, pH, ammonium, nitrite, conductivity and temperature) in the control (DSW) and the highest concentration.

10.8.4 Test implementation (quality test + environmental samples)

- Count the number of neonates in each vial and fill in at t=0 on the score form. Gently add 250 µl test medium in the vials with a pipette subsequently. Check whether any chydorids stick to the glass wall. If so, take care they get immersed in the test medium.
- Cover the vials with a crimp cap to prevent evaporation and incubate for 48 h in a climate room at 20 °C and a light:dark regime of 16:8 h.
- Place the vials under a reverse dissecting microscope after 48 h and determine immobilization by activation of the animals by slightly tapping with a finger to the vial and monitoring them for 30 sec. Take care in observing any floating dead chydorids on the surface. (Note: immobility means not capable of moving any more, but movement of filter bows could be observed).

10.9 Parameters

Since this test is newer than the other tests described in this chapter, not as much experience is gained for this test as for others. Therefore, no detailed information is available yet about the allowable range for the most sensitive parameters. For the time being, we recommend to use the same parameters and ranges as for the Daphnia. A number of parameters have been drawn up for the toxicity test using Daphnia. The following parameters might apply also to this test:

- pH: 5.5-9.5;
- oxygen content: > 20% of saturation value;
- temperature: 20 ± 2 °C;
- ammonium content: < 60 mg/l (at pH=8.0, 20 °C)
  < 15 mg/l (at pH=8.5, 20 °C);
- nitrite content: < 10 mg/l;
- conductivity: < 6500 µS/cm.

10.10 Validity criteria

The following validity criteria apply to the chydotox toxicity test.

- The solvent used must cause less than 10% reduction of mobility relative to the blank.
- The quality test must be valid.

Further validity criteria may need to be investigated for this test.

10.11 Statistical processing

The significance of differences in the average percentage immobility per concentration relative to the blank is assessed by means of a hypothesis test (e.g. Dunnett's or William's test). This is used to determined the values of the NOEC. A parametric (e.g. Maximum Likelihood Probit) or non-parametric (e.g. Spearman-Karber) method is also used to determine the concentration that produces 50% inhibition (EC_{50}) among the test organisms after exposure.

10.12 Reference substance toxicity

An EC_{50}-value of 780 µg/l (95% confidence interval 580 to 980 µg/l) is reported in literature (Pieters et al., 2008). More research is needed to ensure this value is correct. For the time being, it is recommended to accept a series of test when the EC_{50} is between 580 and 980 µg/l.

10.13 Reporting

The report of the test will need to include at least the following:

- pretreatment of sample;
- sample data;
- sample parameter measurements;
- calculated toxicity data for the sample (EC_{50});
- calculated toxicity data for the reference substance (EC_{50});
- shewart chart for the reference substance.

10.14 Quality assurance

To guarantee the quality of the test for application on surface water samples, the test must meet the following quality criteria:

- Repeatability: unknown
- Rounding interval: unknown
- Reproducibility: unknown

10.15 Safety

Always wear suitable protective clothing (lab coat and gloves) when performing the tests.

Regard the contaminated samples as chemical waste and dispose of accordingly.
Handle the reference toxicant potassium dichromate with due care. Read the toxicity data and safety instructions before use.

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10.17 Score form

Scoring (sub)lethal effects Chydorids after 48 hours of exposure to concentrates

Date: Time: t=0

nr\ groep	conc.	A			B			C			D			E			F			G			H		
		d	i	a	d	i	a	d	i	a	d	i	a	d	i	a	d	i	a	d	i	a	d	i	a	d	i	a
1	1000x																											
2	500x																											
3	250x																											
4	125x																											
5	62.5x																											
6	31x																											
7	16x																											
8	8x																											
9	4x																											
10	2x																											
11	1x																											
12	0.5x																											
Total		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

d=dead i= immobile a=active (normal)

Date: Time: t=48 hours

nr\ groep	conc.	A			B			C			D			E			F			G			H		
		d	i	a	d	i	a	d	i	a	d	i	a	d	i	a	d	i	a	d	i	a	d	i	a
1	1000x																								
2	500x																								
3	250x																								
4	125x																								
5	62.5x																								
6	31x																								
7	16x																								
8	8x																								
9	4x																								
10	2x																								
11	1x																								
12	0.5x																								
Total		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

d=dead i= immobile a=active (normal)

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