METHODS FOR DIRECT ESTIMATION OF ECOLOGICAL EFFECT POTENTIAL (DEEEP)

L Slabbert

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METHODS FOR DIRECT ESTIMATION OF ECOLOGICAL EFFECT POTENTIAL (DEEEP)

Report to the Water Research Commission

by

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PREFACE

Experience has shown that substance-specific methods are not in themselves able to fully assess the ecological hazard posed by complex industrial wastewater discharges. To protect the ecological integrity of aquatic ecosystems, a more comprehensive approach is necessary, one that focuses on the potential effects of chemical substances, rather than on the substances themselves.

The effect-based approach typically uses a battery of acute and chronic toxicity tests. Examples of this approach include the Whole Effluent Toxicity (WET) testing used in the USA and the Direct Toxicity Assessment (DTA) used in the UK. These approaches are flexible and can include a variety of tests and test organisms. Several countries have followed suit and selected the most appropriate combination of tests to meet their particular conditions. The TEM [Totale Effluent Milleuhygiene] approach used in the Netherlands is slightly different. In addition to typical acute and chronic toxicity measurements, it also includes other hazard parameters such as oxygen depletion potential, bioaccumulation, and mutagenicity.

In South Africa, the Department of Water Affairs and Forestry (DWAF) has found the TEM approach the most appropriate for ecological hazard assessment, and has adopted a similar approach to manage complex industrial wastewater discharges (DWAF, 2003). The South African approach is called the Direct Estimation of Ecological Effect Potential (DEEEP).

The following ecological hazard parameters and tests to assess the parameters have been selected by DWAF for the DEEEP approach:

Parameter	*Test*
Oxygen demand	COD BOD
Short-term toxicity	Bacterium[1] Alga Invertebrate Fish
Long-term toxicity	Invertebrate
Mutagenicity	Ames *Salmonella*
Bioaccumulation potential	HPLC estimation
Persistence	To be decided

[1] to be added to the manual in later editions

The ideal is to use all the selected parameters. Fortunately, the DEEEP approach is sufficiently flexible to allow the use of those parameters for which tests and capacity are readily available. Parameters and their evaluation techniques will be revised from time to time to ensure that they are up to date and cost-effective.

This manual is intended to introduce DEEEP ecological hazard assessment methods to waste dischargers. It describes selected test methods for oxygen demand, short- and long-term toxicity, and mutagenicity, and aims to provide guidance and to facilitate the use of consistent, appropriate and comprehensive procedures. The test methods described here are not standardised in all respects. In future editions more attention will be given to standardisation and quality control.

This manual does not serve as an introduction to general laboratory protocols but assumes prior laboratory practice or accreditation.

The production of this manual is a collaborative effort within the technical community and between DWAF and stakeholders (wastewater dischargers and other government departments). Comments are welcome and should be addressed to:

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0001

REFERENCE

DWAF. 2003. The Management of Complex Industrial Wastewater Discharges. Introducing the Direct Estimation of Ecological Effect Potential (DEEEP) approach. A discussion document. Institute for Water Quality Studies, Department of Water Affairs and Forestry, Pretoria.

GLOSSARY

Acclimation. Physiological adjustment to controlled laboratory conditions.

Acute. Refers to a stimulus that induces an effect in a short period of time, usually within 96 hours. The effect can be lethal or sublethal.

Auxotroph. Organism that, in addition to a carbon and energy source, also requires organic growth factors.

Axenic. In pure culture (free from foreign organisms).

Base-pair substitution mutations. Change in which one or more nucleotides are replaced by other nucleotides, resulting in incorrect codons and the incorporation of wrong amino acids into protein molecules.

Carcinogen. A toxicant that causes cancer.

Chronic. Refers to a stimulus typically occurring in small doses that induces direct or cumulative sublethal effects over a long period of time.

Complex industrial wastewater discharge. A discharge that contains 10% or more by volume of industrial wastewater (defined in Regulation 1191 of 8 October 1999 under the National Water Act of 1998).

Control. A test which duplicates all the conditions of the exposure treatment, but contains no toxicants. The control is used to determine the absence of measurable toxicity due to basic test conditions, *e.g.* salinity, temperature, health of test organisms, or effects due to handling of test organisms.

Culture. To carry out the procedure of growing organisms.

Dechlorinated water. Water, usually municipal drinking water, that has been treated to remove chlorine and chlorinated compounds from solution.

Definitive test. An experimental technique that estimates the concentration of a toxicant (or dilution of a wastewater) at which a specified percentage or number of organisms exhibit a certain response. The test typically reports a toxicity endpoint, *e.g.* Lethal Concentration (LC), Effect Concentration (EC), No Observed Effect Concentration (NOEC).

Discharge. Water containing waste, released directly or indirectly to a surface water resource.

EC (Effect Concentration). The estimate of the toxicant (or wastewater) concentration at which a specified percentage of the test organisms would be affected, *e.g.* the EC_{10} (10% effect) or EC_{50} (50% effect).

Ephippia. Resting eggs.

Frameshift mutations. Change in which one or more nucleotides have been inserted into (+ shift) or removed (- shift) from DNA.

Hazard. Undesirable or adverse effect.

Histidine. Amino acid.

Homogenate. A homogeneous suspension of smaller pieces of a solid.

Juvenile. Young organism, still sexually immature.

LC (Lethal Concentration). The estimate of the toxicant (or wastewater) concentration at which the specified percentage of organisms die, *e.g.* the LC_{10} (10% lethal) or LC_{50} (50% lethal).

Lethality. The extent to which a toxicant can cause death by direct action.

LOEC (Lowest Observed Effect Concentration). The lowest tested toxicant (or wastewater) concentration that causes a statistically significant sublethal effect on test organisms.

Long-term. When the period of concern for the manifestation of lethal or sublethal toxic effects is a significant portion of the life span of the organism – weeks or more.

Moult. To cast off an outer covering, such as an exoskeleton or carapace.

Mutagenicity. The extent to which a toxicant can damage or change an organism's or cell's genetic material.

NOEC (No Observed Effect Concentration). The highest tested toxicant (or wastewater) concentration that does not cause any statistically significant sublethal effect on test organisms.

Ovoviviparous. Producing effects that are incubated and hatched within the parent's body.

Parthenogenesis. Development of a new individual from an unfertilised egg.

Photoperiod. The duration of illumination and darkness within a 24 hour period.

Plasmid. A generic term for all types of intracellular inclusions that can be considered as having genetic functions.

Prototroph. Organism that, in addition to minerals, needs only a carbon and an energy source.

Reconstituted fresh water. Milli-Q® water (or equivalent) to which reagent grade chemicals are added in order to obtain a desired hardness and pH.

Reference toxicant. Standard chemical used to measure the sensitivity of test organisms to establish confidence in the toxicity data obtained for a test chemical/water/wastewater. A reference toxicant is used to determine the precision of results obtained by a laboratory for that chemical.

RSD. The standard relative variation of a distribution or set of data, defined as the standard deviation (SD) divided by the mean, expressed as a percentage. Also called coefficient of variation (CV).

S9 liver fraction. Liver preparation enriched with cytochrome P450-containing monooxygenases.

Screening test. A toxicity test performed on the water or test sample "as is", *i.e.* without dilution. The test typically reports a percentage effect or a yes/no result.

Short-term. When the period of concern for the manifestation of lethal or sublethal toxic effects is days or less (a short period in relation to the life span of the organism).

Static test. A toxicity test in which test water is not renewed during the test.

Stock culture. The group of healthy parental organisms that are grown under defined and controlled conditions to produce healthy test organisms.

Stock solution. Concentrated water solution of a substance or substances.

Sublethality. The extent to which a toxicant is detrimental without causing death.

Test organism. The biological (typically a specific species), cellular or sub-cellular system (typically associated with a specific species) that is used in a toxicity test.

Toxicant. A chemical pollutant capable of exhibiting a toxic effect.

Toxicity. The inherent potential or capacity of a chemical or group of chemicals to cause adverse effects on living organisms. Adverse effects include lethality or effects which limit an organism's ability to survive in nature. Effects can be acute or chronic.

Toxicity test. A technique that determines the effect of a chemical or water or wastewater on a group of organisms or cellular/subcellular systems, using defined conditions. The test either measures the proportions of test organisms affected (*e.g.* number of fish died) or the degree of effect (*e.g.* percentage inhibition of oxygen uptake) after exposure to specific concentrations of the chemical/water/wastewater. In the toxicological field a toxicity test if often referred to as a toxicity assay or biossay, or a biological toxicity test, or simply a biotest.

Waste. Defined by the National Water Act as any solid material or material that is suspended, dissolved or transported in water (including sediment) and which is spilled or deposited on land or into a water resource in such volume, composition or manner as to cause, or to be reasonably likely to cause, the water resource to be polluted.

Wastewater. Any liquid waste (sewerage, industrial) discharged into a surface water resource. Alternatively referred to as effluent.

ACRONYMS AND SYMBOLS

BOD	biochemical oxygen demand
COD	chemical oxygen demand
CSIR	Council for Scientific and Industrial Research
d	day(s)
DO	dissolved oxygen
EC_{20}	concentration causing a 20% effect
EC_{50}	concentration causing a 50% effect
EPA	Environmental Protection Agency
g	gram(s)
g/ℓ	grams per litre
h	hour(s)
ℓ	litre(s)
LC_{10}	concentration causing 10% lethality
LC_{50}	concentration causing 50% lethality
LOEC	Lowest Observed Effect Concentration
M	molarity
µE	micro-Einstein(s)
µg	microgram(s)
µg/ℓ	micrograms per litre
µℓ	microlitre(s)
µm	micrometre(s)
mg	milligram(s)
mg/ℓ	milligrams per litre
mℓ	milliliter(s)
mm	millimetre(s)
min	minute(s)
N	normality
nm	nanometre
NOEC	No Observed Effect Concentration
RO	reverse osmosis
rpm	revolutions per minute
RSD	standard relative variation
s	second(s)
SD	standard deviation
TYA	trout pellet, yeast and alfalfa
UV	ultraviolet
W	watt(s)
°C	degree(s) Celsius
>	greater than
<	less than
≥	greater than or equal to
≤	less than or equal to
±	plus or minus
%	percentage or percent
~	approximately

A note on the text

Bullet points marked ➢ indicate information and requirements.
Bullet points marked • indicate step by step instructions.
Text boxes provide useful information.

CHAPTER 1: INTRODUCTION

The focus of this manual is on assessing the ecological hazard of complex industrial wastewater discharges and receiving surface water, although the methods described here can be applied to establish the quality of various other mediums, such as drinking water, groundwaters, sediments, sludges, and solid waste.

The methods described are not completely standardised, and some of them differ slightly from methods already in use by laboratories. However in order to succeed with the DEEEP approach, it is necessary to use uniform procedures. It is hoped that this manual will contribute to further development and standardisation.

The manual describes the following hazard assessment methods:

- Determination of chemical oxygen demand (COD)
- Determination of biochemical oxygen demand (BOD) – 5 d BOD test
- Fish (*Poecilia reticulata*) lethality test
- *Daphnia pulex* lethality test
- *Daphnia pulex* reproduction test
- Algal (*Selenastrum capricornutum* Printz) growth inhibition test - Microplate assay
- Algal (*Selenastrum capricornutum* Printz) growth inhibition test - Flask test
- Ames *Salmonella* mutagenicity test – Plate incorporation assay

COD determination has been widely applied in South Africa. Several standard methods are available (described in Chapter 3).

Limited use is made of BOD determination. Traditionally, the standard 5 d bottle test is used (described in Chapter 4). Various commercial oxygen-measuring systems based on the bottle test are also available.

The fish and *Daphnia* lethality tests have been in use in South African since the 1980s (Morgan,1982; Slabbert *et al.*,1998a,b). The protocols are based on US EPA methodologies (US EPA, 1985; 1993). The methods described in Chapters 5 and 6 reflect the collective experience of a number of South African laboratories.

The fish test employs the guppy, *P. reticulata*. This fish is not the ideal test organism, because it is prone to bacterial and fungal infections (as a result of in-breeding) that periodically cause major culture crashes. Another disadvantage is that the numbers of offspring are small, which means that large culturing facilities are required for a constant supply of fish for routine testing. The use of zebra fish (*Brachydanio rerio*) as a test organism is currently being investigated by several laboratories. This fish is widely used in standard methods (Slabbert *et al.*, 1998a) and it is expected that the zebra fish will replace the guppy in future methodology.

The algal (*S. capricornutum* Printz) microplate assay (Chapter 8) is a miniaturised version of the US EPA algal flask test methodology (Slabbert and Hilner, 1990; Slabbert *et al.*,1998a,b). This method has been in routine use at the CSIR laboratories for many years, and is currently being implemented and evaluated by other South African laboratories. The traditional algal flask test, as outlined in standard US EPA (1989) methodologies, is included in Chapter 9.

D. pulex reproduction has been evaluated by a few South African laboratories as a possible tool for the assessment of long-term toxicity. The test is time-consuming and so far has had limited routine application. The test described in Chapter 7 is based on

the ISO *D. magna* Strauss test (1998) with inputs from the US EPA *Ceriodaphnia dubia* method (1989).

The mutagenicity test described in Chapter 10 follows the methodology of Bruce Ames, the developer of the test (Maron and Ames, 1983; US EPA, 1983). Although the current capacity for performing this test is limited, the test has been in use in South Africa since the 1980s (Slabbert *et al.*, 1998a).

Besides describing these tests, the manual also includes general information on sample collection, transport and storage; waste disposal; and safety (Chapter 2).

Finally, five Appendices give methods for fish and *Daphnia* culturing; the preparation of synthetic moderately hard water; sample concentration for the Ames test; and the preparation of reference toxicants, while the sixth Appendix contains a complete reference list.

1.1 REFERENCES

ISO. 1998. Water quality – Determination of long term toxicity of substances to Daphnia magna Straus. Draft International Standard ISO/DIS 10706. International Organisation for Standardisation, Geneva.

Maron, D.M. and Ames, B.N. 1983. Revised methods for the Salmonella mutagenicity test. *Mutation Research* 113, 173-215.

Morgan, W.S.G. 1982. The development of continuous automatic biological monitoring systems for water quality control. DPhil thesis. Faculty of Science, University of Cape Town.

Slabbert, J.L. and Hilner, C.A. 1990. Development of an algal toxicity test for water quality testing. CSIR Report, Project No. 670 27441. Division of Water Technology, Pretoria.

Slabbert, J.L., Oosthuizen, J., Venter, E.A., Hill, E., Du Preez, M. and Pretorius, P.J. 1998a. Development of guidelines for toxicity bioassaying of drinking and environmental waters in South Africa. WRC Report No. 358/1/98. Pretoria.

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US EPA. 1983. Interim procedures for conducting the *Salmonella*/microsomal mutagenicity assay – Ames test. EPA/600/4-82/068. Health Effects Research Laboratory, US Environmental Protection Agency, Triangle Park.

US EPA. 1985. Methods for measuring the acute toxicity of effluents to freshwater and marine organisms. EPA/600/4-85/013. Environmental Monitoring and Support Laboratory, Office of Research and Development, US Environmental Protection Agency, Cincinnati.

US EPA. 1989. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Second Edition. EPA/600/4-89/001. Environmental Monitoring Systems Laboratory, Office of Research and Development, US Environmental Protection Agency, Cincinnati.

US EPA. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Fourth Edition. EPA/600/4-90/027F. Environmental Monitoring and Support Laboratory, Office of Research and Development, US Environmental Protection Agency, Cincinnati.

CHAPTER 2: SAMPLING, WASTE DISPOSAL, AND SAFETY

2.1 SAMPLE COLLECTION, TRANSPORT AND STORAGE

Collect samples in such a way that they reflect the true nature of the water or wastewater (Slabbert *et al.*, 1998). Containers should be clean (free of chemicals and if necessary sterile) and of inert material, preferably glass. Use only glass or aluminium containers for Ames test samples.

Rinse the containers with sample water prior to filling. Fill containers to the brim to eliminate air, and seal well to avoid leaking and loss of volatile chemicals. Label the containers clearly, noting type of sample, source, sample location, sample identification, date and time of collection, and name of sampler. For COD measurements, if delay before analysis is unavoidable (>24 h) the samples must be preserved by acidification to pH ≤2 with concentrated sulphuric acid (APHA-AWWA-WEF, 1992).

Samples must be kept in the dark (cooler bag) at 4°C during transport and before testing (Slabbert *et al.*, 1998). They should be delivered to the test laboratory without delay so as to limit changes in composition. Toxicity tests and BOD analysis should be initiated within 24 to 36 h of sampling. The Ames test samples should be concentrated as soon as possible after sample receipt and extracts stored at -20°C until tested. Keep water and wastewater samples in the dark at 4°C until tests are completed.

> ➢ Do not freeze samples.

2.1.1 References

APHA-AWWA-WEF. 1992. Chemical oxygen demand (COD), in: *Standard Methods for the Examination of Water and Wastewater*. Eighteenth Edition. American Public Works Association, American Water Works Association, and Water Pollution Control Federation, Washington DC. Part 5220, 5-6 to 5-10.

Slabbert, J.L., Oosthuizen, J. Venter, E.A., Hill, E., Du Preez, M. and Pretorius, P.J. 1998. Development of guidelines for toxicity bioassaying of drinking and environmental waters in South Africa. WRC Report No. 358/1/98. Pretoria.

2.2 WASTE DISPOSAL

Waste (water, wastewater, chemicals and chemical solutions) with no or low toxicity can be discarded via the sewer. Dilute toxic waste to non-toxic concentrations and discard via the sewer or collect in a drum and send to a hazardous waste treatment company. Neutralise acidic and basic materials before disposal. Obtain a list of acceptable disposal concentrations from the local authority/municipality to ensure that dilution is adequate. Send waste with significant mutagenic activity to a hazardous waste treatment company (APHA-AWWA-WEF, 1992).

Sterilise bacterial cultures (autoclave for 15 min at 121°C) and dispose via the sewer. Discard used Petri dishes and other disposables in a medical waste container. *Daphnia* and algae are discarded with test/culture water. Remove fish from test containers and discard in a biological waste container if samples were toxic, or in a waste bin if samples were not toxic or displayed low toxicity. Discard sick fish in a medical waste container.

2.2.1 Reference

APHA-AWWA-WEF. 1992. Safety, in: *Standard Methods for the Examination of Water and Wastewater*. Eighteenth Edition. American Public Works Association, American Water Works Association, and Water Pollution Control Federation, Washington DC. Part 1090, 1-34 to 1-42.

2.3 SAFETY

People collecting wastewater samples and conducting ecological hazard assessment tests should take all safety precautions necessary to prevent illness which might result from the ingestion or invasion of infectious organisms or hazardous chemicals, inhalation or absorption of corrosive or harmful substances through skin contact, or asphyxiation as a result of lack of oxygen or the presence of noxious gases (APHA-AWWA-WEF, 1992; US EPA, 1989).

- Use safety equipment as required, such as safety shoes, safety glasses, masks, respirators, gloves, rubber aprons and laboratory coats.
- Use fume cupboards and laminar flow cabinets whenever necessary.
- Ensure that first aid kits and eye fountains are available for cases of emergency.
- Use a pipetting device for pipettes (*Never pipette by mouth*).
- Rinse exposed parts of the body immediately.
- Frequently wash and disinfect hands, and the working surface.
- Label containers clearly to indicate their contents.
- Handle mutagenic/carcinogenic substances with the utmost care.
- Autoclave any material containing *Salmonella* (mutagenicity test) before it is washed.

Detailed information on establishing a laboratory safety programme can be obtained from APHA-AWWA-WEF (1992).

2.3.1 References

APHA-AWWA-WEF. 1992. Safety, in: *Standard Methods for the Examination of Water and Wastewater*. Eighteenth Edition. American Public Works Association, American Water Works Association, and Water Pollution Control Federation, Washington DC. Part 1090, 1-34 to 1-42.

US EPA. 1989. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Second Edition. EPA/600/4-89/001. Environmental Monitoring Systems Laboratory, Office of Research and Development, US Environmental Protection Agency, Cincinnati.

CHAPTER 3: DETERMINATION OF CHEMICAL OXYGEN DEMAND (COD)

3.1 SCOPE

COD is a measure of the oxygen equivalent of the carbonaceous or organic matter content present in wastewater discharge or receiving water that is susceptible to oxidation by a strong chemical oxidant (APHA-AWWA-WEF, 1992).

3.2 PRINCIPLE

Most types of organic matter are oxidised by a boiling mixture of chromic and sulphuric acids. The sample is digested in a strong acid solution with a known excess of potassium dichromate.

After digestion, in the open and closed reflux titrimetric methods, the remaining unreduced dichromate is titrated with ferrous ammonium sulphate to determine the potassium dichromate consumed. In the closed reflux colorimetric method, the remaining unreduced dichromate is measured spectrophotometrically at 420 or 620 nm, depending on the COD concentration range.

The oxidisable organic matter is reported in terms of the oxygen equivalent.

3.3 INTERFERENCES

- Volatile straight-chain aliphatic compounds are not easily oxidised. This is partly because volatile organic chemicals are present in the vapour space and do not come into contact with the oxidising liquid.

  - Silver sulphate can be added as catalyst to overcome this problem.
  - However, if halides are present, precipitates are produced that are oxidised only partially.
  - This can be eliminated by adding mercuric sulphate before the refluxing procedure to complex the halides.
  - Do not use this approach when samples contain more than 2 000 mg/ℓ chloride.

- Nitrate exerts a COD of 1.1 mg oxygen/mg nitrogen (NO_2).

  - Because the concentrations of nitrite in water seldom exceed 1 or 2 mg/ℓ NO_2, the interference is considered insignificant and is usually ignored.
  - To eliminate a significant interference due to nitrite, 10 mg of sulphamic acid may be added for each mg of NO_2 present in the sample volume used
  - The same amount of sulphamic acid should also be added to the blank.

- Reduced inorganic species such as ferrous iron, sulphide, and manganous manganese are oxidised quantitatively under the test conditions.

- If samples contain significant quantities of these species, stoichiometric oxidation can be assumed from the known initial concentration of the interfering species and corrections made to the COD value obtained.

3.4 METHODOLOGY

The dichromate reflux method is preferred over methods using other oxidants, because of its superior oxidising ability, applicability to a variety of samples, and ease of use. Oxidation of most organic compounds is 95 to 100% of the theoretical value (APHA-AWWA-WEF, 1992).

Two different reflux methods are available:

- The open reflux method is applicable to large sample volumes.
- The closed reflux methods use smaller sample volumes and therefore less metallic salt reagents. However, samples containing suspended solids require homogenisation for reproducible results.

The dichromate reflux methodology is described in a number of standard methods (ISO,1989; SANS,1990; APHA-AWWA-WEF,1992; ASTM, 2000). Commercial products are also available in the form of ampules and culture tubes with pre-measured reagents. If these are used, the manufacturer's instructions should be followed.

3.4.1 Sample preparation

- Homogenise samples containing suspended solids for representativeness.
- Make dilutions if COD is high to reduce errors inherent in measuring small sample volumes.

3.5 REFERENCES

APHA-AWWA-WEF. 1992. Chemical oxygen demand (COD), in: *Standard Methods for the Examination of Water and Wastewater*. Eighteenth Edition. American Public Works Association, American Water Works Association, and Water Pollution Control Federation, Washington DC. Part 5220, 5-6 to 5-10.

ASTM. 2000. Standard test methods for chemical oxygen demand (dichromate oxygen demand) of water. D-252-00. American Society for Testing Materials, Philadelphia.

ISO. 1989. Water quality determination of the chemical oxygen demand. International Standard 6060. International Organisation for Standardisation, Geneva.

SANS. 1990. Water chemical oxygen demand. SANS 6048. South African National Standards, South African Bureau of Standards, Pretoria.

CHAPTER 4: DETERMINATION OF BIOCHEMICAL OXYGEN DEMAND (BOD) - 5 d BOD TEST

4.1 SCOPE

BOD is a measure of the amount of oxygen used by microorganisms to oxidise the organic matter in wastewater discharges and receiving waters and to synthesise new cellular material from the wastes.

4.2 PRINCIPLE

The method involves filling an airtight bottle of a specified size to overflowing with a sample and incubating it at a specified temperature for 5 d. Dissolved oxygen is measured at the beginning and end of the incubation period. The BOD is calculated from the difference between the initial and final dissolved oxygen (DO).

> ➢ Because the initial DO is determined immediately after the dilution is made, all oxygen uptake, including that occurring during the first 15 min, is included in the BOD measurement.

4.3 INTERFERENCES

- ➢ Oxidation of reduced nitrogen, mediated by microorganisms, exerts nitrogenous demand. Measurements that include nitrogenous demand generally are not useful for assessing the oxygen demand associated with organic material.
- ➢ High algal loads may lead to incorrect BOD measurements.

> ➢ The interference from nitrogenous demand can now be prevented by an inhibitory chemical.

4.4 METHODOLOGY

The most commonly used test is the 5 d BOD bottle test (APHA-AWWA-WEF, 1992). There are many variations of this test. These include longer or shorter incubation periods, the determination of oxygen uptake rate, and continuous oxygen uptake measurements (APHA-AWWA-WEF, 1992). Alternative seeding, dilution, and incubation conditions can be used to mimic receiving water conditions and thus give an indication of the environmental effects of wastewater discharges.

Various commercial oxygen measuring systems are available that are based on the standard 5 d BOD test. If used, the manufacturer's instructions should be followed.

4.4.1 Sample preparation

- ➢ The BOD concentration in most wastewater discharges exceeds the DO in an air-saturated sample. It is therefore necessary to dilute the sample before testing, to bring the oxygen demand and supply into balance.
- ➢ Add nitrogen, phosphorus and trace metals to dilution water to serve as nutrients for bacteria.
- ➢ The dilution water is also buffered to ensure that the pH of the incubated sample remains within a suitable range for bacterial growth.

4.5 REFERENCE

APHA-AWWA-WEF. 1992. Biochemical oxygen demand (BOD), in: *Standard Methods for the Examination of Water and Wastewater*. Eighteenth Edition. American Public Works Association, American Water Works Association, and Water Pollution Control Federation, Washington DC. Part 5010, 5-1 to 5-6.

CHAPTER 5: FISH (*POECILIA RETICULATA*) LETHALITY TEST

5.1 SCOPE

This method measures the short-term (acute) toxicity of wastewater discharges and receiving waters to the freshwater fish *Poecilia reticulata* (Teleostei, Poeciliidae – common name, guppy).

5.2 PRINCIPLE

Fish, 10 to 21 d old, are exposed to test samples for a period of 96 h in a static test. Lethality is recorded after 24, 48, 72 and 96 h of exposure.

The test is applied directly (screening test) to receiving waters to determine the percentage lethality. Definitive tests (testing dilutions) are carried out on wastewater discharges to determine toxicity endpoints – 96 h LC_{10} (concentration causing 10% lethality – minimum effect concentration) and LC_{50} (concentration causing 50% lethality). A definitive test is usually preceded by a range finding test (testing 10-fold dilutions) to establish the lethal concentration range.

The test is based on US EPA methodology (US EPA, 1985; 1993; Slabbert *et al.*, 1998a,b), with inputs from Canadian (EPS, 2000) and ISO (1992) methods.

5.3 INTERFERENCES

- Dark coloured samples, and samples containing high loads of suspended solids, may impede the observation of organisms.

5.4 TEST ENVIRONMENT

- Use a temperature controlled facility at 23±2°C. The temperature should not vary by more than 2°C during a test.
- Use ambient laboratory illumination (artificial and/or day light:10 to 14 h light).
- The test facility must be separate from the culture facility.
- The facility must be free of vapours, odours and dusts that may be toxic to fish.

5.5 MATERIALS, EQUIPMENT AND REAGENTS

5.5.1 Materials

- ✓ Glass beakers - 50, 100 and 500 mℓ
- ✓ Glass measuring cylinders - 100 mℓ, 1 and 2 ℓ
- ✓ Glass pipettes, graduated - 10 and 25 mℓ
- ✓ Glass reagent bottles - 100 mℓ
- ✓ Polyethylene bottle - 100 mℓ
- ✓ Test containers - 300 to 500 mℓ glass beakers/dishes/flasks (or clear disposable polystyrene containers)
- ✓ Pipettes, front end cut off and fire-polished (opening approximately 5 mm) or equivalent, to remove dead fish from containers.
- ✓ Pasteur pipettes
- ✓ Fish nets (small mesh of soft inert material)
- ✓ Pipette bulbs
- ✓ Plastic/glass sheets

- ✓ Parafilm
- ✓ Whatman No. 1 filter paper
- ✓ Wash bottles

5.5.2 Equipment

- ✓ Dissolved oxygen (DO) meter
- ✓ pH meter
- ✓ Balance
- ✓ Pipettor
- ✓ Equipment to measure free chlorine
- ✓ Thermometers
- ✓ Aeration device

5.5.3 Reagents

Use reagent grade chemicals and water purified by a Millipore Milli-Q® system or equivalent for the preparation of solutions. Use reverse osmosis (RO) or distilled water for rinsing.

Chemicals

- ✓ Sodium hydroxide (NaOH)
- ✓ Hydrochloric acid (HCl)
- ✓ Sodium thiosulphate (NaS_2O_3)

Chemical solutions

Sodium hydroxide (1 N)

NaOH	4 g
Water	100 mℓ

- Store at room temperature in a 100 mℓ polyethylene bottle.

Hydrochloric acid (1 N)

HCl	8.3 mℓ
Water	100 mℓ

- Store at room temperature in a 100 mℓ glass reagent bottle.

Sodium thiosulphate (20%)

NaS_2O_3	20 g
Water	100 mℓ

- Store at room temperature in a 100 mℓ glass reagent bottle.

Dilution and control water

Synthetic moderately hard water, prepared as described in Appendix C. Keep at 23±2°C.

Reference toxicant

Potassium dichromate ($K_2Cr_2O_7$) stock solution (1000 mg/ℓ), prepared as described in Appendix E.

5.6 TEST ORGANISM

P. reticulata, commonly known as the guppy. The maintenance and breeding of this fish is described in Appendix A.

- Test fish should be between 10 and 21 d old, not weighing more than 25 mg.
- Test fish in any particular batch should not differ in age by more than 7 d.
- Test fish should be free of disease or visible malformation, should not have received treatment for disease, and should not have been used in previous tests.
- The fish should be acclimated and maintained for at least 7 d prior to the test in moderately hard water, using specified culturing conditions as described in Appendix A.
- Fish must not be fed during the 24 h before testing.

5.7 TEST SAMPLES

Wastewater discharges and receiving waters. Sample collection, transport and storage is described in Chapter 2.

- The samples must be tested as received, without physical/chemical treatment.
- Allow samples to reach a temperature of 23±2°C before use.
- Measure the pH, DO, temperature and free chlorine (if applicable) before sample preparation.
- Note the appearance of the sample (colour, turbidity, odour).

> - If the sample is high in suspended solids, a parallel test can be carried out on the sample after filtration through Whatman No. 1 filter paper.
> - If the pH and/or oxygen concentration is outside the optimum range for fish (pH:<6 or >8.5; DO <40% of saturation), a parallel test can be carried out after pH adjustment and/or aeration.
> - If the free chlorine is >0.2 mg/ℓ, a parallel test can be carried out after neutralisation with sodium thiosulphate (final concentration: 20 mg/ℓ).

5.8 TEST PROCEDURE

5.8.1 Prepare test solutions

> - Samples should be shaken vigorously to ensure homogeneity and to re-suspend particulate matter.

Receiving water

Water samples are tested directly (screening test). 1 ℓ water will be required.

Wastewater discharges

Wastewater discharges are usually tested after dilution. Approximately 3 ℓ wastewater will be sufficient. Note any changes in appearance of the wastewater during dilution (colour change, precipitation, flocculation, release of volatiles).

10-fold dilutions on wastewater for a range finding test

A range finding test (testing ten-fold dilutions) is usually carried out before a definitive test (testing serial dilutions) to establish the range of concentrations to be used for a definitive test.

The dilutions can be made as follows:

- Transfer 60 mℓ of the wastewater (100%) with a 100 mℓ measuring cylinder to a 1 ℓ measuring cylinder.
- Fill with moderately hard water to the 600 mℓ mark, cover with Parafilm and shake well.
- Transfer 60 mℓ of this solution (10% wastewater) with another clean 100 mℓ measuring cylinder to a second 1 ℓ measuring cylinder.
- Fill with moderately hard water to the 600 mℓ mark (1% wastewater), cover the cylinder with Parafilm and shake well.
- Continue in this way until all the dilutions have been made (two to four dilutions should be sufficient).

Serial dilutions on wastewater for a definitive test

A definitive test should span a concentration range that exhibits 0% lethality, and if possible, 100% lethality. A dilution factor of 0.5 provides good precision and is commonly used in definitive tests.

Dilutions can be made as follows:

- Fill a 2 ℓ measuring cylinder to the 1 ℓ mark with the 100% wastewater or the lowest dilution of the wastewater that exhibited 100% lethality in the range finding test.
- Add moderately hard water to the 2 ℓ mark, cover the cylinder with Parafilm and shake well.
- Transfer 1 ℓ of this solution to a second 2 ℓ measuring cylinder.
- Add moderately hard water to the 2 ℓ mark, cover with Parafilm and shake well.
- Continue in this way until all the dilutions have been made (four to seven dilutions should be sufficient).

5.8.2 Distribute samples in test containers

- Use two test containers per sample for range finding tests and four test containers per sample for screening and definitive tests.
- Distribute 250 mℓ volumes of the samples into each container (total of 500 mℓ for range finding tests and 1 ℓ for screening and definitive tests).
- For each series of samples, prepare control containers in the same way, using moderately hard water (two control containers for range finding tests and four control containers for screening and definitive tests).
- Label containers and place them in rows on a work surface in the test facility.
- Measure the pH and DO in one of the two/four test and control containers.

- If chlorinated samples are tested, also measure the free chlorine.
- Measure the temperature in one container (the control container).

5.8.3 Transfer test fish to test containers

- Move the acclimation tank containing the test fish from the culture facility to the test facility.
- Select the fish at random from the tank and place five fish in each container using a fish net (total of 10 fish for range finding tests or 20 fish for screening and definitive tests).
- Start with the control and highest test dilutions (the least toxic samples).
- Try not to touch the water in the test containers with the net in order to avoid contamination of the fish in the holding tank and cross-contamination of samples. If the net touches the water, rinse with moderately hard water before taking another batch of fish from the holding tank.
- Discard any fish dropped or mishandled during the transfer.
- Cover the containers with plastic or glass plates, and then keep them at 23±2°C for 96 h.

> ➢ Fish are not fed during the test period of 96 h.
> ➢ The loading density of fish per container should be ≤0.4 g/ℓ.

5.8.4 Record the results

- Record the number of dead fish in each container every 24 h during the 96 h exposure period. Death can be assumed if there is no movement on gentle prodding.
- Remove the dead fish after counting.
- Note any atypical/stressed behaviour of the surviving fish.
- Record the temperature in one of the controls every 24 h.
- After recording the results at the end of the 96 h test period (screening and definitive tests only), measure the pH and DO in the control and lower test solutions where all or some of the fish died. If chlorinated samples are tested, also measure the free chlorine.

5.9 DATA ANALYSIS AND EXPRESSION OF RESULTS

- Calculate the percentage lethality for each sample in relation to the total number of fish used:

  $(N_{t0} - N_{tx})/N_{t0} \times 100\%$, where:

  N_{t0}: Number of live fish at t = 0 h
  N_{tx}: Number of live fish at t = 24, 48, 72 and 96 h

  > ➢ Lethality ≥10% indicates toxicity.

- Calculate the 96 h LC_{10} and LC_{50} (toxicity endpoints) and the 95% confidence limits for a wastewater, by applying an appropriate statistical method (*e.g.* probit analysis) to definitive test data.

- Express toxicity endpoints as a percentage, *e.g.* LC_{50}: 20%.
- In cases where lethality is too low to use a statistical method to calculate the toxicity endpoints, endpoints could be given as a concentration range, *e.g.* LC_{10} : 50-100%; or as larger as 100%, *e.g.* LC_{50} >100%.
- Determine the R^2 (R = correlation coefficient) for each data set to establish how closely the calculated regression line fits the measured data.

5.10 TEST PRECISION

A reference toxicant must be used to assess the relative toxicity of the population of fish used in the toxicity test. This also assesses the precision and reliability of data produced by the laboratory personnel for that reference toxicant under standardised test conditions.

- Conduct a definitive test on the reference toxicant $K_2Cr_2O_7$ according to the test procedure in paragraph 5.8.
- Calculate the toxicity endpoints according to paragraph 5.9.
- Verify the sensitivity of the test organism by comparing the calculated endpoints with historical test results (control chart).

> - Do the test at least once a month in parallel with a definitive test on a wastewater when fish are maintained regularly in own facility (fish for these tests must come from the same breeding stock).
> - If test fish are obtained from an outside source, for every new batch of test fish, conduct a test in parallel with definitive tests on wastewater discharges.

5.11 TEST VALIDITY

For a test to be valid:

- Control lethality should be <10%.
- Control DO should not be <40% of saturation.
- Control fish showing atypical/stressed behaviour should be <10%.
- R^2 should be >0.9 (definitive test only).
- 96 h LC_{50} of the reference toxicant should be within 2SDs of the historic geometric mean of the laboratory.

> - If the 96 h LC_{50} of the reference toxicant does not fall within 2SDs of the historic geometric mean, the sensitivity of the fish and overall credibility of the test system are suspect.
> - All aspects of the test should be carefully examined for defects and repeated with a different batch of fish.

5.12 SUMMARY OF TEST CONDITIONS

Table 5.1 summarises the test conditions for the *P. reticulata* lethality test.

Table 5.1: Summary of test conditions for the *P. reticulata* lethality test

Test type	Static
Water temperature	23±2°C
Light quality	Ambient laboratory illumination
Photoperiod	10 to 14 h light
Oxygen concentration	As obtained
pH	As obtained
Feeding	None
Size of test container	300 to 500 mℓ
Volume of test sample	250 mℓ
Age of test organisms	10 to 21 d
Number of organisms per container	5
Number of replicate containers	4 for screening and definitive tests and 2 for the range finding test
Total number of organisms per test	20 for the screening and definitive tests and 10 for the range finding test
Control and dilution water	Moderately hard water
Test duration	96 h
Effect measured	Lethality (no movement on gentle prodding)
Detection limit	10%

5.13 TEST REPORT

The test report should refer to this test method and include the following:

5.13.1 Sample

- The name and location of industry where wastewater and receiving water samples were taken.
- Type of industry (paper mill, acid mine drainage).
- Brief description of sampling point.
- Date and time of sampling.
- Sampling method (grab, composite).
- Name of person collecting the sample/s.
- Appearance and other properties of samples (colour, turbidity, odour).

5.13.2 Test facility

- Name and city of testing laboratory.
- Name/s of person/s performing the test.
- Name/s of person/s verifying the results.
- Date and starting time of screening/definitive test.

5.13.3 Test conditions

- Test type (screening, definitive).
- Details on any deviation from the standard method.
- Origin and age of test organism.
- Percent lethality of test fish during the 7 d acclimation period.
- Number of organisms per test container.
- Estimated loading density (g/ℓ) of fish.
- Concentrations and volumes tested, including controls, and indication of replication.
- Changes in sample during sample dilution (colour, precipitation, flocculation, release of volatiles).
- pH, DO, temperature and free chlorine (if applicable) of samples before sample preparation.

- Confirmation that no adjustment of pH/DO/free chlorine occurred.
- Indication of procedure used for pH/DO/free chlorine adjustment if tests were carried out on both adjusted and non-adjusted samples.
- Measurements of pH, DO and free chlorine (if applicable) of samples at the start and end of the test completion of the test (96 h).
- Measurements of the temperature at the start of the test and every 24 h thereafter.

5.13.4 Results

- The percentage lethality in each test solution, including the control, after 24, 48, 72 and 96 h.
- Percentage of control fish showing stressed behaviour.
- 96 h LC_{10}, LC_{50}, and 95% confidence limits.
- Statistical method used for data analysis.
- R^2.
- Most recent 96 h LC_{50} for $K_2Cr_2O_7$, date test initiated and the historic geometric mean 96 h LC_{50}±2SD.

5.14 REFERENCES

EPS. 2000. Biological test method: Reference method for determining acute lethality of effluents to rainbow trout. Second Edition. EPS 1/RM/13. Method Development and Applications Section, Environmental Technology Centre, Environment Canada, Ottawa.

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Slabbert, J.L., Oosthuizen, J., Venter, E.A., Hill, E., Du Preez, M. and Pretorius, P.J. 1998a. Development of guidelines for toxicity bioassaying of drinking and environmental waters in South Africa. WRC Report No. 358/1/98. Pretoria.

Slabbert, J.L., Oosthuizen, J., Venter, E.A., Hill, E., Du Preez, M. and Pretorius, P.J. 1998b. Development of procedures to assess whole effluent toxicity. WRC Report No. 453/1/98. Pretoria.

US EPA. 1985. Methods for measuring the acute toxicity of effluents to freshwater and marine organisms. EPA/600/4-85/013. Environmental Monitoring and Support Laboratory, Office of Research and Development, US Environmental Protection Agency, Cincinnati.

US EPA. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Fourth Edition. EPA/600/4-90/027F. Environmental Monitoring and Support Laboratory, Office of Research and Development, US Environmental Protection Agency, Cincinnati.

CHAPTER 6: *DAPHNIA PULEX* LETHALITY TEST

6.1 SCOPE

This method measures the short-term (acute) toxicity of wastewater discharges and receiving waters to the freshwater cladoceran *Daphnia pulex* (Crustacea).

6.2 PRINCIPLE

Daphnia, less than 24 h old, are exposed to test samples for a period of 48 h in a static test. Lethality is recorded after 24 and 48 h exposure. The test is applied directly (screening test) to receiving waters to determine the percentage lethality. Definitive tests (testing dilutions) are carried out on wastewater discharges to determine toxicity endpoints – LC_{10} (concentration causing 10% lethality – minimum effect concentration) and LC_{50} (concentration causing 50% lethality). A definitive test is usually preceded by a range finding test (testing 10-fold dilutions) to establish the lethal concentration range.

The test is based on US EPA methodology (US EPA, 1985; 1993; Slabbert *et al.*, 1998a,b).

6.3 INTERFERENCES

- Dark coloured samples, and samples containing high loads of suspended solids, may impede the observation of organisms.
- Samples containing oils and surface tension altering compounds may cause organisms to float.
- Pathogenic and/or predatory organisms in samples may affect survival.

6.4 TEST ENVIRONMENT

- Use a temperature controlled facility at 20±2°C. The temperature should not vary by more than 2°C during a test.
- Use ambient laboratory illumination (artificial and/or day light:10 to 14 h light).
- The test facility must be separate from the culture facility.
- The facility must be free of vapours, odours and dusts that may be toxic to *Daphnia*.

6.5 MATERIALS, EQUIPMENT AND REAGENTS

6.5.1 Materials

- ✓ Glass beakers - 50, 100 and 500 mℓ
- ✓ Glass measuring cylinders - 100 and 250 mℓ
- ✓ Glass pipettes, graduated - 10 and 25 mℓ
- ✓ Polyethylene bottle - 100 mℓ
- ✓ Glass reagent bottles - 100 mℓ
- ✓ Test containers - 50 mℓ glass beakers (or clear disposable polystyrene cups/beakers)
- ✓ Pasteur pipettes or micropipette tips (1 000 µℓ), end cut off and fire-polished (opening approximately 2 mm), or disposable bulb pipettes (opening approximately 2 mm) for handling young *Daphnia*
- ✓ Pasteur pipettes
- ✓ Pipette bulbs
- ✓ Plastic/glass sheets

- ✓ Parafilm
- ✓ Whatman No. 1 filter paper
- ✓ Trays
- ✓ Wash bottles

6.5.2 Equipment

- ✓ Dissolved oxygen (DO) meter
- ✓ pH meter
- ✓ Equipment to measure free chlorine
- ✓ Balance
- ✓ Pipettor
- ✓ Aeration device
- ✓ Light box
- ✓ Magnifying glass
- ✓ Thermometers

6.5.3 Reagents

Use reagent grade chemicals and water purified by a Millipore Milli-Q® system or equivalent for the preparation of solutions. Use reverse osmosis (RO) or distilled water for rinsing.

Chemicals

- ✓ Sodium hydroxide (NaOH)
- ✓ Hydrochloric acid (HCl)
- ✓ Sodium thiosulphate (NaS_2O_3)

Chemical solutions

Sodium hydroxide (1 N)

NaOH	4 g
Water	100 mℓ

- Store at room temperature in a 100 mℓ polyethylene bottle.

Hydrochloric acid (1 N)

HCl	8.3 mℓ
Water	100 mℓ

- Store at room temperature in a 100 mℓ glass reagent bottle.

Sodium thiosulphate (20%)

NaS_2O_3	20 g
Water	100 mℓ

- Store at room temperature in a 100 mℓ glass reagent bottle.

Dilution and control water

Synthetic moderately hard water, prepared as described in Appendix C. Keep at 20±2°C.

Reference toxicant

Cadmium chloride ($CdCl_2$) stock solution (100 mg/ℓ), prepared as described in Appendix E.

6.6 TEST ORGANISM

D. pulex, less than 24 h old (first instars), obtained by acyclic parthenogenesis, using specified culturing conditions as described in Appendix B.

6.7 TEST SAMPLES

Wastewater discharges and receiving waters. Sample collection, transport and storage is outlined in Chapter 2.

- The samples must be tested as received, without physical/chemical treatment. (If predators or other invertebrates are present, filter the samples before testing).
- Allow samples to reach a temperature of 20±2°C before use.
- Measure the pH, DO, temperature and free chlorine (if applicable) before sample preparation.
- Note the appearance of the sample (colour, turbidity, odour).

> - If the sample is high in suspended solids, a parallel test can be carried out on the sample after filtration through Whatman No. 1 filter paper.
> - If the pH and/or oxygen concentration is outside the optimum range for *Daphnia* (pH:<6 and >8.5; DO: <40% of saturation), a parallel test can be carried out after pH adjustment and/or aeration.
> - If the free chlorine is >0.2 mg/ℓ, a parallel test can be carried out after neutralisation with sodium thiosulphate (final concentration: 20 mg/ℓ).

6.8 TEST PROCEDURE

6.8.1 Prepare test solutions

> - Samples should be shaken vigorously to ensure homogeneity and re-suspend particulate matter.

<u>Receiving water</u>

Water samples are tested directly (screening test). Approximately 200 mℓ water will be required.

Wastewater discharges

Wastewater discharges are usually tested after dilution. Approximately 500 mℓ wastewater will be sufficient. Note any changes in appearance of the wastewater during dilution (colour change, precipitation, flocculation, release of volatiles).

10-fold dilutions on wastewater for a range finding test

A range finding test (testing ten-fold dilutions) is usually carried out before a definitive test (testing serial dilutions) to establish the range of concentrations to be used for a definitive test.

The dilutions can be made as follows:

- Transfer 15 mℓ of the wastewater (100%) with a graduated pipette to a 250 mℓ measuring cylinder.
- Fill with moderately hard water to the 150 mℓ mark, cover with Parafilm and shake well.
- Transfer 15 mℓ of this solution (10% wastewater) with another clean graduated pipette to a second 250 mℓ measuring cylinder.
- Fill with moderately hard water to the 150 mℓ mark, cover the cylinder with Parafilm and shake well.
- Continue in this way until all the dilutions have been made (two to four dilutions should be sufficient).

Serial dilutions on wastewater for a definitive test

A definitive test should span a concentration range exhibiting 0% and, if possible, 100% lethality. A dilution factor of 0.5 provides good precision and is commonly used in definitive tests.

The dilutions can be made as follows:

- Fill a 250 mℓ measuring cylinder to the 125 mℓ mark with the 100% wastewater or the lowest dilution of the wastewater that exhibited 100% lethality in the range finding test.
- Add moderately hard water to the 250 mℓ mark, cover the cylinder with Parafilm and shake well.
- Transfer 125 mℓ of this solution to a second 250 mℓ measuring cylinder.
- Add moderately hard water to the 250 mℓ mark, cover with Parafilm and shake well.
- Continue in this way until all the dilutions have been made (five to seven dilutions should be sufficient).

6.8.2 Distribute samples in test containers

- Use five 50 mℓ test containers per sample (water sample or a wastewater and its dilutions).
- Distribute 25 mℓ volumes in each container.
- For each series of samples, prepare control containers in the same way, using moderately hard water.
- Mark each container and place the beakers in rows on a tray with the highest test concentrations furthest away from the control.

- Remove the fifth container of each sample and control and determine the pH and DO.
- If chlorinated samples are tested, also measure the free chlorine.
- Measure the temperature in the control container. (Keep the fifth control container with the other containers for temperature measurements after 24 and 48 h).

6.8.3 Transfer *Daphnia* from culture containers to a holding beaker

- Remove the young (pipette/micropipette tip with 2 mm diameter) from the toxicity test culture containers (Appendix B) and place them in a 50 or 100 mℓ holding beaker, approximately 30 min before the start of a test.
- Reduce the water in the holding beaker by means of a pipette and replace the water with fresh moderately hard water to remove traces of food.
- Concentrate the organisms (if required) by reducing the water in the holding beaker.
- Use the test organisms immediately to prevent them from being stressed.

> ➢ Young *Daphnia* are very susceptible to air entrapment, and should be handled with a pipette rather than being poured.
> ➢ The tip of the pipette should be kept below the surface of the water when the *Daphnia* are released.

6.8.4 Transfer *Daphnia* from the holding beaker to test containers

- Take the holding beaker to the test facility.
- Place five *Daphnia* in each test container (total of 20 *Daphnia* per sample).
- Start with the control and highest test dilutions (the least toxic samples).
- Rinse the pipette/micropipette tip between every transfer with moderately hard water before taking another batch of *Daphnia* from the holding beaker.
- Cover the containers with plastic or glass plates and keep them in the test facility at 20±2°C for 48 h.

> ➢ Introduce *Daphnia* in the smallest possible volume of water to avoid dilution of the sample.
> ➢ If *Daphnia* become less dense in the holding beaker, reduce the volume of water to concentrate the remaining *Daphnia* for easy transfer.
> ➢ If more than five *Daphnia* are placed in a container, do not remove the additional *Daphnia*. Take the number into account when analysing the results.

6.8.5 Record the results

- Record the number of dead *Daphnia* (death indicated by no movement of body or appendices on gentle prodding) in each beaker after 24 and 48 h exposure.
- Note any anomalies in the behaviour of the *Daphnia*.
- Record the temperature in the extra control beaker after 24 and 48 h exposure.

- After counting the *Daphnia* after 48 h exposure (screening and definitive tests only), measure the pH and DO in the control and lower test solutions where all or some of the *Daphnia* died (if necessary, pour the contents of the four containers into one container, taking precautions not to modify the oxygen content). If chlorinated samples are tested, also measure the free chlorine.

6.9 DATA ANALYSIS AND EXPRESSION OF RESULTS

- Calculate the percentage lethality for each sample in relation to the total number of *Daphnia* used:

  $(N_{t0} - N_{tx})/N_{t0} \times 100\%$, where:

  N_{t0}: Number of live *Daphnia* at t = 0 h
  N_{tx}: Number of live *Daphnia* at t = 24 and 48 h

> ➢ Lethality ≥10% indicates toxicity.

- Calculate the 48 h LC_{10} and LC_{50} (toxicity endpoints) and the 95% confidence limits for a wastewater by applying an appropriate statistical method (*e.g.* probit analysis) to definitive test data.
- Express toxicity endpoints as a percentage, *e.g.* LC_{50}: 20%.
- In cases where lethality is too low to use a statistical method to calculate the toxicity endpoints, endpoints could be given as a concentration range, *e.g.* LC_{10}: 50-100%; or as larger as 100%, *e.g.* LC_{50}: >100%.
- Determine the R^2 (R = correlation coefficient) for each data set to establish how closely the calculated regression line fits the measured data.

6.10 TEST PRECISION

A reference toxicant must be used to assess the relative toxicity of the population of *Daphnia* used in the toxicity test. This also assesses the precision and reliability of data produced by the laboratory personnel for that reference toxicant under standardised test conditions.

- Conduct a definitive test on the reference toxicant $CdCl_2$ according to the test procedure described in paragraph 6.8.
- Calculate the toxicity endpoints according to paragraph 6.9.
- Verify the sensitivity of the test organism by comparing the calculated endpoints with historical test results (control chart).

> ➢ When stock cultures are maintained regularly, do the test at least once a month in parallel with a definitive test on a wastewater.
> ➢ Do the test in parallel with definitive tests on each batch of wastewater discharges if cultures are not maintained on a routine basis, or if cultures are obtained regularly from outside sources.

6.11 TEST VALIDITY

For a test to be valid:

- Control lethality should be <10%.
- Control DO should not be <40% of saturation.
- R^2 should be >0.9 (definitive test only).
- 48 h LC_{50} of the reference toxicant should be within 2SDs of the historic geometric mean of the laboratory.

> - If the 48 h LC_{50} of the reference toxicant does not fall within 2SDs of the historic geometric mean, the sensitivity of the *Daphnia* and overall credibility of the test system are suspect.
> - All aspects of the test should be carefully examined for defects and repeated with a different batch of *Daphnia*.

6.12 SUMMARY OF TEST CONDITIONS

Table 6.1 summarises the test conditions for the *Daphnia* lethality test.

Table 6.1: Summary of test conditions for the *Daphnia* lethality test

Test type	Static
Water temperature	20±2°C
Light quality	Ambient laboratory illumination
Photoperiod	10 to 14 h light
Oxygen concentration	As obtained
pH	As obtained
Feeding	None
Size of test container	50 mℓ
Volume of test sample	25 mℓ
Age of test organisms	≤24 h
Number of organisms per container	5
Number of replicate containers	4
Total number of organisms per test	20
Control and dilution water	Moderately hard water
Test duration	48 h
Effect measured	Lethality (no movement on gentle prodding)
Detection limit	10%

6.13 TEST REPORT

The test report should refer to this test method and include the following:

6.13.1 Sample

- The name and location of the industry where wastewater and receiving water samples were taken.
- Type of industry.
- Brief description of sampling point.
- Date and time of sampling.
- Sampling method (grab or composite).
- Name of person collecting the sample/s.
- Appearance and other properties of samples (colour, turbidity, odour).

6.13.2 Test facility

- Name and city of testing laboratory.
- Name/s of person/s performing the test.
- Name/s of person/s verifying the results.
- Date and time for start of test.

6.13.3 Test conditions

- Test type (screening, definitive).
- Details on any deviation from the standard method.
- Origin and age of test organism.
- Number of organisms per test container.
- Concentrations and volumes tested, including controls, and indication of replication.
- Changes in sample during sample dilution (colour, precipitation, flocculation, release of volatiles).
- pH, DO, temperature and free chlorine (if applicable) of samples before sample preparation.
- Confirmation that no adjustment of pH/DO/free chlorine occurred.
- Confirmation that samples have been filtered to eliminate predators or other invertebrates.
- Indication of procedure used for pH/DO/free chlorine adjustment if tests were carried out on both adjusted and non-adjusted samples.
- Measurements of pH, DO and free chlorine (if applicable) at the start and end of the test.
- Measurements of the temperature at the start of the test and every 24 h thereafter.

6.13.4 Results

- The percentage lethality in each test solution, including the control, after 24 and 48 h.
- Any abnormal behaviour of the *Daphnia* during the test.
- 48 h LC_{10}, LC_{50}, and 95% confidence limits.
- Statistical method used for data analysis.
- R^2.
- Most recent 48 h LC_{50} for $CdCl_2$, date test initiated and the historic geometric mean 48 h LC_{50}±2SD.

6.14 REFERENCES

Slabbert, J.L., Oosthuizen, J., Venter, E.A., Hill, E. Du Preez, M. and Pretorius, P.J. 1998a. Development of guidelines for toxicity bioassaying of drinking and environmental waters in South Africa. WRC Report No. 358/1/98. Pretoria.

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CHAPTER 7: *DAPHNIA PULEX* REPRODUCTION TEST

7.1 SCOPE

The method measures the long-term (chronic) sublethal toxicity of wastewater discharges and receiving waters to the freshwater cladoceran *Daphnia pulex* (Crustacea).

7.2 PRINCIPLE

Daphnia, less than 24 h old, are exposed to test samples for a period of 21 d in a semi-static test. The survival of the parents and the number of offspring produced per live parent is recorded and compared to those of control parents. The test is applied directly (screening test) to receiving waters to determine the percentage survival and reproduction. Definitive tests (testing dilutions) are carried out on wastewater discharges to determine toxicity endpoints – EC_{20} (concentration causing 20% inhibition) and EC_{50} (concentration causing 50% inhibition).

The test is based on the ISO *D. magna* Straus test (1998) with inputs from the US EPA *Ceriodaphnia dubia* method (US EPA, 1989).

7.3 INTERFERENCES

- Dark coloured and very turbid samples may impede the observation of organisms.
- Samples containing oils and surface tension altering compounds may cause organisms to float.
- Pathogenic and/or predatory organisms in samples may affect survival.

7.4 TEST ENVIRONMENT

- Use a temperature controlled facility at 20±2°C. The temperature should not vary by more than 2°C during a test.
- Use ambient laboratory illumination (artificial and/or day light:10 to 14 h light; light intensity: 10 to 20 $\mu E/m^2/s$).
- The facility must be separate from the culture facility.
- The facility must be free of vapours, odours and dusts that may be toxic to *Daphnia*.

> - Variations in ambient light intensities and prevailing day/night cycles in most laboratories do not seem to affect *Daphnia* growth and reproduction significantly. If available, artificial light set at 14 h light and 10 h dark (simulating the South African summer reproduction light cycle), can be used.

7.5 MATERIALS, EQUIPMENT AND REAGENTS

7.5.1 Materials

- ✓ Glass beakers - 50, 100 and 500 mℓ
- ✓ Glass measuring cylinders - 100 and 500 mℓ
- ✓ Glass pipettes, graduated - 10 and 25 mℓ
- ✓ Polyethylene bottle - 100 mℓ

- ✓ Glass reagent bottles - 100 mℓ
- ✓ Test containers - 50 mℓ glass beakers (or clear disposable polystyrene cups/beakers)
- ✓ Pasteur pipettes or micropipette tips (1 000 µℓ), end cut off and fire-polished (opening approximately 2 mm), or disposable bulb pipettes (opening approximately 2 mm) for handling young *Daphnia*
- ✓ Pasteur pipettes
- ✓ Pipette bulbs
- ✓ Plastic/glass sheets
- ✓ Parafilm
- ✓ Whatman No. 1 filter paper
- ✓ Racks or trays to hold test containers [*e.g.* styrofoam board into which holes were drilled (14 rows of 10 holes), attached to a wooden board]
- ✓ Wash bottles

7.5.2 Equipment

- ✓ DO meter
- ✓ pH meter
- ✓ Equipment to measure free chlorine
- ✓ Balance
- ✓ Light meter
- ✓ Pipettor
- ✓ Aeration device
- ✓ Light box
- ✓ Magnifying glass
- ✓ Thermometers

7.5.3 Reagents

Use reagent grade chemicals and water purified by a Millipore Milli-Q® system or equivalent for the preparation of solutions. Use RO (reverse osmosis) or distilled water for rinsing.

Chemicals

- ✓ Sodium hydroxide (NaOH)
- ✓ Hydrochloric acid (HCl)
- ✓ Sodium thiosulphate (NaS_2O_3)

Chemical solutions

Sodium hydroxide (1 N)

NaOH	4 g
Water	100 mℓ

- Store at room temperature in a 100 mℓ polyethylene bottle.

Hydrochloric acid (1 N)

HCl	8.3 mℓ
Water	100 mℓ

- Store at room temperature in a 100 mℓ glass reagent bottle.

Sodium thiosulphate (20%)

NaS_2O_3	20 g
Water	100 mℓ

- Store at room temperature in a 100 mℓ glass reagent bottle.

Daphnia food

Trout pellet, yeast and alfalfa (TYA) food and algal suspension (2.5×10^7 cells/mℓ), prepared as described in Appendix B.

Dilution and control water

Synthetic moderately hard water, prepared as described in Appendix C. Keep at 20±2°C.

Reference toxicant

Cadmium chloride ($CdCl_2$) stock solution (100 mg/ℓ), as described in Appendix E.

7.6 TEST ORGANISM

D. pulex, less than 24 h old (first instars), obtained by acyclic parthenogenesis, using specified culturing conditions as described in Appendix B.

7.7 TEST SAMPLES

Wastewater discharges and receiving waters. Sample collection, transport and storage are outlined in Chapter 2.

- The samples must be tested as received, without physical/chemical treatment (If predators are present, filter the samples before testing).
- Allow samples to reach a temperature of 20±2°C before use.
- Measure the pH, DO, temperature and free chlorine (if applicable) before sample preparation.
- Note the appearance of the sample (colour, turbidity, odour).

> - If the sample is high in suspended solids, a parallel test can be carried out on the sample after filtration through Whatman No. 1 filter paper.
> - If the pH and/or oxygen concentration is outside the optimum range for *Daphnia* (pH:<6 and >8.5; DO: <40% of saturation), a parallel test can be carried out after pH adjustment and/or aeration.
> - If the free chlorine is >0.2 mg/ℓ, a parallel test can be carried out after neutralisation with sodium thiosulphate (final concentration: 20 mg/ℓ).

7.8 TEST PROCEDURE

7.8.1 Prepare test solutions

Ideally, fresh samples should be used to replace test solutions in a semi-static test. This will allow the incorporation of variations at the sampling site. This will, however, only be feasible if the test is conducted on site or close to the sampling site. It is expected that the test will mostly be applied to a sample stored at 4°C until completion of the test.

- Samples should be shaken vigorously before use to ensure homogeneity and resuspend particulate matter.
- Test solutions should be renewed every second day (three times per week).

Receiving water

Water samples are tested directly (screening test). Approximately 2.5 ℓ sample is required (10 containers with 25 mℓ sample; renewed eight to nine times).

Wastewater discharges

Wastewater discharges are usually tested after dilution. Approximately 5 ℓ wastewater will be sufficient (10 containers with 25 mℓ sample and dilutions; renewed eight to nine times). Note any changes in appearance of the wastewater during dilution (colour change, precipitation, flocculation, release of volatiles).

- Because of the extended exposure period, a range finding test to select the exposure concentrations for the definitive test is not feasible.
- Instead, conduct a *Daphnia pulex* lethality test (range finding and/or definitive) on the sample or base the selection of concentrations on previous lethality test data.

Serial dilutions on wastewater for a definitive test

A definitive test should span a concentration range where the reproduction in the lowest concentration is not significantly different from the control reproduction (<20%) and the inhibition in reproduction in the highest concentration is >50%. A dilution factor of 0.5 provides good precision and is commonly used in definitive tests.

The dilutions can be made as follows:

- Fill a 500 mℓ measuring cylinder to the 250 mℓ mark with the 100% wastewater or the lowest dilution of the wastewater that will inhibit reproduction by approximately 80%.
- Add moderately hard water to the 500 mℓ mark, cover the cylinder with Parafilm and shake well.
- Transfer 250 mℓ of this solution to a second 500 mℓ measuring cylinder.
- Add moderately hard water to 500 mℓ mark, cover with Parafilm and shake well.

- Continue in this way until all the dilutions have been made (five dilutions should be sufficient).

> ➢ Prepare the same dilutions for each renewal (three times per week).

7.8.2 Add *Daphnia* food to samples

- Before distributing samples into test containers, add 375 µℓ TYA food and 2 mℓ algal suspension to 250 mℓ sample and mix well (concentration in test solution: 2×10^5 cells/mℓ).

7.8.3 Distribute samples in test containers

- Use ten 50 mℓ test containers per sample (water sample or a wastewater and its dilutions).
- Distribute 25 mℓ volumes in each container.
- For each series of samples, prepare control containers in the same way, using moderately hard water.
- Mark each container and place the containers in rows in racks or trays.
- Measure and record the pH, DO and temperature in one of the control containers and one of the highest test concentration (or receiving water) containers, at the beginning of the test and after 2 d exposure, after *Daphnia* have been transferred to fresh solutions.
- If chlorinated samples are tested, also measure the free chlorine.
- Repeat the measurements once a week, at the beginning and end of one of the renewal periods.

> ➢ For every sample renewal, add *Daphnia* food and distribute test solutions in the same way in clean test containers.
> ➢ When a tray with 14 rows of 10 holes is used, the first test containers can be placed in every second row of holes on the tray. During sample renewal, the new containers can be placed in the empty rows, enabling rapid transfer of *Daphnia*.

7.8.4 Transfer *Daphnia* from culture containers to a holding beaker

- Remove the young (pipette/micropipette tip with 2 mm diameter) from the toxicity test culture containers (see Appendix B) and place them in a 50 or 100 mℓ holding beaker, approximately 30 min before the start of a test.
- Reduce the water in the holding beaker by means of a pipette and replace the water with fresh moderately hard water to remove traces of food.
- Concentrate the organisms (if required) by reducing the water in the holding beaker.
- Use the test organisms immediately to prevent them from being stressed.

> ➢ Young *Daphnia* are very susceptible to air entrapment, and should be handled with a pipette rather than being poured.
> ➢ The tip of the pipette should be kept below the surface of the water when the *Daphnia* are released.

7.8.5 Transfer *Daphnia* from the holding beaker to test containers

- Take the holding beaker to the test facility.
- Place one *Daphnia* in each container (total of 10 *Daphnia* per sample).
- Start with the control and highest test dilutions (the least toxic samples).
- Rinse the pipette/micropipette tip between every transfer with moderately hard water before taking another batch of *Daphnia* from the holding beaker.
- Cover the containers with plastic or glass plates and keep them in the test facility at 20±2°C for 21 d.

> ➢ Introduce *Daphnia* in the smallest possible volume of water to avoid dilution of the sample.
> ➢ If *Daphnia* become less dense in the holding beaker, reduce the volume of water to concentrate the remaining *Daphnia* for easy transfer.

7.8.6 Transfer *Daphnia* to fresh test solutions

- Transfer live parents to fresh test solutions (with food) in clean containers every second day (thee times per week). Rinse the pipette/micropipette tip to avoid cross-contamination.

7.8.7 Record the results

- Every second day, when test solutions are renewed (three times per week) during the 21 d exposure period:
  - Count the live offspring produced by each parent.
  - Record the presence of dead parents, offspring, males and ephippia.
- Record the OD, pH and temperature when required (see paragraph 7.8.3).
- Record the light intensity at the surface of the test solutions at least once during the study.

> ➢ Discard the test solutions containing the offspring when measurements are completed.

7.9. DATA ANALYSIS AND EXPRESSION OF RESULTS

- Calculate the percentage adult lethality for each sample solution and control in relation to the total number of *Daphnia* used:

$(N_{t0} - N_{tx})/N_{t0} \times 100\%$, where:

N_{t0}: Number of live *Daphnia* at t = 0 h
N_{tx}: Number of live *Daphnia* at t = 21 d

> ➢ Lethality >20% indicates toxicity.

- Calculate the mean number of offspring (to a single decimal point) produced per live parent in each sample solution and control during the 21 d exposure period.
- Calculate the inhibition in reproduction by surface water:

$(N_c - N_t)/N_c \times 100\%$, where:

N_c: Mean number of offspring per live control parent at t = 21 d
N_t: Mean number of offspring per live test parent at t = 21 d

> ➢ Inhibition >20% indicates toxicity.

- Calculate the effect of wastewater discharges on reproduction in terms of an EC_{20} and EC_{50} (toxicity endpoints) and the 95% confidence limits, by applying an appropriate statistical method (*e.g.* probit analysis) to definitive test data.
- Express toxicity endpoints as a percentage, *e.g.* EC_{50}: 20%.
- In cases where lethality is too low to use a statistical method to calculate the toxicity endpoints, endpoints could be given as a concentration range, *e.g.* EC_{20}: 50-100%; or as larger as 100%, *e.g.* EC_{50}: >100%.
- Determine the R^2 (R = correlation coefficient) for each data set to establish how closely the calculated regression line fits the measured data.

> ➢ If any parent dies before the end of the test or is identified as a male, exclude the replicate from the data analysis for reproduction.

7.10 TEST PRECISION

A reference toxicant must be used to assess the relative toxicity of the population of *Daphnia* used in the toxicity test. This also assesses the precision and reliability of data produced by the laboratory personnel for that reference toxicant under standardised test conditions.

- Conduct a definitive test on the reference toxicant $CdCl_2$ according to the test procedure described in paragraph 7.8.
- Calculate the toxicity endpoints according to paragraph 7.9.
- Verify the sensitivity of the test organism by comparing the calculated endpoints with historical test results (control chart).

> ➢ When stock cultures are maintained regularly, do the test at least once in three months in parallel with a definitive test on a wastewater.
> ➢ Do the test in parallel with definitive tests on each batch of wastewater discharges if cultures are not maintained on a routine basis, or if cultures are obtained regularly from outside sources.

7.11 TEST VALIDITY

For a test to be valid:

- The *Daphnia* have been exposed for 21 d.
- Adult lethality in the control is <20% at the end of the test.
- Living males in the control are ≤20% at the end of the test.
- The mean number of offspring per parent in the controls is >40.
- R^2 is >0.9 (definitive test only).
- 21 d EC_{50} of the reference toxicant is within 2SDs of the historic geometric mean of the laboratory.

> - If the 21 d EC_{50} of the reference toxicant does not fall within 2SDs of the historic geometric mean, the sensitivity of the *Daphnia* and overall credibility of the test system are suspect.
> - All aspects of the test should be carefully examined for defects and repeated with a different batch of *Daphnia*.

7.12 SUMMARY OF TEST CONDITIONS

Table 7.1 summarises the test conditions for the *Daphnia* reproduction test.

Table 7.1: Summary of test conditions for the *Daphnia* reproduction test

Test type	Semi-static
Water temperature	20±2°C
Light quality	Ambient laboratory illumination
Light intensity	10-20 µE/m²/s
Photoperiod	10 to 14 h light
Oxygen concentration	As obtained
pH	As obtained
Feeding	37.5 and 200 µl TYA food and algal suspension, respectively per test container (25 mℓ)
Renewal of test solutions	Three times per week
Size of test container	50 mℓ
Volume of test sample	25 mℓ
Age of test organisms	<24 h
Number of organisms per container	1
Number of replicate containers	10
Total number of organisms per sample	10
Control and dilution water	Moderately hard water
Test duration	21 d
Effect measured	Lethality (no movement on gentle prodding) and effect on reproduction
Detection limit	20%

7.13 TEST REPORT

The test report should refer to this test method and include the following:

7.13.1 Sample

- The name and location of industry where wastewater and receiving water samples were taken.
- Type of industry.

- Brief description of sampling point.
- Date and time of sampling.
- Sampling method (grab or composite).
- Name of person collecting the sample/s.
- Appearance and other properties of samples (colour, turbidity, odour).

7.13.2 Test facility

- Name and city of testing laboratory.
- Name/s of person/s performing the test.
- Name/s of person/s verifying the results.
- Date and time for start of test.

7.13.3 Test conditions

- Test type (screening, definitive).
- Details on any deviation from the standard method.
- Origin and age of test organism.
- Number of organisms per test container.
- Concentrations and volumes tested, including controls, and indication of replication.
- Feeding regime.
- Changes in sample during sample dilution (colour, precipitation, flocculation, release of volatiles).
- pH, DO, temperature and free chlorine (if applicable) of samples.
- Light intensity and photoperiod.
- Confirmation that samples have been filtered to eliminate predators.
- Confirmation that no adjustment of pH/DO/free chlorine occurred.
- Indication of procedure used for pH/DO/free chlorine adjustment if tests were carried out on both adjusted and non-adjusted samples.

7.13.4 Results

- The percentage adult lethality in each test solution, including the control, after 21 d.
- Lethality of offspring.
- Presence of males and ephippia.
- Total number of offspring in each test solution and control.
- Mean number of offspring per live adult in each test solution and control.
- EC_{20}, EC_{50}, and 95% confidence limits.
- Statistical method used for data analysis.
- R^2.
- Most recent EC_{50} for $CdCl_2$, date test initiated and the historic geometric mean EC_{50}±2SD.

7.14 REFERENCES

ISO. 1998. Water quality – Determination of long term toxicity of substances to Daphnia magna Straus. Draft International Standard ISO/DIS 10706. International Organisation for Standardisation, Geneva.

US EPA. 1989. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Second Edition. EPA/600/4-89/001. Environmental Monitoring Systems Laboratory, Office of Research and Development, US Environmental Protection Agency, Cincinnati.

CHAPTER 8: ALGAL (*SELENASTRUM CAPRICORNUTUM* PRINTZ) GROWTH INHIBITION TEST - MICROPLATE ASSAY

8.1 SCOPE

The method measures the sublethal toxicity of wastewater discharges and receiving waters to the freshwater unicellular alga *Selenastrum capricornutum* Printz (Chlorophiceae).

8.2 PRINCIPLE

Logarithmically growing *S. capricornutum* are exposed to test samples over several generations for a period of 72 h, in a static 24-well microplate system, using defined conditions. Growth inhibition is measured as a reduction in growth rate relative to a control carried out under identical conditions. Growth is determined in terms of optical density (OD).

The test is applied directly (screening test) to receiving waters to determine the percentage growth inhibition. Definitive tests (testing serial dilutions) are carried out on wastewater discharges to determine toxicity endpoints – EC_{20} (concentration causing 20% growth inhibition - minimum effect concentration) and EC_{50} (concentration causing 50% growth inhibition). A definitive test is usually preceded by a range finding test (testing 10-fold dilutions) to establish the toxic concentration range.

8.3 BACKGROUND

The assay is a miniaturised version of the standard US EPA flask test (US EPA, 1978, 1989). The test is carried out in 2 mℓ volumes in 24-well microplates as opposed to the 50 to 100 mℓ volumes used in the flask test. The microplate assay uses a larger cell inoculum (200 000 cells per mℓ) than the flask test (10 000 cells per mℓ) to enable OD measurement after 72 h. An improved growth was obtained with 10% BG-11 medium (as opposed to the flask test medium), while the sensitivity of tests was in agreement (Slabbert and Hilner, 1991; Slabbert *et al.*, 1998a,b).

The microplate assay has the advantage that sample handling and processing is simplified – sample volume is small, sample sterilisation quicker and cheaper, and sample dispensing quicker. Other advantages are that incubation space is reduced; a number of replicates can be accommodated per plate; a large number of samples can be tested at one time; and cleaning of glassware is minimised.

8.4 INTERFERENCES

- Dark coloured samples can interfere with OD measurements, and can affect the light intensity, resulting in lower growth.
- High salt concentrations in samples can lead to precipitation upon algal medium addition, which can interfere with OD measurements.
- Volatile substances might inhibit growth of algae in other wells, including that of the control.

8.5 TEST ENVIRONMENT

- Use a temperature controlled room or chamber that will remain at a temperature of 24±2°C at the level of the test solutions. The temperature should not vary by more than 2°C during a test.

- The room or chamber must be fitted with a system of cool white fluorescent light (illumination from above) with a light intensity of 80 to 95 $\mu E/m^2/s$ at the level of the test solutions. The illumination should be continuous.
- The test facility must be separate from the culture area.
- The facility must be well-ventilated and free of vapours, odours and dusts that may be toxic to algae.

8.6 MATERIALS, EQUIPMENT AND REAGENTS

8.6.1 Materials

- ✓ Glass beakers - 10 mℓ, 50 mℓ and 1 ℓ
- ✓ Glass measuring cylinders - 100 mℓ, 500 mℓ and 1 ℓ
- ✓ Volumetric flasks - 100 mℓ, 500 mℓ and 1 ℓ
- ✓ Erlenmeyer flasks - 500 mℓ
- ✓ Sterile Erlenmeyer flasks with air-permeable stoppers - 50,125 and 250 mℓ
- ✓ Sterile filtration flask - 1 ℓ
- ✓ Sterile Schott bottles - 100 mℓ, 250 mℓ, 500 mℓ and 1 ℓ
- ✓ Sterile graduated glass pipettes - 1, 5, 10 and 25 mℓ
- ✓ Sterile glass tubes - 20 x 150 mm, approximately 50 mℓ
- ✓ Polyethylene bottle - 100 mℓ
- ✓ Glass reagent bottles - 100 mℓ
- ✓ Microplates - sterile disposable polystyrene 24-well plates (well volume: 3 mℓ) with lids
- ✓ Sterile Pasteur pipettes
- ✓ Sterile disposable micropipette tips - 100 and 1 000 µℓ
- ✓ Sterile plastic medium reservoirs (if multipipette is used)
- ✓ Sterile disposable Petri dishes - 60 mm diameter
- ✓ Disposable syringes - 25 or 50 mℓ
- ✓ Sterile membrane filters - 0.22 µm
- ✓ Sterile syringe filter units - 0.45 and 0.22 µm
- ✓ Haemacytometer cover glasses
- ✓ Insulation tape
- ✓ Parafilm
- ✓ Aluminium foil
- ✓ Wash bottles

8.6.2 Equipment

- ✓ DO meter
- ✓ pH meter
- ✓ Equipment to measure free chlorine
- ✓ Balance
- ✓ Microscope with contrast (green filter), providing 100 to 400x magnification
- ✓ Cold room (4°C) or refrigerator
- ✓ Autoclave or pressure cooker
- ✓ Incubator
- ✓ Laminar flow cabinet (or work near a flame)
- ✓ 24-well microplate reader
- ✓ Vacuum pump
- ✓ Magnetic stirrer
- ✓ Light meter
- ✓ Thermometers
- ✓ Haemacytometer
- ✓ Filter apparatus (47-mm stainless steel filter holder)

- ✓ Pipettor
- ✓ Adjustable micropipettes (or multipipette) - 100 and 1 000 µℓ
- ✓ Gas supply and burner
- ✓ Magnetic stirring bars
- ✓ Test tube racks
- ✓ Inoculation needle
- ✓ Spatula
- ✓ Calculator

8.6.3 Reagents

Use reagent grade chemicals and water purified by a Millipore Milli-Q® system or equivalent for the preparation of solutions. Use reverse osmosis (RO) or distilled water for rinsing.

Chemicals

- ✓ Calcium chloride ($CaCl_2.2H_2O$)
- ✓ Sodium nitrate ($NaNO_3$)
- ✓ Dipotassium hydrogen phosphate (K_2HPO_4)
- ✓ Magnesium sulphate ($MgSO_4.7H_2O$)
- ✓ Sodium bicarbonate ($NaHCO_3$)
- ✓ Boric acid (H_3BO_3)
- ✓ Manganese chloride ($MnCl_2.4H_2O$)
- ✓ Copper sulphate ($CuSO_4.5H_2O$)
- ✓ Zinc sulphate ($ZnSO_4.7H_2O$)
- ✓ Cobalt nitrate [$Co(NO_3)_2.6H_2O$]
- ✓ Ferri ammonium citrate [$Fe(NH_3)$-citrate]
- ✓ Sodium molybdenum oxide ($Na_2MoO_4.2H_2O$)
- ✓ Citric acid ($C_6H_8O_7.7H_2O$)
- ✓ Sodium EDTA ($Na_2EDTA.2H_2O$)
- ✓ Sodium hydroxide (NaOH)
- ✓ Hydrochloric acid (HCl)
- ✓ Sodium thiosulphate (NaS_2O_3)
- ✓ Agar
- ✓ Nutrient agar

Chemical solutions

Sodium hydroxide (1 N)

NaOH	4 g
Water	100 mℓ

- Store at room temperature in a 100 mℓ polyethylene bottle.

Hydrochloric acid (1 N)

HCl	8.3 mℓ
Water	100 mℓ

- Store at room temperature in a 100 mℓ glass reagent bottle.

Sodium thiosulphate (20%)

NaS_2O_3	20 g
Water	100 mℓ

- Store at room temperature in a 100 mℓ glass reagent bottle.

Algal growth medium

Stock solutions of macro- and micro-nutrients

- Prepare each of the stock solutions A to H, listed in Table 1, by placing the reagent/s in a 1 ℓ volumetric flask and making it up to 1 ℓ with water.
- The micro-nutrient stock solution, F, is prepared by filling a 1 ℓ volumetric flask with approximately 500 mℓ water. The reagents are then added to the volumetric flask in the order listed in Table 8.1. Because the quantities of $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $Co(NO_3)_2.6H_2O$ and $Na_2MoO_4.2H_2O$ are so small, these chemicals are prepared separately in volumetric flasks at 100-times higher concentrations than given in Table 8.1, and then added as 10 mℓ volumes to the 1 ℓ flask (100-fold dilution). Shake the flask in-between additions of reagents to avoid precipitation. Make up to 1 ℓ with water.
- Transfer the solutions to 1 ℓ Schott bottles.
- Store solutions A to E and G at room temperature (20±2°C).
- Keep stock solutions F and H in the dark at 4°C.

Table 8.1: Algal growth medium[1,2]

Stock solution		Reagent	Stock solution concentration (g/ℓ)	10% BG-11 medium		20-times concentrated 10% BG-11 medium	
				Volume (mℓ) of stock solution added per 1 ℓ water	Concentration (mg/ℓ)	Volume (mℓ) of stock solution added per 100 mℓ water	Concentration (mg/ℓ)
Macro-nutrients	A	$CaCl_2.2H_2O$	3.6	1	3.6	2	72
	B	$NaNO_3$	15.0	10	15.0	20	300
	C	K_2HPO_4	4.0	1	4.0	2	80
	D	$MgSO_4.7H_2O$	7.5	1	7.5	2	150
	E	$NaHCO_3$	2.0	1	2.0	2	40
Micro-nutrients	F	H_3BO_3	0.286	1	0.286	2	5.72
		$MnCl_2.4H_2O$	0.181		0.181		3.62
		$CuSO_4.5H_2O$	0.0079		0.0079		0.158
		$ZnSO_4.7H_2O$	0.0222		0.0222		0.444
		$Co(NO_3)_2.6H_2O$	0.00494		0.00494		0.0988
		$Na_2MoO_4.2H_2O$	0.039		0.039		0.78
		Citric acid	0.6		0.6		12.0
	G	$Na_2EDTA.2H_2O$	0.1	1	0.1	2	2.0
	H	$Fe(NH_3)$-citrate	0.6	1	0.6	2	12.0

1 Rippka *et al.* (1979)
2 Prepare fresh stock solutions every three months

10% BG-11 medium (referred to as BG-11 in the text)

- Fill a 1 ℓ volumetric flask with 700 mℓ water.
- Add the stock solutions in the order listed in Table 8.1.
- Shake the flask in-between additions to avoid precipitation.
- Make up to 1 ℓ with water.
- Measure the pH of the medium and if needed, adjust to between 7.1 and 7.2 using NaOH or HCl.
- Sterilise the medium by filtration through a 0.22 µm filter.
- Transfer to a sterile 1 ℓ Schott bottle. Medium can be distributed in smaller volumes (*e.g.* in 250 mℓ Schott bottles) if required.
- Keep at room temperature (20±2°C).
- When required, 50 mℓ volumes of the medium is transferred to sterile 250 mℓ Erlenmeyer flasks fitted with air-permeable stoppers, under sterile conditions (for culture maintenance).

20-times concentrated 10% BG-11 (referred to as 20xBG-11 in the text)

- Fill a 100 mℓ volumetric flask with 50 mℓ water.
- Add the stock solutions in the order listed in Table 8.1.
- Shake the flask in-between additions to avoid precipitation.
- Make up to 100 mℓ with water.
- Measure the pH of the medium and if needed, adjust to between 7.1 and 7.2 using NaOH or HCl.
- Sterilise the medium by filtration through a 0.22 µm syringe filter.
- Transfer to a sterile 250 mℓ Schott bottle. Medium can be distributed in smaller volumes (*e.g.* in 100 mℓ Schott bottles) if required.
- Keep at room temperature (20±2°C).

> ➢ Prepare the BG-11 and 20xBG-11 as required.
> ➢ Do not keep longer than 2 weeks.

BG-11 agar plates

Agar	3 g
BG-11	250 mℓ

- Dissolve the agar in water in a 500 mℓ Erlenmeyer flask.
- Cover with aluminium foil.
- Autoclave for 15 min at 121°C.
- Cool to 45°C.
- Pour into sterile Petri dishes (about 13 mℓ/dish).
- Allow to solidify and store plates upside down in the dark at 4°C (maximum storage 2 weeks).

Nutrient agar plates

Nutrient agar	8 g
Water	250 mℓ

- Dissolve the nutrient agar in water in a 500 mℓ Erlenmeyer flask.
- Cover with aluminium foil.

- Autoclave for 15 min at 121°C.
- Cool to 45°C.
- Pour into Petri dishes (about 13 mℓ/dish).
- Allow to solidify and store plates upside down in the dark at 4°C (maximum storage 2 weeks).

Dilution and control water

Sterile Milli-Q® water (autoclaved for 15 min at 121°C). Keep at 20±2°C.

Reference toxicant

Cadmium chloride ($CdCl_2$) stock solution (100 mg/ℓ), as described in Appendix E.

8.7 TEST ORGANISM

The freshwater unicellular green alga, *S. capricornutum* Printz.

8.7.1 Species

S. capricornutum is a non-motile, crescent-shaped alga, 40 to 60 µm in size. The organism is ubiquitous in most freshwaters. It does not clump because it is free of complex structures and does not form chains. It is therefore easy to count. The alga is easily cultured in the laboratory and is particularly sensitive to metal toxicants.

The alga was isolated in 1948 by Olav Skulberg and has since been renamed to *Raphidocelis subcapitata* (Nygaard *et al.*,1986). However, the species is commonly known in literature and in standard methods as *S. capricornutum*.

> ➢ Until such time as the designation is changed in international standard methods, the name *S. capricornutum* will be retained in this manual.

8.7.2 Source

A liquid or plate culture can be obtained from the Biotoxicology Laboratory, Division of Water, Environment and Forestry Technology, CSIR. The culture was originally obtained from the St. Lawrence Centre, Environment Canada as ATCC 22662.

ATTC 2266 is a reliable American source that can be obtained from the American Type Culture Collection. (Address : 12301 Parklawn Drive, Rockville, Maryland 20852; Tel: 301 881 2600; Fax: 301 231 5826). The ATTC delivers a frozen liquid culture in an ampule that is transported in dry ice. Upon receipt the culture must be suspended in growth medium.

8.7.3 Culture maintenance

Culture facility

- ➢ Use a temperature controlled area that will provide a temperature of 24±2°C at the level of the test solutions.
- ➢ The area should be fitted with a system of cool white fluorescent light with a light intensity of 80 to 95 $\mu E/m^2/s$ at the level of the test solutions. The illumination should be continuous.

- The culture area must be separate from the test area.
- The area must be well ventilated and be free of vapours, odours and dusts that may be toxic to algae.

Stock cultures

- The alga is axenically maintained in 50 mℓ BG-11 in 250 mℓ Erlenmeyer flasks fitted with air-permeable stoppers.

> - 20% algal solution to flask volume ratios are recommended to avoid growth inhibition due to carbon dioxide limitation.

- The alga is subcultured every 3 to 4 d in fresh medium (two to three flasks) using 1 to 2 mℓ algae (glass pipette) per 50 mℓ medium, to have a constant supply of logarithmic growth phase cells for toxicity testing.

> - Sterile protocols are followed during culturing and handling of the algal cultures.

- The purity of the stock culture used for subculturing should be verified at each transfer by:
  - examining a subsample under a microscope for contamination by microorganisms, and
  - transferring approximately 1 mℓ of the stock culture to nutrient agar plates (spread plate) and incubating the plates upside down at 37°C for 48 h (this will reveal the presence of bacteria/fungi that cannot be detected microscopically).

Back-up cultures

Liquid cultures kept at 4°C

Once a month, one or two extra subcultures can be prepared. These are kept in the dark at 4°C for six months. If contamination of stock cultures occurs, these back-up cultures can be used to start fresh cultures.

Plate cultures

Periodically prepare plate cultures to ensure culture purity.

- Under sterile conditions, using streak-plate procedures, transfer algal cells from stock cultures onto sterile BG-11 agar plates.
- Incubate the plates upside down in the culture area until colonies are visible (approximately two weeks).
- Store at 4°C in the dark for future use (cells will remain viable for three months).

- A fresh stock culture should be started every three months from an algal colony isolated from the solid growth medium (put the algae in a small volume of medium and transfer to larger volumes as algae grow).

8.8 TEST SAMPLES

Wastewater discharges and receiving waters. Sample collection, transport and storage is outlined in Chapter 2.

- The samples must be tested as received, without pH or DO adjustments.
- Measure and record the pH, DO and free chlorine (if applicable) before sample preparation.
- Note the appearance of the sample (colour, turbidity, odour).

- If the pH and/or oxygen concentration is outside the optimum range for algae (pH:<6 and >8.5; DO: <40% of saturation), a parallel test can be carried out after pH adjustment and/or aeration.
- If the free chlorine is >0.2 mg/ℓ, a parallel test can be carried out after neutralisation with sodium thiosulphate (final concentration: 20 mg/ℓ).

8.9 TEST PROCEDURE

- Work at room temperature (20±2°C),
- Near a flame or in a laminar cabinet to ensure sterility, and
- Follow standard sterility protocols.

8.9.1 Sample preparation

- Samples should be shaken vigorously to ensure homogeneity and to re-suspend particulate matter.

Sterilise samples

- As soon as possible after sample receipt, filter sterilise approximately 50 mℓ of the sample using a syringe and a sterile 0.22 µm syringe filter.
- Place the sample in a sterile 100 mℓ Schott bottle.
- Keep at 4°C until use.
- Allow samples to reach a temperature of 20±2°C before use.

- When samples are very turbid, first filter the sample with a 0.45 µm syringe filter.
- Sterile filtration do not significantly reduce the toxicity of toxicants in solution.

Prepare test solutions

Receiving water

Water samples are tested directly (screening test).

Wastewater discharges

Wastewater discharges are usually tested after dilution.

10-fold dilutions on wastewater for a range finding test

A range finding test (testing ten-fold dilutions) is usually carried out before a definitive test (testing serial dilutions) to establish the range of concentrations to be used for a definitive test.

Dilutions can be made as follows:

- Place sterile 50 mℓ glass tubes in a tube rack.
- Dispense 9 mℓ sterile dilution water (Milli-Q®) with a sterile 10 mℓ glass pipette in each tube.
- Transfer 1 000 µℓ of the sterilized wastewater (100%) with a micropipette and sterile pipette tip to the first tube.
- Shake the tube to mix the sample.
- Use the same pipette tip to transfer 1 000 µℓ sample from the first tube (10%) to the second tube.
- Shake the tube to mix the sample.
- Continue in this way until all the dilutions are made (2 to 4 dilutions should be sufficient).

Serial dilutions on wastewater for a definitive test

A definitive test should span a concentration range that exhibits no growth inhibition (<20%) and, if possible, complete growth inhibition (100%). A dilution factor of 0.5 provides good precision and is commonly used in definitive tests.

Dilutions can be made as follows:

- Place sterile 50 mℓ glass tubes in a tube rack.
- Dispense 10 mℓ sterile dilution water (Milli-Q®) with a sterile 10 mℓ glass pipette in each tube.
- Transfer 10 mℓ of the sterilised 100% wastewater, or the lowest dilution of the wastewater that exhibited complete growth inhibition in the range finding test (sterilised), with a sterile 10 mℓ pipette to the first tube.
- Use another sterile 10 mℓ pipette to mix the sample in the first tube.
- Then, transfer 10 mℓ of this sample to the second tube.
- Take another sterile 10 mℓ pipette to mix the sample in the second tube.
- Continue in this way until all the dilutions are made (8 to 12 dilutions are usually used).

8.9.2 Distribute samples in microplates

- Remove the required number of sterile 24-well microplates from their plastic covers (keep lids in position) and place them horizontally (six wells across, four wells down) on the work surface.
- Mark the plates on the side (not on top because this will interfere with illumination) according to the configuration given in Figure 8.1.

Screening test

CB	1B	1B	2B	2B	CB
C	1	1	2	2	C
C	1	1	2	2	C
C	1	1	2	2	C

Range finding and definitive test

CB	0.1B	1B	10B	100B	C
C	0.1	1	10	100	C
C	0.1	1	10	100	C
C	0.1	1	10	100	C

Figure 8.1: Configuration for screening, range finding and definitive tests
(C: control; 1, 2: different receiving waters; 100, 10, 1, 0.1: wastewater and dilutions; B: Blank)

> ➢ The top row of each plate is used for blanking and will receive sample and medium only (no algal cells).

- Dispense 1.8 mℓ sterile control water (Milli-Q®) with a micropipette (2x900 µℓ) into the first and last columns (eight wells) of each of the required microplates (Figure 8.1).
- Keep the other wells covered with the lid to avoid contamination.

> ➢ A multipipette can alternatively be used to speed up the dispensing. Use a sterile plastic reservoir for each test concentration or if a single reservoir is used, start with the control and lowest test concentrations progressing towards the highest concentration.

- Dispense 1.8 mℓ sample in the other columns according to the configuration. Eight wells on a plate are used per sample in the case of screening tests and four wells in the case of range finding and definitive tests (Figure 8.1).
- Use a new pipette tip/s for each sample in the screening test.
- In the case of range finding and definitive tests, start with the lowest concentration, and use the same pipette tip/s, working towards the highest concentration.
- Prepare an extra plate with 1.8 mℓ control water in the first two columns (eight wells) for the t=0 h OD reading.
- Leave the plates (covered) on the work surface until inoculation.

> ➢ If volatile samples or samples with strong odours are tested, use separate microplates for the high test concentrations.

8.9.3 Prepare the algal inoculum and medium to be added to samples in microplates

- Use a 3 to 4 d old logarithmic phase algal culture.
- Remove the supernatant medium from the settled algal layer in the algal flask by means of a sterile Pasteur pipette and vacuum pump (do not disturb the algal layer to retain as much of the algal cells as possible).
- Discard the supernatant medium.
- Add 50 mℓ sterile BG-11 with a sterile 25 mℓ pipette to the algal flask (avoid contamination of medium by not touching the flask sides or use a fresh pipette for the second 25 mℓ medium aliquot).
- Use the same pipette to re-suspend the algae (pull up and push out, a number of times).
- Cover the flask with stopper and leave on work surface for preparation of the algal inoculum.
- Dispense 9 mℓ of the BG-11 into a sterile glass tube in a test tube rack, using a sterile 10 mℓ pipette.
- Transfer 1 mℓ of the well-mixed resuspended algae in the flask to the test tube, using a sterile 1 mℓ pipette (a micropipette can cause contamination).
- Mix well.
- Prepare the haemacytometer for cell counting.
- Place the well-mixed cell suspension in the tube with a micropipette in the haemacytometer and count the cells microscopically (at least 10 areas).
- Calculate the number of cells in the resuspended algal culture:
  - Mean cell number x 10^1 (for 10-fold dilution) x 10^4 (factor used for haemacytometer) (*e.g.* 100 x 10 x 10 000 = 10 000 000 cells/mℓ).
- Calculate the algal inoculum volume for the test and control wells (excluding blank wells):

> ➢ 200 000 cells are used per mℓ sample (400 000 cells/well).
> ➢ 0.1 mℓ algal inoculum is used per well.

  - Number of wells: *e.g.* three plates x 18 wells = 54 wells plus six wells = 60 wells (always take a larger number of wells to ensure enough inoculum).
  - Number of cells required for 60 wells: 200 000 cells/mℓ x 2 (2 mℓ per well) x 60 = 24 000 000 cells.
  - Volume of the prepared cell suspension required for inoculation: number of cells required for 60 wells divided by number of cells/mℓ of the prepared cell suspension – *e.g.* 24 000 000 divided by 10 000 000 = 2.4 mℓ.
  - Total cell inoculum to be added to 60 wells: 0.1 mℓ x 60 = 6 mℓ. The prepared cell suspension part of the 6 mℓ is 2.4 mℓ (see previous step), the remaining volume of 3.6 mℓ is supplemented by BG-11.

- Determine the volume of 20xBG-11 to be added to test and control wells (excluding blanks):

> ➢ 0.1 mℓ medium is used per well.

  - Number of wells, *e.g.* three plates x 18 wells = 54 wells plus six wells = 60 wells
  - Total volume of medium to be added to 60 wells: 0.1 mℓ x 60 = 6.0 mℓ
- Combine the algal inoculum and medium in a sterile glass tube (or sterile Schott bottle if larger volumes are prepared) for test and control well inoculation using sterile pipettes and micropipette tips: *e.g.* 6 mℓ 20xBG-11 + 3.6 mℓ BG-11 + 2.4 mℓ well-mixed resuspended algae (from the flask).
- Determine the volumes of 20xBG-11 and BG-11 to be added to blank wells:

> ➢ 0.1 mℓ 20xBG-11 and 0.1 mℓ BG-11 is used per well.

  - Number of wells, *e.g.* three plates x six wells = 18 wells plus four wells = 22 wells
  - Volume of 20xBG-11: 0.1 mℓ x 22 wells = 2.2 mℓ
  - Volume of BG-11: 0.1 mℓ x 22 wells = 2.2 mℓ
- Combine the 20xBG-11 and BG-11 in a sterile glass tube for blank well inoculation using sterile micropipette tips: *e.g.* 2.2 mℓ 20xBG-11 + 2.2 mℓ BG-11.

8.9.4 Dispense the algal inoculum and medium in microplate wells

- Transfer 200 µℓ (0.2 mℓ) of the 20xBG-11 and BG-11 medium mixture to the blank wells (top row of plates) in the different plates (including the extra plate for t=0 t OD reading) using a micropipette or multipipette and sterile tip/s. Keep the other wells covered with the lid.

> ➢ To avoid cross-contamination, do not touch the liquid in the wells with the tip/s. Keep the pipette tip/s at an angle so that the liquid can run down the side of a well.

- Transfer 200 µl of the algal inoculum and medium mixture to test and control wells (including the extra plate for t=0 t OD reading) using a micropipette or multipipette and sterile tip/s. Keep the other wells covered with the lid.

> ➢ Shake the mixture by hand in between transfers to ensure homogeneity.

- Place lids on plates.
- With lid in position, put two layers of dark insulation tape around the edge of each plate (to avoid over-illumination of outer wells). Avoid touching the bottom of the plate.
- Use a blade to cut the tape between the lid and the plate (to allow air circulation during incubation).

8.9.5 Incubate the microplates

- Place the microplates in the temperature and light controlled test facility.
- Incubate for 72 h.

- Move the plates every 24 h to other positions to ensure homogeneous illumination.
- Record the air temperature at the level of the plates at t=0, and thereafter at 24 h intervals.

> ➢ If the growth in the controls do not meet the stated OD validity requirement (paragraph 8.12) after 72 h incubation, plates can be incubated for a further period, but not for longer than 96 h.
> ➢ If the incubation period is extended, the time should be stated in the results.

8.9.6 Record the results

- Determine the OD at t=0 h
  - Shake the extra plate for 2 min on a microplate shaker to suspend the cells.
  - Remove the lid of the plate and read the OD in the microplate reader (450 nm).
- After 72 h incubation, place each microplate on a white background/light box and examine visually and record signs of evaporation, microbial contamination, precipitation, colour, and any other observations.
- Determine the OD at t=72 h
  - Shake each plate on a microplate shaker for 5 min to suspend cells (make sure that all cells are suspended).
  - Remove the lid of each plate and read the OD in the microplate reader (450 nm).
- Measure the pH in test and control wells (one well of each column) using pH indicator paper.

> ➢ If samples interacted with the growth medium to form large precipitates, OD measurement will not be possible.
> ➢ In such cases the algal test should be omitted from the battery of tests applied.

8.10 DATA ANALYSIS AND EXPRESSION OF RESULTS

- Use the OD printouts of the microplate reader to determine:
  - The mean OD of six control wells at t=0 (OD_0).
  - The mean OD (ODC) and coefficient of variation (RSD) of the six control wells on each plate at t=72 h.
  - The mean OD (ODT) of the three (range finding and definitive test) or six test wells (screening test) on each plate at t=72 h.
- Calculate the percentage growth inhibition (or stimulation) using the formula:

$$[(ODC - OD_0) - (ODT - OD_0 - ODB_{>0.005})]/(ODC - OD_0) \times 100, \text{ where}$$

$ODB_{>0.005}$ is the blank OD in the corresponding test wells >0.005 (to compensate for colour and/or precipitation).

> - Growth inhibition ≥20% indicates toxicity, and
> - Growth enhancement ≥20% usually indicates excess nutrients.

- Calculate the 72 h EC_{20} and EC_{50} (toxicity endpoints) for the wastewater by applying linear regression (% inhibition versus log concentration) to the data located within the 16 to 84% growth inhibition range. Do not use the stimulation data in the calculation.
- Express toxicity endpoints as a percentage, *e.g.* EC_{50}: 20%.
- In cases where growth inhibition is too low to use regression analysis to calculate the toxicity endpoints, endpoints could be given as a concentration range, *e.g.* EC_{10}: 50-100%; or as larger as 100%, *e.g.* EC_{50}: >100%.
- Determine the R^2 (R = correlation coefficient) for each data set to establish how closely the calculated regression line fits the measured data.

> - Test concentrations are diluted by a factor of 0.9 with the addition of the algal inoculum and medium. The dilution should be taken into account when calculating the toxicity endpoints (multiply concentrations with 0.9).

8.11 TEST PRECISION

A reference toxicant must be used to assess the relative toxicity of the algal population used in the toxicity test. This also assesses the precision and reliability of data produced by the laboratory personnel for that reference toxicant under standardised test conditions.

- Conduct a definitive test on the reference toxicant $CdCl_2$ (filter sterilised) according to the test procedure described in paragraph 8.9.
- Calculate the toxicity endpoints according to paragraph 8.10.
- Verify the sensitivity of the test organism by comparing the calculated endpoints with historical test results (control chart).

> - Do the test at least once a month in parallel with a definitive test on a wastewater when stock cultures are maintained regularly.
> - Do the test in parallel with every batch of definitive tests on wastewater if cultures are not maintained on a routine basis.

8.12 TEST VALIDITY

For a test to be valid:

- RSD in the control should be <10%.
- OD in the control after 72 h exposure should be 0.150±0.03.
- R^2 should be >0.9 (definitive test only).
- 72 h EC_{50} of the reference toxicant is within 2SDs of the historic geometric mean of the laboratory.

- If the 72 h EC_{50} of the reference toxicant does not fall within 2SDs of the historic geometric mean, the sensitivity of the alga and overall credibility of the test system are suspect.
- All aspects of the test should be carefully examined for defects and repeated with a different batch of algae.

8.13 SUMMARY OF TEST CONDITIONS

Table 8.2 summarises the test conditions for the *S. capricornutum* microplate assay.

Table 8.2: Summary of test conditions for the *S. capricornutum* microplate assay

Test type	Static
Air temperature	24±2°C
Light quality	Cool white fluorescent light (80 to 95 µE/m²/s)
Photoperiod	Continuous
Oxygen concentration	As obtained
pH	As obtained
Feeding	BG-11
Size of test well	350 µℓ (24-well plate)
Volume of test sample	1.8 mℓ (plus 0.2 mℓ inoculum and medium)
Age of algal culture	3 to 4 d
Inoculum size	200 000 cells/mℓ
Number of replicate wells	Six control and screening test, three range finding and definitive test
Control/dilution water	Sterile Milli-Q® water
Test duration	72 h (or until the required OD is reached)
Effect measured	Growth inhibition in terms of OD
Detection limit	20%

8.14 TEST REPORT

The test report should refer to this test method and include the following:

8.14.1 Sample

- The name and location of industry where wastewater and receiving water samples were taken.
- Type of industry.
- Brief description of sampling point.
- Date and time of sampling.
- Sampling method (grab or composite).
- Name of person collecting the sample/s.
- Appearance and other properties of samples (colour, turbidity, odour).

8.14.2 Test facility

- Name and town/city of testing laboratory.
- Name/s of person/s performing the test.
- Name/s of person/s verifying the results.
- Date and time for start of test.

8.14.3 Test conditions

- Test type (screening, definitive).
- Details on any deviation from the standard method.
- Origin and age of test culture.
- Inoculum size.
- Incubation period.
- Concentrations and volumes tested, including controls, and indication of replication.
- pH, DO and free chlorine (if applicable) of samples before sample preparation.
- Confirmation that no adjustment of pH/DO/free chlorine occurred.
- Indication of procedure used for pH/DO/free chlorine adjustment if tests were carried out on both adjusted and non-adjusted samples.
- pH measurements at the end of the test.
- Temperature measurements at the start of the test and every 24 h thereafter.

8.14.4 Results

- The percentage growth inhibition (or growth enhancement) in each test solution after 72 h.
- EC_{20} and EC_{50}.
- Statistical method used for data analysis.
- R^2.
- Most recent EC_{50} for $CdCl_2$, date tested, and the historic geometric mean EC_{50} ±2SD.

8.15 REFERENCES

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CHAPTER 9: ALGAL (*SELENASTRUM CAPRICORNUTUM* PRINTZ) GROWTH INHIBITION TEST - FLASK TEST

9.1 SCOPE

This method measures the sublethal toxicity of wastewater discharges and receiving waters to the freshwater unicellular alga *Selenastrum capricornutum* Printz (Chlorophiceae).

9.2 PRINCIPLE

Logarithmically growing *S. capricornutum* are exposed to test samples over several generations for several days, in a static flask test, using defined conditions. Growth inhibition is measured as a reduction in growth or growth rate relative to a control carried out under identical conditions. Growth is determined in terms of OD, cell density (cell counts), biomass or chlorophyll content.

The test is applied directly (screening test) to receiving waters to determine the percentage growth inhibition. Definitive tests (testing serial dilutions) are carried out on wastewater discharges to determine toxicity endpoints – EC_{20} (concentration causing 20% growth inhibition), EC_{50} (concentration causing 50% growth inhibition), LOEC (lowest concentration tested at which a significant effect occurred) and NOEC (highest concentration tested at which no significant effect occurred). A definitive test is usually preceded by a range finding test (testing 10-fold dilutions) to establish the toxic concentration range.

9.3 INTERFERENCES

- Dark coloured samples can interfere with OD measurements.
- Dark coloured samples can affect the light intensity, resulting in lower growth.
- High salt concentrations in samples can lead to precipitation upon algal medium addition, which can interfere with OD measurements.

> - Interference by colour and high salt content can be eliminated by cell counting or chlorophyll measurements.

9.4 METHODOLOGY

Various standard algal flask tests are available (OECD, 1984; EEC, 1988; ISO, 1989; US EPA, 1989). The major difference between these tests is the composition of the growth medium. The US EPA (1989) test has been used in South African laboratories in the past, and is well documented and described. It is the basis for the microplate assay described in Chapter 8. It is recommended that this procedure is followed when a flask test is applied.

9.5 SUMMARY OF TEST CONDITIONS

Table 9.1 summarises the recommended test conditions for the *S. capricornutum* flask test.

Table 9.1: Summary of test conditions for the *S. capricornutum* flask test

Test type	Static
Temperature	25±1°C
Light quality	Cool white fluorescent light
Light intensity	86 ± 8.6 $\mu E/m^2/s$
Photoperiod	Continuous
Oxygen concentration	As obtained
pH	As obtained
Size of test container	250 mℓ (Erlenmeyer)
Volume of test solution	50 mℓ
Age of algal culture	4 to 7 d
Inoculum size	10 000 cells/mℓ
Number of replicate containers	3
Shaking rate	60 rpm continuous or twice daily by hand
Dilution water	Algal stock culture medium
Test duration	96 h
Effect measured	Growth inhibition (OD, cell counts, chlorophyll, biomass)
Test acceptability	$2x10^5$ cells/mℓ in controls after 96 h (20 fold increase); Control RSD should be <20%
Detection limit	20%

9.6 REFERENCES

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CHAPTER 10: AMES *SALMONELLA* MUTAGENICITY TEST - PLATE INCORPORATION ASSAY

10.1 SCOPE

The Ames *Salmonella* mutagenicity test detects mutagens in wastewater discharges and receiving waters. Mutagenicity is an indication of the presence of carcinogens, since approximately 83% of the mutagens detected by the Ames test have been shown to be carcinogenic in animal tests (Ames and McCann, 1981).

10.2 PRINCIPLE

The test uses two histidine-requiring strains of the bacterium *Salmonella typhimurium*, namely TA98 and TA100. Each tester strain contains a different type of mutation in the operon coding for histidine biosynthesis. When the bacteria are exposed to mutagenic substances, reverse mutation from amino acid (histidine) auxotrophy (dependence) to prototrophy (independence) occurs (Denkhaus, 1979; Maron and Ames, 1983; US EPA, 1983; Slabbert *et al.*,1998). TA98 detects frameshift mutagens and TA100 base-pair substitution mutagens. Since certain chemicals require metabolic activation before exhibiting mutagenic activity, tests are carried out with and without chemically induced rat liver homogenate.

The plate incorporation assay entails combining the test sample, bacterial tester strain, and S9 liver mix (or buffer solution) in soft agar and pouring the mixture onto minimal agar plates. Plates are incubated for 48 to 72 h at 35-37°C. Mutagenicity is determined by comparing the number of revertant colonies on test plates to that on control plates. Results are expressed as mutation ratios (MRs).

The protocols described in this method are based on the methodology of Ames (US EPA, 1983; Maron and Ames, 1983), as optimised by Denkhaus (1979) for water quality testing.

10.3 INTERFERENCES

- Volatile substances might inhibit growth of bacteria in surrounding plates, including that of the control.

10.4 SHORTCOMINGS

- The test fails to detect certain classes of carcinogens, *e.g.* the polychlorinated pesticides (Maron and Ames, 1983) and metal carcinogens (Kfir, 1981).

10.5 TEST FACILITY

- Use a dedicated area in a laboratory or a separate facility for assaying to avoid contamination.
- The facility must be free of vapours, odours and dust that may inhibit bacterial growth or cause microbial contamination (switch air-conditioner off).
- Use a temperature controlled chamber or an incubator that will provide a temperature of 35-37°C.

10.6 MATERIALS, EQUIPMENT AND REAGENTS

10.6.1 Materials

- ✓ Glass beakers - 50, 100 and 500 mℓ
- ✓ Glass measuring cylinders -100 mℓ and 1 ℓ
- ✓ Erlenmeyer flasks - 250 mℓ, 500 mℓ and 1 ℓ
- ✓ Sterile Schott bottles - 250 mℓ, 500 mℓ and 1 ℓ
- ✓ Sterile graduated glass pipettes - 1, 5 and 10 mℓ
- ✓ Sterile glass tubes with caps - 15 mm x 160 mm, 14 mℓ
- ✓ Medical flats - 100 and 250 mℓ
- ✓ Gamma irradiated Petri dishes - 90 mm diameter
- ✓ Sterile Eppendorf vials
- ✓ Sterile syringe filter units - 0.22 µm
- ✓ Disposable syringes - 25 or 50 mℓ
- ✓ Sterile disposable micropipette tips - 100 and 1 000 µℓ
- ✓ Pasteur pipettes
- ✓ Parafilm
- ✓ Aluminium foil
- ✓ Sterile (autoclaved) filter paper discs (Whatman No. 1; 4 mm diameter)
- ✓ Ice and ice box

> ➢ Chemically treated Petri dishes and plasticware should not be used, as such chemicals could be mutagenic.

10.6.2 Equipment

- ✓ pH meter
- ✓ Balance
- ✓ Cold room (4°C) or refrigerator
- ✓ Freezer, -70°C
- ✓ Autoclave or pressure cooker
- ✓ Temperature controlled chamber or incubator
- ✓ Laminar flow cabinet
- ✓ 15 W germicidal lamp
- ✓ Water bath
- ✓ Shaker
- ✓ Vortex
- ✓ Sonicator
- ✓ Colony counter
- ✓ Magnetic stirrer
- ✓ Test tube racks
- ✓ Adjustable micropipettes - 100 and 1 000 µℓ
- ✓ Pipettor
- ✓ Gas supply and burner
- ✓ Thermometers
- ✓ Magnetic stirring bars
- ✓ Inoculation needle
- ✓ Spatula
- ✓ Tweezers

10.6.3 Reagents

Use reagent grade chemicals and water purified by a Millipore Milli-Q® system or equivalent for the preparation of solutions. Use reverse osmosis (RO) or distilled water for rinsing.

Chemicals

- ✓ Magnesium sulphate ($MgSO_4.7H_2O$)
- ✓ Citric acid ($C_6H_8O_7.H_2O$)
- ✓ di-Potassium hydrogen phosphate (K_2HPO_4)
- ✓ Sodium ammonium hydrogen phosphate ($NaNH_4HPO_4.4H_2O$)
- ✓ Magnesium chloride ($MgCl_2.6H_2O$)
- ✓ Potassium chloride (KCl)
- ✓ Sodium chloride (NaCl)
- ✓ Sodium hydroxide (NaOH)
- ✓ Dimethylsulphoxide (DMSO)
- ✓ Acetone
- ✓ 2-aminofluorene
- ✓ Sodium azide (NaN_2)
- ✓ Crystal violet
- ✓ Ampicillin trihydrate
- ✓ Agar
- ✓ Nutrient broth (Oxoid No. 2)
- ✓ D-Biotin
- ✓ L-Histidine-HCl
- ✓ Glucose
- ✓ β-nicotineamide adenine dinucleotide phosphate (NADP)
- ✓ Glucose-6-phosphate
- ✓ Lyophilized SD rat liver fraction S9, Aroclor 1254 induced (Molecular Toxicology Inc, USA, cat. No. 11-01I, Fax: 091 828 264 0103)

> ➢ Nutrient broths extracted from beef at high temperatures are likely to be mutagenic to some extent. Oxoid No. 2 nutrient broth is recommended for use, because this broth does not enhance the reversion frequency of tester strains.

> ➢ Aroclor 1254, used for the preparation of S9, is not available in South Africa for rat liver induction. Lyophilised S9 can be purchased from Molecular Toxicology Inc.

Medium Preparations

Nutrient broth

Nutrient broth	25 g
Water	1 ℓ

- Dissolve nutrient broth in water.
- Aliquot 50 mℓ quantities in medical flats.

- Autoclave, loosely capped, for 15 min at 121°C.
- When cooled, tighten caps.
- Store at 4°C.

Nutrient agar plates

Nutrient broth	2.5 g
Agar	1.5 g
Water	100 mℓ

- Dissolve nutrient broth and agar in water (heat).
- Cover container with aluminium foil.
- Autoclave for 15 min at 121°C.
- Cool to 45°C.
- Pour into Petri dishes (about 30 mℓ/dish).
- Allow to solidify.
- Keep plates upside down at 4°C until use (maximum storage two weeks).

Vogel-Bonner medium

$MgSO_4.7H_2O$	2 g
Citric acid-H_2O	20 g
K_2HPO_4	100 g
$NaNH_4HPO_4.4HO_2$	35 g
Water	1 ℓ

- Dissolve ingredients successively in water.
- Aliquot 100 mℓ quantities in medical flats.
- Autoclave, loosely capped, for 15 min at 121°C.
- When cooled, tighten caps.
- Store at 4°C.

Glucose solution

Glucose	200 g
Water	500 mℓ

- Dissolve glucose in water.
- Aliquot 50 mℓ quantities in medical flats.
- Autoclave, loosely capped, for 15 min at 121°C.
- When cooled, tighten caps.
- Store at 4°C.

Minimal agar plates

Agar	15 g
Water	850 mℓ

- Dissolve agar in water (heat).
- Cover container with aluminium foil.
- Autoclave for 15 min at 121°C.
- Cool to 45°C.

- Add 100 mℓ Vogel-Bonner medium and 50 mℓ glucose solution
- (warmed to 45 °C).
- Mix well.
- Pour into Petri dishes (about 30 mℓ/dish).
- Allow to solidify.
- Keep plates upside down at 4°C until use (maximum storage two weeks).

Histidine solution

Histidine	0.25 g
Water	50 mℓ

- Add histidine to water.
- Stir (or sonicate) to dissolve.
- Filter sterilise (0.22 µm).
- Store in a sterile Schott bottle at 4°C.

Biotin solution

Biotin	30.5 mg
Water	250 mℓ

- Add biotin to water.
- Stir to dissolve.
- Filter sterilise (0.22 µm).
- Store in a sterile Schott bottle at 4°C.

Histidine-biotin minimal agar plates

Agar	1.5 g
Water	83.4 mℓ

- Prepare duplicate containers with agar and water.
- Heat to dissolve.
- Cover containers with aluminium foil.
- Autoclave for 15 min at 121°C.
- Cool to 45°C.
- Add 10 mℓ Vogel-Bonner medium, 5 mℓ glucose solution, 1 mℓ histidine solution and 0.6 mℓ biotin solution (warmed to 45 °C) to the one container.
- Add the same constituents, except the histidine, to the second container. Replace the histidine with 1 mℓ sterile water.
- Mix well.
- Pour into Petri dishes (about 30 mℓ/dish).
- Allow to solidify.
- Keep plates upside down at 4°C until use (maximum storage two weeks).

> ➢ The plates without histidine are used for control testing.

Ampicillin solution

Ampicillin	0.8 g
Sodium hydroxide (0.02 N)	100 mℓ

- Dissolve ampicillin in sodium hydroxide.
- Filter sterilise (0.22 µm).
- Store in a sterile Schott bottle at 4°C.

Ampicillin minimal agar plates

Agar	1.5 g
Water	83.085 mℓ

- Dissolve agar in water (heat).
- Cover container with aluminium foil.
- Autoclave for 15 min at 121°C.
- Cool to 45°C.
- Add 10 mℓ Vogel-Bonner medium, 5 mℓ glucose solution, 1 mℓ histidine solution, 600µℓ biotin solution and 315µℓ ampicillin solution (warmed to 45 °C).
- Mix well.
- Pour into Petri dishes (about 30 mℓ/dish).
- Allow to solidify.
- Keep plates upside down at 4°C until use (maximum storage two weeks).

Crystal violet solution

Crystal violet	0.1 g
Water	100 mℓ

- Dissolve crystal violet in water.
- Filter sterilise (0.22 µm).
- Store at 4°C in a sterile Schott bottle, wrapped in aluminium foil to protect against light.

0.5 mM Histidine-biotin solution

Biotin	30.5 mg
Histidine	26.2 mg
Water	250 mℓ

- Add biotin to water.
- Stir to dissolve.
- Add histidine to solution.
- Continue to stir (or sonicate) until dissolved.
- Filter sterilise (0.22 µm).
- Store in a sterile Schott bottle at 4°C.

> ➢ It may be necessary to stir overnight to ensure complete solution.

Top agar

Agar	6 g
NaCl	5 g
Water	1 ℓ

- Dissolve ingredients in water (heat).
- Aliquot 100 mℓ quantities in medical flats.
- Autoclave, loosely capped, for 15 min at 121°C.
- When cooled, tighten caps.
- Store at 4°C.

Potassium chloride solution

KCl	61.5 g
Water	500 mℓ

- Dissolve chemical in water.
- Aliquot 100 mℓ quantities in medical flats.
- Autoclave, loosely capped, for 15 min at 121°C.
- When cooled, tighten caps.
- Store at 4°C.

Magnesium chloride solution

$MgCl_2.6H_2O$	40.7 g
Water	500 mℓ

- Dissolve chemical in water.
- Aliquot 100 mℓ quantities in medical flats.
- Autoclave, loosely capped, for 15 min at 121°C.
- When cooled, tighten caps.
- Store at 4°C.

0.1 M NADP solution

NADP	1 g
Sterile (autoclaved) water	13 mℓ

- Following sterile procedures, add water directly to reagent bottle.
- Dissolve.
- Store at -20°C.

> ➢ Solutions of NADP stored in the freezer are stable for at least six months.

1 M Glucose-6-phosphate solution

Glucose-6-phosphate	1 g
Sterile (autoclaved) water	1.77 mℓ

- Following sterile procedures, add water to reagent bottle.
- Dissolve.
- Store at -20°C.

> ➢ Solutions of glucose-6-phosphate stored in the freezer are stable for at least six months.

0.2 M Sodium phosphate buffer

Stock solution A

Na_2HPO_4	11.97 g
Water	500 mℓ

- Dissolve in water.

Stock solution B

$NaH_2PO_4.H_2O$	13.8 g
Water	500 mℓ

- Dissolve in water.

Buffer

- Mix ~ 405 mℓ of solution A with ~95 mℓ of solution B.
- Adjust volume of A or B to get to a pH of 7.4.
- Dispense in medical flats.
- Autoclave, loosely capped, for 15 min at 121°C.
- When cooled, tighten caps.
- Store at 4°C.

Negative control and solvent for sample dilution

Acetone.

Positive controls

Sodium azide and 2-aminofluorene.

10.7 CULTURES

10.7.1 Tester strains

The histidine-requiring strains TA98 and TA100 of the bacterium *S. typhimurium* are used for mutagenicity testing (Maron and Ames, 1983). Each strain has a different mutation in the histidine operon (Table 10.1). The *hisD3052* mutation in TA98 is in the *hisD* gene coding for the enzyme histidinol dehydrogenase. TA98 detects various frameshift mutagens. A frameshift mutation restores the correct reading frame for histidine synthesis. This mutation is reverted by mutagens such as 2-aminofluorene and benzo(a)pyrene. The *hisG46* mutation in TA100 is in the *hisG* gene coding for the

first enzyme of histidine biosynthesis. This mutation substitutes proline for leucine in the wild-type organism. This strain detects mutagens that cause base-pair substitutions, *e.g.* sodium azide and ethyl methane sulphonate.

The tester strains also contain other mutations that greatly increase their ability to detect mutagens (Table 10.1). *Rfa* causes partial loss of the lipopolysaccharides in the cell wall and so increases cell permeability to large molecules such as benzo(a)pyrene. *UvrB* is a deletion of a gene coding for the DNA excision repair system, increasing sensitivity to mutagens. The deletion through *uvrB* also includes the biotin genes, and as a consequence these strains also require biotin for growth. Both tester strains contain the R-factor plasmid, pKM101. This plasmid increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA repair system present in the bacterium (Maron and Ames, 1983).

Table 10.1: Genotypes of the tester strains used for mutagenicity testing (y: Yes)

Strain	Histidine mutation	Rfa	UvrB	R-factor
TA98	*hisD3052*	y	y	y
TA100	*hisG46*	y	y	y

S. typhimurium LT2 is the parent strain. This strain is not very virulent and is used by geneticists all over the world. The tester strains are relatively harmless because the *rfa* lowers virulence by orders of magnitude (Maron and Ames, 1983). The pKM101 plasmid with one antibiotic resistance marker for ampicillin should be a minimal hazard as plasmids are very common in the natural occurring enteric population.

10.7.2 Source

Cultures can be obtained from the Biotoxicology Laboratory, Division of Water, Environment and Forestry Technology, CSIR. The cultures were originally obtained from Prof. B.N. Ames of the University of California, Berkeley.

10.7.3 Culturing

- Work in a laminar flow cabinet.
- Wipe the working surface with a strong detergent followed with 70% ethanol before and after use.
- Follow sterile protocols and practices during culturing and handling of bacterial cultures.
- Autoclave any material containing the bacteria before it is washed.
- Discard used disposables in a medical waste container.

Cultures for assaying

Tester strains are grown in nutrient broth to a density of 1-2 x 10^9 cells/mℓ (OD: approximately 0.4 at 600 nm; 1 cm cuvette). Cultures are prepared from frozen permanent cultures kept in Eppendorf tubes at -70°C.

- Thaw the cultures by warming the Eppendorf tubes with your hand.
- Transfer the contents of a tube (TA98: 1 mℓ; TA100: 0.5 mℓ) to 50 mℓ nutrient broth in a medical flat.

- Incubate the medical flats (loosely capped) overnight (16 to 18 h) at 35-37°C on a gyratory shaker (approximately 30 rpm).
- Remove the culture from the shaker when the required cell density is reached.
- Keep the cultures on ice until use.

Permanent cultures

- Prepare overnight cultures as described in 'Cultures for assaying' above.
- Transfer each culture to a sterile Schott bottle.
- For each 1 mℓ culture in the bottle, add 0.09 mℓ DMSO (cryoprotective agent).
- Swirl the bottle gently until DMSO is dissolved.
- Distribute the contents into appropriately labelled sterile Eppendorf tubes (TA98: 1 mℓ; TA100: 0.5 mℓ), kept upright in finely crushed ice.
- Keep the cultures in the ice until frozen.
- Store the cultures in a -70°C freezer.

> - Keep a few tubes apart as master copies. These cultures are only used when frozen stocks require regeneration.
> - The other cultures are used sequentially to prepare overnight cultures.
> - If freezer space is available, large numbers of permanent cultures can be prepared at one time.
> - Frozen permanent cultures can be stored unopened for three or more years without losing viability or genetic markers.

Re-isolation of tester strains

Contamination or an accumulation of back mutations by repeated subculturing may lead to abnormally high spontaneous reversion of tester strains. In this case the strain may be recovered by re-isolation from a frozen master copy.

- Thaw a frozen master copy by warming the Eppendorf tube with your hand.
- Using an inoculation loop and streak plate techniques, transfer the culture to two ampicillin minimal agar plates.
- Discard the remaining culture.
- Invert the plates and incubate for 48 h at 35-37°C.
- Pick a well-isolated colony from one of the plates.
- Transfer the colony to nutrient broth in a medical flat.
- Incubate the culture as described in 'Cultures for assaying' above.
- Prepare fresh permanent cultures as described in 'Permanent cultures' above.

10.7.4 Confirmation tests

Tester strain genotypes should be confirmed in the following cases:

- When receiving cultures from an outside source.
- When fresh permanent cultures are prepared.
- When the number of spontaneous revertants per plate falls outside the specified range.
- When there is a loss of sensitivity to reference mutagens.

Histidine requirement

The *his* character of tester strains is confirmed by growth on minimal agar plates containing histidine.

- With a sterile inoculation loop, streak an overnight nutrient broth culture across the surface of a control plate (without histidine) and then across the histidine-biotin plate. (Different tester strains can be streaked in parallel stripes onto one plate).
- Invert the plates and incubate overnight at 35-37°C.
- Examine the histidine-biotin plate for growth.
- No growth should occur on the plate without histidine.

Rfa mutation

The *rfa* character is demonstrated by means of crystal violet sensitivity.

- Add 0.1 mℓ overnight nutrient broth culture to 2 mℓ molten top agar in a tube held in a 45°C water bath.
- Vortex for 3 s at low speed and pour onto a nutrient agar plate (or rotate between palms of the hands).
- Tilt and rotate the plate to allow even distribution of the agar.
- Place the plate on a level surface and allow to solidify.
- Pipette 10 µℓ crystal violet solution onto a sterile filter paper disc (4 mm diameter).
- Place the disc on the seeded plate using a sterile tweezers.
- Press the disc lightly with the tweezers to embed it in the top agar.
- Invert the plate and incubate at 35-37°C.
- After 12 h incubation a clear zone of inhibition (approximately 14 mm) appears around the disc, indicating the presence of the *rfa* mutation that permits large molecules such as crystal violet to enter and kill bacteria.

UvrB mutation

The *uvrB* deletion is quite stable and not easily lost from bacteria. However, its presence can be confirmed by demonstrating ultraviolet (UV) sensitivity.

- With a sterile wire loop, streak an overnight nutrient broth culture across the surface of a nutrient agar plate (Different tester strains can be streaked in parallel stripes onto one plate).
- Place a piece of cardboard over the uncovered plate so that half of the bacterial streak is covered.
- Irradiate the plate for 8 s with a 15 W germicidal lamp at a distance of 33 cm.
- Cover the plate.
- Invert the plate and incubate at 35-37° for 12 to 24 h.
- The tester strains will grow only on the non-irradiated side of the plate.

R-factor

The R-factor plasmid is somewhat unstable and can be lost from bacteria. An ampicillin resistance test is carried out to test for the presence of the plasmid in the tester strains.

- With a sterile inoculation loop, streak an overnight nutrient broth culture across the surface of an ampicillin plate. (Different tester strains can be streaked in parallel stripes onto one plate).
- Invert the plate and incubate at 35-37°C for 12 to 24 h.
- Examine the plate for growth along the streak.

10.8 TEST SAMPLES

Acetone extracts of wastewater discharges and receiving waters (2 000x concentrated – *e.g.* 4 ℓ water concentrated to 2 mℓ). Sample collection, transport and storage is outlined in Chapter 2.

- Note the appearance of the samples (colour, turbidity, odour).
- Concentrate the samples as soon as possible after sample receipt following the procedures outlined in Appendix D.
- Store the prepared acetone extracts at -20°C until use.
- Test acetone extracts directly (screening test).

> ➢ Acetone is not toxic to the tester strains at the concentration (5%) used in plates (reversion similar to that of Milli-Q® water).

10.9 TEST PROCEDURE

The test sample (acetone extract) is tested with both tester strains, with and without S9-mix. Both negative and positive control plates, as well as plates for sterility control, are included in the assay.

> ➢ Negative controls contain acetone (no test chemical) and are required to establish the spontaneous reversion of each tester strain
> ➢ Positive controls contain standard mutagens and are used to confirm the reversion properties and specificity of each tester strain.

10.9.1 Minimal agar plates

- Take minimal agar plates out of cold room and allow to reach room temperature.
- Label plates.
- Triplicate plates are used for each tester strain, with and without S9 (12 plates per sample and negative control).
- Single plates are used for positive controls and sterility checks.

10.9.2 Cultures

- Place the fresh nutrient broth cultures (kept on ice) at room temperature.

10.9.3 Test samples

- Keep the test samples and the negative control (acetone) in finely ground ice in an ice box.

- Work at room temperature (20±2°C) near a flame.
- Limit access to the laboratory to maintain sterile conditions.
- Follow sterile protocols and practices during culturing and handling of bacterial cultures.
- Wipe the working surface with a strong detergent followed with 70% ethanol before and after use.
- Autoclave any material containing the bacteria before it is washed.
- Discard used disposables in a medical waste container.

10.9.4 S9-mix

- Place the ingredients of the S9-mix (Table 10.2) in finely ground ice in an ice box.
- Determine the required volume of S9-mix by multiplying the number of plates to receive S9 with 500 µℓ (volume S9-mix required per plate).
- Prepare the S9-mix according to Table 10.2 in a sterile Schott bottle.
- Keep the S9-mix in ice until use.

Table 10.2: Volume of each ingredient required to prepare the S9-mix

Number of plates	2	4	10	20	40	60	80	100
Volume S9-mix required (mℓ)[1]	1	2	5	10	20	30	40	50
Volume of each ingredient to prepare S9-mix								
Magnesium chloride solution	0.02	0.04	0.1	0.2	0.4	0.6	0.8	1.0
Potassium chloride solution	0.02	0.04	0.1	0.2	0.4	0.6	0.8	1.0
Phosphate buffer solution	0.5	1.0	2.5	5.0	10	15	20	25
Glucose-6-phosphate solution	0.005	0.01	0.025	0.05	0.1	0.15	0.2	0.25
NADP solution	0.04	0.08	0.2	0.4	0.8	1.2	1.6	2.0
S9	0.07	0.14	0.35	0.7	1.4	2.1	2.8	3.5
Sterile water	0.345	0.69	1.725	3.45	6.9	10.35	13.8	17.25

[1] Other S9-mix volumes can be prepared by adding or subtracting volumes

- Any leftover S9-mix should be discarded at the end of the assay (use medical waste container).

10.9.5 Top agar

- Melt top agar in autoclave (121°C) for approximately 5 min.
- Stabilise temperature at 45°C in water bath.
- Add 10 mℓ histidine-biotin solution to 100 mℓ top agar before use.
- Place sterile test tubes in a tube holder in the water bath.
- For tests without liver activation:
  - Distribute 2.5 mℓ of the top agar mixture into the test tubes.
  - Add100 µℓ overnight culture, 100µℓ sample/acetone and 500µℓ phosphate buffer solution.
- For tests with liver activation:
  - Distribute 2.5 mℓ of the top agar mixture into the test tubes.
  - Add 100µℓ overnight culture, 100µℓ sample/acetone and 500µℓ S9-mix.

> - Bacteria can remain at 45°C for a few minutes without loss of viability.
> - The S9-mix should not be left at this temperature for more than a few seconds.

For positive controls:

- Replace the 100µℓ sample/acetone with phosphate buffer solution.
- Vortex for 3 s at low speed and pour onto a nutrient agar plate (or rotate between palms of the hands).
- Pour the contents onto a minimal agar plate.
- Quickly tilt and rotate the uncovered plate to ensure uniform distribution.
- Cover the plate and allow to solidify on a level surface (few minutes).
- Continue in this way until all the plates have been prepared.

> - The mixing, pouring and distribution of top agar should be carried out in <20 s.
> - The top agar can start to harden in mid operation if not adhering to the time limit.
> - This will result in a stippled surface which will interfere with the scoring of revertants.

10.9.6 Sterility checks

- Include the following plates:
    - Minimal agar plate;
    - Minimal agar plate left open for a few seconds;
    - Minimal agar plate with top agar; and
    - Minimal agar plate with top agar containing S9 mix.

10.9.7 Positive controls

> - Mutagenic chemicals should be handled with the utmost care (wear disposable gloves and do not spill).

Sodium azide spot test

Sodium azide is a base-pair substitution mutagen which induces mutations in TA100 without liver activation.

- Place a few crystals of the mutagen on one side of a TA100 plate with and without S9.

2-aminofluorene spot test

This chemical is a frameshift mutagen which induces mutations in TA98 with liver activation.

- Place a small quantity of the mutagen in the middle of a TA98 plate with and without S9.

10.9.8 Incubation

- Invert all the plates within an hour of preparation.
- Incubate the plates for 48 to 72 h at 35-37°C.

10.9.9 Results

Test and negative control plates

> - The trace of histidine in the top agar allows all the bacteria on the plate to undergo several divisions to produce a faint background lawn that is visible to the naked eye.
> - A lawn that is thin or granular compared to the negative control plate indicates bacterial toxicity.
> - Colonies that appear on a plate with no background lawn are not revertants and should not be scored. These colonies arise from the surviving bacteria that live off the histidine present in the top agar.

- Count the colonies (revertants to histidine prototrophy) on the test and negative control plates.
- Confirm the light background lawn of growth (auxotrophic bacteria) on all plates.

Sterility controls

- Confirm the absence of contamination.

Positive controls

Sodium azide

- Observe a clear halo (no background bacteria) on the side of the plate where the chemical was placed, as result of toxicity.
- Observe the large number of revertant colonies on the perimeter of the halo on the plate without S9.

2-Aminofluorene

- Observe a clear spot around the chemical (no background bacteria), as a result of toxicity
- Observe the large number of revertant colonies around the spot on the plate containing S9.

10.10 DATA ANALYSIS AND INTERPRETATION

- Determine the mean number of colonies on triplicate negative control and test plates (each tester strain, with and without S9).
- Calculate the mutation ratio (MR) (each tester strain, with and without S9) using the formula:

$$MR = \frac{\text{Mean number of colonies on test plate}}{\text{Mean number of colonies on negative control plate}}$$

- The presence of mutagens is indicated by an increase in the number of revertant colonies on test plates when compared to the negative control plates.
- A MR ≥2.0 indicates mutagenicity.
- If a sample is toxic (background lawn thin or absent) the test should be repeated on sample dilutions (*e.g.* 25 and 50%).

10.11 TEST ACCEPTABILITY

- The number of colonies on negative control plates should be between 20 and 50 in the case of TA98, and between 100 and 250 in the case of TA100 (the numbers on plates with S9 might be slightly higher).
- Sterility checks should be negative (no contamination).
- Positive controls should show appropriate responses.

10.12 TEST REPORT

The test report should refer to this test method and include the following:

10.12.1 Sample

- The name and location of industry where wastewater and receiving water samples were taken.
- Type of industry.
- Brief description of sampling point.
- Date and time of sampling.
- Date when sample concentration started.
- Sampling method (grab or composite).
- Name of person collecting the sample/s.
- Appearance and other properties of samples (colour, turbidity, odour).

10.12.2 Test facility

- Name and town/city of testing laboratory.
- Name/s of person/s performing the test.
- Name/s of person/s verifying the results.
- Date and time of start of test.

10.12.3 Test conditions

- Test type (screening or test on dilutions).
- Details on any deviation from the standard method.
- Origin of tester strains.
- Age of cultures.
- Concentrations tested.
- Controls included.
- Replication.
- Incubation time and temperature.

10.12.4 Results

- The MRs obtained with the two tester strains, with and without S9.
- Number of colonies on negative control plates and confirmation that numbers were within the stated range.
- Results of sterility checks.
- Results of positive controls.

10.13 REFERENCES

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APPENDIX A: *POECILIA RETICULATA* CULTURING

A.1 BACKGROUND

Poecilia reticulata (common name guppy) is an exotic fish found in freshwaters from the Caribbean Islands to the northern part of South America. It was first described in 1859 and named *Lebistes reticulates*. It is a common aquarium fish, and specific instructions for culturing and care can be found in standard reference books on tropical fish (Wainwright, 1983; Axelrod and Schultz, 1990).

The male is little over 2.5 cm in length, but the female can be twice as long. The male has a colourful appearance, his body covered in patches of red, orange, yellow, green, violet and sporadic black spots, and possessing a bright colourful tail. The wild female guppy lacks the bright colours and is largely whitish. Guppies live to the age of two to three years.

The guppy is prolific, matures rapidly, is active, thrives well in small aquaria, can stand a wide range of temperatures and can live in poor quality water. It eats aquatic insects, algae and the eggs of other fish.

Fertilisation is internal, part of the anal fin of the male being converted into a gonopodium for the transfer of sperm to the oviduct of the female. Sperm can be stored, and females kept separate from males can have as many as eight broods of young from a single mating. The guppy is ovoviviparous, *i.e.* eggs hatch just before leaving the mother's body and the young are born alive. As the time for this draws near, the female's abdomen becomes swollen and a dark patch appears near the anal fin.

Once a female has given birth it will be at least 23 d before she does so again. Large females may have as many as 100 offspring. Females eat their young, and thus for optimal breeding it is important to keep them in breeding cages from which the young can escape.

A.2 SOURCE OF ORGANISMS

Guppies can be obtained from fish farms. It is important to make use of a reputable source to ensure that the animals are disease free. New stock should be kept separate from existing stock to avoid the possible spread of disease.

In recent years the guppy has become very prone to disease as a consequence of in-breeding. As soon as the fish is stressed, *e.g.* during transport, it is prone to a variety of microbial infections resulting in large kills among the young. Because of this it will be difficult to establish a central facility from which young fish could be supplied to biotoxicology laboratories. It will be the best to establish a culturing facility at each test laboratory.

A.3 CULTURE FACILITY

- Use a temperature controlled facility at 23±2°C (optimum temperature).
- Equip the facility with an automatic light switch to maintain the photoperiod at
- 14 h light and 10 h dark (simulating the South African summer reproduction light cycle).
- Fit the facility with an aeration system to aerate fish tanks and drums or containers with culture water.

- Keep the culture facility separate from the test facility.
- The facility must be free of vapours, odours and dusts that may be toxic to fish.

A.4 MATERIALS, EQUIPMENT AND REAGENTS

A.4.1 Materials

- ✓ Glass tanks - 10 and 100 ℓ
- ✓ Plastic drums with lids - 200 ℓ
- ✓ Teflon tubing
- ✓ Glass tubes/pipettes
- ✓ Plastic covers or shade nets with small mesh
- ✓ Plastic breeding baskets/cages - 4 mm mesh, approximate dimensions: 25 cm x 25 cm x 8 cm (swimming space of approximately 1 ℓ)
- ✓ Fish nets (various sizes)
- ✓ Water plants
- ✓ Clean river sand
- ✓ Stones

A.4.2 Equipment

- ✓ Activated carbon filters
- ✓ Aerators
- ✓ Activated carbon water purification system
- ✓ Thermometers
- ✓ Heaters
- ✓ Scrapers

A.4.3 Reagents

Chemicals

Fish food

- ✓ Tetramin has been proven to be a very reliable and high quality fish food. The food is available for adults (large flakes) and young fish (small flakes).

Antibiotics

Examples of antibiotics that can be used to control disease:

- ✓ Ovalene B (6 mg methylene blue/mℓ) is an aid in the prevention and treatment of external fungus (*Saprolegnia*) and certain protozoan diseases (*Costia, Chilodonella, Trichodina, Cryptobia* and *Trypanosoma*) in ornamental freshwater fish.
- ✓ Baytril (10%) is used to treat bacterial infections.

Salt

Coarse salt to disinfect tanks and nets.

Culture water

- ✓ Synthetic moderately hard water, as prepared in Appendix C.
- ✓ Dechlorinated tap water (aerated) or water purified by a carbon filtration system.

A.5 FISH STOCK MAINTENANCE AND BREEDING

Each laboratory should establish its own maintenance and breeding practices. The methods given below are examples of how this could be achieved (US EPA, 1985; 1993; ISO, 1992). What is important is that the test fish meets the requirements outlined in the test method (Chapter 5, paragraph 5.6).

Five different types of tanks can be set up, namely breeding, quarantine, maternity, holding and acclimation.

A.5.1 Breeding tanks

- 100 ℓ tanks are used for maintenance and breeding.
- Clean river sand and stones, and water plants, can be placed in tanks to simulate natural conditions conducive to breeding.
- The tanks are filled with about 80 ℓ dechlorinated tap water or water purified by a carbon filtration system.
- Aerators (at least two) are placed in the corners of the tanks to ensure sufficient DO.
- Tanks are equipped with activated carbon filters to prevent the accumulation of toxic metabolic waste (mainly nitrite and ammonia) in the water.
- Heaters are placed in tanks to keep the water at 26±1°C to stimulate breeding.
- Each tank is stocked with about 20 adults (*e.g.* five males and 15 females) (loading: ≤0.4 g/ℓ).
- Ten to 20 breeding tanks should provide an adequate amount of test fish per week for toxicity testing.
- Plastic covers/netting are placed over tanks to prevent fish from jumping out.

Maintenance

Daily maintenance (Monday to Friday)

- Observe the fish in breeding tanks for abnormal appearance and behaviour or signs of disease.
- If bacterial/fungal infection is noticed, add antibiotics to the tanks following the supplier's instructions. Continue treatment until the infection has cleared up.
- Move individual fish with infections to a separate tank (quarantine tank) for treatment.
- Move females that are close to giving birth (very swollen abdomen and very dark patch near anal fin) to maternity tanks.
- If newborn fish are noticed in breeding tanks that are not treated with antibiotics, transfer them to the test fish holding tanks.

> ➢ Newborn fish in tanks containing antibiotics are discarded.

- Remove dead fish from tanks.
- Add water to tanks to compensate for evaporation.
- Ensure that aerators and carbon filters are working.
- Replace aerators and activated carbon in filters as needed.
- Measure and record the DO and pH (and ammonia if necessary).
- Record the temperature and adjust heaters if required.

- Feed fish twice a day. Food should be carefully controlled to prevent overfeeding and accumulation of debris.
- Place nets used for handling fish in a salt solution before use in the next tank, and after use to sterilise.

> ➢ The DO should be >80% of saturation.
> ➢ The pH should be between 6 and 8.5.

Weekly maintenance

- Scrape walls of tanks to remove excessive build-up of algal growth.
- Siphon the debris (food, detritus, waste) accumulating in tanks from the bottom of the tanks, using a glass tube/pipette attached to Teflon tubing.
- Remove approximately 20% of the water and replace with fresh water.

Long-term maintenance

- Remove fish, plants and equipment from tanks.
- Drain all the water from tanks and remove the sand and stones.
- Wash the tanks.
- Wash the sand and stones/or replace.
- Clean the equipment and replace if needed.
- Set up the clean tank as described in paragraph A.5.1.

A.5.2 Quarantine tanks

- Fill a required number of 10 ℓ tanks with 8 ℓ dechlorinated tap water or water purified by a carbon filtration system.
- Fit the tanks with aerators only.
- Place infected fish in tanks (not more than ten per tank to avoid stress).
- Treat with antibiotics according to supplier's instructions.
- Cover tanks to prevent fish from jumping out.

Maintenance

Daily maintenance (Monday to Friday)

- Observe the fish to establish if the infection is clearing up.
- Fish that have totally recovered can be reintroduced into breeding tanks.

> ➢ Heavily diseased fish should be discarded.

- Remove dead fish from tanks.
- Discard any newborn fish.
- Add water to tanks to compensate for evaporation.
- Ensure that aerators are working.
- Measure and record the DO, pH and temperature.

- Feed fish twice a day. Food should be carefully controlled to prevent overfeeding and accumulation of debris.
- Place nets used for handling fish in a salt solution before use in the next tank, and after use to sterilise.

Weekly maintenance

- Siphon the debris (food, detritus, waste) accumulating in tanks from the bottom of the tanks using a glass tube/pipette attached to Teflon tubing.
- Remove approximately 20% of the water and replace with fresh water.
- Add antibiotics as required.

Long-term maintenance

- Drain all the water from tanks.
- Wash and disinfect (with coarse salt) the tanks and aerators.
- When required, set up the clean tank as described in paragraph A.5.2.

A.5.3 Maternity tanks

- Fill a required number of 100 ℓ tanks with 80 ℓ dechlorinated tap water/water purified by a carbon filtration system.
- Fit each tank with three plastic breeding cages/baskets.
- Place ≤5 females in each basket.
- Place aerators (at least two) in the corners of the tanks.
- Equip tanks with activated carbon filters.
- Cover tanks to prevent fish from jumping out.

Maintenance

Daily maintenance (Monday to Friday)

- Return females that have given birth to breeding tanks.
- Remove dead fish from tanks.
- Transfer the newborn fish to a test fish holding tank.
- Add water to tanks to compensate for evaporation.
- Ensure that aerators and filters are working.
- Measure and record the DO, pH and temperature.
- Feed fish twice a day.
- Place nets used for handling fish in a salt solution before use in the next tank, and after use to sterilise.

Weekly maintenance

- Siphon the debris (food, detritus, waste) accumulating in tanks from the bottom of the tanks using a glass tube/pipette attached to Teflon tubing.
- Remove approximately 20% of the water and replace with fresh water.

Long-term maintenance

- Drain all the water from tanks.
- Wash the tanks.

- Clean the equipment and replace as needed.
- When required, set up the clean tank as described in paragraph A.5.3.

A.6 TEST FISH MAINTENANCE

A.6.1 Holding tanks

- Fill a required number of 10 ℓ tanks with a mixture of 4 ℓ moderately hard water and 4 ℓ dechlorinated tap water/water purified by a carbon filtration system.
- Fit the tanks with aerators to ensure sufficient DO.
- Stock each tank with between 200 and 300 newly born fish (loading: <0.4 g/ℓ) taken from breeding tanks and maternity tanks during a one week period (*e.g.* Monday to Friday).
- After the stocking period (usually on the following Monday), transfer the fish to acclimation tanks.
- Place plastic covers/netting over tanks to prevent fish from jumping out.
- Start with new holding tanks every Monday.

Daily maintenance (Monday to Friday)

- Observe the fish for abnormal appearance and behaviour or signs of disease.
- If signs of bacterial/fungal infection are noticed, discard all the fish.

> ➢ Do not treat test fish with antibiotics.

- Remove dead fish from tanks.
- Add water to tanks to compensate for evaporation.
- Ensure that aerators are working.
- Measure and record the DO, pH and temperature.
- Feed fish twice a day. Food should be carefully controlled to prevent overfeeding and accumulation of debris.
- Siphon debris from the bottom of the tanks using a glass tube/pipette attached to Teflon tubing.
- At the end of the 7 d holding period, after fish have been transferred to acclimation tanks, drain the water from the tanks, wash tanks, clean aerators and set up for following batch of fish.
- Place nets used for handling fish in a salt solution before use in the next tank, and after use to sterilise.

A.6.2 Acclimation tanks

- Fill a required number of 10 ℓ tanks with 8 ℓ moderately hard water.
- Fit the tanks with aerators to ensure sufficient DO.
- Stock each tank with between 200 and 300 fish from holding tanks.
- Maintain the fish for 7 d before use in toxicity tests.
- Place plastic covers/netting over tanks to prevent fish from jumping out.

Daily maintenance (Monday to Friday)

- Observe the fish for abnormal appearance and behaviour or signs of disease.
- If signs of bacterial/fungal infection are noticed, discard the fish.

> ➢ If <5% of the test fish die during the 7 d period before testing, the batch of fish can be used.
> ➢ If between 5 and 10% die, acclimation can continue for an additional 7 d.
> ➢ If >10% die, discard the batch of fish.

- Record and remove dead fish from tanks
- Add water to tanks to compensate for evaporation.
- Ensure that aerators are working.
- Measure and record the DO, pH and temperature.
- Feed fish twice a day.

> ➢ Do not feed fish during the 24 h period before use in toxicity tests.

- Siphon debris from the bottom of the tanks using a glass tube/pipette attached to Teflon tubing.
- Place nets used for handling fish in a salt solution before use in the next tank, and after use to sterilise.
- After fish have been used in toxicity tests drain the water from the tanks, wash tanks, clean aerators and set up for following batch of fish.

> ➢ Fish that are not used in toxicity tests can be kept for future breeding stock, or can be discarded.

A.7 REFERENCES

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APPENDIX B: *DAPHNIA PULEX* CULTURING

B.1 BACKGROUND

Daphnia pulex is widely distributed in South Africa, in ponds and dams and slow flowing streams. The adult has a maximum length of 3.5 mm. During most of the year, populations of *Daphnia* consist almost exclusively of females. The production of males appears to be induced by high population densities and the subsequent accumulation of excretory products, and/or a decrease in food, which lowers the metabolism of the parent. These conditions may also induce the formation of resting eggs (ephippia) that are cast off during the next moult. Males are smaller than females, have larger antennules, a modified post-abdomen, and first legs that are armed with stout hooks used in clasping. Males and ephippia will only appear in laboratory cultures when cultures are neglected (US EPA, 1985;1993).

The average life span of *D. pulex* is 50 d at 20°C. There are four distinct phases in the life cycle – the egg, juvenile, adolescent and adult. Minutes after moulting, a clutch of eggs is released into the brood chamber. The eggs hatch in the brood chamber and the juveniles, which are similar in form to adults, are released in approximately 2 d when the female moults (casts off her exoskeleton or carapace). Growth occurs immediately after each moult while the new carapace is still elastic. The time required to reach maturity (produce first offspring) varies from 6 to 10 d. The growth rate of the organism is the greatest during its juvenile stages (early instars), and the body size may double during each of these stages. *D. pulex* has 3 to 4 juvenile instars. The adolescent period is very short and consists of only one instar. It is during this instar that the first clutch of eggs reaches full development in the ovary.

The adult phase consists of 18 to 25 instars. The duration of instars increases with age. A given instar lasts approximately 2 d under favourable conditions but may be as long as one week under unfavourable conditions. *D. pulex* may produce as many as 30 young during each adult instar, but more commonly the number is 6 to 10. The number of young released during the adult instars reaches a maximum at the tenth instar with a gradual decrease thereafter (US EPA, 1985; 1993).

B.2 SOURCE OF ORGANISMS

D. pulex is available from the Biotoxicology Laboratory, Division of Water, Environment and Forestry Technology, CSIR, Pretoria. The organism was isolated from a pond in the Pretoria area in 1985 and identified by taxonomists at the CSIR (Slabbert *et al.*, 1998a,b). Only a small number of organisms (20 to 30) are needed to start a culture.

B.3 CULTURE FACILITY

- Use a temperature controlled facility at 20±2°C (optimum temperature).
- Use ambient laboratory illumination (artificial and/or day light:10 to 14 h light).
- Keep the culture facility separate from the test facility.
- The facility must be free of vapours, odours and dusts that may be toxic to *Daphnia*.

- Cultures should be protected from sudden changes in temperature, *e.g.* when an air-conditioner breaks down, because this may cause death.
- The variations in ambient light intensities, and prevailing day/night cycles in most laboratories do not seem to affect *Daphnia* growth and reproduction significantly. If available, artificial light set at a desired photoperiod can be used.

B.4 MATERIALS, EQUIPMENT AND REAGENTS

B.4.1 Materials

- ✓ Glass beakers - 50 and 500 mℓ and 1 ℓ
- ✓ Glass measuring cylinders - 1 ℓ
- ✓ Glass pipettes, graduated - 5 mℓ
- ✓ Sterile Erlenmeyer flasks with air-permeable stoppers - 1 ℓ
- ✓ Sterile filtration flask - 1 ℓ
- ✓ Sterile graduated glass pipettes - 10 and 25 mℓ
- ✓ Sterile glass test tubes - 20 x 150 mm, approximately 50 mℓ
- ✓ Sterile Pasteur pipettes
- ✓ Sterile membrane filters - 0.22 µm
- ✓ Polystyrene bottles with caps - 50 mℓ
- ✓ Culture containers - 3 to 4 ℓ glass beakers or equivalent
- ✓ Pasteur pipettes or micropipette tips (1 000 µℓ), end cut off and fire-polished (opening approximately 2 and 4 mm), or disposable bulb pipettes (opening approximately 2 and 4 mm) for handling *Daphnia*
- ✓ Pipette bulbs
- ✓ Micropipette tips - 1 000 µℓ
- ✓ Teflon tubing covered with fine mesh netting around the open end
- ✓ Paper towel/sponge
- ✓ Plastic/glass sheets

- Clear glass beakers are recommended for culturing to allow easy observation of organisms.

B.4.2 Equipment

- ✓ Freezer, -20°C
- ✓ Cold room or refrigerator, 4°C
- ✓ Laminar flow cabinet (alternatively, work near a flame)
- ✓ Vacuum pump
- ✓ Filter apparatus (47-mm stainless steel filter holder)
- ✓ Pipettor
- ✓ Blender
- ✓ Micropipette - 1 000 µℓ
- ✓ Balance
- ✓ Sterile dispensing equipment (adjustable) - 50 and 100 mℓ
- ✓ Light box
- ✓ Gas supply and burner
- ✓ Haemacytometer

- ✓ Electrical light
- ✓ Magnifying glass
- ✓ Thermometers
- ✓ Calculator

B.4.3 Reagents

Use ingredients of known origin and water purified by a Millipore Milli-Q® or equivalent for preparation of solutions and suspensions. Use reverse osmosis (RO) or distilled water for rinsing.

Culture water

Synthetic moderately hard water, prepared as described in Appendix C. Keep at 20±2°C.

Trout pellet, yeast and alfalfa (TYA) food

Ingredients

- ✓ Trout pellets (EPOL, obtainable from certain pet shops)
- ✓ Dried yeast (Anchor, obtainable from supermarkets)
- ✓ Alfalfa (tablets obtainable from supermarkets, pharmacies or health shops)

Preparation

- Place 12.6 g trout pellets, 5.2 g dried yeast and 1.0 g alfalfa in a blender.
- Add 1 ℓ water.
- Mix at high speed for 5 min.
- Place at 4°C and allow to settle for 1 h.
- Decant the top 600 mℓ into a clean beaker.
- Discard the remainder.
- Distribute 20 to 30 mℓ volumes of the food suspension in 50 mℓ polystyrene bottles with screw caps.
- Store at -20°C.

> ➢ The TYA food is easily prepared and provides sufficient nutrition for organisms used in acute toxicity tests (US EPA, 1985; 1993).

Algal food suspension (for *Daphnia* reproduction test only)

Components

- ✓ Logarithmic growth phase *S. capricornutum* Printz, maintained in 50 mℓ 10% BG-11 medium (BG-11) in 250 mℓ Erlenmeyer flasks for 3 to 4 d, according to the procedures described in Chapter 8.
- ✓ Sterile BG-11, prepared as described Chapter 8.
- ✓ Sterile synthetic moderately hard water sterilised by means of a 0.22 µm filter.

Algal culturing

- Distribute 200 mℓ volumes of the BG-11 into sterile 1 ℓ Erlenmeyer flasks fitted with air-permeable stoppers (20% medium to flask volume ratio to avoid growth inhibition due to carbon dioxide limitation).
- Inoculate the medium with 8 mℓ logarithmic growth phase cells (verify sterility according to the procedures described in Chapter 8).
- Incubate the cultures for 7 to 10 d at 24±2°C under cool white fluorescent light (light intensity of 80 to 95 $\mu E/m^2/s$) in the algal culture facility (Chapter 8).
- Shake the cultures twice a day by hand to enhance growth.

> ➢ Allow the algae to settle for 2 d before preparation of the algal food suspension.

Preparation of algal suspension

- Remove the supernatant medium from the settled algal layer in the algal flask by means of a sterile Pasteur pipette and vacuum pump (do not disturb the algal layer to retain as much of the algal cells as possible).
- Add 200 mℓ sterile moderately hard water to the algal flask.
- Resuspend the algae by means of a sterile 25 mℓ pipette (pull up and push out, a number of times).
- Cover the flask with the stopper and allow the algae to settle in a cold room (in the dark) for a few days.
- Once again, remove the supernatant water by means of a sterile Pasteur pipette and vacuum pump (to remove as much as possible of the algal growth medium).
- Add 40 mℓ sterile moderately hard water to the flask (instead of 200 mℓ) to concentrate the cells.
- Resuspend the algae with a sterile 25 mℓ pipette.
- Count and calculate the number of cells in the algal concentrate using a haemacytometer and microscope, following the steps described in Chapter 8, *e.g.* 43 000 000 cells/mℓ.

> ➢ An algal concentrate of 25 000 000 (2.5×10^7) cells/mℓ is required to have 200 000 (2×10^5) cells/mℓ after addition of 1 mℓ algal concentrate to 125 mℓ culture water or test solution in the *Daphnia* reproduction test.

- Determine the dilution of the algal concentrate to have 2.5×10^7 cells/mℓ as follows:
  - Divide the number of cells required per mℓ by the number of cells counted per mℓ in the concentrate, *e.g.* $2.5 \times 10^7/4.3 \times 10^7$.
  - The result shows that 0.5814 mℓ cell concentrate contains 2.5×10^7 cells.
  - 0.4186 mℓ sterile moderately hard water should, therefore, be added to each 0.5814 mℓ algal concentrate to have 2.5×10^7 cells/mℓ.
  - If the flask contains 40 mℓ algal concentrate, add 28.7994 mℓ moderately hard water to the flask to have a suspension of 2.5×10^7 cells/mℓ.
- Add the required volume of moderately hard water to the flask and store in a cold room in the dark.

B.5 *DAPHNIA* SUBCULTURING

The water in stock culture containers should be replaced each week with fresh water (US EPA, 1985; 1993).

> ➢ It is important to maintain several culture containers to ensure a sufficient stock for the growing of test organisms and to avoid the lost of an entire laboratory culture in the event of a population crash.

- Thaw portions of the TYA food as needed.
- Siphon approximately 2.7 ℓ of the old water out of a culture container, using Teflon tubing covered with fine mesh netting at the open end. Alternatively, allow the *Daphnia* to move to one side of the beaker using sunlight/electrical light attraction. Rapidly pour about 2.7 ℓ water out of the beaker.
- Pour the remaining 300 mℓ water containing the adult *Daphnia* and offspring into a temporary holding beaker.

> ➢ Water containing adult *Daphnia* can be poured from one container to another without the risk of air becoming trapped under their carapaces.

- Clean the culture container as follows:
  - Wash with warm water and wipe with a paper towel (or sponge) to remove accumulated food and debris.
  - Rinse with tap water, followed by RO water and moderately hard water before reuse.
  - Once a month, wash with detergent, and rinse with tap water, RO water and moderately hard water.
- Fill the cleaned beaker with approximately 3 ℓ moderately hard water.
- Add 4.5 mℓ of the thawed TYA food to the water.
- ***In the case of cultures for the reproduction test, also add 24 mℓ of the prepared algal suspension.***
- Gently pour part of the contents of the temporary holding beaker into the container with the fresh water and food, to have about 30 adults (and offspring) in the beaker. Alternatively, transfer the *Daphnia* by using a pipette (disposable bulb or Pasteur) or pipette tip (end cut off and fire polished), with an opening of 4 mm.
- Continue in this way to subculture all the stock cultures.
- Cover each container with a clear plastic sheet or glass plate to exclude dust and dirt and minimise evaporation.
- Feed every second day with 4.5 mℓ thawed TYA food ***and 24 mℓ algal suspension in the case of cultures for reproduction tests*** (*e.g.* Monday, Wednesday and Friday).

- Although the pH of the water will rise by as much as 0.5 units as the population increases, it will remain within a pH range of 7.0 to 8.5. It is therefore not necessary to monitor or to adjust the pH during culture maintenance.
- Oxygen concentrations in the stock cultures are expected to be above 40% of saturation. Unless the cultures are too crowded or overfed, aeration is not necessary.

B.6 *DAPHNIA* CULTURES FOR TOXICITY TESTING

- Remove adult females (>21 d old) bearing embryos in their brood pouches from stock cultures, 24 h before the start of a toxicity test. Use a pipette (disposable bulb or Pasteur) or micropipette tip (end cut off and fire polished), with an opening of 2 mm.

- Females should not be taken from cultures that are producing ephippia.
- A good time to start cultures for toxicity testing is during subculturing.

- Place the organisms in glass beakers containing 250 mℓ moderately hard water and 375 µℓ thawed food suspension. ***In the case of reproduction tests also add 2 mℓ algal suspension.*** Two beakers, each with 25 adults, should supply enough first instars after 24 h for one toxicity test.
- Some of the *Daphnia* will not produce young within the first 24 h of culturing. Remove all the young from the culture if another batch of *Daphnia* ≤24 h is required on the following day.
- Cover beakers with a plastic or glass sheet to exclude dust and dirt.

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APPENDIX C: SYNTHETIC MODERATELY HARD WATER

C.1 GENERAL

Moderately hard water is used for:

- Culturing and maintenance of *Daphnia* (Appendix B).
- Maintenance of fish before use in toxicity tests (Appendix A).
- Dilution of wastewater discharges for fish and *Daphnia* tests (Chapters 5, 6 and 7).
- Control testing in fish and *Daphnia* tests (Chapters 5, 6 and 7).

The use of synthetic water is recommended because it is easily prepared, it is of known quality, produces predicable results and allows adequate growth and reproduction of test organisms.

The water is prepared according to US EPA methodology (1985; 1993).

C.2 MATERIALS, EQUIPMENT AND REAGENTS

C.2.1 Materials

- ✓ Glass bottles/carboys - 20 ℓ
- ✓ Glass beakers - various sizes
- ✓ Glass measuring cylinders - 100 mℓ
- ✓ Volumetric flasks - 100 mℓ and 1 ℓ
- ✓ Schott bottles - 1 ℓ
- ✓ Polyethylene bottles - 100 mℓ and 1 ℓ
- ✓ Glass reagent bottles - 100 mℓ
- ✓ Pasteur pipettes
- ✓ Pipette bulbs

C.2.2 Equipment

- ✓ Dissolved oxygen meter
- ✓ pH meter
- ✓ Balance
- ✓ Sonicator
- ✓ Aeration device
- ✓ Spatula

C.2.3 Reagents

Use reagent grade chemicals and water purified by a Millipore Milli-Q® system or equivalent to prepare solutions. Use reverse osmosis (RO) or distilled water for rinsing.

Chemicals

- ✓ Sodium hydroxide (NaOH)
- ✓ Hydrochloric acid (HCl)
- ✓ Potassium chloride (KCl)
- ✓ Magnesium sulphate ($MgSO_4$)

- ✓ Sodium bicarbonate ($NaHCO_3$)
- ✓ Calcium sulphate dihydrate ($CaSO_4.2H_2O$)

Chemical solutions

Sodium hydroxide (1 N)

NaOH	4 g
Water	100 mℓ

- Store at room temperature in a reagent bottle.

Hydrochloric acid (1 N)

HCl	8.3 mℓ
Water	100 mℓ

- Store at room temperature in a reagent bottle.

Potassium chloride stock

KCl	8 g
Water	1 ℓ

- Place KCl in a 1 ℓ volumetric flask.
- Make up to 1 ℓ.
- Store in the dark at room temperature in a 1 ℓ polyethylene bottle.

Magnesium sulphate stock

$MgSO_4$	120 g
Water	1 ℓ

- Place $MgSO_4$ in a 1 ℓ volumetric flask.
- Make up to 1 ℓ.
- Store in the dark at room temperature in a 1 ℓ polyethylene bottle.

Sodium bicarbonate stock

$NaHCO_3$	96 g
Water	1 ℓ

- Place $NaHCO_3$ in a 1 ℓ volumetric flask.
- Make up to 1 ℓ.
- Store in the dark at room temperature in a 1 ℓ polyethylene bottle.

C.3 PREPARATION OF MODERATELY HARD WATER (20 ℓ)

- Pour approximately 17 ℓ water in a 20 ℓ container.
- Place 1.2 g $CaSO_4.2H_2O$ in a 1 ℓ Schott bottle.
- Add 1 ℓ water to the Schott bottle.
- Close the bottle (no leaking) and shake vigorously to dissolve the $CaSO_4.2H_2O$, or place in a sonicator.

- Without dripping, add the solution to the 20 ℓ container (rinse the bottle a number of times to ensure complete transfer of the contents).
- If not completely dissolved, allow to settle and decant clear supernatant into the 20 ℓ container. Add more water to the Schott bottle and shake again until completely dissolved.
- Shake 20 ℓ container after addition of $CaSO_4.2H_2O$ solution to mix.
- Add the following volumes of stock solution to the 20 ℓ container and mix well after each addition:

  10 mℓ KCl
  10 mℓ $MgSO_4$
  20 mℓ $NaHCO_3$

- Make up to 20 ℓ.
- Aerate vigorously for 2 h (or until DO has reached saturation and the pH has stabilised).
- Allow water to stand overnight before use. Do not aerate the water further before use.
- Measure the pH before use. If necessary, adjust the pH to 7.6±0.2 with NaOH solution or HCl
- Use the water within one week of preparation.
- The final salt concentrations and resulting water quality is shown in Table C.1.

Table C.1: Moderately hard water

KCl	$MgSO_4$	$NaHCO_3$	$CaSO_4.2H_20$	pH[1]	Dissolved oxygen	Hardness	Alkalinity
mg/ℓ					mg/ℓ	As mg/ℓ $CaCO_3$	
4	60	96	60	7.4-7.8	≥6.8	80-100	60-70

[1] Approximate equilibrium pH after aeration

> - Other volumes of water can be prepared. Adjust the volumes of the stock solutions and the weight of the $CaSO_4.2H_2O$ accordingly.
> - If preferred, the water can be prepared by adding the salts directly to the water (omitting stock solutions), following the steps outlined.

C.4 REFERENCES

US EPA. 1985. Methods for measuring the acute toxicity of effluents to freshwater and marine organisms. EPA/600/4-85/013. Environmental Monitoring and Support Laboratory, Office of Research and Development, US Environmental Protection Agency, Cincinnati.

US EPA. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Fourth Edition. EPA/600/4-90/027F. Environmental Monitoring and Support Laboratory, Office of Research and Development, US Environmental Protection Agency, Cincinnati.

APPENDIX D: SAMPLE CONCENTRATION BY MEANS OF SOLID PHASE ADSORPTION

D.1 INTRODUCTION

Testing wastewater discharges and receiving waters for mutagens consists in most cases of an initial stage of passing a large volume of water through a column of adsorbing medium in order to concentrate any mutagens that may be present. The larger the sample volume, the more sensitive the test will be.

In this method, Amberlite XAD-7, a moderately polar acrylic resin, is used for sample concentration. This resin can adsorb a wide range of organic substances, some of which may be mutagens. The adsorbed organic substances are recovered by elution (desorption) using acetone. The eluant is concentrated by rotary evaporation prior to evaluation by the Ames *Salmonella* mutagenicity test. The procedure is based on US EPA methodology (1985).

D.2 MATERIALS, EQUIPMENT AND REAGENTS

D.2.1 Materials

- ✓ Glass beakers - 25 and 100 mℓ
- ✓ Glass measuring cylinders - 100 mℓ and 2 ℓ
- ✓ Erlenmeyer flasks - 250 mℓ
- ✓ Large mouth glass pipette - 10 mℓ
- ✓ Graduated stoppered glass tubes - 20 mℓ
- ✓ Glass columns, each fitted with a ground glass stopper and Teflon stopcock - approximately 15 x 170 mm; 25 mℓ
- ✓ Glass aspirator bottles, fitted with a ground glass opening at the bottom - 5 ℓ
- ✓ L-shaped ground glass joints, fitted with a Teflon stopcock (connecting aspirator bottle and glass column)
- ✓ Glass sample bottles - 5 ℓ
- ✓ Round bottomed glass flasks to fit rotary evaporator - 250 mℓ
- ✓ Glass funnels
- ✓ Sintered glass funnels
- ✓ Pasteur pipettes
- ✓ Vacutainers
- ✓ Silanised glass wool
- ✓ Cork rings for round bottom flasks
- ✓ Length of wire
- ✓ Filter paper - Whatman No. 1; 40 cm diameter
- ✓ Elastic bands

D.2.2 Equipment

- ✓ Balance
- ✓ Freezer, -20°C
- ✓ Rotary evaporator
- ✓ Compressed air supply
- ✓ Desiccator with silica gel
- ✓ Stopwatch
- ✓ Pipettor
- ✓ Test tube racks
- ✓ Spatula

D.2.3 Reagents

Use reagent grade chemicals and water purified by a Millipore Milli-Q® system or equivalent.

- ✓ Anhydrous sodium sulphate (Na_2SO_4)
- ✓ Amberlite XAD-7 resin (Röhm & Haas, supplied by BDH or Sigma), prepared according to the manufacturer's instructions and slurried in acetone
- ✓ Acetone

D.3 PROCEDURE

> ➢ Depending on the availability of equipment, a number of samples can be prepared at one time.

D.3.1 Prepare sample

- If sample contains excess suspended solids (including algae), allow solids to settle.
- Filter the supernatant sample through Whatman No. 1 filter paper (or through a plug of silanised glass wool placed in the neck of a large glass funnel).

D.3.2 Pack XAD-7 column

- Mount a glass column on a burette stand.
- Close the stopcock.
- Push a plug of silanised glass wool into the column with a length of wire to form a plug at the bottom sufficiently compact to prevent resin from escaping when column is packed.
- Pour in XAD-7 slurry and allow to settle (use a beaker or large mouth pipette).
- Open stopcock to allow acetone to drain out slowly (into Erlenmeyer with XAD-7 slurry).

> ➢ Do not allow column to run dry.

- Top up repeatedly with XAD-7 slurry until column has been packed to approximately 50% of its length (approximately 10 mℓ).
- Drain the acetone to the top level of the XAD-7 bed.
- Close the stopcock.
- Fill the column with Milli-Q® water.
- Tilt to mix and remove air bubbles.
- Continue draining, filling and mixing until the acetone has been completely replaced by water.
- Insert another loose, silanised glass wool plug to protect the surface of the resin bed.

> ➢ Suspended solids <5% are trapped by the glass wool plug.

- Top up column with water.
- Close column with glass stopper.

D.3.3 Connect column to aspirator bottle

- Place the aspirator bottle on the front side of a laboratory bench.
- Connect the column to the aspirator bottle with L-shaped ground glass joint.
- Secure the connections with elastic bands.
- Close the stopcock on the L-shaped joint.

D.3.4 Transfer sample to aspirator bottle

- Fill the aspirator bottle with sample up to the opening with the L-shaped joint.
- Add 4 ℓ sample to the bottle.

D.3.5 Adsorb organic chemicals

- Open the stopcocks on the aspirator bottle and column and adjust until water drips out at a rate of approximately 2 drops/s (9 mℓ/min). Check with a stopwatch and measuring cylinder.
- Check regularly to ensure a constant flow rate.

> ➢ Columns should not run dry during adsorption.
> ➢ Bubbles should not form.

D.3.6 Wash column

- When the 4 ℓ sample has passed through the column (column will probably have gone dry), disconnect the column and place in burette stand.
- Remove the upper glass wool plug with a hooked wire.
- Add Milli-Q® water to the column, pouring in small volumes to re-slurry the resin and tilting as necessary to remove air bubbles.
- When all bubbles have been removed, place the column in an upright position in the burette stand and wash the resin bed with 3 x 10 mℓ water (approximately 40 mℓ).

D.3.7 Dry column

- Connect the column to a compressed air device.
- Open the stopcock.
- Open the air and allow the resin column to blow dry with a gentle stream of air (usually overnight).

D.3.8 Elute column

- Close the air and remove the column.
- Place the column in burette stand.
- Close the stopcock.
- Add acetone to re-slurry the resin, tilting as required to remove entrapped air.
- Place a bed of anhydrous sodium sulphate (approximately 7.5 g) in a sintered glass funnel (to dry the eluate).
- Place the funnel on a round bottomed flask (on cork ring) underneath the XAD-7 column in the burette stand.
- Run out the acetone into the flask.

> ➢ Do not allow to run dry.

- Elute with 5 x 10 mℓ portions of acetone, collecting the acetone in the round bottomed flask.
- Wash the sodium sulphate bed with acetone, adding the acetone to the round bottomed flask.

D.3.9 Concentrate the eluate

> ➢ If the residue remains aqueous, all the water was not taken up by the sodium sulphate.
> ➢ Add about 50 mℓ acetone to flask and redry over anhydrous sodium sulphate as described in paragraph D.3.8.

- Place the round bottomed flask on a rotary evaporator.
- Evaporate the contents to dryness at 30°C.

D.3.10 Dissolve the residue

> ➢ The residue is dissolved to a final volume of 2 mℓ.

- Add approximately 1 mℓ acetone to the flask and dissolve.
- Transfer the contents to a graduated glass tube using a Pasteur pipette.
- Rinse the flask with small volumes of acetone, adding the volumes to the glass tube.
- Make up to 2 mℓ with acetone.
- Transfer the contents to a Vacutainer.
- Store at -20°C until assaying.

D.4 REFERENCE

US EPA. 1985. Guidelines for preparing environmental and waste samples for mutagenicity (Ames) testing: Interim procedures and panel meeting proceedings. EPA/600/4-85/058. Office of Research and Development, US Environmental Protection Agency, Cincinnati.

APPENDIX E: REFERENCE TOXICANTS

E.1 INTRODUCTION

The ability of a laboratory to obtain precise, consistent and reliable results must be demonstrated with reference toxicants (US EPA, 1989; EPS, 1999). These are standard chemicals used to measure the precision of results for a given test method within the laboratory. The results are compared with historical data to confirm acceptability and demonstrate satisfactory laboratory performance. Results that do not fall within an acceptable range indicate a change in the test organism's health or genetic sensitivity, or a method inconsistency, or a combination of these factors.

Chemicals used as reference toxicants include: sodium chloride (NaCl), potassium chloride (KCl), potassium dichromate ($K_2Cr_2O_7$), cadmium ($CdCl_2$), copper ($CuSO_4$), zinc ($ZnSO_4$), phenol (C_6H_5OH), sodium dodecylsulphate ($C_{12}H_{25}O_4SNa$) and pentachlorophenate (Cl_5C_6ONa) (EPS, 1992; 1999; US EPA, 1985; 1989). The following criteria are used to select appropriate reference toxicants (EPS, 1992; 1999):

- Previous use.
- Available in a pure form.
- Readily water soluble.
- Stable in aqueous solution.
- Stable shelf life.
- Easy to measure analytically.
- Stable toxicity with normal changes in laboratory water quality.

Cadmium is recommended as reference toxicant for the algal tests described in Chapters 8 and 9, and the *Daphnia* tests described in Chapters 6 and 7. Potassium dichromate is used for the fish test described in Chapter 5.

E.2 MATERIALS, EQUIPMENT AND REAGENTS

E.2.1 Materials

- ✓ Glass beakers - 25 and 100 mℓ
- ✓ Volumetric flasks - 1 ℓ
- ✓ Schott bottles - 1 ℓ
- ✓ Pasteur pipettes
- ✓ Pipette bulbs
- ✓ Parafilm

E.2.2 Equipment

- ✓ Balance
- ✓ Cold room (4°C) or refrigerator
- ✓ Spatula
- ✓ Desiccator

E.2.3 Reagents

Use reagent grade chemicals and water purified by a Millipore Milli-Q® system.

- ✓ Cadmium chloride
- ✓ Potassium dichromate

> ➢ Dry the chemicals at approximately 100°C and store in a desiccator.

E.3 PREPARATION OF STOCK SOLUTIONS

E.3.1 Cadmium stock (100 mg/ℓ)

$CdCl_2$	163.1 mg
Water	

- Transfer the chemical to a 1 ℓ volumetric flask.
- Make up to 1 ℓ.
- Store in Schott bottle at 4°C.
- Prepare a fresh solution once a month.

E.3.2 Potassium dichromate stock (1 000 mg/ℓ)

$K_2Cr_2O_7$	1 000 mg
Water	

- Transfer the chemical to a 1 ℓ volumetric flask.
- Make up to 1 ℓ.
- Store in Schott bottle at 4°C.
- Prepare a fresh solution once a month.

> ➢ Conduct a chemical analysis on the stock solutions at the beginning of each test to confirm the concentrations (APHA-AWWA-WEF, 1992).

E.4 CONTROL CHART

- Conduct 10 or more definitive tests with each toxicity test on the reference toxicant over a period of time as described in the toxicity test methods (Chapters 5 to 9).
- Calculate the toxicity endpoints (LC_{50} or EC_{50}) for each definitive test.
- Construct a control chart for each toxicity test, where the x-axis represents the test date or number, and the y axis the toxicity endpoint (log concentration).

> ➢ The control chart can be constructed with either the measured or nominal toxicity endpoints, but not with a mixture of both.

- Calculate the mean and SD of the accumulated toxicity endpoints.
- Plot the mean and control limits (mean±2SDs) as lines parallel to the x-axis on the control chart.
- Recalculate the mean and SD with each successive point until the statistics stabilise.

> ➢ The calculations and plotting could be done by hand on semi-logarithmic paper, or a spreadsheet could be set up on the computer for calculations with sequential replotting of the endpoints and limits.

- Use the control chart (historical data) to assess the validity of the results from subsequent tests.

> ➢ Values that fall outside the upper and lower control limits and trends of increasing or decreasing sensitivity are readily identified.

- Update the control chart once a year.

E.5 REFERENCES

EPS. 1992. Biological test method: Growth inhibition test using the freshwater alga *Selenastrum capricornutum*. EPS 1/RM/25. Environmental Protection, Conservation and Protection, Environment Canada, Ottawa.

EPS. 1999. Guidance document on application and interpretation of single-species tests in environmental toxicology. EPS 1/RM/34. Method Development and Application Section, Environmental Technology Centre, Environment Canada, Ottawa.

US EPA. 1985. Methods for measuring the acute toxicity of effluents to freshwater and marine organisms. EPA/600/4-85/013. Environmental Monitoring and Support Laboratory, Office of Research and Development, US Environmental Protection Agency, Cincinnati.

US EPA. 1989. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Second Edition. EPA/600/4-89/001. Environmental Monitoring Systems Laboratory, Office of Research and Development, US Environmental Protection Agency, Cincinnati.

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