Pergamon

Wat. Sci. Tech. Vol. 39, No. 10–11, pp. 367–373, 1999

0273–1223/99 $20.00 + 0.00

PII: S0273-1223(99)00300-5

BIOLOGICAL ASSAYS FOR AQUATIC TOXICITY TESTING

J. L. Slabbert and E. A. Venter

Division of Water, Environment and Forestry Technology, CSIR, P.O. Box 395, Pretoria 0001, South Africa

ABSTRACT

A number of aquatic toxicity tests have been established for South African use, which include fish and *Daphnia* lethality tests, microbiotests, and short-term chronic tests. Studies on effluents and surface waters showed that all the tests have a viable role to play in water quality management. The most advantageous use of the tests is in battery form, so that tests can complement each other. The fish and *Daphnia* lethality tests, and algal growth inhibition test are recommended for regulatory and management purposes of effluents. If receiving water is used for drinking water purposes, the Ames *Salmonella* mutagenicity and toad embryo teratogenicity tests should be included in the battery of tests. Some of the rapid microbiotests, e.g. the protozoan oxygen uptake test, bacterial growth test and enzyme tests, could be valuable screening tools to identify and categorize toxic effluents. © 1999 Published by Elsevier Science Ltd on behalf of the IAWQ. All rights reserved

KEYWORDS

Biological assays; effluents; mutagenicity; surface water; teratogenicity; toxicity tests

INTRODUCTION

The deliberate discharge and accidental release of harmful chemical compounds into the environment has the potential to disrupt the structure and functioning of natural ecosystems. The ability to detect adverse chemical activity is, therefore, a prerequisite to effective environmental management.

The conventional approach to control harmful chemicals in the aquatic environment is to use a set of global physical-chemical parameters. However, water pollution is a complex situation that involves a vast number and diversity of chemical substances, many of which are unknown. Chemical procedures alone can, therefore, not provide sufficient information on the potential harmful effects of chemicals on the aquatic environment. Since living material responds to the total effect of actual and potential disruptions, biological assays have become important tools to assess harmful chemical activity (Blaise *et al.*, 1988). Biological toxicity testing is now a rapidly-expanding field involving numerous bioanalytical techniques developed and applied at the sub- and multicellular levels of biological organization (Slabbert *et al.*, 1996). Environmentally relevant biotests provide information on the initial levels of damage and disasters and assist in developing precautionary measures and strategies for environmental management.

Various countries are using toxicity tests as part of their water quality monitoring programme. In South Africa, where industrial effluent and hazardous waste are posing a growing problem, a number of biological assays have been developed and evaluated for aquatic toxicity testing (Slabbert, 1996). This paper outlines

the toxicity tests and discusses their potential as diagnostic tools and decision making aids by presenting the results of studies on surface water and effluents.

TOXICITY TESTS

The toxicity tests measure acute and chronic toxicity and corresponding lethal and sublethal effects, detect a wide range of integrated effects of substances released into the freshwater environment, and utilize organisms from different levels of the aquatic food chain as well as cellular and subcellular systems. Acute toxicity tests include standard aquatic tests, e.g. fish and *Daphnia* lethality tests and microbiotests, *e.g.* a protozoan oxygen uptake test, algal and bacterial growth inhibition tests, and urease and acetylcholinesterase enzyme tests. The sensitivities of the acute tests to a number of chemicals are shown in Table 1 (Slabbert, 1996). Short-term chronic toxicity tests include the Ames *Salmonella* mutagenicity test and a toad embryo teratogenicity test.

Table 1. Sensitivities of acute toxicity tests to chemicals

Chemical	Fish test[1]	*Daphnia* test[2]	Protozoan assay[3]	Algal test[4]	Bacterial test[5]	Urease test[6]	Acetylcholin-esterase test[7]
Cadmium	1.85	0.319	1.0	0.076	0.08	10.0	-
Carbofuran	-	-	-	-	-	-	0.049
Copper	0.55	0.031	0.5	0.061	0.1	1.0	-
Cyanide	0.13	-	0.014	0.382	0.018	-	-
Malathion	-	-	-	-	-	-	2.0
Manganese	354	32.05	-	-	-	>130	-
Mercury	0.2	0.004	0.5	0.303	0.038	0.05	-
Phenol	15.0	-	90.0	>100	15.1	-	-
Propoxur	3.54	0.001	-	4.01	-	-	20.0
Zinc	-	-	0.5	0.015	0.15	5.0	-

[1] 96-h LC_{50} (mg/l)
[2] 48-h LC_{50} (mg/l)
[3] Minimum inhibiting concentration (mg/l)
[4] 48-h EC_{50} (mg/l)
[5] 6-h EC_{10} (mg/l)
[6] Inhibition of 0.5 mg/ml enzyme (mg/l)
[7] 15-min EC_{10} (µg/l)
- Not tested

Fish (*Poecilia reticulata*) lethality test

The test is based on standard methodology (US EPA, 1991). Ten fish (2 to 3 weeks old) are used per test concentration. Tests are carried out at 23±2°C for 96 h.

Daphnia pulex lethality test

Standard procedures are followed (US EPA, 1991). Twenty organisms, 24 h or less in age, are used per test concentration. Tests are carried out at 20±1°C for 48 h.

Protozoan (*Tetrahymena pyriformis*) oxygen uptake inhibition test

The protozoan test was locally developed and has the outstanding feature of providing results within the short period of 10 min (Slabbert and Morgan, 1982; Slabbert, 1988). The test utilizes the unicellular protozoan, *T. pyriformis*. For bioassaying, cells are suspended in an osmotically balanced salt solution using gravity filtration (filter pore size: 8 µm). Tests are carried out with a standard biological oxygen monitoring system (Yellow Springs Instrument Co) at 27±0.5°C. Oxygen uptake of a cell suspension (3 ml) is monitored continuously before, during and after test sample (3 ml) addition. Results are determined as a ratio of the oxygen uptake rate (slope) after sample addition to that prior to sample addition, and expressed as percentage inhibition (or stimulation).

Algal (*Selenastrum capricornutum*) growth inhibition test

The algal growth inhibition test (Slabbert and Hilner, 1990) is a modification of the standard algal flask test described by the US EPA (1978). The miniaturized test is carried out in 24-well tissue culture plates. Algal cultures 4 to 6 days old are used for testing. Algae are added at a ratio of 1:1 to a 20-times concentrate of modified 10% BG-11 medium (Rippka *et al.*, 1979) and used as 200 µl volumes for inoculation of 1.8 ml test sample (final concentration: $2x10^5$ cells/ml). The tissue culture plates are covered with lids and sealed with parafilm. Plates are incubated at 25±1°C for 48 to 72 h (no agitation), using continuous illumination (cool white fluorescent light: approximately 95 $\mu E/m^2/s$). Growth is measured in 96-well microplates (280 µl volumes) using a microplate reader (450 nm). Results are expressed as percentage inhibition (or stimulation).

Bacterial (*Pseudomonas putida*) growth inhibition test

The test is carried out in 50 ml medical flats in 25 ml minimal medium (Slabbert, 1986, 1988). An overnight culture of *P. putida* is diluted with fresh medium to an optical density (OD) of approximately 0.8. OD measurements are carried out spectrophotometrically at 600 nm using a 4 cm flow-cell. The cell suspension is added at a ratio of 1:4 to a 12.5-times concentration of the minimal medium, and used as 2.5 ml volumes for inoculation of 22.5 ml test sample. Cultures are incubated at 27±1°C for the short period of 6 h after which growth is measured in terms of OD. Effects are expressed in terms of percentage inhibition (or stimulation).

Urease enzyme inhibition test

This rapid biotest is selectively sensitive to heavy metals and provides results within 1 h (Metelerkamp, 1986). Tests are carried out in 96-well microplates, using 160 µl sample and 40 µl enzyme. After 30 min incubation at 25±1°C, 40 µl of urea is added, followed by 40 µl phenolphthalein (after 15 min). Ammonia is formed during the enzyme-substrate interaction. This causes an alkaline pH which results in a pink colour in the presence of phenolphthalein. The reaction is inhibited when heavy metals are present, and the mixture remains colourless. Enzyme activity is measured immediately with a microplate reader at 450 nm and results are expressed as percentage inhibition (or increase).

Acetylcholinesterase enzyme inhibition test

The assay detects organophosphate and carbamate pesticides (Venter, 1990). For each test, 1.9 ml of test sample, 200 µl of potassium phosphate buffer, and 100 µl of enzyme solution are added consecutively to a cuvette, mixed and kept at 37±0.5°C. After an incubation period of 15 min, 100 µl of Ellman's reagent is added to the reaction mixture, followed by 100 µl of substrate (S-acetylthiocholiniodide). After a further incubation period of 1 min, the enzyme reaction rate is monitored by recording the OD of the mixtures for a 2 min period with a spectrophotometer at 405 nm. In the case of latent inhibitors (certain organophosphates) a test is also conducted on an oxidized sample [50 µl N-bromosuccinimide (NBSI) added to 1.9 ml sample]. After stirring for 20 min, the excess NBSI is reduced with 50 µl ascorbic acid. The enzyme reaction rate (slope) is used to calculate results. Results are expressed as percentage inhibition (or increase).

Ames *Salmonella* mutagenicity test

The Ames test is carried out according to a standard plate incorporation assay (US EPA, 1983), using *Salmonella typhimurium* tester strains TA98 and TA100. TA98 detects frame shift mutagens, whilst TA100 detects base-pair substitution mutagens. In the test 100 µl of concentrated test sample is introduced into the top-agar (2 ml) which is poured onto nutrient agar plates. Tests are carried out with and without S9 liver preparation (used for metabolic activation of chemicals which would otherwise be non-mutagenic). Plates are incubated at 37±1°C for 48 h whereafter the number of revertant colonies on plates is counted. Results are expressed as mutation ratios (MR's), calculated as the number of colonies on test plates/number of colonies on control plates.

Toad (*Xenopus laevis*) embryo teratogenicity test

Toads are given a primer injection (100 µl) of Human Chorionic Gonadotrophin (HGC) to stimulate fertility (Genthe and Edge, 1988) and placed in pairs in spawning tanks in dechlorinated tap water. Forty eight hours later toads receive a HGC booster injection (females: 300 µl; males: 100 µl). After fertilization, eggs are transferred to 500 ml glass containers with 200 ml test sample. One hundred eggs are used per test (50 eggs in duplicate containers). After 48 h exposure at 23±2°C the developing embryos are counted and examined under a dissection microscope for abnormalities. Features examined for malformation include embryo development (size and length of embryos), pigmentation, head shape, and form of spines and tails. Results are expressed as percentage deformation and lethality.

Data analysis

Effects 10% in the *Daphnia*, algal, bacterial, urease, acetylcholinesterase and toad embryo (lethality) tests; >10% in the fish test; and 5% in the protozoan test indicate toxic activity. A mutation ratio 2.0 indicates mutagenicity and toad embryo deformation 20% indicates teratogenicity.

Effective concentrations of effluents, e.g. LC_{50}'s (concentrations causing 50% lethality) and EC_{50}'s (concentrations causing 50% inhibition) are statistically derived (linear regression) using dose-response curves (% effect versus log concentration). When toxicity is too low to use linear regression, endpoints are presented as: LC_{50}: >100% or LC_{50}: 50-100%. No effect concentrations (LC_0's/EC_0's - highest concentrations tested not exceeding detection limits) are derived from the concentrations tested.

STUDIES ON SURFACE WATER AND EFFLUENTS

Test samples

Samples were collected from dams/rivers/streams and effluent dischargers in the Johannesburg-Pretoria area. Six dam and six river/stream waters were studied. Three types of effluents were evaluated, namely secondary treated sewage effluent from a sewage works receiving both domestic and industrial effluent, paper mill effluent, and effluent from a metal refinery. The sewage works receives effluent from some 70 major and approximately 400 small industrial dischargers. The treated chlorinated sewage effluent is discharged into a river where very little dilution takes place for the largest part of the year. River water upstream and downstream of the point of discharge was collected for ambient toxicity testing. The paper mill effluent was sampled before and after treatment (clarification). The treated effluent is discharged into a dam which also receives water from a small stream. The dam has a retention period of 30 to 40 days. Water was taken from the stream and from the overflow of the dam to examine ambient toxicity. The metal plating effluent is discharged directly into the sewer after lime treatment.

Control and dilution water

The control water for the different biotests were:

- Sterile (autoclaved) deionized water - microbial and enzyme tests; and
- Moderately hard water (US EPA, 1991) - Fish, *Daphnia* and toad embryo test.

Sample preparation

Samples used in microbial and enzyme tests (except Ames test) were filter sterilized (0.22 µm). Ames test samples were concentrated by passing 20 l water/effluent through XAD-7 resin. The absorbed chemicals were extracted by means of acetone, which was concentrated to a final volume of 10 ml. The acetone extracts were tested directly.

Results

All the tests, except the Ames test, detected adverse chemical activity in one or more of the surface water samples (Table 2). Three of the samples were toxic in the algal test, the urease test and the toad embryo lethality and deformation tests; two in the fish test; and one in the *Daphnia*, protozoan, bacterial and acetylcholinesterase tests. Effects ranged from slight (20%) to high (100%). No adverse chemical activity was detected in Roodeplaat Dam, Hartbeespoort Dam, Lakefield Dam, Moreleta Stream and Fountains Stream water. The Hennops River and Illiondale Stream showed the highest toxicity and exhibited responses in four and six tests, respectively.

Table 2. Effect of surface water (% effect/MR)

Sample	F test	D test	P test	A test	B test	UE test	AE test	TE test		Ames test
								Leth.	Def.	
Rietvlei Dam	10	<10	<5	+48	+19	+33	<10	21	24	<2.0
Bon Accord Dam	10	<10	+7	+53	25	+13	<10	<10	21	<2.0
Roodeplaat Dam	10	<10	<5	+70	<10	<10	<10	<10	<20	<2.0
Hartbeespoort Dam	10	<10	<5	+66	<10	+10	<10	<10	<20	<2.0
Centurion Lake	10	<10	<5	+70	<10	19	<10	<10	<20	<2.0
Lakefield Dam	10	<10	<5	+46	+24	+14	<10	<10	<20	<2.0
Hennops River	100	<10	<5	37	+29	+23	<10	100	100	<2.0
Moreleta Stream	10	<10	<5	<10	<10	+11	<10	<10	<20	<2.0
Jukskei River	10	<10	<5	+28	+49	+33	+13	15	<20	<2.0
Illiondale Stream	20	100	51	84	<10	34	67*	<10	<20	<2.0
Pienaars River	10	<10	<5	23	+68	30	+15	<10	<20	<2.0
Fountains Stream	10	<10	<5	<10	+11	<10	<10	<10	<20	<2.0

* After oxidation; A Algal; B Bacterial
D *Daphnia*; F Fish; P Protozoan
Def. Deformation; Leth. Lethality; + Enhanced activity
AE Acetylcholinesterase enzyme; MR Mutation ratio
UE Urease enzyme (0.5 µl/ml); TE Toad embryo

Table 3 summarizes the toxicity of the different effluents (range finding tests). All the acute toxicity tests, except the protozoan and enzyme tests, showed positive results with the sewage effluent and paper mill effluent before treatment. The sewage effluent also showed chronic toxicity, causing toad embryo deformation. None of the effluents were mutagenic. Only three of the acute toxicity tests, namely the algal, bacterial and toad embryo lethality tests showed positive results with the paper mill effluent after treatment. No embryo deformation was noticed and mutagenicity was detected only on one occasion. All the toxicity tests, except the Ames test, showed positive results with metal plating effluent. The acetylcholinesterase enzyme test was not applied as no pesticide pollution was suspected.

Definitive tests (testing serial dilutions) with fish, *Daphnia*, algal, bacterial and toad embryo tests showed that the EC_{50}'s and LC_{50}'s of sewage effluent were >100% and the EC_0's and LC_0's were 50%. The highest sensitivity was exhibited by the toad embryo test. The EC_{50}'s of both the paper mill effluents were between 50 and 100% and the EC_0's were 25%. The bacterial growth inhibition test was found to be the most sensitive test. Definitive tests on metal plating effluent showed that the *Daphnia* and algal tests were the most sensitive (EC_{50} and LC_{50}: 0.1%), followed in order of magnitude by the urease test (EC_{50}: 0.2%), the fish test (LC_{50}: 0.3%), the protozoan test (EC_{50}: 2.1%), the toad embryo lethality test (LC_{50}: >5%) and the bacterial test (EC_{50}: 10.7%). Teratogenicity occurred at the 5% effluent concentration. Based on the results of the most sensitive test (algal EC_0: 0.01%) a 100 000-fold dilution of metal plating effluent would be required to avoid adverse effects on aquatic life.

The river water upstream and downstream of the sewage effluent discharge did not exhibit toxicity on any of the sampling occasions. The stream water flowing into the dam into which paper mill effluent is discharged was toxic, affecting the protozoan (inhibition: 9%), algal (inhibition: 51%), bacterial (inhibition: 71%), urease (inhibition: 35%) and toad embryo tests (lethality: 36%; deformation: 54%). Usually, the water upstream of discharge should be free of toxicants if used for dilution of effluents to establish relative toxicity (to simulate effluent/receiving water interaction), which implied that the stream water would not be suitable for dilution purposes. The water from the outflow of the dam receiving paper mill effluent was toxic to the bacterial growth test on one sampling occasion.

Table 3. Effective concentration range (%) of effluents*

Toxicity test	Sewage effluent	Paper mill effluent - before clarification	Paper mill effluent - after clarification	Metal plating effluent
Fish test	10->100	10->100	>100	0.1-10
Daphnia test	10->100	1.0->100	>100	0.1-10
Protozoan test	>100	>100	>100	1.0->100
Algal test	10->100	10->100	100->100	0.001->10
Bacterial test	10->100	1.0->100	10->100	0.1-100
Urease test	>100	>100	>100	1.0-10
Acetylcholinesterase test	>100	>100	>100	not tested
Toad embryo test - lethality	10->100	10->100	10->100	1.0-100
Toad embryo test - Deformation	10-100	no deformation	no deformation	0.1-100
Ames test	not mutagenic	not mutagenic	one sample positive	not mutagenic

* Three data sets; dilution with control water

Some of the surface water (Table 2) and effluent samples showed enhanced activity in the microbiotests. This is usually attributed to the presence of nutrients. However, it is also possible that the stimulating effects were due to low levels of toxic chemicals, a phenomenon known as hormesis (Stebbing, 1982). In the case of the enzyme tests, the higher spectrophotometric readings could have been due to precipitation. Some of the water and effluent samples caused precipitation in the algal and bacterial growth inhibition tests. This interference was particularly high in the case of the paper mill effluent before treatment.

The positive results of the urease test (Tables 2 and 3) indicated the presence of heavy metals. Chemical analyses on the Illiondale Stream water (Table 2), where the acetylcholinesterase test showed a positive response, indicated that pesticides were absent but identified a fire retardant, ethylenechlorophosphate. In general, the surface water samples and sewage and paper mill effluents contained relatively low levels of potentially toxic chemicals, indicating that effects were probably due to a combination of pollutants. The stream flowing into the dam receiving paper mill effluent contained relatively high levels of manganese (2.1 mg/l), cyanide (0.178 mg/l) and phenol (0.233 mg/l), which individually or in combination could have caused toxicity. The metal plating effluent contained metals at high concentrations, e.g. cadmium (13.8 mg/l), chromium (12.6 mg/l), copper (7.3 mg/l), iron (94 mg/l), nickel (11.5 mg/l), zinc (206 mg/l), as well as cyanide (9.8 mg/l).

DISCUSSION

Studies on water and effluents indicated that all the tests have a viable role to play in water quality monitoring and control in the country. The studies demonstrated that there is no single method that can satisfy a comprehensive approach to aquatic life protection. For this reason, toxicity tests should be applied in battery form so that tests can complement each other. For example, when surface waters are monitored, tests should be selected to include organisms from different trophic levels. If metal and pesticide contamination is suspected the enzyme tests could be valuable screening tools.

The fish and *Daphnia* lethality tests and the algal growth inhibition test have been found to be the most suitable for regulatory and management purposes of effluents. These tests are sensitive, relatively simple to conduct, are compatible with the effluents, and are similar to aquatic tests used elsewhere. The urease test showed a high sensitivity to metal plating effluent and could be calibrated against the algal test to provide a simple and inexpensive test for routine use. Although chronic tests such as the Ames *Salmonella* mutagenicity and teratogenicity tests are more complex and too time consuming to use on a routine basis, they will be necessary where receiving water is used for drinking water purposes. In general, the rapid microbiotests, e.g. protozoan, bacterial growth and enzyme tests, could be valuable screening tools to identify and categorize toxic effluents.

The studies showed that the toxicity of effluents discharged into the environment is low. In order to effectively monitor such effluents and receiving waters, additional chronic tests should be established. The Ames test is not sensitive to metal containing pollutants. Alternative genotoxicity/mutagenicity tests are, therefore, required.

ACKNOWLEDGEMENT

The financing of the project by the Water Research Commission is greatly appreciated.

REFERENCES

Blaise, C., Sergy, G., Wells, P., Bermingham, N. and van Coillie, N. (1988). Biological testing - Development, application, and trends in Canadian Environmental Protection laboratories. *Tox. Assess.: An Intern. J.*, **3**, 385-406.

Genthe, B. and Edge, P. R. (1988). *Teratogenicity studies using the African clawed toad Xenopus laevis.* Progress Report for the Department of Health and Population Development, Division of Water Technology, Pretoria, South Africa.

Metelerkamp, N. C. (1986). *The development of an enzyme test system, using urease, to detect toxic levels of heavy metals in drinking water, under (a) laboratory simulated, and (b) field conditions.* CSIR Report, Project No. 620/9341/4, Pretoria, South Africa.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. and Stanier, R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.*, **110**, 1-61.

Slabbert, J. L. (1986). Improved bacterial growth test for rapid water toxicity screening. *Bull. Environ. Contam. Toxicol.*, **37**, 565-569.

Slabbert, J. L. (1988). Microbial toxicity assays used for water quality evaluation in South Africa. *Tox. Assess: An Intern. J.*, **3**, 101-115.

Slabbert, J. L. (1996). *Guidelines for toxicity bioassaying of waters and effluents in South Africa.* Contract Report for the Water Research Commission, Project No. K5/358/0/1, Division of Water Technology, Pretoria, South Africa.

Slabbert, J. L. and Morgan, W. G. S. (1982). A bioassay technique using *Tetrahymena pyriformis* for rapid assessment of toxicants in water. *Wat. Res.*, **16**, 517-523.

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, South Africa.

Slabbert, J. L., Oosthuizen, J., Venter, E. A., Hill, E. and du Preez, M. (1996). *Development of guidelines for toxicity bioassaying of drinking and environmental waters in South Africa.* Contract Report for the Water Research Commission, Project No. K5/358/0/1, Division of Water Technology, Pretoria, South Africa.

Stebbing, A. R. D. (1982). Hormesis - The stimulation of growth by low levels of inhibitors. *Sci. Tot. Environ.* **22**, 213-234.

US EPA (1978). *The Selenastrum capricornutum Printz algal assay bottle test: Experimental design, application and data interpretation protocol.* EPA/600/9-78/018, US Environmental Protection Agency, Corvallis, Oregon.

US EPA (1983). *Interim procedures for conducting the Salmonella/microsomal mutagenicity assay - Ames test.* EPA/600/4-85/013, US Environmental Protection Agency, Cincinnati, Ohio.

US EPA (1991). *Methods for measuring the acute toxicity of effluents to aquatic organisms.* 4th ed., EPA/600/4-90/027, US Environmental Protection Agency, Cincinnati, Ohio.

Venter, E. A. (1990). *Selected methodology for the acetylcholinesterase (isolated from electric eel) enzyme assay.* CSIR Report, Division of Water Technology, Pretoria, South Africa.