DEVELOPMENT OF GUIDELINES FOR TOXICITY BIOASSAYING OF DRINKING AND ENVIRONMENTAL WATERS IN SOUTH AFRICA

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JL SLABBERT, J OOSTHUIZEN, EA VENTER, E HILL,
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AND

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JL SLABBERT

Environmentek. CSIR

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Water Research Commission

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EXECUTIVE SUMMARY

Various countries are currently applying toxicity tests to assess and control water pollution. In South Africa, where industrial effluent and hazardous waste are manifesting a growing problem, the demand for biological tests for water toxicity testing is also rapidly increasing.

The importance of toxicity as parameter for the evaluation of water quality has recently been acknowledged by the Department of Water Affairs and Forestry. It is expected that the new approach of the Department will result in an increased use of such tests in the country. In order to ensure that standardized protocol and procedures will be introduced for use in South Africa it is essential to establish guidelines for toxicity testing. The aim of this project was to formulate biotoxicity guidelines which will assist decision makers in their future policies on water quality management.

Locally available toxicity bioassays were applied to different types of water to evaluate the efficiency and applicability of the tests. Acute toxicity was established by means of the following tests: fish and water flea lethality tests; a protozoan oxygen uptake test; algal and bacterial growth inhibition tests; luciferase, urease and acetylcholinesterase enzyme inhibition tests; and a mammalian cell colony formation test. The Ames *Salmonella* mutagenicity assay and a toad embryo teratogenicity test were used for chronic toxicity detection.

Tests were carried out on raw and treated drinking water, ground water, and river and dam water. Reference toxicants *e.g.* cadmium, pentachlorophenol, carbofuran and malathion (acetylcholinesterase enzyme test only), and sodium selenite and methyl viologen (toad embryo test only) were included in each series of tests. In addition, synthetically prepared moderately hard, hard and tap water were evaluated as alternative controls.

With the exception of the water flea, luciferase and urease tests, all the acute toxicity tests detected toxicity in one or more of the drinking water samples. In general the effects on the test systems were slight. However, the algal, mammalian cell, and toad embryo tests showed high levels of toxicity for a number of samples. Toxicity was detected in both raw and treated water. No specific pattern in the responses and sensitivities of the test systems were observed. The negative results obtained with the urease and acetylcholinesterase enzyme tests indicated that heavy metals and organophosphate and carbamate pesticides were either absent or present at low concentrations. Mutagenicity was detected in three raw water samples, while none of the samples showed teratogenicity.

All the acute toxicity tests, except the oxygen uptake and luciferase tests, were adversely affected by the groundwater samples. The water flea test proved to be highly sensitive to groundwater, showing adverse activity in 75% of the samples. A large number of samples were also toxic to algae, bacteria and fish. For some of the tests the effects were slight, but a high toxicity was generally observed with the water flea, algal, bacterial and luciferase enzyme tests. Two of the groundwater samples were slightly mutagenic while one of the samples caused teratogenicity. In a few instances three to four of the standard aquatic toxicity tests showed toxicity, indicating some pattern in the responses.

All the bioassays detected toxicity in one or more of the surface water samples. The highest toxicity was obtained with the Illiondale Stream water, which exhibited toxic effects on nine of the bioassays. The BGM mammalian cell test showed the highest sensitivity, detecting toxicity in six of the samples. Effects exhibited by the samples ranged from slight to high. The

mutagenicity and teratogenicity tests showed positive results on three occasions.

Chemical analyses showed that, in general, potentially toxic chemicals in groundwater and surface water samples were low, and that effects were probably due to a combination of chemicals. Some of the groundwater samples contained low oxygen levels which could have contributed to adverse effects. The Winterveld 2 sample contained a high zinc level which could have caused the toxicity detected by several bioassays. The Illiondale sample contained high levels of zinc, cadmium, iron, manganese and cyanide, as well as an organic compound, ethylenechlorophosphate (fire retardant), which individually or in combination could have caused the toxicity in test systems. Although chemical data were available it was found that toxic effects could not be explained by simple comparison. The high adverse activity of groundwater was of particular concern. As alternative, chemical equilibrium modelling was used to interpret toxicity results. The findings of this evaluation are presented in a report entitled **Application of chemical equilibrium modelling to interpret the toxic effects of borehole water** (Pretorius, 1994), which is attached as Appendix A.

Many of the tests showed enhanced activity when exposed to the water samples. This is usually attributed to the presence of nutrients in the water. It is also possible that the stimulating effects were due to low levels of toxic chemicals. Stimulation in sublethal responses such as growth (Stebbing, 1982) and respiration (Slabbert and Morgan, 1982) has been reported when organisms and cellular systems were exposed to low levels of individual toxic chemicals. This phenomenon is known as hormesis. However, extensive research is required to verify that stimulation by water samples which contain complex mixtures of chemicals can be attributed to toxic activity. In the case of the urease test, the high density readings could have been due to precipitation rather than increased activity. Some of the samples caused precipitation in the algal and bacterial growth inhibition tests, which could have interfered with the interpretation of results.

A good reproducibility was found with most of the tests (CV: <10%). The reproducibility was in agreement with the precision of tests used in other countries, or even exceeded it.

The good agreement established between results calculated as percentage effects and by means of Student's t-test indicates that the use of detection limits could be adequate to decide whether or not effects are significant. In a number of instances detection limits might provide false positive results. However, the possibility that significant effects might not be picked up by using detection limits are limited.

In general, both the acute and chronic tests showed an appropriate response with the reference chemicals, indicating that the tests were successfully applied. Some degree of variation was observed in the sensitivity of the tests, which generally reflected the natural variation in the sensitivity of test organisms. Methyl viologen was found to be more suitable as positive control in the toad embryo teratogenicity test than sodium selenite.

Reference chemicals are used to establish the validity of toxicity data generated by laboratories. It is recommended that all laboratories involved in toxicity testing should carry out tests with recommended reference chemicals so that inter- and intra-laboratory precision can be monitored.

The evaluation of alternative controls indicated that moderately hard and hard water did not drastically change responses in the majority of tests. However, large variations occurred in

some of the tests (algal and urease enzyme tests). It is, therefore, recommended to continue to use deionized water as control in microbial, enzyme and mammalian cell tests. Tap water showed inhibition in several instances, indicating that this water was unsuitable as control in these tests. The results obtained with fish and toads indicated that moderately hard water was an ideal substitute for tap water as control.

Most of the biological tests used in this study were well established. However, in certain cases a need for optimization was indicated. Most of the changes that were recommended were carried out. Apart from the luciferase test, which was found to be unsuitable for water testing, the algal test still showed a low growth in certain instances. This was mainly due to deficient lighting, erratic subculturing, and insufficient nutrients as a result of medium precipitation. Problems were experienced with the reproduction of fish and toads, particularly in winter months. Furthermore, fish were very prone to disease.

In order to solve breeding and disease problems with fish, it is recommended that fish for toxicity testing purposes are bred and supplied from a central facility. Although satisfactory results are obtained with guppies, attention should be given to the development of procedures employing indigenous species. The algal test problems can be rectified by appropriate optimization. Algal growth was measured at 450 nm instead of at the standard wavelengths of 600-650 nm used in the USA and Europe. This wavelength was selected for density determinations because studies conducted by Slabbert and Hilner (1990) during technique development showed that higher and more acceptable readings can be obtained for microplate use. A wavelength of 450 nm is also used by Canada in their miniaturized algal test (Environment Canada, 1992a). Any interferences by organic or other chemicals at this wavelength should be detected in the blanks. In order to ensure that 450 nm is the most appropriate wavelength for future use, it is recommended that studies are carried out to establish the effect of wavelength on test results. In order to improve fertility of toads, culturing conditions could be revised and alternative hormone treatment could be investigated.

The study showed that, with the exception of the luciferase enzyme test, all the biological toxicity tests employed have a viable role to play in water quality monitoring and control in the country.

A separate document **Guidelines for toxicity bioassaying of drinking and environmental waters in South Africa** (Slabbert, 1994) has been compiled, outlining specific guidelines on test methodologies, data analysis, sampling and application.

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Dr S A Mitchell	Water Research Commission (Chairman)
Mr F P Marais	Water Research Commission (Secretary, 1991 and 1992)
Mrs A M du Toit	Water Research Commission (Secretary from 1993)
Dr M J Pieterse	Water Research Commission
Prof J J Alexander	University of the Witwatersrand
Mr M C Steynberg	Rand Water
Dr P L Kempster	Department of Water Affairs and Forestry
Dr H van Vliet	Department of Water Affairs and Forestry
Mr W v d Merwe	Department of National Health and Population Development
Dr P Ashton	Division of Water Technology, CSIR
Dr R Kfir	Division of Water Technology, CSIR

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1. INTRODUCTION AND LITERATURE STUDY

1.1 Introduction

Since the start of industrialization in the nineteenth century, technological progress has had negative as well as positive consequences. While the use and development of new energy sources were intended to enhance human welfare, they also have been the major cause of environmental pollution. Incidents like the mercury poisoning in Minimata, Japan, dioxin exposure in Sevesco, Italy, and the illnesses due to various contaminants in Love Canal, New York, have demonstrated how harmful chemicals in the environment can threaten human lives (Blaise *et al.*, 1985).

Pollution of the environment means the contamination of soil, air and water. As water is essential for life, its pollution will not only endanger aquatic life, but also terrestrial organisms that need water for their existence, including man (Kfir, 1981).

Harmful chemicals may enter the aquatic environment through domestic and industrial waste discharges, and if not eliminated might reach drinking water systems. Even groundwater may become polluted through seepage from wastewater or by rain containing soluble pollutants entering the ground (Kfir, 1981). In order to adequately protect human health and the aquatic environment from exposure to harmful chemicals, effective detection procedures are needed.

Traditionally physical-chemical analytical procedures are used to monitor and control water pollution (Trussel and Umphres, 1978; Hattingh, 1979). Some of the chemical detection systems available today are highly sophisticated, very sensitive and accurate. However, physical-chemical measures have certain disadvantages when applied to control harmful chemicals in water, particularly in complex effluents (Sergy, 1987). These disadvantages can be summarized as follows:

- Water pollution is a complex situation that involves a vast number and diversity of chemical substances, and many harmful chemical pollutants are unknown (Bradfield and Rees, 1978)

- Chemical procedures cannot detect all possible harmful chemicals which might be present in water

- Chemical characterization of water or effluents is very expensive

- Chemical procedures cannot predict the effect of exposure due to combinations of substances and cannot account for changes in effects resulting from reactions within the matrix of constituents (synergism, antagonism and addition), and

- Chemical analysis cannot predict the potential long-term effects of toxicants.

Living material responds to the total effect of actual and potential disruptions in water and, therefore, the use of biological toxicity tests has become an important approach to complement chemical analysis to monitor and control harmful chemicals in water (Blaise *et al.*, 1988).

Various countries are currently applying biological toxicity tests to assess and control water

pollution. For example, in the United States of America (USA) the Environmental Protection Agency (EPA) uses an integrated hazard assessment scheme in which biological toxicity tests play a key role (US EPA, 1991a). The use of bioassays to control toxicants in industrial effluents and related receiving waters is also advocated by international organizations such as the Organization of Economic Co-operation and Development (OECD, 1987). In South Africa, where industrial effluent and hazardous waste are manifesting a growing problem, the demand for biological tests for water toxicity testing is rapidly increasing. The importance of toxicity as parameter in the evaluation of water quality has recently been acknowledged by the Department of Water Affairs and Forestry (DWA&F). The Department has identified whole effluent toxicity testing as an appropriate tool to assess the suitability of hazardous effluents for discharge into receiving waters (DWA&F, 1991). In addition, the Department has started to request industry to have their effluent evaluated using toxicity tests.

Biological tests for water toxicity testing have been developed and evaluated by the Division of Water Technology (DWT) since the late 1970's (Morgan, 1982; Grabow *et al.*, 1985; Slabbert, 1988). These tests have been primarily used to test drinking water and to evaluate drinking water treatment processes. Recently these tests have also been applied to evaluate impacts on surface waters, *e.g.* the Sappi Ngodwana spill into the Elands River, and to establish safe levels of chemical products and effluents for discharge into receiving waters. The locally available toxicity tests measure both acute and chronic toxicity with their corresponding lethal and sublethal effects, and include representative species of different trophic levels of the aquatic food chain (*e.g.* fish, water flea, algae, etc.), as well as mammalian cell culture and enzyme tests.

It is expected that international exposure and the new approach of the DWA&F will result in an increased use of bioassay techniques in the country. While the usefulness of biological tests has clearly been proven, it is essential to establish guidelines for toxicity bioassaying of drinking and environmental waters to ensure that standardized protocol and procedures will be introduced in South Africa.

This project was aimed at the formulation of biotoxicity guidelines which will assist decision makers, *e.g.* the Department of National Health and Population Development, the DWA&F, and the Department of Environmental Affairs in their future policies on biotoxicological water quality issues. *Specific tasks included:*

* A literature study on a) recent policies and strategies of other countries on the use of toxicity bioassays in the water field, and b) toxicity bioassays in use in South Africa and elsewhere in the world; and

* The application of different bioassays to various types of water to study their applicability and efficiency.

This report includes the literature study and presents the findings of the toxicity evaluation. The biotoxicity guidelines (toxicity tests, sampling and quality control) are outlined in a separate document **Guidelines for toxicity bioassaying of waters and effluents in South Africa** (Slabbert, 1996). Equilibrium modelling was carried out to interpret toxicity test results. The results are discussed in the document **Application of chemical equilibrium modelling to interpret the toxic effects of borehole water** (Pretorius, 1994), which is attached as Appendix A.

1.2 Literature study

The literature study was carried out to review policies and strategies of other countries and international organizations with reference to biological toxicity tests. The toxicity tests currently in use overseas to regulate and control toxicants in water are briefly reviewed and the tests used in South Africa are discussed and compared with those technologies.

1.2.1 Policies and strategies on the use of biological toxicity tests for fresh water toxicity testing

1.2.1.1 United States of America (USA)

The Federal Water Pollution Control Act of 1972, as amended by the Clean Water Act (CWA) of 1977 and by the Water Quality Act of 1987 (USA, 1987), specifies the objectives of restoring and maintaining the chemical, physical, and biological integrity of the nation's waters. Protection of aquatic life and human health from impacts caused by the release of toxicants to surface waters is called for by the Act, which states that "it is the national policy that the discharge of toxic pollutants in toxic amounts be prohibited."

The National Pollutant Discharge Elimination System (NPDES) permit programme, mandated by the Act, regulates the discharge of pollutants from point sources. In order to assess and control the discharge of toxic substances through the NPDES permit programme, the United States Environmental Protection Agency (US EPA) has issued a national policy statement entitled "Development of Water Quality-Based Permit Limitations for Toxic Pollutants" in 1984 (US EPA, 1984). The policy supports an integrated strategy consisting of both chemical and biological methods to address toxic and non-conventional pollutants from industrial and municipal sources.

The EPA's *surface toxics control* regulation, issued on 2 June 1989 (US EPA, 1989a), established specific requirements that an integrated approach be used in *water quality-based toxics control*. For the protection of aquatic life, the integrated approach consists of whole effluent and chemical-specific testing. As techniques are made available for implementing biocriteria (direct measure of ambient aquatic life and overall biological integrity of a water body), they too will be integrated into the *water quality-based toxics control*. Each approach has its limitations and for this reason exclusive use of one approach alone cannot ensure the required protection. For the protection of human health, technical constraints do not yet allow for full reliance on an integrated strategy, and thus primarily chemical-specific assessment and control techniques are employed (US EPA, 1991a).

The integrated approach to *water quality-based toxics control* relies on the water quality standards that each State has adopted. All States have water quality standards consisting of both chemical-specific numerical norms for individual pollutants, and narrative "free from toxics in toxic amounts" criteria. The use of toxicity testing and whole effluent toxicity limits is based on a State's narrative water quality criterion and/or in some cases, a State numeric criterion for toxicity (US EPA, 1991a).

1.2.1.1.1 Whole effluent toxicity testing approach

The whole effluent toxicity testing approach for the protection of aquatic life involves the use of acute and chronic toxicity tests to measure the toxicity of wastewaters. The EPA has

published extensive written protocols listing numerous plant, invertebrate and vertebrate species for toxicity testing (US EPA, 1991b,c).

At various points during testing the number of organisms affected is counted and the lowest effluent concentration that causes an effect is calculated. This concentration, referred to as the endpoint concentration, becomes a quantified measure of the concentration that would cause instream impact if exceeded for a particular period of time (US EPA, 1991a). It is usually stated either as an LC_{50} (the concentration at which 50% of the test organisms are killed) or a No Observed Effect Concentration (NOEC) (the highest effluent concentration at which no unacceptable effect will occur even at continuous exposure).

The toxicity measurement can then be used to limit the discharge of toxicants in an effluent. Toxicity itself is used as the effluent parameter. The toxicants creating that toxicity need not be specifically identified or controlled where the effluent's toxicity is limited.

Acute (TU_a) and chronic (TU_c) toxicity units are used as a mechanism for quantifying instream toxicity when using the whole effluent approach. The number of toxic units in an effluent is defined as follows:

$TU_a = 100/LC_{50}$, and
$TU_c = 100/NOEC$, where

100 = the whole effluent toxicity (no dilution) expressed as percentage (100%); and both LC_{50} and NOEC are calculated as percentage dilution of the whole effluent.

The procedure to implement the narrative criteria using a whole effluent approach should specify the testing procedure, the duration of the tests (acute or chronic), the test species, and the frequency of testing (US EPA, 1991a).

The EPA's recommended criteria for whole effluent toxicity are as follows: to protect aquatic life against chronic effects, the ambient (in stream) toxicity should not exceed 1,0 TU_c to the most sensitive of at least three different test species. For the protection against acute effects, the ambient toxicity should not exceed 0,3 TU_a to the most sensitive of at least three different test species.

1.2.1.2 Canada

In Canada the use of biological toxicity procedures has evolved from data acquisition on acute toxicities, to physicochemical and biological parameters being regulated and monitored by industrial sectors by the 1970's, and finally to compliance monitoring and hazard assessments being conducted in the 1980's (Blaise *et al.*, 1988). Environmental Protection Service Laboratories have the capacity to perform a number of toxicity tests in support of a variety of objectives, programmes and intervention activities. Such a capability satisfies responsibilities under several statutes (Fisheries Act; International Boundary Waters Treaty Act, Environmental Contaminants Act) (Sergy, 1987).

Canada implicitly supports biological testing within ecotoxicological approaches, aimed at effluent characterization and control, and has reviewed its national biotesting capabilities to implement uniform assessment on a national scale (Blaise *et al.*, 1988). The Ontario Ministry of the Environment reassessed strategies for effluent control by way of the Municipal and

Industrial Strategy for Abatement (MISA) programme. Although the emphasis is on toxicants, MISA controls conventional pollutants as well (Sergy, 1987). Critical assessments were made on the use of biological tests in the MISA programme. The removal of acute lethal toxicity *via* Best Available Technology Economically Achievable is considered to be the first step. The 96-h rainbow trout and the *Daphnia* lethality tests are the most likely regulatory tests. Other biomonitoring tools which measure chronic, genotoxic and sublethal effects were also under evaluation at MISA pilot site studies. These would play a role in the definition of water quality-based controls. The MISA Working Group stated: "As a minimum, industrial and municipal discharges must be non-acutely lethal to fish. Since the impact of toxic discharges on aquatic organisms ranges from acute lethality through sublethal toxicity leading to adverse chronic effects, further appropriate effluent biomonitoring tests should be applied (and/or developed) on an industry or sector-specific basis" (Sergy,1987).

In 1987 the Canadian Environmental Protection Act received first reading in the House of Commons. Both the spirit and letter of the Act demanded the use of toxicity tests and biomonitoring procedures (Sergy, 1987). (No further information on the outcome of this legislation is available).

1.2.1.3 Member States of the European Community (EEC)

Community standards for water quality (surface water, water for human consumption, etc.) and dangerous substances (including hazardous effluents) are given in Directives. Toxicity testing is not included as test parameter, which means that Member States are not obliged by Community legislation to use bioassays. However, it is open to Member States to use bioassay techniques where they judge this appropriate (Mandl, communication by letter, 1991).

1.2.1.3.1 France

France controls effluent discharges by means of a number of legal and regulatory measures using various technical means, including biological toxicity tests (OECD, 1987). Within the framework of the fishing law, for example, the 24-h *Daphnia magna* acute toxicity test is regularly carried out, especially in the case of pollution accidents. River Basin Agencies (regulating authorities) are using the *Daphnia* test to calculate industrial pollution charges (toxicity is established in terms of the maximum dilution necessary to bring the effluent below the lethal dose for *Daphnia*). The standard zebra fish and rainbow trout tests are also used to assess the LC_{50} of effluents. Such tests are particularly relevant in the case of short-term accidental pollution events, which permit authorities to estimate the duration/concentration of short-term pollution which may be dangerous for fish populations (OECD, 1984).

1.2.1.3.2 Germany

In Germany water quality is controlled by the Federal Water Act of 1957, as amended in 1976 (Harris, 1992). States have set up water authorities who are responsible for river water quality. Local authorities are responsible for discharges into sewers. The General Administrative Directive for effluents (Allgemeine Rahmen-Verwaltungsvorschrift über Mindestanforderungen an das Einleiten von Abwasser in Gewässer) (GMBl, 1989) includes guidelines on fish toxicity for some effluents, expressed in terms of a dilution factor (for which all the fish survive under the conditions specified in the standard method) (DIN, 1989b). Recent information (German Technology Report, 1992) indicates that water authorities will in future rely more extensively

on a range of biological tests, which will move Germany away form its current strict reliance on emission parameters, towards a Quality Standards for Receiving Waters Approach. It is envisaged that fish, *Daphnia*, algal and luminescent bacterial tests will be used in a few years time to monitor all waters.

1.2.1.3.3 Ireland and the United Kingdom

In Ireland guidelines for restrictions on the discharge of toxic effluents, expressed in terms of toxicity, are developed on an industry-specific basis. These guidelines are then incorporated on a case-by-case basis in individual permits issued to dischargers. The guidelines recognize the importance of mixing conditions by stipulating that at least a factor of 20 dilutions must be available in the immediate vicinity of a discharge for each toxic unit discharged. Compliance monitoring is carried out annually or bi-annually on representative samples of effluents. The test species most commonly used is the rainbow trout, *Oncorhynchis mykiss.* Confirmation of the efficacy of toxicity limits is obtained through biological surveys of receiving waters at least once every three years, particularly in areas of special biological importance or sensitivity (OECD, 1987).

The United Kingdom has drawn up a scheme for the biological monitoring and control of point source discharges. Three standard acute toxicity tests using species from three taxonomic groups, namely fish, invertebrates and algae will be used to derive toxic based consents. A luminescent bacterial test will be calibrated against the most sensitive of the three test species to provide a simple and relatively inexpensive test for routine use (OECD, 1987; Hunt *et al.;* 1992).

1.2.1.4 Organization for Economic Co-operation and Development (OECD)

The OECD has published a document promoting the use of biological tests for water pollution assessment and control (OECD, 1987) in industrialized and developing countries. The guidance document suggests desirable approaches but does not specify what test methods are to be used or the toxicity levels at which certain cautionary actions should be taken. The OECD states that as a result of the differing needs and circumstances within member countries, determination of these matters must be left to the judgement of individual countries. The OECD concluded "that toxicity testing procedures for complex effluents are founded on a sound and generally accepted scientific basis. Evaluation of effluent toxicity, particularly when analyzed in conjunction with engineering, chemical and ecological data, can provide a valid indication of the effects of toxic effluents on receiving systems, and can significantly improve the development of regulatory requirements to protect aquatic life" (OECD, 1987).

1.2.2 Biological tests used for water toxicity testing

1.2.2.1 Overview

An extensive number and variety of biological assays are at present available for water toxicity testing, and new developments in this field are regularly published. Organisms from various levels of the aquatic food chain, including fish, metazoa, algae, protozoa and bacteria are being used (Little, 1976; Kingsbury and Rees, 1978). Since the 1970's there has been a shift from the traditional acute toxicity test (Sprague, 1969) to methods employing sublethal responses (chronic effects) such as growth, reproduction, metabolism and behaviour (Hunter, 1978; Kingsbury and Rees, 1978; Cairns and van der Schalie, 1980). Because of the demand

for rapid, sensitive, and simple tests, great emphasis has been placed on the development of microbial toxicity tests in the last decade (Bitton, 1983; Liu and Dutka, 1984; Slabbert; 1988). These include short-term microbial tests to detect adverse chemical activity such as mutagenicity and genotoxicity (US EPA, 1983; 1985b; Quillardet *et al.*, 1985) which are aimed at the protection of human health against chronic toxicity. Rapid procedures have also extended to the use of enzymes (Bitton, 1983; Obst *et al.*, 1988) and subcellular particles (Knobeloch *et al.*, 1989). Currently, a new trend in the field of biotoxicity testing is the development of biosensors which will allow for much more rapid and simple monitoring (Rawson *et al.*, 1987).

To establish the adverse chemical activity in water on human health, a number of advanced techniques employing mammalian cell cultures have been developed (US EPA, 1979; Kfir, 1981; Hsie *et al.*, 1981; Slabbert *et al.*, 1992). For compliance monitoring a number of very useful electronic continuous monitoring systems using fish and invertebrates have been designed and established (Cairns and van der Schalie, 1980; Morgan, 1982; OECD; 1987).

Currently countries throughout the world are using biological toxicity assays for water quality testing. As in South Africa, a large part of this application is still aimed at research and development. For effective quality control standardized methods are required. Fish, water flea and algal tests have been successfully standardized so far. Various organizations are also currently working on the standardization of the bacterial luminescent bioassay. Countries like the USA, Germany, and France have their own standard methods for biological toxicity tests for water. These closely resemble the standard methods of organizations such as the International Organization for Standardization (ISO), the Organization for Economic Co-Operation and Development (OECD) and the American Society for Testing Materials (ASTM).

1.2.2.2 Biological toxicity tests used by other countries

The tests currently used by some countries for regulatory purposes, or which are recommended by international organizations, are discussed below.

1.2.2.2.1 USA

Whole effluent toxicity tests employ the use of standardized, surrogate freshwater vertebrates, invertebrates, and plants. The EPA has published extensive written protocols listing numerous organisms for toxicity testing (US EPA, 1991b,c). The following are examples of freshwater fish and invertebrates recommended by the EPA for acute lethal toxicity determination of effluents (US EPA, 1985a):

Cold water fish: Rainbow trout (*O. mykiss)*
Warm water fish: Fathead minnow (*Pimephales promelas*)
Cold water invertebrates: Cladocera (*D. magna/pulex*; *Ceriodaphnia* spp.)

Traditionally, chronic tests are either full life-cycle tests or a shortened test of about 30 days, known as an early life stage test. However, the duration of most of the EPA chronic toxicity tests has been shortened to 7 days by focusing on the most sensitive life-cycle stages. These tests are therefore called short-term chronic tests (Table 1).

TABLE 1: Short-term chronic toxicity methods

Species/Common name	Test duration	Test parameter
C. dubia Cladoceran	Approximately 7 days (until 60 percent of control has three broods)	Survival, reproduction
P. promelas Fathead minnow	7 days	Larval growth, survival
P. promelas Fathead minnow	7-9 days	Embryo-larval survival, percent hatch, percent abnormality
Selenastrum capricornutum Freshwater algae	96 h	Growth

The following tests are currently in use or under development for non-threshold human health toxicants (assessing carcinogenicity or mutagenicity):

- *Salmonella typhimurium* assay (Ames Test) [US EPA, 1983; 1985b] - endpoint: gene mutation, response measured in revertant colonies/ℓ effluent;
- *Escherichia coli* SOS assay (SOS Chromotest) [Quillardet *et al.*, 1985] - endpoint: DNA damage, response measured as the change in optical density;
- Sister-chromatid exchange assay (SCE) [Eckl *et al.*, 1987] - endpoint: DNA damage, response measured in SCE per chromosome/ℓ effluent;
- Chinese hamster ovary cell assay [Hsie *et al.*, 1981] - endpoint: gene mutation, response measured as % survival/ℓ effluent;
- Medaka fish tumour assay [US EPA, 1988; 1989b] - endpoint: tumour formation, response measured in frequency of tumours at a given site/effluent concentration.

1.2.2.2.2 Canada

A wide range of biological tests are conducted in the Canadian Federal Environmental Protection Laboratories. Generally, the tests employed measure both acute and chronic toxicity with their corresponding lethal and sublethal effects and include several representative species of different trophic levels (Table 2) (MacGregor and Wells, 1984; Sergy, 1987). The amount, manner and effectiveness of use of the tests is not the same in the different laboratories. This is because of different federal regional strategies, mandates, expertise and budgets (Blaise *et al.*, 1988). The types of tests are as consistent as possible with OECD guidelines, US EPA and ASTM methods.

The application of the biological tests in environmental protection activities includes four major steps: problem identification; problem assessment; control or intervention; and control evaluation (Sergy, 1987).

The application of bioassays for drinking water protection in Canada is limited to research institutions like the National Water Research Institute, Canada Centre for Inland Waters, Burlington. The battery approach is followed using a range of tests, including several microbial tests (Dutka and Kwan, 1981; 1988).

TABLE 2: Types of current fresh water biological tests conducted by Environment Canada[1]

Test type[2]	Test parameter
Lethality	
Trout - *Oncorhynchis*[3]	Mortality
Water flea - *Daphnia*	Mortality
Sub-lethality	
Trout - *Oncorhynchis*	ATP energy stress
Water flea - *Daphnia/Ceriodaphnia*	Reproduction
Algae - *Selenastrum*[4]	Growth inhibition
Bacteria - *Photobacterium*[5]	Light inhibition
Genotoxicity	
Dark mutant test	Mutagenicity
SOS chromotest	Genotoxic potential
AMES test	Mutagenicity

1 Blaise *et al.* (1988)
2 No test duration was specified
3 Standard procedure (Environment Canada, 1980)
4 Standard US EPA test (1978) and recommended Environment Canada test (1992a)
5 Recommended Environment Canada test (1992b)

1.2.2.2.3 France

The French standard methods are government approved guidelines and can be included in contracts and/or used in the event of litigation. The following standard tests are available for water toxicity testing:

- Fish (*Brachydanio rerio* and *O. mykiss*) lethality test - static and flow through tests (NF, 1985a,b)
- *D. magna* mobility inhibition test (NF, 1983)
- Algal (*Scenedesmus subspicatus*) growth inhibition test (NF, 1980)

These methods are very similar to ISO standards.

1.2.2.2.4 Germany

A number of German standard methods have been prepared for the examination of water, wastewater and sludge. General guidelines for the planning, performance and evaluation of biological tests are also outlined (DIN, 1982a). Possible uses are:

- comparison of the toxicity of various substances or mixtures of substances relative to one another under predetermined test conditions
- comparison of the specific sensitivity of various organisms relative to the same pollutants
- prediction of the effect of wastewater and wastewater constituents on the balance of substances in the receiving water
- supervision of the functioning of detoxification and wastewater cleaning plants
- supervision of water quality
- supervision of water resource quality
- determination of and warning against water resource contamination
- checking for observance of inlet conditions
- calculation of wastewater discharge in terms of the biological polluting action

The following standard methods are used:

- Fish (golden orfe - *Leuciscus idus*) lethality test (DIN, 1980; 1982b; 1989b)

- *D. magna* mobility inhibition test (DIN, 1982c; 1989a)

- Algal (*S. subspicatus*) growth inhibition and chlorophyll fluorescence inhibition tests (DIN, 1989c; 1991a)

- Bacteria (*Pseudomonas putida*) cell multiplication inhibition test (DIN, 1991b)

- Bacteria (*Photobacterium phosphoreum*) luminescence inhibition test (DIN, 1991c)

The fish, *Daphnia* and algal (growth inhibition) tests are similar to ISO standards. A method based on urease enzyme inhibition is currently being standardized (personal communication).

1.2.2.2.5 ISO Standards

The following ISO standard methods are available for water quality testing:

- Fish (*B. rerio*) lethality test - static, semi-static, and flow-through methods (ISO, 1984a,b,c)

- Water flea (*D. magna*) mobility inhibition test (ISO, 1989b)

- Algal (*S. subspicatus* and *S. capricornutum*) growth inhibition tests (ISO, 1989a)

1.2.2.2.6 OECD Guidelines

The following tests, applicable in the water field, are recommended in "OECD Guidelines for Testing of Chemicals" (1981):

- Acute lethal fish test
- Chronic 14 day fish test
- *Daphnia* immobilization test
- *Daphnia* reproduction test
- Algal growth inhibition test

The OECD guidelines also include a list of tests for human health effect assessment of chemicals. Some of the recommended genetic toxicology tests which are applied in the water field are: the *S. typhimurium* assay; the *E. coli* reverse mutation assay; and the *in vitro* sister chromatid exchange assay using mammalian cells.

1.2.2.2.7 ASTM Standards

The following ASTM standard methods developed for biological effect and environmental fate testing can be used for water testing:

- Acute toxicity tests on aqueous effluents with fish, macro-invertebrates and amphibians (ASTM, 1988a,b)
- Early life-stage toxicity tests with fish (ASTM, 1988c)
- Static acute toxicity tests on wastewaters with *D. magna* (ASTM, 1984)
- Renewal life-cycle toxicity tests with *D. magna* (ASTM, 1987a)
- Renewal toxicity tests with *C. dubia* (ASTM, 1989)
- Algal growth potential testing with *S. capricornutum* (ASTM, 1987b)

1.2.2.2.8 Summary of bioassays used by some countries

Table 3 summarizes the bioassays used by the USA, Canada, France and Germany.

1.2.2.3 Biological toxicity tests used in South Africa

1.2.2.3.1 Background

Biological toxicity tests have been under development and evaluation at the DWT for many years. These developments were initially aimed at establishing tests for the evaluation of drinking water quality. Because of South Africa's limited water supplies, reuse of water is inevitable and toxicity testing has always been considered indispensable to ensure that our drinking water is safe. The rationale behind the use of a battery of tests for drinking water is that the basic similarity of living organisms regarding biological functions and structure is such, that there is no reason to believe that health-hazardous chemicals might occur in water, which cannot be detected by one bioassay or another (Grabow *et al.*, 1985).

Since the late 1970's the emphasis was on the development of microbial toxicity tests, resulting in the establishment of several rapid tests (Slabbert, 1988). Development of rapid enzyme test systems, mainly for field application by the Defence Force, started during the mid 1980's (Grabow *et al.*,1985). In order to ensure protection against chronic chemical activity, the Ames *Salmonella* mutagenicity test was evaluated and introduced for use between 1979 and 1981 (Denkhaus *et al.*, 1980; Kfir *et al.*, 1982). In addition, certain mammalian cell culture techniques were evaluated during the same time (Kfir, 1981). The tests proved to be highly sensitive for the detection of toxicants and potential carcinogens.

Fish was used for toxicity testing mainly in continuous monitoring systems (Morgan, 1982).

TABLE 3: Freshwater tests conducted by the USA, Canada, France and Germany

Type of test	USA	Canada	France	Germany
Acute	Rainbow trout/fathead minnow lethality test	Rainbow trout lethality test	Rainbow trout and *Brachydanio* (static and flow through) lethality tests	Golden orfe lethality test
	Daphnia/Ceriodaphnia lethality test	*Daphnia* lethality test	*Daphnia* mobility inhibition test	*Daphnia* mobility inhibition test
	Algal (*Selenastrum*) growth inhibition test[1]	Algal (*Selenastrum*) growth inhibition test	Algal (*Scenedesmus*) growth inhibition test	Algal (*Scenedesmus*) growth and chlorophyll fluorescence inhibition test
				Bacterial (*Pseudomonas*) cell multiplication inhibition test
		Bacterial (*Photobacterium*) light inhibition test		Bacterial (*Photobacterium*) light inhibition test
Chronic (short-term)	Fathead minnow embryo-larval survival/growth test	Rainbow trout ATP energy stress test		
	Ceriodaphnia survival/reproduction test	*Daphnia/Ceriodaphnia* reproduction test		
Genotoxic	Ames *Salmonella* mutagenicity test	Ames *Salmonella* mutagenicity test		
		SOS chromotest		
		Dark mutant mutagenicity test		

[1] Algal test considered to be a chronic test if exposure period is 4 days and longer

These systems have been applied both for drinking water and wastewater monitoring. Fish lethality tests were occasionally used to evaluate effluents for industry since the beginning of the 1970's and for research purposes (Morgan, 1982). In 1986 the water flea lethality test was introduced primarily to evaluate the sensitivities of newly developed rapid tests. However, since the demand for toxicity tests on effluents, environmental water and chemical products increased in the late 1980's, fish and water flea tests became well established components of the battery of tests used by the DWT. Since 1987 biological toxicity tests have been applied to a wide range of waters, and are also used for chemical product and material testing. An emergency service has also been established to rapidly evaluate acute toxicity (Slabbert, 1989).

Currently, three institutions, namely, the DWT, the DWA&F's Institute for Water Quality Studies (IWQS) and Rand Water, have the facilities and infrastructure to conduct routine freshwater toxicity tests. The latter two laboratories mainly handle in-house testing. Tests established for local use are presented in Table 4.

1.2.2.3.2 Toxicity tests

The biological toxicity tests used at the DWT are described in detail in Section 2 (Toxicity Test Methodologies).

Fish and water flea tests are carried out according to US EPA (1985a) procedures, which are in good agreement with the standard tests employed in other countries. However, the fish species is different (*Poecilia reticulata* - guppy). Although this test organism has not been included in some of the standard procedures discussed previously, it is fairly regularly used in some European countries, as well as in Brazil (personal communication). The guppy has been selected as test organism because it is easy to breed and maintain, and it is relatively sensitive. The organism used in the water flea test (*D. pulex*) has been locally obtained. According to literature this test species is slightly more sensitive than other *Daphnia* species (Elnabawary *et al.*, 1986). The sensitivities of the tests are shown in Table 5.

The algal (*S. capricornutum*) growth inhibition test (Slabbert and Hilner, 1990) is based on the standard algal test of the US EPA (1978), which is similar to the algal tests used in some of the other countries. The major differences between different standard algal tests are the composition of the growth medium, the test species, the inoculum size, the growth measurement and the exposure period. In order to simplify the algal test and to make it more cost-effective for use, the standard test has been miniaturized for our use (Slabbert and Hilner, 1990). Instead of using flasks, tests are carried out in 24-well tissue culture plates, and growth measured with a microplate reader. A microplate technique has also been developed by Blaise *et al.* (1986), which is now in use in Canada (Environment Canada (1992a). During technique development it was found that the use of different media affected the sensitivity of the toxicity test (Slabbert and Hilner, 1990) (Table 5). It was, therefore, recommended to use both the US EPA (1978) growth medium and a modified BG-11 medium (Rippka *et al.*, 1979) to enhance the sensitivity of the test. In general, studies showed a good agreement between the sensitivity of the microplate assay and the traditional flask test (Blaise, *et al.*, 1986; Slabbert and Hilner, 1990) (Figure 1).

The bacterial (*P. putida*) growth inhibition test (Slabbert, 1986) which was developed in the DWT laboratories (Table 5) is similar to the standard test employed in Germany. Although bacterial growth tests are also used in countries such as Canada (Dutka and Kwan, 1981),

TABLE 4: Locally available bioassays for freshwater testing

Laboratory	Type of test	Bioassay	Test organism/mammalian cells/enzyme specificity	Method	Exposure time
DWT[1]	Acute	Fish lethality test	*Poecilia reticulata* - guppy	EPA	96 h
		Daphnia lethality test	*Daphnia pulex*	EPA	48 h
		Protozoan oxygen uptake assay	*Tetrahymena pyriformis*	Developed at DWT	10 min
		Algal growth inhibition assay	*Selenastrum capricornutum*	Miniaturized assay based on EPA flask test	48-72 h
		Bacterial growth inhibition assay	*Pseudomonas putida*	Developed at DWT	6 h
		Urease enzyme inhibition test	Heavy metals	Developed at DWT	15 min
		Acetylcholinesterase enzyme inhibition test	Organophosphate and carbamate pesticides	Developed at DWT	15 min
		Mammalian cell colony formation inhibition test	BGM and V79 cells	Developed at DWT	6-8 days
	Chronic	Toad embryo teratogenicity test	*Xenopus laevis*	Developed at DWT	48 h
	Genotoxic	Ames mutagenicity test	*Salmonella typhimurium* (strains 98 and 100)	EPA	48 h
		Cell transformation assay	Hamster embryo cells	Developed at DWT	8 days
Rand Water	Acute	Fish lethality test	*P. reticulata* - guppy	EPA	96 h
		Daphnia lethality test	*D. pulex*	EPA	48 h
		Algal growth inhibition assay	*S. capricornutum*	EPA	72 h
		Bacterial growth inhibition assay	*P. putida*	Developed at DWT	6 h
	Genotoxic	Ames mutagenicity test	*S. typhimurium* (strains 98 and 100)	EPA	48 h
IWQS[2]	Acute	Fish lethality test	*P. reticulata*	EPA	96 h
		Daphnia/Ceriodaphnia lethality test	*D. pulex/C. dubia*	EPA	24/48 h
		Algal growth inhibition assay	*S. capricornutum*	EPA	72 h
		Bacterial growth inhibition assay	*P. putida*	Developed at DWT	6 h
	Chronic	Invertebrate reproduction test	*D. pulex/C. dubia*	EPA	7-21 days
CCEP[3]	Acute	*Daphnia* lethality test	*D. pulex*	EPA	48 h
		Algal growth inhibition assay	*S. capricornutum*	EPA	96 h

[1] Division of Water Technology
[2] Institute for Water Quality Studies
[3] Catchment and Coastal Environmental Programme, CSIR - Durban

TABLE 5: Sensitivities of bioassays to a number of toxic chemicals

Chemical	Fish lethality test[1]	Water flea lethality test[2]	Protozoan oxygen uptake assay[3]	Algal growth inhibition test[4]		Bacterial growth inhibition test[5]	Luciferase enzyme test[6]	Urease enzyme test[7]	Acetylcholin-esterase enzyme test[8]	BGM mammalian cell test[9]
	96-h LC_{50} (mg/ℓ)	48-h LC_{50} (mg/ℓ)	Minimum inhibiting concentration (mg/ℓ)	48-h EC_{50} (mg/ℓ)		6-h EC_{10} (mg/ℓ)	Inhibition at 0,5 mg/ℓ chemical (%)	Concentration inhibiting 0,5 mg/ml enzyme (mg/ℓ)	15-min EC_{10} (µg/ℓ)	Survival at concentration chemical (mg/ℓ) (in parenthesis) (%)
				AAM[10]	BG-11[10]					
Aldicarb	-	-	-	-	-	-	-	-	16,0	-
Azinphosmethyl	-	-	-	-	-	-	-	-	0,5	-
Cadmium	1,65	0,319	1,0	0,145	0,078	0,08	+18	10,0	-	89,6 (0,065)
Carbofuran	-	-	-	-	-	-	-	-	0,049	-
Cobalt	-	-	-	-	-	-	-	10,0	-	-
Copper	0,55	0,031	0,5	0,083	0,061	0,1	75	1,0	-	30,2 (0,025)
Cyanide	0,13	-	0,014	0,227	0,382	0,018	+18	-	-	-
Demeton-S-methyl	-	-	-	-	-	-	-	-	373	-
Lead	-	2,003	-	-	-	-	+8	>30,0	-	37,7 (0,3)
Malathion	-	-	-	-	-	-	-	-	2,0	-
Manganese	-	-	-	-	-	-	-	>100	-	-
Mercury	0,2	0,004	0,5	0,472	0,303	0,025-0,05	78	0,05	-	57,9 (0,005)
Nickel	-	-	-	-	-	-	53	10,0	-	-
Phenol	15,0	-	90,0	73,8	>100	15,1	-	-	-	32,4 (0,5)
Propoxur	-	-	-	-	-	-	-	-	20,0	-
Silver	-	0,002	-	-	-	-	87	0,1	-	-
Zinc	-	-	0,5	0,024	0,015	0,15	-	5,0	-	-

1 Morgan (1982)
2 Elnabarawy *et al.* (1985)
3 Slabbert and Morgan (1982)
4 Slabbert and Hilner (1990)
5 Slabbert (1986)
6 Meintjies *et al.* (1990)
7 Meterlerkamp (1986)
8 Venter (1991)
9 Kfir (1981)
10 Growth medium
\+ Stimulation
\- No results

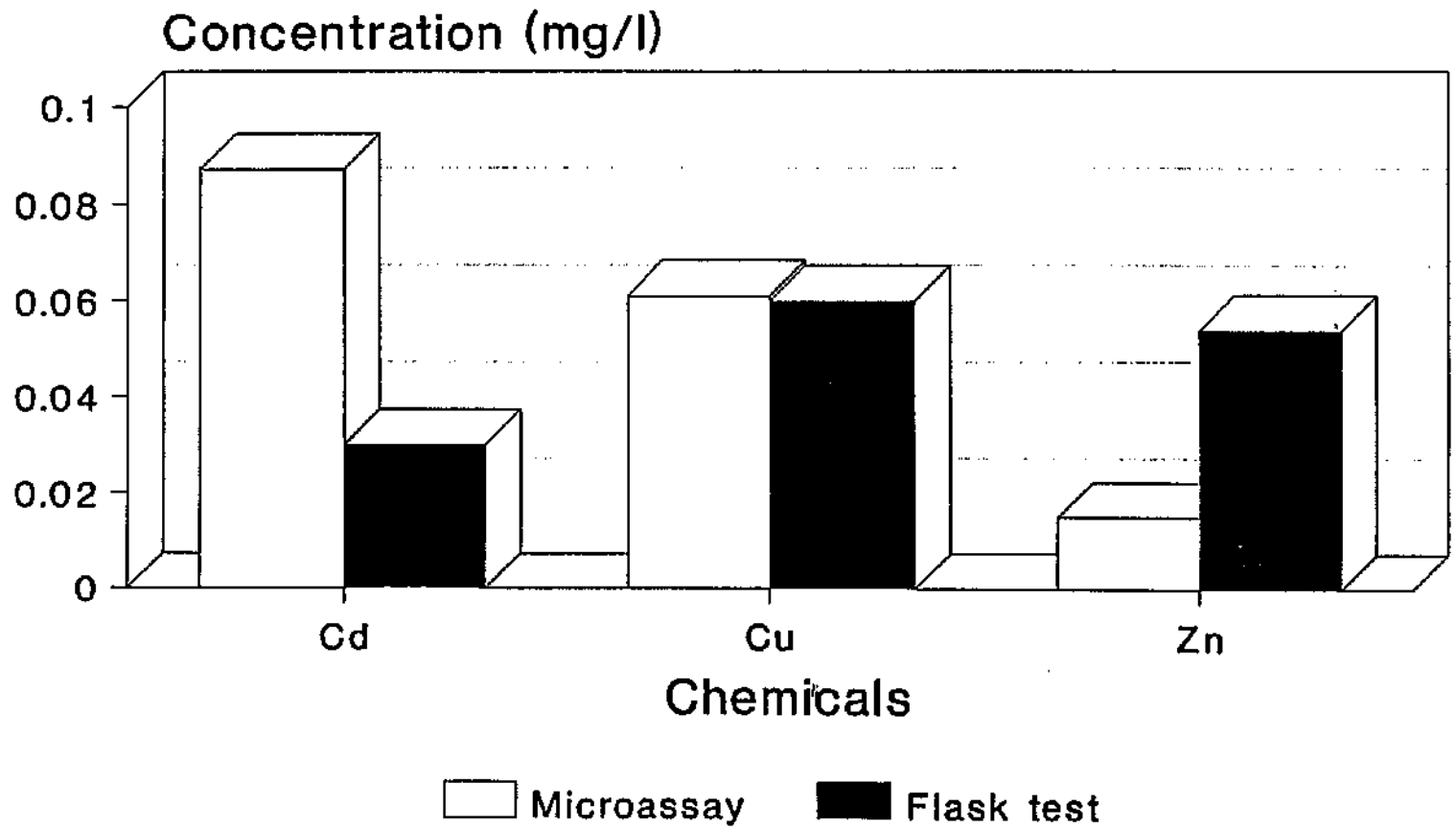

FIGURE 1: **Comparison between the sensitivity of microassay (48-h EC_{50}) and 'classic' flask test (72-h EC_{50})**

only the German test has been standardized. *P. putida* is cultured under modified tests conditions to have a more rapid, but equally sensitive test than the German test. Instead of the very complex medium used in the German test, the local test uses minimal medium prepared from a few chemicals. The incubation period has been reduced from 16 to 6 h. In general, a good agreement was found between the sensitivities of the two growth tests (Slabbert, 1986) (Table 6). This bioassay has also been miniaturized (Slabbert, 1988), using *P. putida* and *Aeromonas punctata* as test organisms. However, the reproducibility of these tests is not as good as that of the usual test and their use is, therefore, limited to specific studies.

TABLE 6: Sensitivities of two bacterial growth inhibition tests[1]

Chemical	6 h *Pseudomonas putida* test[2]		16 h *Pseudomonas putida* test[3]
	EC10	EC50	Minimum EC[4]
Copper	0,1	1,05	0,03
Cadmium	0,08	0,72	0,08
Mercury	0,025[5]	0,05[6]	0,01
Cyanide	0,018	0,69	0,001
Phenol	15,1	244	64
Acetone	594	4 385	1 700

1 mg/ℓ
2 Slabbert (1986)
3 Bringmann and Kühn (1980)
4 Concentration exhibiting 3% inhibition
5 No effect
6 100% inhibition

The protozoan (*Tetrahymena pyriformis*) oxygen uptake test was developed for rapid toxicity screening purposes (Slabbert and Morgan, 1982). This bioassay has the outstanding feature of providing results within the short period of 10 min, and has been found invaluable in emergency situations when rapid information on acute toxicity is needed. This bioassay has recently been modified to carry out a number of tests simultaneously (unpublished information). The sensitivity of this bioassay compares well with that of the standard fish test (Table 5). As alternative bacteria (*P. putida*) (Slabbert, 1988) and mammalian cells (Slabbert *et al.*, 1982) have been used as test material in this test system, but because increased sensitivity was not observed their use is limited to specific studies. Respiration tests similar to the *T. pyriformis* test have been used in other research studies. A few standardized oxygen uptake tests exist, but these are used for biodegradation studies (*e.g.* activated sludge respiration inhibition tests) (OECD, 1981).

The enzyme tests are rapid tests which can provide information on toxicity within less than 1 h. The sensitivities of these tests are presented in Table 5. Similar tests are used in

countries such as Germany (Obst *et al.*, 1988) and the USA (Army). Test kits for enzyme tests are also available. The luciferase test currently applied in our laboratory is still under development. Luciferase, a protein isolated from firefly, catalyses the following reaction:

$Enz\text{-}LH_2 + ATP \quad ---> \quad Enz - LH_2AMP + PPi$
$Enz - LH_2AMP + O_2 \quad ---> \quad hv + CO_2 + AMP + Oxyluciferin$

Enz	Luciferase
LH_2	Luciferin
ATP	Adenosine triphosphate
$Enz - LH_2AMP$	Luciferin adenylate complex
Ppi	Pyrophosphate
hv	Light energy (550 nm)

The first reaction requires Mg^{2+} as co-factor.

Luciferase undergoes a large conformational change, rendering a product which can be directly and precisely measured by the amount of light given off by the reaction.

The urease enzyme test is specific for the detection of heavy metals (Metelerkamp, 1986). The test is carried out in microplates which allows field testing. Microplates also allow for quantitative data calculation using a microplate reader at 450 nm. The enzyme reaction is as follows:

$NH_2 - CO - NH_2$ (Urea) + H_2O (water) ---> CO_2 (carbon dioxide) + $2NH_3$ (ammonia)

The ammonia causes an alkaline pH, resulting in a pink colour in the presence of the pH indicator phenolphthalein. When heavy metals are present the above mentioned reaction is inhibited and the mixture remains colourless.

Acetylcholinesterase is an enzyme involved in the transmission of nerve impulses. This enzyme is selectively inhibited by organophosphates and carbamates (Venter, 1990). The reaction is as follows:

Acetylthiocholine ---> (Enzyme) thiocholine + acetic Acid

Thiocholine + Ellman's Reagent (2,2'-dinitro-5-5'-dithiodibenzoic acid) --->
Thiocholine-2-nitro-5-thiobenzoic acid + 2-nitro-5-thiobenzoic acid

The enzyme reaction rate is measured in terms of OD at 405 nm with a spectrophotometer.

The mammalian cell colony formation (cloning efficiency) test has been developed primarily for drinking water toxicity testing (Kfir, 1981), and has proved to be highly sensitive to various chemicals (Table 5). Two different cells are currently employed in the test, namely Buffalo Green monkey (BGM) kidney cells and Chinese hamster V79 cells to compare their sensitivities. Mammalian cell culture techniques are used in other countries (Germany, England, the Netherlands) for water toxicity testing, but other test parameters are utilized.

A mammalian cell culture transformation assay, using hamster embryo cells, is available for

the detection of potential carcinogens (Kfir, 1981) in water. Currently, mammalian cell culture techniques are being developed for the detection of tumour promoters (chemicals which are not carcinogenic on their own, but which can promote tumour formation when exposed to after exposure to a subcritical dose of a carcinogen) in water (Slabbert *et al.*, 1992).

The *Salmonella* mutagenicity assay (Ames test) is carried out according to US EPA (1983; 1985) standard procedures [similar to OECD (1981) procedures]. A modified plate incorporation assay, in which the growth medium is prepared with the test sample, is usually used in the DWT laboratories for drinking water testing (Kfir *et al.*, 1982). Water is tested unconcentrated, and also 2x and 4x concentrated (concentration by means of flash-evaporation). Alternatively, XAD-resin and dichloromethane extracts of water samples can be tested. These are incorporated into the top-agar of plates (Kfir *et al.*, 1982). The *E coli* SOS assay (SOS Chromotest) [Quillardet *et al.*, 1985] which detects DNA damage is being evaluated for use. This is a quick test in which effects are measured in terms of OD. The test is available in kit form. Various other countries are at present evaluating this test for use in the water field (Section 1.2.2.2).

The toad (*Xenopus laevis*) embryo test detects teratogens. A teratogen is defined as any agent capable of causing the formation of congenital anomalies or monstrosities. Thalidomide is a well-known teratogen. The toad embryo test has been used in several research studies in the water field (Genthe and Edge, 1988). A similar test is under evaluation for use in the Netherlands (personal communication). The USA utilizes the sheepshead minnow embryo test for the detection of teratogens in water (US EPA, 1985a).

Development and evaluation of biotoxicity tests are continuing. High on the priority list are chronic *Daphnia/Ceriodaphnia* tests. The establishment of local fish tests also requires attention.

2. TOXICITY TEST METHODOLOGIES

A range of acute and chronic toxicity tests were evaluated for applicability and efficiency, aimed at the development of guidelines for toxicity testing. The acute toxicity tests included: fish and water flea lethality tests; a protozoan oxygen uptake test; algal and bacterial growth inhibition tests; luciferase, urease and acetylcholinesterase enzyme inhibition tests; and a mammalian cell colony formation (cloning efficiency) test. The Ames mutagenicity assay and a toad embryo teratogenicity test were used for chronic toxicity detection.

The fish, water flea and mutagenicity tests were carried out according to standard procedures (US EPA, 1985a,b), and microbial, enzyme, mammalian cell and teratogenicity tests according to procedures developed by the DWT (Section 1.2.2.3.2)

Although the tests are relatively well established, a need for optimization was indicated in certain cases as the study progressed (Conclusions and Recommendations - Section 3.1.3). The modifications carried out to optimize bioassays are summarized in Table 10.

2.1 Test protocols

2.1.1 Fish (*Poecilia reticulata* - guppy) lethality test

Tests were carried out with 1 - 2 weeks old fish. Test conditions are summarized in Table 7.

TABLE 7: Fish bioassay test conditions[1]

Temperature	22°C
Light quality	Laboratory illumination
Photoperiod	Approximately 12 h daylight
Feeding regime	No feeding
Oxygen concentration	As obtained (>40% of saturation)
pH	As obtained
Size of test vessel	500 mℓ
Volume of test sample	350 mℓ
Number of fish/vessel	5
Number of replicate vessels	2
Total number of fish/test	10
Control water	Dechlorinated tap water
Test duration	96 h
Effect measured	% Lethality, calculated in relation to control

[1] According to US EPA (1985a) procedure

2.1.2 Water flea (*Daphnia pulex*) lethality test

Organisms 24 h or less in age were used for toxicity testing. In order to obtain the necessary number of young for a test, adult females bearing embryos in their brood pouches were removed from the stock cultures 24 h preceding the initiation of a test, and placed in 100 mℓ beakers containing 50 mℓ moderately hard water (Table 8) and food suspension (trout chow, alfalfa and yeast).

Test conditions are summarized in Table 9. Test organisms were transferred to a small intermediary holding beaker and from there to the test beakers.

TABLE 8: Moderately hard reconstituted water[1]

Reagent added[2] (mg/ℓ)	$NaHCO_3$	96,0
	$CaSO_4.2H_2O$	60,0
	$MgSO_4$	60,0
	KCl	4,0
Nominal water quality range[3]	pH	7,4 - 7,8 (8,2)
	Hardness[4]	80 - 100 (89)
	Alkalinity	60 - 70 (59)

1 US EPA (1985a)
2 Prepared with deionized water
3 Measured value in parenthesis
4 As mg/ℓ $CaCO_3$

TABLE 9: Water flea bioassay test conditions[1]

Temperature	22°C
Light quality	Laboratory illumination
Photoperiod	Approximately 12 h daylight
Feeding regime	No feeding
Oxygen concentration	As obtained (>40% of saturation)
pH	As obtained
Size of test beaker	50 mℓ
Volume of test sample	25 mℓ
Number of organisms/beaker	5
Number of replicate beakers	4
Total number of organisms/test	20
Control water	Moderately hard water
Test duration	48 h
Effect measured	% Lethality (no movement of body or appendages on gentle prodding), calculated in relation to control

1 According to US EPA (1985a) procedure

2.1.3 Protozoan (*Tetrahymena pyriformis*) oxygen uptake assay

T. pyriformis strain W was cultured in proteose peptone broth at 27°C for 18 h. For bioassaying, cells were suspended in an osmotically balanced salt solution using gravity filtration and suspended to a concentration yielding an oxygen uptake rate of 6 - 8%/min (Slabbert and Morgan, 1982).

Tests were carried out with a modified biological oxygen monitoring system (Yellow Springs Instrument Co, Yellow Springs, OH) consisting of an electronic unit, modified to accommodate four oxygen probes, and a self-designed bath assembly with four test chambers (to carry out four tests simultaneously), each fitted with a standard oxygen probe. The electronic unit was

connected to a data-logger which produced a printout of the percentage dissolved oxygen in each test chamber at 15 sec intervals (unpublished data). During bioassaying the monitoring system, cell suspension and test samples were maintained at 27°C using a constant temperature water circulator.

For each set of tests 3 mℓ of the cell suspension was aerated for 5 min in the test chambers provided with magnetic stirrers (Slabbert and Morgan, 1982; Slabbert, 1988). Dissolved oxygen was then monitored continuously before (reference), during and after test sample addition, for a period of approximately 10 min. Test samples (3 mℓ) were introduced after a monitoring period of between 3 and 4 min. Sterile (autoclaved) deionized water was used for control testing. Each test and control was carried out in triplicate. Results were determined as a ratio of the oxygen uptake rate after sample addition (test slope) to that prior to sample addition (reference slope) (Slabbert, 1988). Effects are expressed as percentage inhibition (or stimulation), calculated in relation to control tests.

2.1.4 Algal (*Selenastrum capricornutum*) growth inhibition test

The unicellular alga *S. capricornutum* was maintained axenically in Erlenmeyer flasks according to standard procedures (US EPA, 1978). Culturing was carried out in a constant temperature room at 22°C, without shaking, using continuous illumination. Algae were subcultured weekly to have a constant supply of logarithmic growth phase cells for bioassaying. Two different media, namely algal assay medium (AAM 30%) (US EPA, 1971) and 10% modified BG-11 (Rippka *et al.*, 1979), were used for culture maintenance and toxicity testing.

Toxicity tests were carried out in five-fold in sterile 24-well tissue culture plates (Slabbert and Hilner, 1990). The algal suspension was prepared by removing the supernatant medium and resuspending cells in fresh medium to a concentration yielding an optical density (OD) of 0,02 (2 - 6 x 10^5 cells/mℓ). OD readings were carried out on a microplate reader at 450 nm. The algal suspension was added at a ratio of 1:1 to a 20-times concentrate of the culture medium and used as 200 $\mu\ell$ volumes for inoculation of 1,8 mℓ sample in test wells (well volume: 3,5 mℓ). Sterile (autoclaved) deionized water was used for control testing. Single wells which received sample and medium only, were used for blanking of the microplate reader. Plates were covered with lids and incubated for 48 or 72 h at 22°C under continuous illumination. At the end of the incubation period cells were re-suspended. Three hundred microlitres of the cell suspension were removed from each well and transferred to sterile microplate wells (96-well flat-bottomed microplates) for OD readings. Results are expressed as percentage inhibition (or stimulation), calculated as follows:

$$100\% - \left[\frac{ODT - OD_0}{ODC - OD_0} \times 100\%\right]$$

where

ODC = Optical density of control
ODT = Optical density of test
OD_0 = Optical density at time 0

2.1.5 Bacterial (*Pseudomonas putida*) growth inhibition assay

Tests were carried out in minimal medium in 50 mℓ medical flats (Slabbert, 1988). A culture of *P. putida* strain Berlin 33/2, grown overnight at 27°C, was diluted with fresh minimal medium to an OD of 0,8, 30 min before inoculation of test samples (Slabbert, 1986). OD measurements were carried out spectrophotometrically at 600 nm. The cell suspension was added at a ratio of 1:4 to a 12,5-times concentrate of the minimal medium, and used as 2,5 mℓ volumes for inoculation of 22,5 mℓ test samples. Each test and control was carried out in five-fold. Sterile (autoclaved) deionized water was used for control testing. Cultures were incubated at 27 °C for 6 h. Growth was measured spectrophotometrically at 600 nm. Effects are expressed as percentage inhibition (or stimulation), determined in relation to control results.

2.1.6 Luciferase enzyme inhibition test

A commercially available crude enzyme extract, consisting of luciferase and its substrate luciferin (firefly lantern extract - Sigma), was used for toxicity testing. An enzyme stock solution of 40 mg/mℓ) was prepared by reconstituting the freeze-dried extract with sterile deionized water. The freeze-dried adenosine triphosphate (ATP) was reconstituted with 5 mℓ sterile deionized water. The preparations were kept on ice during testing and stored at 4°C.

The enzyme stock solution was diluted with sterile (autoclaved) dechlorinated tap water to a concentration providing an initial light level of approximately 2 000 mV (milli volt) (working solution) just before each series of toxicity tests (10 - 30 µℓ enzyme/mℓ tap water). The working solution was introduced as 500 µℓ aliquots into cuvettes containing 500 µℓ test sample, and mixed well. After an incubation period of 15 min at room temperature 50 µℓ of the ATP solution was added to the water-enzyme mixture and mixed well. The light produced by the ATP firefly reaction was then (within 10 sec of ATP addition) monitored for a period of 20 sec with a standard luminometer. Luminescence readings were printed out by the instrument at 1 sec intervals. Initial light level and the decay rate (slope) were used for calculation of results. Each test and control was carried out in triplicate. Sterile dechlorinated tap water was used as control. Effects are expressed as percentage inhibition (stimulation), calculated in relation to control tests.

2.1.7 Urease enzyme inhibition test

Three enzyme concentrations (0,5; 1,0; and 2,0 mg/mℓ) were used to detect different levels of heavy metal pollution (Metelerkamp, 1986). The test was found to be the most sensitive at the 0,5 mg/mℓ enzyme concentration. At this concentration the enzyme detects a number of metals at the recommended limits for drinking water (Kempster and Smith, 1985).

Tests were carried out in 96-well microplates. Samples were added in 200 µℓ volumes to 50 µℓ enzyme. An exposure period of 30 min at room temperature (20°C) was allowed. Urea (substrate) and phenolphthalein (pH indicator) were then added consecutively (50 µℓ each), and 15 min were allowed for enzyme-substrate interaction. Each test and control was carried out in triplicate. Deionized water served as control.

The enzyme interacts with the substrate to form ammonia. This causes an alkaline pH, resulting in a dark pink colour development in the presence of phenolphthalein. Heavy metals inhibit this reaction and the mixture remains colourless. Results are determined qualitatively

by colour observation and reported as follows:

Total inhibition:	colourless	+++
Moderate inhibition:	very light pink	++
Slight inhibition:	light pink	+
No inhibition:	pink	-

2.1.8 Acetylcholinesterase enzyme inhibition test

For each test, 1,9 mℓ of test sample, 200 $\mu\ell$ of potassium phosphate buffer (0,5 M), and 100 $\mu\ell$ of enzyme solution [200 μg enzyme (1 enzyme unit)/mℓ 0,05 M potassium phosphate buffer] were added consecutively to a cuvette, mixed and kept at 37°C (Venter, 1990). After an incubation period of 15 min, 100 $\mu\ell$ of 10 mM Ellman's reagent was added to the reaction mixture, followed by 100 $\mu\ell$ of substrate (30 mM S-acetylthiocholiniodide). After a further incubation period of 1 min, the enzyme reaction rate was monitored by recording the OD of the mixtures (measured at 28 sec intervals) for a 2 min period with a spectrophotometer at 405 nm. Each test and control was carried out in triplicate. Deionized water was used as control. The enzyme reaction rate (slope) was used to calculate results. Results are expressed as percentage inhibition/stimulation, calculated in relation to control tests.

2.1.9 Mammalian cell colony formation test

Two different mammalian cell lines were used for toxicity testing, namely Buffalo green monkey (BGM) kidney and Chinese hamster V79. These cell lines were cultured in Dulbecco's modified Eagle medium (DME) with 10% foetal calf serum (FCS) and in minimal essential medium (MEM) with 5% FCS, respectively. Cultures were maintained in 250 mℓ flasks at 37°C in a humidified incubator supplied with a constant flow of 7% carbon dioxide in air. Before reaching the confluent stage cells were trypsinized and recultured in fresh medium in culture flasks (Kfir, 1982). At this growth stage cells were also used for toxicity tests.

Toxicity tests were carried out in 6-plate culture dishes (diameter: 35 mm). Two hundred cells were seeded per plate in 4,0 mℓ medium. After approximately 18 h incubation at 37°C, the medium was removed from each plate and replaced with fresh medium prepared with test samples. Fresh medium prepared with deionized water was used as control. Each test and control was carried out in six-fold. Plates were incubated for a further 5 to 7 days. At the end of the incubation period the medium was removed. The plates were washed with phosphate buffer solution and colonies were fixed with methanol and stained with Giemsa stain (Kfir, 1982). Colonies of cells were counted under a dissection microscope at a magnification of 25x. Results are expressed as percentage colony formation inhibition calculated according to the following formula:

$$100\% - \left[\frac{\text{Average number of colonies on treated plates}}{\text{Average number of colonies on controlplates}} \times 100\%\right]$$

2.1.10 Ames *Salmonella* mutagenicity assay

Mutagenicity was tested by means of a plate incorporation assay (Kfir *et al.*, 1982). *Salmonella typhimurium* tester strains, TA98 and TA100, were used. TA98 detects frame shift

mutagens whereas TA100 detects base-pair substitution mutagens. Tests were carried out with and without S9 liver preparation (used for metabolic activation of chemicals which would otherwise be non-mutagenic). Water samples were either unconcentrated or concentrated 2- and 4-fold by means of flash evaporation. The concentration of samples was found to improve the test sensitivity. Each test was carried out in triplicate. Results are expressed as mutation ratios, calculated as follows:

$$\text{Mutation ratio} = \left[\frac{\text{Number of mutants per test sample}}{\text{Number of spontaneous mutants}}\right]$$

2.1.11 Toad (*Xenopus laevis* - African clawed toad) embryo teratogenicity test

Three days before testing three pairs of toads were given a primer injection (100 $\mu\ell$) of Human Chorionic Gonadotrophin (HGC) to stimulate fertility (Genthe and Edge, 1988). After 48 to 72 h the toads received a HGC booster injection (females: 300 $\mu\ell$; males: 100 $\mu\ell$) and were transferred in pairs to spawning tanks. After fertilization (18 h later), eggs were removed from the spawning tanks and transferred in batches of 50 or 100 (depending on the total number of fertilized eggs) to 500 $m\ell$ glass containers with 200 $m\ell$ test sample. Tests were carried out at 22 °C, in duplicate. Dechlorinated tap water was used for control testing. After 3 to 5 days the developing embryos were counted and examined under a dissection microscope for abnormalities. Test embryos were compared to control embryos. Features examined for malformations were embryo development (size and length), pigmentation, head shape, and form of spines and tails.

2.2 Data analysis

The results of tests were interpreted by means of detection limits. The detection limits specified in the standard protocols were used for the fish, water flea and mutagenicity tests. A lethality ≥10% was taken as an indication of toxicity in the case of the fish and water flea tests. For a test to be valid, a lethality of <10% is specified for control tests (this means 1 out of 20 organisms can be killed). Because of limited sources only 10 fish per sample (in some instances only 5 fish) could be used. When one of the fish in the control died (10% lethality) tests were not considered to be valid. In order to allow for 1 death/control test (10% lethality), a detection limit of >10% was applied during the last part of the study (groundwater and surface water testing). In the case of the mutagenicity test a mutation ratio ≥2 indicated mutagenic activity.

Detection limits for the microbial, enzyme, and mammalian cell toxicity tests, and the toad embryo teratogenicity test, calculated as percentage inhibition/stimulation/lethality/ deformation, were selected on the basis of the reproducibility of the tests. The reproducibility of the microbial, enzyme and mammalian cell tests was established by calculating the coefficient of variation (CV) of the controls of each set of tests:

$$CV = \left[\frac{\text{Standard deviation}}{\text{Average}} \times 100\right]$$

In addition, another method, *i.e.* Student's t-test (Clarke, 1969), was used to establish whether test and control results differed significantly at the P = 0,05 level. The two methods used for evaluation of the results were compared in order to test the validity of the results obtained and to make recommendations on procedures for data analysis. The reproducibility of the toad embryo teratogenicity test was established by calculating the averages and standard deviations of the controls of different sets of tests.

TABLE 10: Summary of modifications made to bioassays as study progressed

Bioassay	Modification
Fish test	Temperature for maintenance and toxicity testing increased from 22°C to 25°C
Water flea test	Temperature for maintenance and toxicity testing decreased from 22°C to 20°C
Protozoan oxygen uptake test	Modified electronic unit accommodating four oxygen probes replaced by standard equipment which was originally used for technique development (Slabbert and Morgan, 1982; Slabbert, 1988). Because repetitive testing is very time-consuming only one test was carried out per sample. Control tests were carried out in triplicate
Algal growth test	Maintenance and toxicity testing carried out at a constant temperature of 25° (instead of 22°C). Lights were positioned in such a way to have a constant illumination of approximately 95 $\mu E/m^2/s$. Microscopic counting was used in addition to density measurement to standardize the inoculum concentration (200 000 cells/mℓ). The initial density ranged from 0,005 to 0,012. Because of differences in well volumes of different batches of microplates, the 300 $\mu\ell$ of cell suspension used for OD readings were changed to 280 $\mu\ell$ to avoid contact with the probe
Bacterial growth test	Cell suspensions with densities of between 0,8 and 0,95 (600 nm) were used for inoculation. Sample and test preparation was carried out in a constant temperature room at 20°C. The incubation temperature was increased from 27°C to 28°C
Luciferase enzyme test	The enzyme was used at a slightly lower concentration with an initial light level of approximately 1 000 mV (instead of 2 000 mV). The ATP solution was diluted by 50% before use and introduced as 100 $\mu\ell$ quantities (instead of 50 $\mu\ell$) into the enzyme-sample mixture to reduce variation due to sample volume
Urease enzyme test	The assay was carried out in a constant temperature room at 25°C. Enzyme activity was established by means of spectrophotometric measurement with a microplate reader at 450 nm. Effects are expressed as percentage inhibition (or stimulation), determined in relation to control results. Because of differences in well volumes of different batches of microplates, the volumes used in the test were changed to avoid contact with the probe: 160 $\mu\ell$ sample; 40 $\mu\ell$ enzyme; 40 $\mu\ell$ urea and 40 $\mu\ell$ phenolphthalein
Mutagenicity test	Flash evaporation was replaced by XAD resin extraction to concentrate water. The acetone extracts were incorporated into the top-agar (100 $\mu\ell$/2 mℓ agar) following standard protocol (Kfir *et al.*, 1982; Maron and Ames, 1983). Acetone was used as additional control testing
Teratogenicity test	A fixed number of eggs, namely 100, were used per test. Eggs were placed in duplicate containers, each containing 50 eggs. An exposure period of 48 h was used. Temperature for maintenance and testing increased from 22°C to 25°

3. APPLICATION AND EVALUATION OF BIOASSAYS

3.1 Drinking water

3.1.1 Water samples

Water samples were collected from the following locations:

Schoemansville Water Works;
Rietvlei Dam Water Works;
Parys Water Works;
Klipgat area north of Pretoria; and
Apies River.

The raw water source, treated water before chlorination, and final drinking water from the first three sampling locations were tested. The Klipgat samples included water from the Sand River (raw source) and a seepage well (filtered water) on the river-bank. Two raw water samples were collected from the Apies River, namely, south of Pretoria North and south of Onderstepoort. Each location was sampled twice (periods: July - September; and October - November 1991).

The pH of all the water samples and free and total chlorine levels in final drinking water were measured on site. The measured chlorine levels were confirmed upon arrival of the samples at the laboratory using a titration technique. Immediately after receipt final drinking water samples were dechlorinated (except samples used for mutagenicity testing). Usually free chlorine in water samples was reduced by means of aeration to non-toxic levels (<0,2 mg/ℓ). However, this procedure delayed testing by 24 to 48 h. The addition of sodium thiosulphate to water samples to neutralize free chlorine was, therefore, investigated. The Schoemansville and Rietvlei Dam final drinking water samples collected during the July - September sampling period, were dechlorinated using both procedures to compare results. All the samples collected thereafter were neutralized with sodium thiosulphate (concentration: 20 mg/ℓ). Samples were stored at 4°C before testing.

The water samples used for the microbial and mammalian cell toxicity tests were sterilized by filtration through a 0,22 µm membrane filter (samples in this form could be stored for a period of time). The samples tested in the *Salmonella* mutagenicity test were used to prepare the nutrient agar plates. The agar mixture was autoclaved before plates were poured. Plates prepared with deionized water were used for control testing. For the mammalian cell toxicity tests, tissue culture media were prepared with each water sample (100 mℓ) by adding DME (BGM cells) or MEM (V79 cells) medium powder (DME medium: 1,36 g DME + 0,37 g $NaHCO_3$; MEM medium: 0,97 g MEM + 0,2 g $NaHCO_3$) and antibiotics (0,5 mℓ antibiotic-antimycotic). The medium was decontaminated by filtration through a 0,22µm membrane filter (fitted on a syringe). Control media were prepared in a similar way using deionized water.

3.1.2 Results and discussion

The pH values of the water samples ranged between 6,2 and 8,5, which were within the limits required for aquatic animal tests. The microbial, enzyme and mammalian cell toxicity tests were carried out in media or buffer solutions, which ensured that the pH of test samples were adjusted to optimum pH. The total and free chlorine in final drinking water ranged between

1 and 2,1 mg/ℓ, and 0,5 and 2,1 mg/ℓ, respectively. Aeration and thiosulphate addition reduced the free chlorine levels to ≤0,2 mg/ℓ.

3.1.2.1 Fish and water flea tests

Table 11 presents the results of fish and water flea tests. The water flea test did not detect toxicity in any of the water samples (lethality: <10%). Nine water samples caused lethality with the fish test. The effects were slight ranging from 10 to 20% lethality. The Rietvlei Dam samples of 05-08-1991 were tested using 5 fish per sample. The aerated final drinking water sample resulted in a 20% lethality. However, due to the small number of fish used, the reliability of this result is in doubt. Problems were experienced with a fungal disease during the August sampling period, resulting in fish lethality in controls. The results given for Parys (27-08-1991) were established only after a number of tests.

3.1.2.2 Microbial, enzyme and mammalian cell toxicity tests

The reproducibility (precision) of the different microbial, enzyme and mammalian cell toxicity bioassays is shown in Table 12. The bacterial growth inhibition test was the most reproducible with an average coefficient of variation (CV) of 4,6%, with the algal growth inhibition tests being the least reproducible, with average CV's of 12,0% and 13,0% for tests carried out with BG-11 and AAM growth medium, respectively. The protozoan oxygen uptake test, the bacterial growth inhibition test, the luciferase enzyme test (using immediate luminescence as parameter), the acetylcholinesterase enzyme test, and the V79 mammalian cell colony formation test, generally showed CV's <10% (>70% of results). As a result a 10% change in activity/function was selected as the detection limit for each of these bioassays.

The CV's of the algal growth inhibition tests, the luciferase (decay rate) enzyme test, and the BGM mammalian cell colony formation test, were mostly >10%. For these bioassays a detection limit of 20% was selected. The detection limits selected for bacterial and algal tests were in agreement with those recommended in previous studies (Slabbert, 1988; Slabbert and Hilner, 1990). The protozoan oxygen uptake test carried out with the modified oxygen monitoring apparatus proved to be less reproducible (CV: 10%) than when tests were carried out with the standard equipment (CV: 5%) (Slabbert, 1988).

Table 13 presents the control values obtained for the bioassays. A fair amount of variation occurred between control results. Variations like these might affect the sensitivity of bioassays, and where necessary, attention should be given to standardize tests. On a few occasions very low control growth was obtained with algae, even though a further incubation period was allowed (density readings of 0,08 to 0,1 should be aimed at). It has been demonstrated that enzyme tests are more sensitive if low enzyme concentrations are used for toxicity testing (Metelerkamp, 1986). It is expected that the luciferase test might show a considerable increase in sensitivity if the enzyme concentration is reduced to have a maximum light level of 1 000 mV for the control. A comparison between immediate luminescence values and decay rate showed a linear relation (correlation coefficient: 0,92). Although 200 cells were seeded in the mammalian cell colony formation assay, not all the cells will attach to plates. The average cloning efficiency (number of cells seeded on control plates/number of cells developing into colonies x 100%) for the BGM and V79 cell assays were 47,5% (range: 24%-61%) and 80,5% (range: 62%-102%), respectively.

The results of the microbial toxicity tests are presented in Table 14. Percentage inhibition/

TABLE 11: Effect of raw and treated drinking water samples on fish and water flea

Sampling location	Date	Sample	Fish test % Lethality after exposure time:				Water flea test % Lethality after exposure time:	
			24 h	48 h	72 h	96 h	24 h	48 h
Schoemansville	29-07-1991	Raw water - Dam	0	0	0	10	0	0
		Pre-chlorinated water	0	10	10	10	0	0
		Final water - aerated	10	20	20	20	0	0
		Final water - neutralized	0	0	0	10	0	0
	30-09-1991	Raw water - Dam	0	0	0	0	0	0
		Pre-chlorinated water	0	10	10	10	0	0
		Final water - neutralized	0	0	0	0	0	0
Rietvlei Dam	05-08-1991	Raw water - Dam	0	0	0	0	0	0
		Pre-chlorinated water	0	0	0	0	0	0
		Final water - aerated	0	0	0	20	0	0
		Final water - neutralized	0	0	0	0	0	0
	07-10-1991	Raw water - Dam	0	0	0	0	0	0
		Pre-chlorinated water	0	0	0	0	0	0
		Final water - neutralized	0	0	0	0	0	0
Parys	27-08-1991	Raw water - River	0	0	0	0	0	0
		Pre-chlorinated water	0	0	0	0	0	0
		Final water - neutralized	0	0	0	0	0	0
	28-10-1991	Raw water - River	0	0	0	0	0	0
		Pre-chlorinated water	0	0	0	0	0	0
		Final water - neutralized	0	0	0	0	0	0
Klipgat	17-09-1991	Raw water - River	0	0	0	0	0	0
		Seepage well water	0	0	0	10	0	0
	06-11-1991	Raw water - River	0	0	0	0	0	0
		Seepage well water	0	0	0	0	0	0
Apies River	17-09-1991	Raw water 1 - River	0	0	0	10	0	0
		Raw water 2 - River	0	0	0	10	0	0
	06-11-1991	Raw water 1 - River	0	0	0	0	0	0
		Raw water 2 - River	0	0	0	0	0	0

TABLE 12: Reproducibility of microbial, enzyme and mammalian cell toxicity tests

	Protozoan oxygen uptake test	Algal growth test		Bacterial growth test	Luciferase enzyme test		Acetyl-cholin-esterase enzyme test	Mammalian cell colony formation test	
		AAM growth medium	BG-11 growth medium		Immediate lumines-cence	Lumines-cence decay rate		BGM cells	V79 cells
CV's[1] for controls of all sets of samples tested (%)	1; 3; 4; 6; 7; 8; 8; 9; 19; 20	7; 8; 11; 12; 15; 25	5; 6; 8; 12; 16; 25	1; 1; 1; 2; 2; 2; 3; 4; 5; 5; 6; 6; 8; 8; 15	2; 3; 4; 6; 6; 6; 8; 9; 11; 12; 16	1; 2; 8; 9; 11; 11; 12; 14; 16; 16; 17; 24	0; 1; 2; 2; 2; 3; 3; 3; 3; 3; 5; 5; 6; 7; 7; 7; 8; 9; 10; 10; 10; 11; 13; 13; 15	3; 4; 6; 6; 6; 7; 7; 9; 11; 12; 13; 14; 14; 17; 19	3; 3; 4; 4; 5; 5; 6; 7; 7; 7; 7; 7; 8; 14; 14; 17
Average CV ± SD[2] (%)	8,5 ± 6,3	13,0 ± 6,5	12,0 ± 7,6	4,6 ± 3,8	7,6 ± 4,2	11,8 ± 6,4	6,3 ± 4,2	9,9 ± 4,8	7,4 ± 4,1

1 Coefficient of variation
2 Standard deviation

TABLE 13: Control results of different bioassays

	Protozoan oxygen uptake test (ratio of test to reference slope)	Algal growth test (OD)		Bacterial growth test (OD)	Luciferase enzyme test (light level/slope)		Acetyl-cholin-esterase enzyme test (slope)	Mammalian cell colony formation test (number of cells)	
		AAM growth medium	BG-11 growth medium		Imme-diate lumines-cence	Lumines-cence decay rate		BGM cells	V79 cells
Average control values	0,67	0,051	0,04	0,455	3274	11,71	0,237	95	181
Control value range	0,59-0,79	0,030-0,069	0,019-0,052	0,323-0,835	834-4857	2,9-17,4	0,139-0,412	48-122	124-204

stimulation, as well as the significance of results, are given. The oxygen uptake test detected toxicity in 4 water samples (inhibition: ≥10%), the algal growth test using AAM medium in 10 samples, the algal test using BG-11 medium in 3 samples (inhibition: ≥20%), and the bacterial growth test in 5 samples (inhibition: ≥10%). Oxygen uptake inhibition was slight, ranging from 10 to 15%, and bacterial growth inhibition ranged from slight (10%) to moderate (23%). In general, high levels of inhibition were obtained for algal growth, varying between 22 and 100%. The three samples showing toxicity towards algae using BG-11 growth medium, were also toxic to algae grown in AAM medium. It was expected that the two algal tests would give similar results, because of their similar sensitivities. The differences observed could be attributed to problems experienced with low growth (standardization needed). A large number of samples stimulated microbial activity (oxygen uptake: 11 samples; algal growth AAM medium: 15 samples; algal growth BG-11 medium: 18 samples; and bacterial growth: 16 samples), indicating the presence of nutrients in the waters. It can be seen in Table 14 that those bioassays showing toxicity/presence of nutrients when applying detection limits (protozoa and bacteria: 10% and algae: 20%), were not always significant when applying Student's t-test. On the other hand, some results below detection limits, and interpreted as an absence of toxicity/nutrients, were significant. Table 15 compares the two methods of result interpretation. A large percentage of the results were in agreement (64 - 86%). Conversely, a small percentage of the results showed an absence of toxicity/stimulation using detection limits, while Students t-test proved the effect to be significant (protozoa: 0%; algae - AAM medium: 4%; algae - BG-11 medium: 3%; and bacteria: 14%). A relatively large number of the results of the protozoan test and the algal test using AAM medium showed toxicity or stimulation when detection limits were applied, while the t-test indicated that effects were not significant (36 and 25%, respectively). These differences are due to the large variation between repetitive tests (test and control) in certain cases, rendering a negative result with the t-test. The large variation in the control results of the oxygen uptake test occurred because tests were carried out consecutively and not simultaneously as with the other bioassays (acetylcholinesterase tests were also carried out consecutively). In the case of the algal test using BG-11 medium and the bacterial test, only a small percentage of the results showed toxicity or stimulation while the t-test was negative.

None of the water samples inhibited luciferase or urease enzyme activity, indicating an absence of toxicity (Table 16). The negative results obtained with the urease test indicated

TABLE 14: Effect of raw and treated drinking water samples on protozoa, algae and bacteria (+ indicates stimulation)

Sampling location	Date	Sample	Protozoan oxygen uptake test		Algal growth test				Bacterial growth test	
			% Inhibition	Result significant (y/n)	AAM growth medium		BG-11 growth medium		% Inhibition	Result significant (y/n)
					% Inhibition	Result significant (y/n)	% Inhibition	Result significant (y/n)		
Schoemans-ville	29-07-1991	Raw water - Dam	10	n	22	y	+43	y	+24	y
		Pre-chlorinated water	10	n	62	y	+49	y	+25	y
		Final water - aerated	+5	n	51	y	10	n	+12	n
		Final water - neutralized	15	y	33	y	13	y	+7	n
	30-09-1991	Raw water - Dam	+8	n	+2	n	+8	n	+33	y
		Pre-chlorinated water	0	n	31	y	31	y	+38	y
		Final water - neutralized	2	n	+3	n	+22	y	+8	y
Rietvlei Dam	05-08-1991	Raw water - Dam	5	n	+21	n	+259	n	10	y
		Pre-chlorinated water	9	n	70	n	100	n	23	y
		Final water - aerated	5	n	+42	n	+83	n	10	n
		Final water - neutralized	13	n	54	n	+9	n	6	y
	07-10-1991	Raw water - Dam	+21	y	+45	y	+35	y	+39	y
		Pre-chlorinated water	+17	n	+51	y	+8	n	+46	y
		Final water - neutralized	+17	y	+52	y	+4	n	+11	y
Parys	27-08-1991	Raw water - River	3	n	79	n	+188	y	+16	y
		Pre-chlorinated water	2	n	93	n	+239	y	+11	y
		Final water - neutralized	8	n	+114	y	+596	y	+24	y
	28-10-1991	Raw water - River	+11	n	+20	n	+85	y	+4	y
		Pre-chlorinated water	+17	n	+46	y	+78	y	4	y
		Final water - neutralized	+8	n	42	y	50	y	0	n
Klipgat	17-09-1991	Raw water - River	+8	n	+69	y	+116	y	19	y
		Seepage well water	8	n	17	y	+7	n	+14	y
	06-11-1991	Raw water - River	+12	y	+86	y	+85	y	+8	n
		Seepage well water	+19	n	+82	y	+30	y	+28	y
Apies River	17-09-1991	Raw water 1 - River	+10	n	+209	y	+359	y	+11	y
		Raw water 2 - River	+10	n	+48	y	+92	y	11	y
	06-11-1991	Raw water 1 - River	+14	y	+151	y	+179	y	+31	y
		Raw water 2 - River	+16	n	+65	y	+42	y	+28	y

TABLE 15: Comparison between results calculated as percentage effect and Student's t-test

	Protozoan oxygen uptake test	Algal growth test		Bacterial growth test	Luciferase enzyme test		Acetyl-cholin-esterase test	Mammalian cell colony formation test	
		AAM growth medium	BG-11 growth medium		Immediate lumines-cence	Lumines-cence decay rate		BGM cells	V79 cells
Results of Student's t-test and percentage effect calculated in agreement[1]	64%	71%	86%	79%	96%	92%	92%	93%	82%
Result significant but effect below detection limit[2]	0%	4%	3%	14%	0%	0%	4%	7%	7%
Result not significant but effect above detection limit[2]	36%	25%	11%	7%	4%	8%	4%	0%	11%

[1] Calculated effect ≥ detection limit = result significant; calculated effect < detection limit = result not significant

[2] Detection limit for algal, luciferase (decay rate) and BGM mammalian cell test: 20%; Detection limit for all other tests: 10%

TABLE 16: Effect of raw and treated drinking water samples on enzyme tests (+ indicates stimulation with luciferase and acetylcholinesterase tests)

Sampling location	Date	Sample	Luciferase enzyme test [(-) - No results]				Urease enzyme test			Acetylcholinesterase enzyme test	
			Immediate luminescence		Luminescence decay rate		Inhibition (+); No effect (-)				
			% Inhibition	Result significant (y/n)	% Inhibition	Result significant (y/n)	2,0 mg/ml enzyme	1,0 mg/ml enzyme	0,5 mg/ml enzyme	% Inhibition	Result significant (y/n)
Schoe-mansville	29-07-1991	Raw water - Dam	+82	y	+100	y	-	-	-	0	n
		Pre-chlorinated water	+88	y	+45	y	-	-	-	7	n
		Final water - aerated	+45	y	+12	n	-	-	-	+1	n
		Final water - neutralized	+50	y	+77	y	-	-	-	10	y
	30-09-1991	Raw water - Dam	+84	y	+89	y	-	-	-	+19	y
		Pre-chlorinated water	+52	y	+63	y	-	-	-	+8	n
		Final water - neutralized	+24	y	+41	y	-	-	-	12	y
Rietvlei Dam	05-08-1991	Raw water - Dam	+124	y	+141	y	-	-	-	+34	y
		Pre-chlorinated water	+145	y	+196	y	-	-	-	+15	y
		Final water - aerated	+116	y	+188	y	-	-	-	+19	y
		Final water - neutralized	+137	y	+124	y	-	-	-	+18	y
	07-10-1991	Raw water - Dam	+38	y	+39	n	-	-	-	+6	n
		Pre-chlorinated water	+85	y	+93	y	-	-	-	+1	n
		Final water - neutralized	+103	y	+87	y	-	-	-	+8	n
Parys	27-08-1991	Raw water - River	-	-	-	-	-	-	-	0	n
		Pre-chlorinated water	-	-	-	-	-	-	-	+8	n
		Final water - neutralized	-	-	-	-	-	-	-	+5	n
	28-10-1991	Raw water - River	+129	y	+115	y	-	-	-	+17	n
		Pre-chlorinated water	+65	y	7	n	-	-	-	+13	y
		Final water - neutralized	+102	y	+95	y	-	-	-	12	y
Klipgat	17-09-1991	Raw water - River	+11	n	+51	y	-	-	-	1	n
		Seepage well water	+120	y	+185	y	-	-	-	1	n
	08-11-1991	Raw water - River	+25	y	+27	n	-	-	-	+2	n
		Seepage well water	+72	y	+104	y	-	-	-	8	y
Apies River	17-09-1991	Raw water 1 - River	+108	y	+183	y	-	-	-	+20	y
		Raw water 2 - River	+85	y	+153	y	-	-	-	+19	y
	08-11-1991	Raw water 1 - River	+62	y	+117	y	-	-	-	4	n
		Raw water 2 - River	+46	y	+114	y	-	-	-	0	n

that heavy metals were present at non-toxic levels. Three samples were toxic to acetylcholincholinesterase (inhibition: ≥10%). Effects were slight and ranged from 10 to 12%. In all instances the toxicity was caused by final water neutralized with thiosulphate, suggesting that the enzyme was possibly sensitive to this chemical. It is unlikely that the effects were due to pesticides, since similar effects would have been observed with the pre-chlorinated samples. Nine samples stimulated acetylcholinesterase activity, and almost all samples stimulated luciferase activity. The increase in enzyme activity is possibly due to changes in enzyme conformation (structure), resulting in more binding sites. It is not clear which chemicals in the water caused these effects, and the role of toxic chemicals cannot be ruled out. In general, similar results were obtained with the luciferase enzyme test, whether immediate luminescence or decay rate was used. The results of the luciferase and acetylcholinesterase tests were also evaluated using Student's t-test. Table 16 indicates in which instances effects were positive (toxicity/stimulation), and when results of Student's t-test were in agreement with these findings. Between 92 and 96% of the results calculated by means of detection limits were in agreement with the results of the t-test (Table 15). None of the results of the luciferase test and only 4% of that of the acetylcholinesterase test were significant when no toxicity/stimulation was detected using detection limits. Furthermore, only a small percentage of the results showed toxicity/stimulation (luciferase - immediate luminescence: 4%; luciferase - decay rate: 8%; and acetylcholinesterase: 4%).

The effect of raw and treated water samples on mammalian cells is shown in Table 17. Nine samples were toxic to BGM cells (lethality: ≥20%) and 3 to V79 cells (lethality ≥10%). In general, effects on BGM cells ranged between 20 and 36%. On one occasion (Schoemansville raw water - 30-09-1991) a lethality of 51% was detected. The effects on V79 cells ranged from slight (10%) to moderate (27%). Two of the samples showing toxicity towards V79 cells, were also toxic to BGM cells. Certain samples stimulated colony formation (BGM: 6 samples; V79: 5 samples), indicating that the water contained growth promoters. Table 17 indicates which samples tested positive for toxicity/growth promoters, and in which instances these results were also significant. The results of Table 15 show that with both cell lines, more than 80% of the results which were positive when using detection limits were also significant, and less than 10% of the samples which were negative showed significance. In the case of V79 cells 11% of the samples were not significant but showed effects ≥10%.

3.1.2.3 Ames *Salmonella* mutagenicity test

Control plates contained between 19 and 68 colonies in the case of tester strain TA98 and between 149 and 304 colonies in case of tester strain TA100. No mutagenic activity was observed (Table 18) with either tester strains, with and without metabolic activation, except for the following raw water samples: Rietvlei Dam - 07-10-1991; Klipgat - 06-11-1991; and Apies River - 06-11-1991. In some instances concentration of the water samples resulted in increased MR values, but MR values were still <2, indicating no significant mutagenic activity (except for the 3 samples mentioned).

The Rietvlei Dam raw water showed marginal mutagenicity (MR = 2,0) on 4x concentration without metabolic activation, using tester strain TA98. Unconcentrated, as well as 2x and 4x concentrated Klipgat raw water displayed mutagenic activity, using tester strain TA98 in the presence of liver extract. Concentration resulted in increased MR values (1x: 2,3; 2x: 2,6; and 4x: 3,8). The unconcentrated Apies River water showed slight mutagenicity (MR: 2,1), while the effect of the 4x concentrate was larger (MR: 2,6), using TA98 with metabolic activation. No effect was observed with the 2x concentrate. Levels of mutagenic activity between 2,0 and

TABLE 17: Effect of raw and treated drinking water samples on mammalian cells (+ indicates stimulation)

Sampling location	Date	Sample	Buffalo green monkey (BGM) cells		Chinese hamster V79 cells	
			% Inhibition	Result significant (y/n)	% Inhibition	Result significant (y/n)
Schoemansville	20-07-1991	Raw water - Dam	+5	n	2	n
		Pre-chlorinated water	9	n	6	n
		Final water - aerated	25	y	27	y
		Final water - neutralized	4	n	0	n
	30-09-1991	Raw water - Dam	51	y	6	n
		Pre-chlorinated water	+21	y	0	n
		Final water - neutralized	+20	y	+4	n
Rietvlei Dam	05-08-1991	Raw water - Dam	+83	y	8	n
		Pre-chlorinated water	+80	y	10	n
		Final water - aerated	+70	y	+7	n
		Final water - neutralized	+69	y	+11	y
	07-10-1991	Raw water - Dam	+17	y	+2	n
		Pre-chlorinated water	+14	n	+16	y
		Final water - neutralized	+2	n	+9	y
Parys	27-08-1991	Raw water - River	+10	y	+6	n
		Pre-chlorinated water	+5	n	+26	y
		Final water - neutralized	0	n	+26	y
	28-10-1991	Raw water - River	+18	n	7	n
		Pre-chlorinated water	+1	n	5	n
		Final water - neutralized	14	n	4	n
Klipgat	17-09-1991	Raw water - River	28	y	+10	n
		Seepage well water	34	y	11	n
	06-11-1991	Raw water - River	36	y	4	n
		Seepage well water	22	y	+3	n
Apies River	17-09-1991	Raw water 1 - River	32	y	+3	n
		Raw water 2 - River	5	n	+4	n
	06-11-1991	Raw water 1 - River	24	y	+9	y
		Raw water 2 - River	20	y	+2	n

TABLE 18: Effect of raw and treated drinking water on *Salmonella* tester strains

Sampling location	Date	Sample	Concentration	Tester strains			
				TA98-S9	TA98+S9	TA100-S9	TA100+S9
				Mutation ratio			
Schoemansville	29-07-1991	Raw water - Dam	1x	0,7	0,7	0,7	0,6
			2x	1,0	0,7	0,8	0,7
			4x	0,8	0,6	1,1	0,9
		Pre-chlorinated water	1x	0,8	0,6	1,1	0,7
			2x	0,8	0,7	0,8	0,6
			4x	1,2	0,5	1,0	0,6
		Final water - chlorinated	1x	0,8	0,7	0,8	0,6
			2x	0,8	0,7	0,8	0,6
			4x	0,8	0,7	1,0	0,6
Rietvlei Dam	05-08-1991	Raw water - Dam	1x	0,7	0,8	0,8	1,1
			2x	0,8	0,5	0,7	0,9
			4x	0,8	0,7	0,8	1,2
		Pre-chlorinated water	1x	1,1	0,9	0,9	1,2
			2x	0,9	0,9	0,7	1,4
			4x	0,9	1,0	0,6	1,2
		Final water - chlorinated	1x	0,7	0,8	0,7	1,3
			2x	0,9	0,9	0,8	0,9
			4x	1,1	0,8	1,2	1,3
Parys	27-08-1991	Raw water - River	1x	1,0	0,8	1,1	0,7
			2x	1,2	0,9	0,9	1,0
			4x	1,0	0,9	1,3	1,2
		Pre-chlorinated water	1x	1,3	0,7	1,3	1,2
			2x	1,1	0,9	1,2	0,8
			4x	1,0	0,7	1,3	1,3
		Final water - chlorinated	1x	0,8	0,8	0,8	0,7
			2x	1,4	0,8	0,8	0,8
			4x	1,7	0,9	1,2	1,0
Klipgat	17-09-1991	Raw water - River	1x	1,3	0,7	0,6	0,8
			2x	1,1	0,9	0,7	0,7
			4x	1,7	0,9	0,6	0,5
		Seepage well water	1x	1,1	0,7	0,8	0,7
			2x	1,6	0,9	0,7	0,9
			4x	1,8	0,9	0,9	0,9
Apies River	17-09-1991	Raw water 1 - River	1x	1,1	0,5	0,5	0,7
			2x	1,3	0,9	0,8	1,0
			4x	1,7	0,8	0,8	1,2
		Raw water 2 - River	1x	1,2	0,6	0,6	0,1
			2x	1,6	1,0	0,8	0,9
			4x	1,3	0,9	0,7	1,1

TABLE 18: Effect of raw and treated drinking water on *Salmonella* tester strains (Continue)

Sampling location	Date	Sample	Concentration	Tester strains			
				TA98-S9	TA98+S9	TA100-S9	TA100+S9
				Mutation ratio			
Schoemansville	30-09-1991	Raw water - Dam	1x	1,5	0,9	1,0	1,0
			2x	1,3	0,9	1,3	1,2
			4x	1,6	1,1	1,4	1,1
		Pre-chlorinated water	1x	1,3	1,1	1,1	1,3
			2x	1,5	1,2	0,8	1,0
			4x	1,4	1,0	1,0	1,3
		Final water - chlorinated	1x	1,1	1,2	1,1	1,0
			2x	1,3	1,1	1,1	0,8
			4x	1,4	1,2	1,3	1,0
Rietvlei Dam	07-10-1991	Raw water - Dam	1x	1,5	0,9	1,0	1,3
			2x	1,8	1,2	1,2	1,3
			4x	**2,0**	1,2	1,2	1,4
		Pre-chlorinated water	1x	1,7	1,0	1,1	0,9
			2x	1,7	1,1	0,9	0,8
			4x	1,3	1,0	1,2	1,1
		Final water - chlorinated	1x	1,2	0,9	1,0	-
			2x	1,7	1,1	1,1	-
			4x	1,6	0,8	1,1	-
Parys	28-10-1991	Raw water - River	1x	1,0	0,5	0,9	0,5
			2x	1,0	0,8	1,1	0,4
			4x	0,8	0,8	1,4	1,2
		Pre-chlorinated water	1x	0,9	0,7	1,0	0,7
			2x	0,7	0,6	1,0	0,8
			4x	0,8	1,0	1,2	1,4
		Final water - chlorinated	1x	0,6	0,7	1,1	0,9
			2x	0,6	0,7	1,1	0,9
			4x	0,8	0,8	0,9	0,5
Klipgat	06-11-1991	Raw water - River	1x	0,9	**2,3**	0,6	0,6
			2x	0,8	**2,5**	0,9	0,8
			4x	0,8	**3,8**	1,0	1,1
		Seepage well water	1x	0,8	1,2	0,6	0,7
			2x	0,9	1,0	0,9	1,0
			4x	1,1	1,7	1,0	1,1
Apies River	06-11-1991	Raw water 1 - River	1x	1,0	1,7	0,5	0,5
			2x	0,9	1,9	0,8	0,8
			4x	1,1	1,7	1,0	1,0
		Raw water 2 - River	1x	1,1	**2,1**	0,6	0,6
			2x	0,9	1,9	0,7	0,7
			4x	1,2	**2,6**	0,9	0,9

- No growth

Positive results in bold

3,8 were recorded occasionally in conventionally treated water in other studies.

3.1.2.4 Toad embryo teratogenicity test

Problems were experienced with this bioassay. Although three pairs of toads were injected, the production of toad eggs could not always be guaranteed. In some instances eggs were produced, but they were not always fertilized. For a large part of the first sampling period no eggs were produced. The only samples tested during this period were Schoemansville and Rietvlei Dam.

Two sets of tests were carried out with the first Schoemansville and Rietvlei Dam samples. Each set used eggs from a different pair of toads (two pairs of toads produced a large number of eggs in each case). Because of the problems with fertilization and egg production experienced, thereafter, eggs from different spawning tanks were pooled for testing.

In general, the percentage of eggs that hatched in control tests ranged from 19 to 38% (Table 19), which indicated low levels of fertility. The only exception was the control of the first set of Schoemansville samples where all eggs hatched.

This bioassay was not well quantified for toxicity testing, as only approximate numbers of eggs were placed in test containers (eggs were not counted). Furthermore, eggs were removed in batches from spawning tanks without proper mixing, and certain batches of eggs could have been less fertilized than others. Because of the variation in numbers of eggs used, a result was interpreted as a toxic effect when ≥50% of the eggs in test containers (compared to controls) did not hatch.

The results of the two sets of Schoemansville (29-07-1991) samples differed. In the first test three samples showed toxicity, while in the second no sample was toxic. On the other hand, similar results were obtained with the two sets of Rietvlei Dam (05-08-1991) tests. In total 7 of the 21 water samples tested showed toxicity.

The number of spontaneous deformities in controls and deformities in water samples were in agreement (0 - 5), indicating an absence of teratogenicity. In general, the type of deformities which occurred in water samples were also similar to those observed in the controls (Table 19).

3.1.2.5 Summary of toxicity test data

The toxicity data obtained with the different bioassays are summarized in Table 20. The water flea, luciferase and urease tests did not detect toxicity in any of the water samples. On the other hand, a relatively large number of samples showed toxicity with the fish, algal AAM medium, and BGM mammalian cell tests. None of the following water samples were toxic: raw, pre-chlorinated, and final Rietvlei Dam water sampled on 07-10-1991; Parys final water sampled on 07-10-1991; and Parys raw and pre-chlorinated water sampled on 28-10-1991. Eight samples showed toxicity with one bioassay only; four samples with two bioassays; five samples with three bioassays; three samples with four bioassays; and two with five bioassays. No specific pattern in the responses and sensitivities of the test systems was observed. For most of the bioassays effects were slight. High toxicity was observed in a number of instances using the algal, BGM mammalian cell, and toad embryo tests.

TABLE 19: Effect of raw and treated drinking water samples on toad embryos

Sampling location	Date	Sample	% Eggs hatched	Number of deformities	Type of deformities
Schoe-mansville	29-07-1991	Control	113	3	underdeveloped, no pigment, curved spine
		Raw water - Dam	32	2	underdeveloped, no pigment
		Pre-chlorinated water	33	2	curved spine
		Final water - aerated	101	2	underdeveloped, curved spine
		Final water - neutralized	31	2	underdeveloped, flattened body, no tails
	29-07-1991	Control	25	2	curved spine
		Raw water - Dam	0	0	
		Pre-chlorinated water	17	2	curved spine
		Final water - aerated	21	2	curved spine, no pigment
		Final water - neutralized	23	2	tails underdeveloped
	30-09-1991	Control	38	0	
		Raw water - Dam	35	0	
		Pre-chlorinated water	41	0	
		Final water - neutralized	40	2	tails curved
Rietvlei Dam	05-08-1991	Control	21	5	underdeveloped, loss of swimming ability
		Raw water - Dam	2	0	
		Pre-chlorinated water	5	0	
		Final water - aerated	3	1	underdeveloped, curved spine
		Final water - neutralized	0	0	
	05-08-1991	Control	19	3	underdeveloped, curved spine and tails
		Raw water - Dam	4	2	underdeveloped
		Pre-chlorinated water	6	2	underdeveloped, tails curved
		Final water - aerated	0	0	
		Final water - neutralized	2	0	
	07-10-1991	Control	38	0	
		Raw water - Dam	25	0	
		Pre-chlorinated water	30	0	
		Final water - neutralized	51	0	
Parys	28-10-1991	Control	38	0	
		Raw water - River	50	0	
		Pre-chlorinated water	59	1	tail underdeveloped
		Final water - neutralized	31	0	
Klipgat	06-11-1991	Control	19	0	
		Raw water - River	19	1	underdeveloped
		Seepage well water	12	0	
Apies River	06-11-1991	Control	19	0	
		Raw water 1 - River	30	0	
		Raw water 2 - River	19	0	

TABLE 20: Raw and treated drinking water exhibiting toxic effects on bioassays (percentage inhibition/lethality)

Sampling location	Date	Sample	Fish test	Water flea test	Proto-zoan test	Algal test		Bacterial test	Luciferase test		Urease test	Acetyl-cholin-esterase test	Mammalian cell test		Toad embryo test
						AAM	BG-11		Lum	Dec			BGM	V79	
Schoemans-ville	29-07-1991	Raw	10		10	22									72
		Pre-chl	10		10	62									70
		Chl aer	20			51							25	27	
		Chl neutr	10		15	33						10			73
	30-09-1991	Raw											51		
		Pre-chl	10			31	31								
		Chl neutr										12			
Rietvlei Dam	05-08-1991	Raw						10							90
		Pre-chl				70	100	23						10	76
		Chl aer	20					10							88
		Chl neutr			13	54									100
	07-10-1991	Raw													
		Pre-chl													
		Chl neutr													
Parys	27-08-1991	Raw				79									
		Pre-chl				83									
		Chl neutr													
	28-10-1991	Raw													
		Pre-chl													
		Chl neutr				42	50					12			
Klipgat	17-09-1991	Raw						19					28		
		Well	10										34	11	
	08-11-1991	Raw											38		
		Well											22		
Apies River	17-09-1991	Raw 1	10										32		
		Raw 2	10					11							
	08-11-1991	Raw 1											24		
		Raw 2											20		

Pre-chl: Pre-chlorination; Chl aer: Chlorination - aeration; Chl neutr: Chlorination - neutralization; Lum: Luminescence; Dec: Decay rate

The adverse effect of the final aerated water on some of the test systems could have been due to traces of free chlorine still present in the water. On the other hand, the effects of the final water neutralized by thiosulphate could be attributed to an excess of the chemical. Chemicals such as aluminium, iron and zinc, present in flocculants, could have been the cause of toxicity in pre-chlorinated water samples. Such chemicals were occasionally present in Windhoek reclaimed water after the flocculation step, when toxicity was detected (CSIR, 1989).

3.1.3 Conclusions and recommendations

With the exception of the water flea, and luciferase and urease enzyme tests, all the acute toxicity tests detected toxicity in one or more of the water samples. In general, the effects on the test systems were slight. However, the algal, mammalian cell, and toad embryo tests showed high levels of toxicity for a number of samples. Toxicity was detected in both raw and treated water. No specific pattern in the responses and sensitivities of the test systems were observed. The results obtained with the urease and acetylcholinesterase enzyme tests indicate that heavy metals and organophosphate and carbamate pesticides were either absent or present at low concentrations. Mutagenicity was detected in three raw water samples, while none of the samples showed teratogenicity.

A good reproducibility was found with most of the tests (CV: <10%). This was found to be in agreement with the precision obtained for tests used in other countries, or even exceeded it.

The good agreement between results calculated as percentage effects and by means of Student's t-test (Table 15) indicates that the use of detection limits could be adequate to decide whether or not effects are significant. In a number of instances detection limits might provide false positive results. However, the possibility that significant effects might not be picked up by using detection limits are limited.

Most of the biological tests applied are well established. However, in certain cases optimization is necessary. For example, the luciferase test was not well evaluated previously. Some changes might be required to enhance the sensitivity of the test, *e.g.* the use of a lower enzyme concentration. The protozoan oxygen uptake test was carried out with the modified oxygen monitoring apparatus which allows for simultaneous testing of samples. However, the test was found to be less reproducible (which reduced the sensitivity level) than when using the standard equipment. This shortcoming might be overcome by optimizing the modified test equipment. Inoculum concentration plays an important role in the sensitivity of microbial tests. Attention should be given to the standardization of these inocula. In general algal growth was low, indicating that parameters such as temperature and light should be optimized. Many of the discrepancies which occurred in the toad embryo test were due to the fact that approximate numbers of eggs were used for tests. It is recommended that a fixed number of eggs be used in tests (50 or 100, depending on availability of eggs). It is also recommended to introduce quantitative determination of urease results using a microplate reader.

Only the Ames *Salmonella* assay is currently used as short-term test to detect potential carcinogens. Although extraction of water samples is costly and time-consuming, a higher sensitivity might be obtained with the Ames test if extracted samples were used.

It is recommended that thiosulphate should be used to neutralize free chlorine in drinking

water in future application, mainly because it does not cause delays in testing as experienced with aeration. The optimum concentration required to neutralize free chlorine should be established to minimize effects on test systems.

Many of the tests showed enhanced activity when exposed to the water samples. This is usually attributed to nutrients in the water. The control of such tests are usually carried out with deionized water. It is recommended to evaluate synthetically prepared water as alternative controls.

The findings of the study indicated that both acute and chronic tests were efficient, and although some modifications are needed the tests were found suitable for the evaluation of water toxicity.

3.2 Ground- and surface water

3.2.1 Water samples

Twelve groundwater samples, six dam/lake water samples and six river water samples were collected. The sample information is summarized in Tables 21 and 22. Each location was sampled once (period: June - November 1992). It was envisaged to collect six groundwater samples from hazardous waste sites/areas. This was, however, not possible as the boreholes on such sites are usually not fitted with pumps, and sampling by hand was not feasible because large volumes of water were required. As an alternative, samples were taken from boreholes where chemical contamination was suspected (*e.g.* industrial area, plant park, near petrol depot and refuse site).

The pH, dissolved oxygen content and temperature of the water samples were measured on site. Samples for chemical analyses were taken in separate containers and preserved as required in standard methods. The chemical data of the different waters are presented in Tables 39 and 40.

Immediately after receipt, the water samples used for the microbial, enzyme and mammalian cell toxicity tests were sterilized by filtration through a 0,22 µm membrane filter. Although sterilization was not necessary for the enzyme tests, it had the advantage that samples could be kept for an extended period without changes in quality, if tests could not be carried out immediately (*e.g.* luciferase test). Initial testing of unfiltered surface water samples with the urease test indicated that particulate matter interfered with density readings and that prefiltration was necessary if samples were not filter sterilized. Test and control media for the mammalian cell tests were prepared according to the procedure described in Section 3.1.1.

Samples which were suspected to be contaminated with latent organophosphates were oxidized before being tested with the acetylcholinesterase enzyme test. For oxidation N-bromosuccinimide was added to the water sample and control.

The samples for the Ames *Salmonella* mutagenicity test were concentrated by means of flash evaporation (2x and 4x) (Section 2.1.10) and XAD resin extraction (US EPA, 1985b). Two methods were followed to prepare the nutrient agar plates with unconcentrated and flash evaporated samples. In the one method the nutrient agar was prepared with the sample and autoclaved as described in Section 3.1.1. In the second method one fifth of the sample was used to prepare the agar which was autoclaved. Four fifths of the sample was used to

TABLE 21: Sample information - groundwater

Sample	Sampling date	Sampling location and use	Physical observation
CSIR	30-06-1992	Southern side of campus - irrigation and maintenance fish ponds	Clear
Wonderboom	01-07-1992	Situated in private garden - irrigation	Clear
Winterveld 1	09-11-1992	Rural area - drinking	Clear
Winterveld 2	09-11-1992	Rural area - drinking	Clear
Moreletta Park	09-11-1992	Situated in private garden - irrigation	Yellow colour - plants and walls stained
Waterkloof	10-11-1992	Situated in private garden - irrigation	Water slightly turbid with small solid particles, very bad rotting smell - bees have nested in the borehole
ISCOR	18-11-1992	Near reservoir, hole in ground about 600 metres from borehole - irrigation and industrial	Clear with black particles, mothball smell
Silverton 1	18-11-1992	On grounds of SPCA, petrol depot across the road - animal use and irrigation	Clear
Derdepoort	18-11-1992	On smallholding between Eersterus refuse site and quarry next to Pietersburg highway - irrigation and drinking	Clear, changes to light brown
Silverton 2	18-11-1992	In private garden close to Moreletta Stream and a garage - drinking and irrigation	Clear
Fochville	22-11-1992	In private garden - irrigation	Clear
Annlin	24-11-1992	Situated in plant park, possible presence of pesticides - irrigation and drinking	Clear

dissolve the nutrients. This solution was then filter sterilized (0,22 μm filter). The two preparations were combined after the agar had cooled down. XAD extracts were prepared by pumping 20 ℓ of sample through a column containing XAD-7 resin. Organic chemicals adsorbed onto the resin were then extracted by means of acetone, which was concentrated to a final volume of 10 mℓ.

All the samples were stored at 4° C before testing.

3.2.2 Results and discussion

3.2.2.1 Fish and water flea tests

Tables 23 and 24 present the results of fish and water flea tests. While control tests were carried out with 10 fish, the majority of test samples were evaluated with 5 fish (information in Tables). This was necessary because of limited numbers of fish. Six of the borehole and three of the surface water samples caused lethality with the fish test (lethality: >10%). With

TABLE 22: Sample information - surface water

Sample	Sampling date	Sampling location	Physical observation
Rietvlei Dam	30-06-1992	At inflow of Hennops River, taken from side	Water looks clean
Bon Accord Dam	03-08-1992	Near dam outlet, taken from side	Water looks clean, crustacea present
Roodeplaat Dam	03-08-1992	5-6 metres from side of jetty - recreational area	Abundance of algae and crustacea
Hartbeespoort Dam	03-08-1992	Taken from side near boat club, 3-4 kilometres from dam wall	High concentrations of algae present. Dead fish on side of dam
Verwoerdburg Lake	29-09-1992	At bridge near outlet	Grey colour, algae present
Lakefield Lake	29-09-1992	Taken from side near bridge - recreational area, Alphen Park, Benoni	Grey colour, algae present
Hennops River	30-06-1992	5 kilometres below discharge point of Kempton Park Sewage Works - middle of stream	Green colour, abundance of algae, sewage and animal dung smell
Moreletta Stream	03-08-1992	North of Silverton, in Waltloo and Eersterus area - middle of stream	Look clean, sewage smell
Jukskei River	29-09-1992	At weir below crushers and close to bridge across old Pretoria Road, Halfway House. Taken from middle of stream	Green colour, contains foam, musty-fishy smell
Illiondale Stream	29-09-1992	Before confluence with stream from Kelvin power station. Sample from middle of stream	Grey colour, contains foam, pesticide/cyanide smell
Pienaars River	23-11-1992	At bridge - old Bronkhorstspruit Road	Water muddy - sampled during a thunder storm
Fountains Stream	24-11-1992	Recreation site - Fountains, just inside fence on southern side. Taken from middle of stream	Water muddy - sampled during a thunder storm

the exception of the Hennops River sample which caused 100% lethality within an exposure period of 24 h, all the effects were small (20% lethality). In instances where only 5 fish were used the significance of a 20% lethality (1/5 fish died) is in doubt. In general, the lethality of control fish was ≤10%, indicating that fish were in good health. However, when tests were carried out on CSIR and Wonderboom groundwater, and Rietvlei Dam water, a control lethality of 30% was observed. These results are, therefore, not valid.

Nine of the borehole samples caused lethality with the water flea test (lethality: ≥10%) (Table 23). Lethality ranged between 15 and 100% after 48 h exposure. Because it was suspected that effects were due to chemicals with a high oxygen demand, tests were repeated on water samples (1 to 3 weeks after sampling) after an aeration period of 4 h. The results (in parenthesis) in Table 23 indicate that toxicity was removed or reduced in five of the nine samples. On the other hand, a larger lethality was observed for two of the samples. Only

TABLE 23: Effect of groundwater on fish[1] and water flea

Sample	Fish test [(-) - No results]				Water flea test	
	% Lethality after exposure time:				% Lethality after exposure time:	
	24 h	48 h	72 h	96 h	24 h	48 h
CSIR	0	0	0	10[2]	0	0
Wonderboom	0	0	0	20[2]	0	0
Winterveld 1	0	0	0	0	100 (0)	100 (90)
Winterveld 2	0	0	0	20	100 (0)	100 (100)
Moreletta Park	0	0	0	0	0 (0)	0 (0)
Waterkloof	20	20	20	20	40 (0)	40 (0)
ISCOR	0	0	20	20	85 (0)	100 (45)
Silverton 1	0	0	0	0	5 (0)	25 (5)
Derdepoort	0	0	0	0	10 (0)	75 (95)
Silverton 2	0	0	0	0	5 (0)	15 (0)
Fochville	0	0	-	20	80 (0)	80 (35)
Annlin	0	0	-	20	15 (0)	40 (95)

[1] Control, CSIR and Wonderboom samples: 10 fish; Other samples: 5 fish

[2] Control lethality: 30%

Results of repeat tests on samples aerated for 4 h in parenthesis

TABLE 24: Effect of surface water on fish[1] and water flea

Sample	Fish test [(-) - No results]				Water flea test	
	% Lethality after exposure time:				% Lethality after exposure time:	
	24 h	48 h	72 h	96 h	24 h	48 h
Rietvlei Dam*	0	0	20	20[2]	0	0
Bon Accord Dam*	0	0	0	0	0	0
Roodeplaat Dam*	0	0	0	0	0	0
Hartbeespoort Dam*	0	0	0	0	0	0
Verwoerdburg Lake	0	0	0	0	0	0
Lakefield Lake	0	0	0	0	0	0
Hennops River*	100	100	100	100[2]	0	0
Moreletta Stream*	0	0	0	0	0	0
Jukskei River	0	0	0	0	0	0
Illiondale Stream	0	0	0	20	100	100
Pienaars River	0	0	-	0	0	0
Fountains Stream	0	0	-	0	0	0

1 Control and samples marked *: 10 fish; Other samples: 5 fish
2 Control lethality: 30%

one of the surface water samples, namely the Illiondale Stream water, was toxic to water flea, causing 100% lethality within 24 h.

3.2.2.2 Microbial, enzyme and mammalian cell toxicity tests

The reproducibility (precision) of the different microbial, enzyme and mammalian cell toxicity bioassays is shown in Table 25. The protozoan oxygen uptake test was the most reproducible with an average CV of 1,9%, and the BGM mammalian cell colony formation (cloning efficiency) test and luciferase enzyme inhibition test based on decay rate the least reproducible, with CV's of 11,0 and 11,7%, respectively. A very good reproducibility was also found with the algal and bacterial growth inhibition tests and the urease and acetylcholinesterase enzyme inhibition tests, with CV's ranging between 4,0 and 5,8%. A comparison with the results obtained when testing drinking water (Section 3.1.2.2) showed a large improvement in reproducibility for the protozoan oxygen uptake (8,5 to 1,9%) and the algal growth inhibition test (AAM: 13,0 to 4,0%; BG-11: 12,0 to 5,8%). The results of the protozoan test improved drastically because the standard equipment was used. The improvement in the algal test reproducibility was due to modifications in test conditions (Table 10). The CV's of the other tests were in agreement with those obtained in Section 3.1.2.2. CV's of the protozoan test were always <5%, therefore a 5% change in oxygen uptake rate was selected as the detection limit for this bioassay. The algal and bacterial growth inhibition tests, the luciferase (based on immediate luminescence), urease and acetylcholinesterase enzyme tests, and the V79 mammalian cell cloning efficiency test generally showed CV's <10% (luciferase test: 64% of results; all other tests ≥80% of results). For these tests a 10% change in activity/function was selected as the detection limit. The CV's of the luciferase (decay rate) enzyme test and the BGM mammalian cell cloning efficiency test, were mostly >10% (luciferase test: 64% of results; mammalian cell test: 80% of results). A detection limit of 20% was selected for these bioassays. With the exception of the algal test (Slabbert and Hilner, 1990), the selected detection limits were in agreement with those recommended previously (Slabbert, 1988; Section 3.1.2.2).

Table 26 presents the control values obtained for the bioassays. Very little variation is noted between the control results of the protozoan oxygen uptake test, which is in agreement with the findings of previous studies when standard equipment was used (Slabbert, 1988). The control values were lower than when the modified equipment (Section 2.1.3) was used (0,495 versus 0,67) (Tables 13 and 26), probably because the modified equipment was not properly standardized against the standard equipment. Compared with the previous study there was a great improvement in algal growth. In general, a better growth was obtained with the BG-11 medium. Although every effort was made to control the growth, a fair amount of variation still occurred. Initially growth was measured after 48 h incubation, which resulted in the desired control values of between 0,08 and 0,10. Suddenly problems were experienced to obtain the same growth. As a result, plates were incubated for 72 h, hence the very high values. In some instances relatively large variations were noted between the controls of different plates. This is an indication that the illumination was not uniform. Although a few high and low control values were obtained with the bacterial growth test, the majority of values ranged between the envisaged density of 0,350 and 0,450. The reduction in variation between control values was achieved by density measurement of overnight cultures to use a standard inoculum. Attempts were made to standardize the concentration of the luciferase enzyme test to have a maximum light level of 1 000 mV for the control. This was, however, not easy to achieve. The light level ranged between 333 and 1980 mV, which was a considerable improvement on the range of values obtained in the previous evaluation (Section 3.1.2.2, Table 13). A slight

TABLE 25: Reproducibility of microbial, enzyme and mammalian cell toxicity tests

	Protozoan oxygen uptake test	Algal growth test		Bacterial growth test	Luciferase enzyme test		Urease enzyme test			Acetyl-cholin-esterase enzyme test	Mammalian cell cloning efficiency test	
		AAM growth medium	BG-11 growth medium		Immediate lumines-cence	Lumines-cence decay rate	0,5 mg/ml enzyme	1,0 mg/ml enzyme	2,0 mg/ml enzyme		BGM cells	V79 cells
CV's[1] for controls of samples tested (%)	1; 1; 1; 2; 2; 2; 2; 2; 2; 4; 4	2; 2; 2; 2; 3; 3; 3; 3; 3; 3; 4; 4; 4; 4; 5; 5; 5; 6; 7; 7; 9	2; 2; 2; 3; 3; 4; 4; 4; 4; 6; 6; 6; 6; 6; 7; 8; 9; 9; 9; 10; 10; 11	1; 2; 3; 3; 4; 4; 4; 4; 5; 5; 6; 6; 6; 6; 7; 7; 7; 8; 10; 10	4; 4; 5; 5; 6; 6; 6; 10; 11; 13; 15	7; 7; 9; 9; 10; 10; 11; 15; 16; 17; 18	0; 1; 2; 2; 3; 3; 3; 3; 3; 3; 3; 3; 4; 4; 4; 4; 4; 5; 6; 6; 7; 7; 8; 8; 9	0; 1; 1; 1; 1; 1; 1; 1; 2; 2; 2; 3; 3; 3; 3; 4; 4; 5; 5; 6; 7; 7; 7; 10; 10	0; 1; 1; 1; 1; 1; 1; 2; 2; 2; 2; 2; 2; 2; 2; 3; 4; 4; 5; 6; 6; 8; 11; 16	0; 2; 2; 2; 2; 2; 2; 2; 2; 2; 3; 3; 3; 4; 5; 6; 8; 8; 8; 9; 10	5; 10; 10; 13; 17	4; 5; 6; 9; 16
Average CV ± SD[2] (%)	1,9±0,8	4,0±1,0	5,8±2,9	5,5±2,4	8,1±3,3	11,7±4,1	4,2±2,3	3,6±2,9	3,6±3,7	4,1±2,9	11,0±4,4	8,0±4,9

1 Coefficient of variation
2 Standard deviation

TABLE 26: Control results of microbial, enzyme and mammalian cell tests

	Protozoan oxygen uptake test (ratio of test to reference slope)	Algal growth test (OD)		Bacterial growth test (OD)	Luciferase enzyme test (light level/slope)		Urease enzyme test (OD)			Acetyl-cholin-esterase enzyme test (OD)	Mammalian cell cloning efficiency test (number of cells)	
		AAM growth medium	BG-11 growth medium		Immediate lumines-cence	Lumines-cence decay rate	0,5 mg/ml enzyme	1,0 mg/ml enzyme	2,0 mg/ml enzyme		BGM cells	V79 cells
Range of controls	0,485; 0,486; 0,490; 0,491; 0,491; 0,492; 0,494; 0,496; 0,498; 0,501; 0,507; 0,508	0,045; 0,052; 0,069; 0,080; 0,084; 0,086; 0,090; 0,091; 0,092; 0,092; 0,094; 0,098; 0,101; 0,101; 0,103; 0,105; 0,107; 0,112; 0,129; 0,032; 0,166; 0,172; 0,173	0,046; 0,050; 0,056; 0,063; 0,074; 0,074; 0,080; 0,082; 0,097; 0,102; 0,106; 0,109; 0,115; 0,131; 0,132; 0,146; 0,164; 0,167; 0,172; 0,173; 0,176; 0,192; 0,193	0,293; 0,295; 0,325; 0,336; 0,349; 0,351; 0,354; 0,357; 0,363; 0,369; 0,371; 0,372; 0,387; 0,407; 0,424; 0,425; 0,450; 0,472; 0,510; 0,551; 0,568	333; 522; 1003; 1370; 1387; 1582; 1605; 1731; 1904; 1956; 1980	2,144; 3,335; 5,956; 6,155; 7,406; 9,879; 10,036; 10,915; 10,968; 11,218; 11,505	0,027; 0,034; 0,035; 0,035; 0,036; 0,038; 0,038; 0,040; 0,046; 0,055; 0,056; 0,058; 0,067; 0,069; 0,074; 0,074; 0,077; 0,077; 0,078; 0,080; 0,081; 0,082; 0,083; 0,084; 0,084	0,045; 0,052; 0,053; 0,053; 0,054; 0,054; 0,057; 0,062; 0,062; 0,070; 0,071; 0,075; 0,078; 0,080; 0,080; 0,084; 0,086; 0,087; 0,088; 0,091; 0,092; 0,095; 0,096; 0,102; 0,104	0,060; 0,062; 0,063; 0,066; 0,066; 0,068; 0,069; 0,069; 0,070; 0,074; 0,074; 0,080; 0,080; 0,088; 0,088; 0,092; 0,093; 0,094; 0,095; 0,096; 0,101; 0,101; 0,107; 0,111; 0,118	0,155; 0,180; 0,199; 0,229; 0,238; 0,251; 0,257; 0,265; 0,275; 0,279; 0,279; 0,281; 0,282; 0,286; 0,289; 0,291; 0,299; 0,300; 0,309; 0,312; 0,319	83; 106; 118; 120; 138	109; 129; 137; 139; 143
Average control ± standard deviation (SD)	0,495 ±0,007	0,103 ±0,033	0,118 ±0,048	0,397 ±0,077	1398 ±562	8,138 ±3,338	0,060 ±0,020	0,075 ±0,018	0,084 ±0,017	0,266 ±0,044	113±20	131±14

improvement in the variation in luminescence decay rate was also observed (value range: 2,144-11,505 as compared to 2,9-17,4). A comparison between immediate luminescence and decay rate showed a linear relation (correlation coefficient: 0,93). The control values of the urease enzyme test showed a relatively large variation, indicating that attention should be given to standardization. The cloning efficiency (number of cells seeded on control plates/number of cells developing into colonies x 100%) for the BGM and V79 cell assays were 56,5±10% and 65,5±7%, respectively. Compared with the results obtained in Section 3.1.2.2, the variation between control results of the acetylcholinesterase enzyme test and the mammalian cell tests were considerably reduced.

The results of the microbial toxicity tests are presented in Tables 27 and 28. Percentage inhibition/stimulation, as well as the significance of results, are given. None of the groundwater samples inhibited protozoan oxygen uptake (Table 27). The algal growth test using AAM medium showed toxicity with eight groundwater samples, while the test using BG-11 medium detected toxicity in four samples (inhibition: ≥10%). Six of the groundwater samples were toxic to bacteria (inhibition: ≥10%). Algal and bacterial growth inhibition ranged from low (algae: 15%; bacteria: 18%) to high (algae: 87%; bacteria: 94%). The oxygen uptake test detected toxicity in one surface water sample (inhibition: ≥5%) (Table 28), the algal growth test using AAM medium, in two samples, the algal test using BG-11 medium, in three samples, and the bacterial growth test in one sample. Bacterial growth inhibition was moderate (25%) and oxygen uptake inhibition relatively high (51%). Adverse effects on algal growth ranged from moderate (23%) to high (98%). The four groundwater samples showing toxicity towards algae when using BG-11 medium, were also toxic to algae grown in AAM medium, while the two samples showing toxicity to algae when using AAM medium were also toxic in the BG-11 medium. It was expected that the two algal tests would give similar results, because of their similar sensitivities. The differences in sensitivity could be due to the lower growth experienced in AAM medium.

Some of the groundwater samples stimulated microbial activity (oxygen uptake: 4 samples; algal growth - AAM medium: 2 samples; algal growth - BG-11 medium: 4 samples; bacterial growth: 4 samples), indicating the presence of nutrients in the water. Only one of the surface water samples stimulated oxygen uptake, while a large number of the samples stimulated growth (algal growth - AAM medium: 8 samples; algal growth - BG-11 medium: 7 samples; bacterial growth: 6 samples). Stimulation in oxygen uptake was small (5 - 15%). Stimulation in algal growth ranged from 10 to 70% and that of bacterial growth from 11 to 113%. Three of the groundwater samples caused precipitation in the bacterial growth test which interfered with the interpretation of results (densities: 0,038 - 0,468). Effects after subtraction of the background density are given in parenthesis (Table 27). Four of the surface water samples caused low precipitation (0,011-0,015) in the algal tests (Table 28). One of the samples also showed a high precipitation in the bacterial growth test (density: 0,470). The effect after subtraction of the background density is shown in parenthesis.

It can be seen in Tables 27 and 28 that those bioassays showing toxicity/presence of nutrients when applying detection limits were not always significant when applying Student's t-test. On the other hand, some results below detection limits, and interpreted as an absence of toxicity/nutrients, were significant. Table 29 compares the two methods of result interpretation. A large percentage of the results were in agreement (87,5 - 96%). In contrast to this, a small percentage of the results showed an absence of toxicity/stimulation using detection limits, while Students t-test proved the effect to be significant (algae - AAM medium: 4%; algae - BG-11 medium: 13,5%; and bacteria: 13,5%). None of the tests showed toxicity or stimulation

TABLE 27: Effect of groundwater on protozoa, algae and bacteria (+ indicates stimulation)

Sample	Protozoan oxygen uptake test	Algal growth test				Bacterial growth test	
	% Inhibition	AAM growth medium		BG-11 growth medium		% Inhibition	Result significant (y/n)
		% Inhibition	Result significant (y/n)	% Inhibition	Result significant (y/n)		
CSIR	+2	+12	y	+43	y	+9	y
Wonderboom	+4	+3	n	+36	y	+21	y
Winterveld 1	+5	+14	y	+7	y	93	y
Winterveld 2	+15	87	y	46	y	53	y
Moreletta Park	+1	54	y	45	y	94	y
Waterkloof	+8	4	n	+11	y	83 (91)	y
ISCOR	+7	52	y	48	y	39	y
Silverton 1	1	72	y	+7	y	+189 (+67)	y
Derdepoort	+1	72	y	22	y	18	y
Silverton 2	+4	15	y	+1	n	+94 (+73)	y
Fochville	+2	41	y	+5	n	+113	y
Annlin	2	41	y	+17	y	6	n

Results after subtraction of background density in parenthesis

TABLE 28: Effect of surface water on protozoa, algae and bacteria (+ indicates stimulation)

Sample	Protozoan oxygen uptake test	Algal growth test				Bacterial growth test	
		AAM growth medium		BG-11 growth medium			
	% Inhibition	% Inhibition	Result significant (y/n)	% Inhibition	Result significant (y/n)	% Inhibition	Result significant (y/n)
Rietvlei Dam	2	+54[1]	y	+48[1]	y	+19	y
Bon Accord Dam	+7	+10	y	+53	y	25	y
Roodeplaat Dam	1	+15	y	+70	y	4	n
Hartbeespoort Dam	3	+11	y	+66	y	7	n
Verwoerdburg Lake	1	+11	y	+70	y	7	y
Lakefield Lake	2	+44	y	+46	y	+24	y
Hennops River	1	34[1]	y	37[1]	y	+29	y
Moreletta Stream	2	+17	y	+6	y	+4	n
Jukskei River	2	+4[1]	y	+28[1]	y	+49 (80)	y
Illiondale Stream	51	98[1]	y	84[1]	y	8	y
Pienaars River	+1	1	n	23	y	+68	y
Fontains Stream	+4	+26	y	2	n	+11	y

[1] Precipitation
Results after subtraction of background density in parenthesis

TABLE 29: Comparison between results calculated as percentage effect and Student's t-test

	Algal growth test		Bacterial growth test	Luciferase enzyme test		Urease enzyme test			Acetyl-cholin-esterase test	Mammalian cell cloning efficiency test	
	AAM growth medium	BG-11 growth medium		Imme-diate lumines-cence	Lumines-cence decay rate	0,5 mg/mℓ enzyme	1,0 mg/mℓ enzyme	2,0 mg/mℓ enzyme		BGM cells	V79 cells
Results of Student's t-test and effect calculated in agree-ment[1]	96%	87,5%	87,5%	91%	87%	79%	75%	71%	79%	87,5%	92%
Result significant but effect below detection limit[2]	4%	13,5%	13,5%	0%	0%	4%	8%	8%	17%	12,5%	0%
Result not significant but effect above detection limit[2]	0%	0%	0%	9%	13%	17%	17%	21%	4%	0%	8%

[1] Calculated effect ≥ detection limit = result significant; calculated effect < detection limit = result not significant

[2] Detection limit for luciferase test (decay rate) and BGM mammalian cell test: 20%; Detection limit for all other tests: 10%

when the t-test was negative. The t-test was not applied to the results of the oxygen uptake test because in most instances replicate tests were not carried out on test samples.

Three of the groundwater samples inhibited the luciferase enzyme (Table 30). The effects were detected with both immediate luminescence and decay rate measurement (immediate luminescence: ≥ 10%; decay rate: ≥20%). Urease enzyme activity was inhibited by three samples at the 0,5 mg/mℓ concentration (inhibition at all three concentrations: ≥10%). Two of these samples also inhibited the enzyme at the 1,0 mg/mℓ level. One of the groundwater samples inhibited the enzyme at the 1,0 mg/mℓ concentration, while no effect was detected at the 0,5 mg/mℓ concentration. No inhibition was detected at the 2,0 mg/mℓ concentration. The acetylcholinesterase enzyme was not inhibited by any of the groundwater samples (inhibition <10%).

Three of the samples which showed toxicity with other bioassays (Winterveld 1 and 2, and Moreletta Park) were oxidized for the detection of latent organophosphates. However, these results were also negative (results given in parenthesis), ruling out the presence of this group of pesticides. Four surface water samples showed toxicity with the luciferase enzyme test when using immediate luminescence (Table 31). Only three of these inhibited the enzyme when measuring decay rate. The urease enzyme was inhibited by three surface water samples when using an enzyme concentration of 0,5 mg/mℓ. Only two of the samples inhibited the enzyme at the 1,0 mg/mℓ concentration and none at the 2,0 mg/mℓ concentration. None of the surface water samples inhibited the acetylcholinesterase enzyme when tested directly. However, after oxidation the Illiondale Stream sample was toxic (67% inhibition), indicating the presence of a latent organophosphate/s. The effects of ground- and surface waters on the luciferase enzyme test ranged from moderate (immediate luminescence: 24%; decay rate: 23%) to high (immediate luminescence: 99%; decay rate: 95%). The results in tables 30 and 32 show that the urease enzyme test was the most sensitive when using 0,5 mg/mℓ enzyme. Inhibition was relatively small and ranged from 11 to 34%, and from 13 to 20%, with 0,5 and 1,0 mg/mℓ enzyme, respectively. The positive results obtained with the urease enzyme test is an indication of heavy metal pollution. In general, a good agreement was found between the quantitative (density) and qualitative (colour observation) determination of urease enzyme activity. However, in a few instances density measurement showed slight inhibition, while this could not be detected by the eye. In instances where precipitation occurred, colour observation was a very useful attribute to the test.

Enzyme activity was stimulated by a number of the samples (Tables 30 and 31). Fourteen of the samples stimulated luciferase enzyme activity. The effects were particularly large with the groundwater samples. A larger number of surface water samples (0,5 mg/mℓ: 7; 1,0 mg/mℓ: 6; 2,0 mg/mℓ: 10) than groundwater samples (0,5 mg/mℓ: 4; 1,0 mg/mℓ: 1; 2,0 mg/mℓ: 8) stimulated urease enzyme activity. The acetylcholinesterase was stimulated by only two groundwater samples and three surface water samples. The increase in enzyme activity is possibly due to changes in enzyme conformation (structure), resulting in more binding sites. It is not clear which chemicals in the water caused these effects, and the role of toxic chemicals cannot be ruled out. In the case of the urease test, the higher density values could also have been due to interaction between the enzyme/reagents and salts in the water resulting in some degree of precipitation. As particulate material can result in increased density readings falsely indicating stimulation, it is important to prefilter water samples. When the effect of deionized water and tap water/buffer/synthetic fresh water on the luciferase enzyme test was compared during previous studies, it was found that luminescence was considerably lower in water other than deionized water. This effect was contributed to the

TABLE 30: Effect of groundwater on enzyme tests (+ indicates stimulation)

Sample	Luciferase enzyme test				Urease enzyme test						Acetylcholinesterase enzyme test	
	Immediate luminescence		Luminescence decay rate		0,5 mg/m*l* enzyme		1,0 mg/m*l* enzyme		2,0 mg/m*l* enzyme		% inhibition	Result significant (y/n)
	% inhibition	Result significant (y/n)	% inhibition	Result significant (y/n)	% inhibition	Result significant (y/n)	% inhibition	Result significant (y/n)	% inhibition	Result significant (y/n)		
CSIR	+283	y	+417	y	+15	n	9	n	+22	n	0	n
Wonderboom	+179	y	+264	y	+21	y	+6	n	+14	n	1	n
Winterveld 1	+337	y	+257	y	11	n	+6	n	+17	y	5	n
Winterveld 2	6	n	0	n	26	y	19	y	+6	y	+12	n
Moreletta Park	+219	y	+212	y	+22	y	+4	y	+8	n	+5	n
Waterkloof	+517	y	+514	y	+107	y	+44	y	+22	y	+1	n
ISCOR	+88	y	2	n	5	n	2	n	+5	y	2	n
Silverton 1	94	y	92	y	6	n	6	y	+14	y	2	n
Derdepoort	81	n	78	y	4	n	+3	n	+10	y	8	y
Silverton 2	24	n	23	n	4	n	4	n	+8	y	+2	n
Fochville	5	n	28	n	9	n	10	n	+13	n	+24	y
Annlin	+26	n	+60	y	21	y	20	y	+10	y	+7	y

TABLE 31: Effect of surface water on enzyme tests (+ indicates stimulation)

Sample	Luciferase enzyme test [(-) - No results]				Urease enzyme test						Acetylcholinesterase enzyme test	
	Immediate luminescence		Luminescence decay rate		0,5 mg/m*l* enzyme		1,0 mg/m*l* enzyme		2,0 mg/m*l* enzyme		% inhibition	Result significant (y/n)
	% inhibition	Result significant (y/n)	% inhibition	Result significant (y/n)	% inhibition	Result significant (y/n)	% inhibition	Result significant (y/n)	% inhibition	Result significant (y/n)		
Rietvlei Dam	+25	y	+50	y	+33	y	+25	n	+32	y	+4	n
Bon Accord Dam	+70	y	+104	y	+13	y	+12	n	+16	n	+2	n
Roodeplaat Dam	+201	y	+315	y	+1	n	+1	n	+16	n	+7	y
Hartbees-poort Dam	+161	y	+235	y	+10	y	+13	n	+29	y	+5	n
Verwoerd-burg Lake	+21	y	+89	y	19	y	13	n	+9	n	8	n
Lakefield Lake	+93	y	+141	y	+14	y	+14	y	+14	y	1	n
Hennops River	-	-	-	-	+23	y	+20	y	+31	y	+7	y
Moreletta Stream	+77	y	+120	y	+11	n	+5	n	+15	y	+8	n
Jukskei River	52	y	23	n	+33	y	+25	y	+37	y	+13	y
Illiondale Stream	46	y	40	y	34	y	+3	n	+28	y	+34 (67)	y
Pienaers River	34	y	3	n	30	n	17	y	+7	n	+15	y
Fountains Stream	99	y	95	y	9	y	1	n	+16	y	6	n

Result after oxidation in parenthesis

interaction of the normal salts in water with the enzyme. After extensive experimentation tap water was selected as control for this test (enzyme also prepared with tap water). Although the luciferase enzyme test is meant to indicate the presence of toxicants when the activity is lower in test samples than in the tap water, it could well be that the lower activity found in this study was due to high salt concentrations rather than to toxicants. Likewise, the increased luminescence could have been due to low salt concentrations (in comparison to the control).

The results of the enzyme tests were also evaluated using Student's t-test. Tables 30 and 31 indicate in which instances effects were positive (toxicity/stimulation), and when results of Student's t-test were in agreement with these findings. Between 71 and 91% of the results calculated by means of detection limits were in agreement with the results of the t-test (Table 29). None of the results of the luciferase test, 4 to 8% of the results of the urease test, and 17% of the results of the acetylcholinesterase test were significant when no toxicity/stimulation was detected using detection limits. A relatively large number of the results of the urease test showed toxicity/stimulation while the t-test was negative (5 mg/mℓ: 17%; 1,0 mg/mℓ: 17%; and 2,0 mg/mℓ: 21%). These differences could be due to the large variation between repetitive tests in certain cases. On the other hand, a small percentage of the results of the luciferase and the acetylcholinesterase test results showed toxicity/stimulation, while the t-test indicated that effects were not significant (luciferase - immediate luminescence: 9%; luciferase - decay rate: 13%; and acetylcholinesterase: 4%).

The effects of ground- and surface water samples on mammalian cells are shown in Tables 32 and 33. Three groundwater and six surface water samples were toxic to BGM cells (inhibition: ≥20%). The effects ranged between 21 and 71%. V79 cell cloning efficiency was inhibited by four groundwater samples and one surface water sample (inhibition: ≥10%). The effects of three of the groundwater samples were small (11-14 %). Contrary to this, a high inhibition was detected with the other groundwater sample and the surface water sample (76 and 96%, respectively). Three of the samples showing toxicity towards V79 cells, were also toxic to BGM cells. A few of the samples (5) stimulated the cloning efficiency of V79 cells, indicating that the water contained growth promoters. Tables 32 and 33 indicate which samples tested positive for toxicity/growth promoters, and in which instances these results were also significant. The results of Table 29 show that more than 85% of the results of the mammalian cell tests, which were positive when using detection limits, were also significant and less than 10% of the samples which were positive were not significant. In the case of BGM cells, 12,5% of the results were significant but showed effects <20%.

3.2.2.3 Ames *Salmonella* mutagenicity test

Control plates contained between 13 and 47 colonies in the case of tester strain TA98 and between 117 and 310 colonies in the case of tester strain TA100 (Table 34). A colony number of 13 is considered too low and indicates undesirable growth for the tester strain. Ideally the number of colonies for TA98 should be between 25 and 50, and that for TA100 between 100 and 200. The results in Table 34 indicate that in some instances too many colonies were obtained with TA100.

Water samples were tested directly and/or after concentration. Initially, concentration was carried out by means of flash evaporation and XAD resin extraction to compare results. Flash evaporation was later discontinued, because XAD extraction was considered a more reliable concentration technique. The unconcentrated and flash evaporated samples were tested in a modified plate incorporation assay, using two methods of media preparation *e.g.* autoclaving

TABLE 32: Effect of groundwater on mammalian cells and toad embryos (+ indicates stimulation)

Sample	BGM cell cloning efficiency test		V79 cell cloning efficiency test		Toad embryo teratogenicity test			
	% Inhibition	Result significant (y/n)	% Inhibition	Result significant (y/n)	% Eggs hatched[1]	%[2] Deformation	% Lethality[3]	Description of deformities
CSIR	6	n	+30	y	85	11	0	no pigment, curved spine, underdeveloped, in sack
Wonderboom	9	y	+8	n	79	6	0	no pigment, curved spine, eyes close together, in sack
Winterveld 1	3	n	+7	n	85	9	18	curved spine, underdeveloped, wormlike
Winterveld 2	+1	n	+4	n	106	20	0	curved spine, underdeveloped, wormlike
Moreletta Park	17	y	11	n	93	17	9	no pigment, curved spine, underdeveloped
Waterkloof	50	y	76	y	93	19	0	curved spine, wormlike
ISCOR	34	y	14	y	86	8	3	curved spine, enlarged stomach
Silverton 1	7	n	+4	n	81	6	8	curved spine, underdeveloped, wormlike
Derdepoort	10	n	1	n	88	13	0	no pigment, curved spine, underdeveloped
Silverton 2	0	n	3	n	90	11	0	curved spine, underdeveloped
Fochville	+6	n	13	y	103	7	0	curved spine, wormlike
Annlin	+3	n	3	n	102	12	0	curved spine, wormlike

1 Dead and surviving embryos
2 Calculated in terms of dead and surviving embryos in test/control
3 Calculated in relation to the surviving embryos in control test

TABLE 33: Effect of surface water on mammalian cells and toad embryos (+ indicates stimulation)

Sample	BGM cell cloning efficiency test		V79 cell cloning efficiency test		Toad embryo teratogenicity test			
	% Inhibition	Result significant (y/n)	% Inhibition	Result significant (y/n)	% Eggs hatched[1]	%[2] Deformation	% Lethality[3]	Description of deformities
Rietvlei Dam	37	y	+29	y	71	24	21	no pigment, curved spine, under-developed, eyes misplaced, wormlike
Bon Accord Dam	31	y	+8	n	83	21	0	no pigment, curved spine, underdeveloped
Roodeplaat Dam	21	y	2	n	89	18	0	curved spine, underdeveloped, wormlike
Hartbees-poort Dam	22	y	+30	y	85	8	0	no pigment, curved spine, underdeveloped, enlarged stomach
Verwoerd-burg Lake	2	n	+18	n	99	11	0	no pigment, curved spine
Lakefield Lake	4	n	+21	y	92	11	3	no pigment, in sack
Hennops River	17	y	+8	n	47	100	100	curved spine, wormlike
Moreletta Stream	12	n	+3	n	74	11	7	no pigment, curved spine, underdeveloped
Jukskei River	71	y	96	y	84	13	15	no pigment, underdeveloped
Illiondale Stream	26	y	+5	n	94	9	0	underdeveloped
Pienaars River	+1	n	3	n	102	10	0	curved spine, underdeveloped
Fountains Stream	16	n	6	n	99	11	0	curved spine, underdeveloped, wormlike

[1] Dead and surviving embryos

[2] Calculated in terms of dead and surviving embryos in test/control

[3] Calculated in relation to the surviving embryos in control test

TABLE 34: Control results of the mutagenicity and teratogenicity tests

	Ames *Salmonella* mutagenicity test (number of colonies)				Toad embryo teratogenicity test		
	TA98-S9	**TA98+S9**	**TA100-S9**	**TA100+S9**	**Number of eggs hatched**	**Number of embryos survived**	**% Deformation**
Range of controls	17; 22; 25; 25; 25; 27; 35; 47	13; 22; 23; 36; 39; 39; 40; 47	117; 118; 168; 177; 244; 245; 250; 310	124; 139; 148; 159; 235; 240; 272; 276	70; 78; 81; 88; 93; 95; 101; 102	67; 77; 77; 88; 93; 95; 100; 102	6; 6; 9; 9; 10; 17; 22; 22
Average control ± standard deviation (SD)	28±9	32±12	204±69	199±63	89±11	87±13	12,6±6,7

and filtration. The latter method was used to limit the effect of heat, which could change the chemical composition of samples. The XAD extracts were incorporated into the top-agar. Tables 35 and 36 presents the results of the mutagenicity test. No mutagenic activity was observed with either tester strains, with and without metabolic activation, except for the following samples: CSIR groundwater; Wonderboom groundwater; Rietvlei Dam water; Hennops River water; and Moreletta Stream water. In some instances concentration by flash evaporation resulted in increased MR values, but these values were still <2,0 indicating that mutagenic activity was not significant (except for the 3 samples mentioned). None of the XAD preparations showed mutagenicity.

Both the groundwater samples which were mutagenic were concentrated by flash evaporation. The filtered preparation was mutagenic in case of the CSIR sample and the autoclaved preparation in case of the Wonderboom sample. The CSIR sample showed marginal mutagenicity on 2x concentration with metabolic activation, using tester strain TA100 (mutation ratio: 2,0), and on 4x concentration with metabolic activation, using tester strain TA98 (mutation ratio: 2,1). The Wonderboom sample was marginally mutagenic (MR: 2,1) at the 2x concentration using TA100 with metabolic activation. The surface waters showed mutagenicity upon direct testing. In this instance, the autoclaved preparation was mutagenic in the case of the Rietvlei Dam sample and the filtered preparations in the case of the Hennops River and Moreletta Stream samples. The Rietvlei Dam and Moreletta Stream samples showed mutation ratios of 2,4 and 2,3, respectively, using TA100 with metabolic activation. The Hennops River sample displayed a mutation ratio of 2,6, with TA98 and metabolic activation. Mutation ratios of between 2,0 and 2,6 were occasionally recorded in raw waters in other studies as well as in Section 3.1.2.3 (Table 18).

It is possible that the mutation ratios ≥2,0 observed with some of the samples were due to the presence of histidine. Analyses were, however, not carried out to confirm this notion. The presence of histidine is eliminated when XAD extraction is used.

3.2.2.4 Toad embryo teratogenicity test

Table 34 shows that between 70 and 102 of the approximately 100 eggs used per control test hatched. This was a major improvement compared to the study of the previous year where only 19 to 38% of the eggs hatched. The number of eggs hatched was calculated by counting dead and surviving embryos. In most instances (75% samples) more than 80 eggs hatched and all the embryos survived (lethality: <5%). Based on these results and the interpretation of effects in fish and water flea tests, 10% lethality was selected as an indication of toxic activity. Deformation in control tests ranged from 6 to 22%. The majority of results (75%) were below 20% deformation. A 20% effect was, therefore, selected as the detection limit for the bioassay.

The effects of the water samples on toad embryos are shown in Tables 32 and 33. In most instances (>80% of samples) more than 80% of the eggs in test samples hatched (dead and surviving embryos). Four of the samples (one borehole and four surface water) exhibited lethality (lethality ≥10%). Most of the effects were small (15 to 21% lethality). However, Hennops River water caused 100% lethality. Only four samples showed a deformation ≥20% (Winterveld 2: 20%; Rietvlei Dam: 24%; Bon Accord Dam: 21% and Hennops River: 100%). The type of deformities which occurred are described in Tables 32 and 33. Similar deformities were noticed in control tests, *e.g.* no pigment, curved spine, eyes close together, underdeveloped, wormlike, and organism in sack.

TABLE 35: Effect of groundwater on *Salmonella* tester strains

Sample	Sample preparation	Concentration	Tester strains			
			TA98-S9	TA98+S9	TA100-S9	TA100+S9
			Mutation ratio			
CSIR	XAD extraction[1]	2 000x	0,8	1,0	1,0	0,9
	Flash evaporation and autoclavation	1x	1,0	0,9	1,0	1,2
		2x	1,0	1,1	1,3	1,3
		4x	1,1	1,4	1,4	-[2]
	Flash evaporation and filtration	1x	-[2]	1,4	1,6	1,8
		2x	0,8	1,2	1,3	**2,0**
		4x	-[2]	**2,1**	1,7	1,4
Wonderboom	XAD extraction	2 000x	1,0	0,7	1,0	0,9
	Flash evaporation and autoclavation	1x	1,0	1,4	1,4	1,2
		2x	1,3	1,2	1,8	**2,1**
		4x	1,6	1,3	1,6	1,7
	Flash evaporation and filtration	1x	-[2]	1,2	1,3	1,5
		2x	0,9	0,7	1,4	1,7
		4x	1,3	1,3	0,9	1,5
Winterveld 1	XAD extraction	2 000x	1,3	1,2	1,2	1,4
Winterveld 2	XAD extraction	2 000x	1,4	1,4	1,2	1,4
Moreletta Park	XAD extraction	2 000x	1,3	1,3	1,4	1,4
Waterkloof	XAD extraction	2 000x	1,4	1,5	1,5	1,5
ISCOR	XAD extraction	2 000x	1,8	1,7	1,8	1,6
Silverton 1	XAD extraction	2 000x	1,3	1,3	1,4	1,4
Derdepoort	XAD extraction	2 000x	1,4	1,5	1,4	1,4
Silverton 2	XAD extraction	2 000x	1,4	1,3	1,2	1,4
Fochville	XAD extraction	2 000x	1,4	1,4	1,5	1,4
Annlin	XAD extraction	2 000x	1,4	1,2	1,2	1,3

[1] 100x concentrated after addition to top-agar
[2] No growth
Positive results in bold

TABLE 36: Effect of surface water on *Salmonella* tester strains

Sample	Sample preparation	Concentration	Tester strains			
			TA98-S9	TA98+S9	TA100-S9	TA100+S9
			Mutation ratio			
Rietvlei Dam	XAD extraction[1]	2 000x	1,0	0,9	0,9	1,0
	Autoclavation	1x	1,0	1,3	1,3	**2,4**
	Filtration	1x	0,7	1,0	1,4	1,7
Bon Accord Dam	XAD extraction	2 000x	1,0	1,0	1,3	0,9
	Autoclavation	1x	1,3	1,1	1,3	1,2
	Filtration	1x	1,3	1,1	1,6	1,5
Roodeplaat Dam	XAD extraction	2 000x	1,0	1,1	1,1	1,1
	Autoclavation	1x	0,9	1,1	1,5	1,7
	Filtration	1x	1,2	1,1	1,5	1,6
Hartbees-poort Dam	XAD extraction	2 000x	1,2	1,2	1,0	1,1
	Autoclavation	1x	1,1	1,1	1,2	1,2
	Filtration	1x	1,8	1,4	1,8	1,8
Verwoerburg Dam	XAD extraction	2 000x	1,5	1,9	0,8	0,6
Lakefield Lake	XAD extraction	2 000x	0,8	1,2	0,9	0,8
Hennops River	XAD extraction	2 000x	0,8	0,9	1,1	1,0
	Autoclavation	1x	1,1	1,6	1,2	1,5
	Filtration	1x	0,9	**2,6**	1,6	1,8
Moreletta Stream	XAD extraction	2 000x	1,0	1,2	1,0	1,3
	Autoclavation	2 000x	1,2	0,9	1,0	1,7
	Filtration	2 000x	1,7	1,1	1,7	**2,3**
Jukskei River	XAD extraction	2 000x	1,1	1,7	0,8	0,5
Illiondale Stream	XAD extraction	2 000x	1,1	1,9	0,7	0,5
Pienaars River	XAD extraction	2 000x	1,6	1,4	1,4	1,4
Fountains Stream	XAD extraction	2 000x	1,4	1,3	1,4	1,4

[1] 100x concentrated after addition to top-agar
Positive results in bold

3.2.2.5 Summary of toxicity test data

The toxicity data obtained with the different bioassays are summarized in Tables 37 and 38. The oxygen uptake test and acetylcholinesterase enzyme test did not detect toxicity in any of the groundwater samples. The water flea test proved to be highly sensitive to groundwater, detecting toxicity in 75% of the samples. A large number of samples also showed toxicity with the algal AAM medium test (67%), the bacterial growth inhibition test (50%) and the fish test (42%). No toxicity was detected in the CSIR and Wonderboom samples. However, both samples showed marginal mutagenicity. The Winterveld 2 sample exhibited a low level of teratogenicity. Two of the samples showed toxicity with three tests; three with four tests; three with five tests; one with six tests (Winterveld 2); and one with seven tests (ISCOR) (considering the luciferase and urease test as single tests). For six of the samples, three or four of the standard aquatic tests, namely fish, water flea, algae and bacteria showed toxicity, indicating some pattern in the responses. For some tests, effects were slight, *e.g.* the fish, urease enzyme test and toad embryo test. High toxicity was generally observed with the water flea test, the algal AAM medium test, the bacterial growth inhibition test, and the luciferase test.

All the bioassays detected toxicity in one or more of the surface water samples (Table 38). Six of the samples were toxic to the BGM cell test, four to the luciferase test, and three each to the algal BG-11 medium test, the urease test, and the toad embryo test. The Rietvlei Dam, Bon Accord Dam and the Hennops River showed teratogenicity. Three of the samples exhibited slight mutagenicity (Rietvlei Dam, Hennops River and the Moreletta Stream). No toxicity was detected in the Lakefield Lake and Moreletta Stream water. Four samples showed toxicity with one bioassay, two with two bioassays, one with three bioassays, two with four bioassays, and one with nine bioassays (Illiondale Stream). The toxicity caused by the Hennops River, Jukskei River and Illiondale Stream waters was generally high. With the exception of the Illiondale Stream sample no specific pattern could be established in the responses of the bioassays.

3.2.2.6 Chemical data

Although extensive chemical analyses were carried out (Tables 39 and 40), the data will not be discussed in detail, but will merely be used to try and explain toxic effects detected with the bioassays. As detailed organic analyses were not carried out it will not be possible to attribute toxicity to particular organic chemicals. The Illiondale sample was the only one which was analyzed for a specific group of organic compounds. An analysis was required because the acetylcholinesterase enzyme indicated the presence of organophosphates. Upon sampling a pesticide-like smell was also evident. Chemical analysis indicated the absence of organochlorine and organophosphate pesticides, but identified ethylenechlorophosphate (commercial name: Fyrol), a fire retardant. It is likely that this chemical contributed to the toxicity detected with at least eight of the tests. Table 39 shows that it is unlikely that pH could have been responsible for adverse effects in groundwater. The Derdepoort sample had a low oxygen content which could have contributed to the effect on water flea. In general, potential toxic chemicals in the CSIR, Wonderboom, Silverton 2 and Fochville samples were below chemical detection limits. The other samples contained one or more of the following chemicals at relatively low to high levels: aluminium, iron, manganese, zinc and phenol. It is expected that in most instances toxicity was due to a combination of the chemicals. However, the zinc level in the Winterveld 2 sample was high and was probably the cause for toxicity in six of the bioassays, including the urease test. Iron and manganese were present at high

TABLE 37: Groundwater samples exhibiting toxicity, mutagenicity and teratogenicity with bioassays

Sample	F test	W test	OU test	AG test		BG test	LE test		UE test			AE test	MC test		TE test		Ames Test			
				AAM	BG-11		Lum	Dec	0,5 µg	1,0 µg	2,0 µg		BGM cells	V79 cells	Leth	Def	98-	98+	100-	100+
1																		2,1		2,0
2																				2,1
3		100				93			11						18					
4	20	100		87	46	53			26	19						20				
5				54	45	94								11						
6	20	40				83							50	76						
7	20	100		52	48	39							34	14						
8		25		72			94	92												
9		75		72	22	18	81	78												
10		15		15			24	23												
11	20	80		41						10				13						
12	20	40		41					21	20										

1 CSIR (30-06-1993)
2 Wonderboom (01-07-1993)
3 Winterveld 1 (09-11-1993)
4 Winterveld 2 (09-11-1993)
5 Moreletta Park (09-11-1993)
6 Waterkloof (10-11-1993)
7 ISCOR (18-11-1993)
8 Silverton 1 (18-11-1993)
9 Derdepoort (18-11-1993)
10 Silverton 2 (18-11-1993)
11 Fochville (22-11-1993)
12 Annlin (24-11-1993)

F Fish
W Water flea
OU Oxygen uptake
AG Algal growth
BG Bacterial growth
LE Luciferase enzyme
UE Urease enzyme
AE Acetylcholinesterase enzyme
MC Mammalian cell
TE Toad embryo
Lum Luminescence
Dec Decay rate
Leth Lethality
Def Deformation

TABLE 38: Surface water samples exhibiting toxicity, mutagenicity and teratogenicity with bioassays

Sample	F test	W test	OU test	AG test		BG test	LE test		UE test			AE test	MC test		TE test		Ames test			
				AAM	BG-11		Lum	Dec	0,5 µg	1,0 µg	2,0 µg		BGM cells	V79 cells	Leth	Def	98-	98+	100-	100+
1													37		21	24				2,4
2						25							31			21				
3													21							
4													22							
5									19	13										
6																				
7	100			34	37										100	100				
8																				2,3
9							52	23					71	96	15					
10	20	100	51	98	84		46	39	34			67[13]	26							
11					23		34		30	17										
12							99	95												

1	Rietvlei Dam (30-06-1993)	2	Bon Accord Dam (03-08-1993)
3	Roodeplaat Dam (03-08-1993)	3	Hartbeespoort Dam (03-08-1993)
5	Verwoerdburg Lake (29-09-1993)	6	Lakefield Lake (29-09-1993)
7	Hennops River (30-06-1993)	8	Moreletta Stream (03-08-1993)
9	Jukskei River (29-09-1993)	10	Illiondale Stream (29-09-1993)
11	Pienaars River (23-11-1993)	12	Fountains Stream (24-11-1993)
F	Fish	W	Water flea
OU	Oxygen uptake	AG	Algal growth
BG	Bacterial growth	LE	Luciferase enzyme
UE	Urease enzyme	AE	Acetylcholinesterase enzyme
MC	Mammalian cell	TE	Toad embryo
Lum	Luminescence	Dec	Decay rate
Leth	Lethality	Def	Deformation

[13] After oxidation

TABLE 39: Chemical quality of groundwater

Determinand	Sample											
	1	2	3	4	5	6	7	8	9	10	11	12
pH	7,3	7,7	6,2	6,3	6,6	6,2	6,3	7,2	7,4	7,5	7,3	7,6
Dissolved oxygen*	-	-	8,9	11,2	8,3	4,2	5,3	4,3	3,1	5,1	8,1	6,9
Temperature (°C)	-	-	24	20	24	21	22	23	24	22	-	21
Electrical cond. (mS/m)	-	-	-	-	-	-	156	80	152	65	-	-
Hardness ($CaCO_3$)*	-	-	22	123	87	162	332	386	208	290	415	155
Alkalinity ($CaCO_3$)*	-	-	41	3,0	69	76	<2,0	295	277	121	146	104
Calcium (Ca)*	-	-	6,0	32	22	56	43	77	37	67	70	47
Magnesium (Mg)*	-	-	2,0	11	8,0	6,0	54	47	28	30	58	9,0
Ammonia (N)*	-	-	<0,2	<0,2	<0,2	8,8	0,8	<0,2	0,4	<0,2	<0,2	0,2
Nitrate-nitrite (N)*	-	-	3,1	44	<0,2	<0,2	3,0	1,3	<0,2	6,2	5,4	0,2
Sulphate (SO_4)*	-	-	9,0	9,0	18	11	111	42	22	24	29	118
Total phosphate (P)*	-	-	<0,2	<0,2	<0,2	1,1	<0,2	<0,2	<0,2	<0,2	<0,2	<0,2
Ortho-phosphate (P)*	-	-	<0,2	<0,2	<0,2	1,0	<0,2	<0,2	<0,2	<0,2	<0,2	<0,2
Chloride (Cl)*	-	-	<5,0	64	5,0	<5,0	281	24	197	32	40	107
COD*	<10	12	10	17	17	67	<10	<10	<10	<10	<10	<10
DOC*	4,1	6,4	1,6	1,7	4,6	18	5,0	27	18	17	8,4	17
Aluminium (Al)**	<100	<100	<100	<100	<100	<100	272	161	104	<100	<100	111
Arsenic (As)**	<5,0	<5,0	5,0	<5,0	<5,0	5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0
Cadmium (Cd)**	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0
Copper (Cu)**	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
Iron (Fe)**	<30	<30	30	<30	390	5200	80	<30	30	<30	<30	440
Mercury (Hg)**	<1,0	<1,0	<1,0	2,0	<1,0	4,0	2,0	1,0	2,0	<1,0	1,0	1,0
Manganese (Mn)**	<25	<25	<25	<25	520	1180	590	<25	50	<25	<25	<25
Lead (Pb)**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
Selenium (Se)**	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0
Zinc (Zn)**	40	<25	190	2380	30	<25	220	30	620	<25	50	<25
Cyanide (CN)**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
Phenol**	<10	<10	<10	<10	<10	298	<10	<10	<10	<10	<10	<10

1 CSIR (30-06-1993)
2 Wonderboom (01-07-1993)
3 Winterveld 1 (09-11-1993)
4 Winterveld 2 (09-11-1993)
5 Moreletta Park (09-11-1993)
6 Waterkloof (10-11-1993)
7 ISCOR (18-11-1993)
8 Silverton 1 (18-11-1993)
9 Derdepoort (18-11-1993)
10 Silverton 2 (18-11-1993)
11 Fochville (22-11-1993)
12 Annlin (24-11-1993)
* mg/ℓ
** µg/ℓ

TABLE 40: Chemical quality of surface water

Determinand	Sample											
	1	2	3	4	5	6	7	8	9	10	11	12
pH	7,7	8,4	8,2	8,4	7,8	9,3	7,5	8,0	7,9	7,7	8,2	8,8
Dissolved oxygen*	-	12,5	12,2	11,2	5,8	10,8	-	11,3	6,6	7,2	5,0	7,5
Temperature (°C)	-	8,7	9,0	9,0	20	24	-	9,0	19	19	22	20
Electrical cond. (mS/m)	-	-	-	-	79	39	-	-	105	51	215	38
Hardness ($CaCO_3$)*	-	-	-	-	159	88	-	-	290	122	2267	11
Calcium (Ca)*	-	-	-	-	34	21	-	-	73	22	46	3,0
Magnesium (Mg)*	-	-	-	-	18	9,0	-	-	26	16	522	<1,0
Ammonia (N)*	-	-	-	-	5,2	0,2	-	-	12	0,5	<0,2	<0,2
Nitrate-nitrite (N)*	-	-	-	-	2,5	<0,2	-	-	<0,2	1,0	25	0,4
Total phosphate (P)*	-	-	-	-	2,8	0,3	-	-	1,2	1,5	<0,2	0,4
Ortho-phosphate (P)*	-	-	-	-	2,8	0,8	-	-	1,1	0,4	<0,2	0,3
COD*	52	62	49	54	31	123	60	78	100	115	<10	<10
DOC*	18	15	10	13	15	11	21	13	24	28	7,1	2,7
Aluminium (Al)**	<100	<100	<100	<100	117	<100	<100	<100	<100	<100	<100	395
Arsenic (As)**	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0
Cadmium (Cd)**	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	22	<5,0	<5,0
Copper (Cu)**	<25	30	<25	30	<25	<25	<25	<25	<25	30	<25	<25
Iron (Fe)**	60	960	70	120	70	80	80	500	880	540	<30	1230
Mercury (Hg)**	<1,0	<1,0	<1,0	<1,0	<1,0	<1,0	<1,0	<1,0	<1,0	2,0	1,0	2,0
Manganese (Mn)**	<25	340	<25	30	<25	<25	<25	190	1630	180	<25	50
Lead (Pb)**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
Selenium (Se)**	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0	<5,0
Zinc (Zn)**	<25	80	40	50	<25	<25	<25	50	<25	2640	280	30
Cyanide (CN)**	<50	<50	<50	<50	<50	67	<50	<50	99	738	<50	<50
Phenol**	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

1 Rietvlei Dam (30-06-1993)
2 Bon Accord Dam (03-08-1993)
3 Roodeplaat Dam (03-08-1993)
3 Hartbeespoort Dam (03-08-1993)
5 Verwoerdburg Lake (29-09-1993)
6 Lakefield Lake (29-09-1993)
7 Hennops River (30-06-1993)
8 Moreletta Stream (03-08-1993)
9 Jukskei River (29-09-1993)
10 Illiondale Stream (29-09-1993)
11 Pienaars River (23-11-1993)
12 Fountains Stream (24-11-1993)
* mg/ℓ
** µg/ℓ

levels in the Waterkloof sample, and this combination probably caused the toxicity in five of the bioassays. This sample also showed a relatively high organic content. No clear explanation could be found for the toxicity of the other samples, particularly the ISCOR sample which affected seven bioassays.

The Lakefield Lake had a relatively high pH (9,3), but no adverse effects on test systems were detected (Table 40). The Illiondale sample contained a high level of zinc, and relatively high levels of cadmium, iron, manganese and cyanide which explains the toxicity detected with nine of the bioassays. The other samples contained one or more of the following chemicals at low to high levels: aluminium, iron, manganese, and zinc. In several instances the organic content was also high. A combination of these chemicals could have been responsible for the toxicity detected.

3.2.3 Conclusions and recommendations

With the exception of the oxygen uptake and luciferase enzyme tests, all the acute toxicity tests detected toxicity in groundwater samples. The water flea test proved to be highly sensitive to groundwater, showing adverse activity in 75% of the samples. A large number of samples were also toxic to algae, bacteria and fish. For some of the tests the effects were slight, but a high toxicity was generally observed with the water flea, algal (AAM medium), bacterial and luciferase enzyme tests. Two of the groundwater samples were slightly mutagenic while one of the samples caused teratogenicity. In a few instances three to four of the standard aquatic toxicity tests showed toxicity, indicating some pattern in the responses.

All the bioassays detected toxicity in one or more of the surface water samples. The Illiondale Stream water exhibited toxic responses with nine of the bioassays. The BGM cell test showed the highest sensitivity, detecting toxicity in six of the samples. Effects exhibited by the samples ranged from slight to high. The mutagenicity and teratogenicity tests showed positive results on three occasions.

Many of the tests showed enhanced activity when exposed to the water samples. This is usually attributed to nutrients in the water. In the case of the urease test, the high density readings could have been due to precipitation rather than increased activity. Some of the samples caused precipitation in the algal and bacterial growth inhibition tests, which could have interfered with the interpretation of results.

Chemical analyses showed that, in general, potential toxic chemicals in groundwater and surface water samples were low, and that effects were probably due to a combination of chemicals. Some of the groundwater samples contained low oxygen levels which could have contributed to adverse effects. The Winterveld 2 sample contained a high zinc level which could have caused the toxicity detected by several bioassays. The Illiondale sample contained high levels of zinc, cadmium, iron, manganese and cyanide, as well as an organic compound, ethylenechlorophosphate (fire retardant), which individually or in combination could have caused the toxicity in test systems.

A good reproducibility was found with most of the tests (CV: <10%). The reproducibility of the protozoan test was drastically improved (CV: <5%) because the modified test system was replaced by standard equipment. The algal test also showed a large improvement (CV: <10%), probably due to modifications in the test conditions. The reproducibility was found to be in agreement with the precision of tests used in other countries, or even exceeded it.

The good agreement between results calculated as percentage effects and by means of Student's t-test, indicates that the use of detection limits could be adequate to decide whether or not effects are significant. In a number of instances detection limits might provide false positive results. However, the possibility that significant effects might not be picked up by using detection limits are limited.

Most of the changes that were recommended after the evaluation of drinking water (Section 3.1.3) to improve reproducibility and sensitivity, and to optimize tests, were carried out. Due to unreliable results, the modified equipment used in the protozoan oxygen uptake test was replaced by the standard system. The inocula of the algal and bacterial tests were standardized and a standard number of eggs were used in the toad embryo teratogenicity test. Quantitative measurement of urease enzyme activity using a microplate reader was also introduced. However, visual observation remains an important attribute, particularly in cases of precipitation. Attempts were made to standardize the concentration of the luciferase enzyme test to have a maximum light level of 1 000 mV. This was not easy to achieve, because of differences in the light levels of different batches of the enzyme. The light level in this study ranged between 333 and 1980 mV, which was a considerable improvement on the range of values obtained in the previous study. The temperature for the algal test was optimized and attempts were made to introduce uniform illumination. However, in some instances growth was still low and further optimization is required. In order to obtain a higher sensitivity with the Ames test, flash evaporation was replaced by XAD resin extraction. The extracts were directly incorporated into the top-agar following standard protocol.

The findings of the study once again indicated that both acute and chronic tests were efficient and suitable for the evaluation of water toxicity. The only exception was the luciferase test, which did not prove to be a viable test.

3.3 Reference chemicals and control waters

3.3.1 Test samples

3.3.1.1 Reference chemicals

Reference chemicals (also called positive controls) are standard chemicals used to measure the sensitivity of test organisms in order to establish confidence in the toxicity data obtained for a water sample. Reference chemicals/toxicants are tested to determine the sensitivity of the organisms at the time a water sample is tested, and the precision of the results obtained by the test laboratory for that chemical. Such a chemical should have a stable shelf life, be highly soluble in water and be stable in solution.

Cadmium ($CdCl_2$) and pentachlorophenol (PCP) were selected as reference chemicals for the fish, water flea, protozoan, algal, bacterial, urease, luciferase, and mammalian cell toxicity tests. These chemicals, particularly PCP, are used by other laboratories as reference toxicants. Stock solutions (initially 1 000 mg/ℓ, and later 100 mg/ℓ) were prepared with deionized water. Solutions were kept at 4°C and examined regularly to eliminate changes in chemical activity due to precipitation or microbial contamination. Dilutions were made with aerated tap water, moderately hard water, or deionized water, depending on the requirements of the bioassay. Sodium selenite (Na_2SeO_3) and methyl viologen ($C_{12}H_{14}N_2Cl_2$) were used in the teratogenicity test [demonstrated to give reproducible effects when tested by Genthe and Edge (1988)]. Fresh solutions were prepared for each test. Carbofuran (concentration: 2 µg/ℓ) and

malathion (concentration: 50 µg/ℓ) were used in the acetylcholinesterase enzyme test. Malathion was used as reference for oxidized samples. In a few instances where pesticide contamination was suspected, samples were also oxidized to activate latent organophosphates, thus converting indirect inhibitors into direct inhibitors to simulate *in vivo* metabolic processes (Venter, 1991). Positive controls used in the mutagenicity assay included 2-amino-anthracene (2AA) (tested with TA98+S9) and sodium azide (tested with TA100) using spot testing (standard methodology) (Maron and Ames, 1983).

3.3.1.2 Control waters

Microbial, enzyme and mammalian cell toxicity tests show enhanced activity (stimulation) when exposed to certain water samples (Sections 3.1.2.2 and 3.2.2.2). This is usually attributed to nutrients in the water. Traditionally, deionized water (nutrients added) is used for control testing in these tests (except for the luciferase test). In this study additional controls were evaluated as possible alternatives. The controls included synthetic moderately hard and hard water, and dechlorinated tap water. The hard water was prepared by using twice the concentration of chemicals specified in Table 8. The pH of the hard water ranged between 7,6 and 8,0; the hardness between 160 and 180 mg/ℓ ($CaCO_3$); and alkalinity between 110 and 120 mg/ℓ.

Dechlorinated (aerated) tap water is used as positive control for the fish and toad embryo teratogenicity tests. However, it can happen that chlorine products are still present in dechlorinated water, resulting in lethality in control tests. For this reason, a few tests were also carried out with moderately hard water as additional control for comparison.

3.3.2 Results and discussion

3.3.2.1 Reference chemicals

Reference chemicals were usually not included in toxicity tests before this study. Because the sensitivity levels of a number of the bioassays to the selected chemicals were not known (see Table 5), the application of the chemicals involved an amount of trail and error.

During the first year of study (Section 3.1.1) a range of dilutions of the reference chemicals were used to select a suitable test concentration/s for each bioassay. Due to the large number of samples which had to be accommodated at one time the number of replicate tests (Section 2.1) per sample was reduced (two/three replicates) in the case of the protozoan, algal, bacterial and mammalian cell tests. The results of the toxicity tests on reference chemicals, carried out in parallel with the water samples (Section 3.1.1), are presented in Tables 41 to 45. The results indicated that for both acute and chronic tests some of the selected test concentrations showed an appropriate response. However, some degree of variation was observed in the sensitivity of the tests. Large variations could have been due to changes in organism sensitivity, experimental error, or changes in the chemical composition of the stock solutions (*e.g.* precipitation).

Only one concentration of carbofuran (2 µg/ℓ) was used as reference chemical in the acetylcholinesterase test (Table 43). A very good reproducibility of results was noticed with this test. The cadmium and PCP test concentrations were changed from time to time when the mammalian cell toxicity tests (Table 44) were carried out to narrow down effective concentration ranges. Because of its selective sensitivity towards heavy metals, the urease

TABLE 41: Effect of reference chemicals (cadmium and pentachlorophenol) on fish and water flea

Reference chemical for:	Cadmium (mg/ℓ)	Fish test % Lethality after exposure time:				Water flea test % Lethality after exposure time:		Pentachlorophenol (mg/ℓ)	Fish test % Lethality after exposure time:				Water flea test % Lethality after exposure time:	
		24 h	48 h	72 h	96 h	24 h	48 h		24 h	48 h	72 h	96 h	24 h	48 h
Schoemansville samples (29-07-1991)	0,1	20	20	20	40	0	85	0,1	0	0	20	20	0	25
	1,0	0	0	0	60	15	100	1,0	0	0	20	40	45	100
	10,0	40	60	60	60	-	-	10,0	100	100	100	100	-	-
Schoemansville samples (30-09-1991)	0,1	0	0	20	20	25	60	0,1	0	0	0	20	0	30
	1,0	0	40	60	80	80	100	1,0	0	40	60	60	80	100
	10,0	100	100	100	100	-	-	10,0	100	100	100	100	-	-
Rietvlei Dam samples (05-08-1991)	0,1	-	-	0	0	5	20	0,1	-	-	0	0	5	25
	1,0	-	-	40	80	100	100	1,0	-	-	0	0	90	95
	10,0	-	-	40	40	-	-	10,0	-	-	100	100	-	-
Rietvlei Dam samples (07-10-1991)	0,1	-	-	-	-	15	40	0,1	-	-	-	-	0	0
	1,0	-	-	-	-	100	100	1,0	-	-	-	-	0	15
	10,0	-	-	-	-	-	-	10,0	-	-	-	-	-	-
Parys samples (27-08-1991)	0,1	-	20	40	60	60	80	0,1	-	0	0	20	0	10
	1,0	-	100	100	100	100	100	1,0	-	0	20	40	60	100
	10,0	-	80	100	100	-	-	10,0	-	100	100	100	-	-
Parys samples (26-10-1991)	0,1	0	0	0	0	5	5	0,1	0	0	0	0	5	5
	1,0	0	0	0	10	45	65	1,0	70	100	100	100	15	35
	10,0	0	0	0	10	-	-	10,0	100	100	100	100	-	-
Klipgat and Apies River samples (17-09-1991)	0,1	0	0	0	80	10	25	0,1	0	0	20	20	0	25
	1,0	0	60	80	100	95	100	1,0	0	40	60	80	90	100
	10,0	20	20	60	100	-	-	10,0	100	100	100	100	-	-
Klipgat and Apies River samples (06-11-1991)	0,1	0	0	0	0	-	-	0,1	0	0	0	0	-	-
	1,0	0	0	0	0	-	-	1,0	30	70	90	100	-	-
	10,0	0	0	0	0	-	-	10,0	100	100	100	100	-	-

\- No results

TABLE 42: Effect of reference chemicals (cadmium and pentachlorophenol) on microbial tests (+ indicates stimulation)

Reference chemical for:	Cadmium (mg/ℓ)	Protozoan oxygen uptake test (% inhibition)	Pentachlorophenol (mg/ℓ)	Protozoan oxygen uptake test (% inhibition)	Cadmium (mg/ℓ)	Algal growth test (% inhibition)		Pentachlorophenol (mg/ℓ)	Algal growth test (% inhibition)		Cadmium (mg/ℓ)	Bacterial growth test (% inhibition)	Pentachlorophenol (mg/ℓ)	Bacterial growth test (% inhibition)
						AAM	BG-11		AAM	BG-11				
Schoemansville samples (29-07-1991)	1,0	+18	1,0	+76	0,1	87	52	0,1	72	53	0,1	11	0,1	6
	10,0	+32	10,0	12	1,0	100	100	1,0	80	81	1,0	36	1,0	+11
	100	56	100	94	10,0	100	100	10,0	100	100	10,0	87	10,0	0
Schoemansville samples (30-09-1991)	1,0	14	1,0	+65	0,1	+8	20	0,1	+6	+14	0,1	30	-	-
	10,0	30	10,0	+81	1,0	74	86	1,0	20	37	1,0	43	50,0	95
	100	49	100	83	10,0	65	69	10,0	38	46	10,0	96	100,0	99
Rietvlei Dam samples (05-08-1991)	1,0	+5	1,0	+49	0,1	95	63	0,1	20	+389	0,1	2	-	-
	10,0	3	10,0	14	1,0	100	100	1,0	100	+133	1,0	2	100,0	67
	100	31	100	85	10,0	100	100	10,0	100	100	10,0	13	500,0	98
Rietvlei Dam samples (07-10-1991)	1,0	14	1,0	+65	0,1	+8	20	0,1	+6	+14	0,1	6	10,0	+2
	10,0	30	10,0	+81	1,0	74	86	1,0	20	37	1,0	36	50,0	89
	100	49	100	83	10,0	65	69	10,0	38	46	10,0	92	100,0	98
Parys samples (27-08-1991)	1,0	+5	1,0	+38	0,1	95	63	0,1	20	+389	0,1	18	50,0	94
	10,0	7	10,0	+24	1,0	100	100	1,0	100	+133	1,0	40	100,0	97
	100	61	100	86	10,0	100	100	10,0	100	100	10,0	95	500,0	95
Parys samples (28-10-1991)	1,0	7	1,0	+13	0,1	+8	20	0,1	+6	+14	0,1	5	10,0	1
	10,0	9	10,0	+56	1,0	74	86	1,0	20	37	1,0	41	50,0	95
	100	14	100	73	10,0	65	69	10,0	38	46	10,0	94	100,0	97
Klipgat and Apies River samples (17-09-1991)	1,0	9	1,0	+22	0,1	+8	20	0,1	+6	+14	0,1	36	-	-
	10,0	25	10,0	+88	1,0	74	86	1,0	20	37	1,0	58	50,0	84
	100	44	100	64	10,0	65	69	10,0	38	46	10,0	94	100,0	98
Klipgat and Apies River samples (06-11-1991)	1,0	7	1,0	+13	0,1	83	71	0,1	+38	+5	0,1	6	10,0	17
	10,0	9	10,0	+56	1,0	100	100	1,0	4	0	1,0	15	50,0	93
	100	14	100	73	10,0	100	100	10,0	30	44	10,0	98	100	96

TABLE 43: Effect of reference chemicals (cadmium, pentachlorophenol and carbofuran) on enzyme systems (+ indicates stimulation with luciferase test)

Reference chemical for:	Cadmium (mg/ℓ)	Luciferase enzyme test		Pentachlorophenol (mg/ℓ)	Luciferase enzyme test		Cadmium (mg/ℓ)	Urease enzyme test			Carbofuran (µg/ℓ)	Acetylcholinesterase enzyme test (% inhibition)
		Immediate luminescence (% inhibition)	Luminescence decay rate (% inhibition)		Immediate luminescence (% inhibition)	Luminescence decay rate (% inhibition)		Total inhibition:+++; Moderate inhibition:++; Slight inhibition:+; No inhibition:-				
								2,0 mg/ml	1,0 mg/ml	0,5 mg/ml		
Schoemansville samples (29-07-1991)	0,2	+38	+80	0,2	+80	+127	1,0	-	-	+	-	-
	2,0	67	42	2,0	+80	+128	10,0	+	++	+++	-	-
	20,0	-	100	20,0	47	40	100,0	+++	+++	+++	-	-
Schoemansville samples (30-09-1991)	0,2	+1	1	0,2	+23	+24	1,0	-	-	-	2	28
	2,0	78	60	2,0	3	+21	10,0	-	-	-	2	37
	20,0	88	70	20,0	76	74	100,0	+++	+++	+++	-	-
Rietvlei Dam samples (05-08-1991)	0,2	+38	+80	0,2	+80	+127	1,0	-	-	-	-	-
	2,0	67	42	2,0	+80	+128	10,0	+	++	+++	-	-
	20,0	-	100	20,0	47	40	100,0	+++	+++	+++	-	-
Rietvlei Dam samples (07-10-1991)	0,2	+1	1	0,2	+23	+24	1,0	-	-	-	2	22
	2,0	78	60	2,0	3	+21	10,0	-	++	+++	-	-
	20,0	88	70	20,0	76	74	100,0	+++	+++	+++	-	-
Parys samples (27-08-1991)	0,2	-	-	0,2	-	-	1,0	-	-	+	2	28
	2,0	-	-	2,0	-	-	10,0	-	+++	+++	-	-
	20,0	-	-	20,0	-	-	100,0	+++	+++	+++	-	-
Parys samples (28-10-1991)	0,2	+134	+164	0,2	+111	+145	1,0	-	-	-	2	25
	2,0	24	+3	2,0	+106	+167	10,0	-	-	-		
	20,0	-	100	20,0	31	36	100,0	-	-	-	-	-
Klipgat and Apies River samples (17-09-1991)	0,2	+1	1	0,2	+23	+24	1,0	-	-	-	2	30
	2,0	76	60	2,0	3	+21	10,0	++	+++	+++	2	60
	20,0	88	70	20,0	76	74	100,0	+++	+++	+++	-	-
Klipgat and Apies River samples (08-11-1991)	0,2	+5	+68	0,2	+59	+69	1,0	-	-	-	2	31
	2,0	40	5	2,0	+42	+85	10,0	++	+++	+++	-	-
	20,0	-	-	20,0	54	48	100,0	+++	+++	+++	-	-

\- No results

TABLE 44: Effect of reference chemicals (cadmium and pentachlorophenol) on mammalian cells (+ indicates stimulation)

Reference chemical for:	Cadmium (mg/ℓ)	BGM cells	V79 cells	Pentachlorophenol (mg/ℓ)	BGM cells	V79 cells
		% inhibition	% inhibition		% inhibition	% inhibition
Schoemansville samples (29-07-1991)	0,01	+4	11	0,01	+13	12
	0,1	+4	39	0,1	6	14
	1,0	0	4	1,0	+13	13
	10,0	+4	100	10,0	+6	10
Schoemansville samples (30-09-1991)	0,1	+3	+16	10,0	+14	2
	0,5	-	+10	12,0	-	1
	1,0	91	99	14,0	3	20
	2,0	100	-	16,0	-	61
	-	-	-	18,0	32	-
	-	-	-	20,0	46	-
Rietvlei Dam samples (05-08-1991)	1,0	72	100	1,0	+47	+9
	5,0	100	100	5,0	+58	+8
	10,0	100	100	10,0	+43	+1
	50,0	100	100	50,0	100	100
Rietvlei Dam samples (07-10-1991)	0,5	25	23	14,0	15	37
	1,0	57	92	16,0	32	44
Parys samples (27-08-1991)	0,5	10	12	14,0	15	100
	1,0	53	98	16,0	18	100
Parys samples (28-10-1991)	0,01	-	+19	10,0	5	+5
	0,1	-	+3	20,0	100	100
	0,5	14	-	30,0	100	100
	1,0	49	100	40,0	100	100
	5,0	100	100	-	-	-
	10,0	100	-	-	-	-
Klipgat and Apies River samples (17-09-1991)	0,1	+23	1	10,0	+51	17
	0,5	17	44	14,0	+39	24
	1,0	41	98	18,0	2	100
	2,0	76	-	20,0	+31	100
Klipgat and Apies River samples (17-09-1991)	0,5	8	+2	14,0	4	100
	1,0	48	84	16,0	50	100

- No results

enzyme was only exposed to cadmium. Only sodium selenite was evaluated in the toad embryo test. The results were inconsistent and the number of deformities were lower than expected (Table 45). This could be attributed to the fact that the test was not optimized and because problems were experienced with fertility. The inconclusive results necessitated further testing.

TABLE 45: Effect of sodium selenite on toad embryos

Reference chemical for:	Concentration (mg/ℓ)	% Eggs hatched	Number of deformities
Schoemansville (29-07-1991)	Control	25	2
	0,1	0	0
	1,0	0	0
	10,0	0	0
	100	0	0
Rietvlei Dam (05-06-1991)	Control	19	3
	0,001	15	2
	0,01	19	7
	0,1	18	4
	1,0	0	0
Schoemansville (30-10-1991); Rietvlei Dam (07-10-1991); and Parys (28-10-1991)	Control	38	0
	0,001	25	6
	0,01	15	1
	0,1	25	8
	1,0	24	5
Klipgat and Apies River (08-11-1991)	Control	18	0
	0,001	12	0
	0,01	8	0
	0,1	8	0
	1,0	18	0

Table 46 summarizes the sensitivity of the various bioassays to cadmium and PCP. Effective concentrations are given as single values or as a range. The effects obtained at each concentration are shown in parenthesis. The fish, water flea and algal tests were the most sensitive to cadmium (0,1 mg/ℓ), followed by the mammalian cell tests (0,5-1,0 mg/ℓ), the bacterial growth test (0,1-10,0 mg/ℓ), the luciferase enzyme tests (2,0-20,0 mg/ℓ), the urease enzyme test (10,0 mg/ℓ) and the protozoan oxygen uptake test (100 mg/ℓ). Fish, water flea and algal tests were also the most sensitive to the organic chemical PCP. The algal AAM medium test showed the highest sensitivity towards PCP (0,1 mg/ℓ), followed by the water flea test (0,1-1,0 mg/ℓ), the fish test and algal BG-11 medium test (1,0 mg/ℓ), the V79 mammalian cell test (10,0-14,0 mg/ℓ), the BGM mammalian cell test (16,0-20,0 mg/ℓ), the luciferase enzyme test (20,0 mg/ℓ), bacterial growth test (10,0-50,0 mg/ℓ) and the oxygen uptake test (10,0-100 mg/ℓ).

TABLE 46: Sensitivity of bioassays to cadmium and pentachlorophenol

Bioassay		Chemical (mg/ℓ)	
		Cadmium	Pentachlorophenol
Fish lethality test		0,1 (lethality: <10 - 80%)	1,0 (lethality: <10 - 100%)
Water flea lethality test		0,1 (lethality: <10 - 85%)	0,1-1,0 (lethality: <10-100)
Protozoan oxygen uptake test		100 (inhibition: 14 - 61%)	10,0-100 (stimulation: 88% to inhibition: 94%)
Algal growth test	AAM growth medium	0,1 (inhibition: <20 - 95%)	0,1 (inhibition: <20 - 100%)
	BG-11 growth medium	0,1 (inhibition: 20 - 71%)	1,0 (stimulation: 133% to inhibition: 81%)
Bacterial growth test		0,1-10,0 (inhibition: <10 - 98%)	10,0-50,0 (inhibition: <10 - 95%)
Urease enzyme test		10,0	-
Luciferase enzyme test	Immediate luminescence	2,0 (inhibition: 24 - 76%)	20,0 (inhibition: 31 - 76%)
	Luminescence decay rate	2,0-20,0 (inhibition: <10 - 100%)	20,0 (inhibition: 36 - 74%)
Mammalian cloning efficiency test	BGM cells	0,5-1,0 (inhibition: <10 - 91%)	16,0-20,0 (inhibition: 18 - 100%)
	V79 cells	0,5-1,0 (inhibition: <10 - 100%)	10,0 - 14,0 (inhibition: <10 - 100%)

\- No results

During the second year of study (Section 3.2.1) only one concentration of each of the chemicals was selected to include as reference with each set of water samples. The selection was based on the results presented in Table 46. However, it was found that some of the concentrations had to be increased or reduced to obtain desirable effects. In order to improve reproducibility, replicate tests were carried out as specified in the test methods (Section 2.1). Because of the inconclusive results obtained with the teratogenicity test a range of concentrations of sodium selenite was once again tested. Methyl viologen was used as additional control to compare the effects of the two teratogens.

The results of the reference chemicals, PCP and cadmium, are presented in Table 47. Fish and water flea were the most sensitive test organisms to PCP and were exposed to concentrations of 0,1 and 1,0 mg/ℓ, respectively. Ten milligram per litre of PCP was used in the protozoan and algal tests, 15 mg/ℓ in the V79 mammalian cell test, 20 mg/ℓ in the luciferase enzyme and BGM mammalian cell tests, and 50 mg/ℓ in the bacterial growth test. The cadmium concentrations used ranged from 1,0 to 20 mg/ℓ. Fish, water flea, algae and mammalian cells were exposed to 1,0 mg/ℓ cadmium, protozoa, bacteria, and the urease enzyme to 10 mg/ℓ, and luciferase to 20 mg/ℓ. Although most of the chemicals were not tested extensively, a relatively good reproducibility was obtained with the number of tests carried out. Variation between sets of tests ranged from small to moderate and reflected the natural variation in sensitivity of test systems.

Table 48 presents the results obtained with the acetylcholinesterase and toad embryo tests for the reference chemicals. The positive controls included in the acetylcholinesterase test were carbofuran (2 μg/ℓ) and malathion (50 μg/mℓ). In both cases a very good reproducibility of results was noticed. A range of concentrations (0,1 to 100 mg/ℓ) of sodium selenite and methyl viologen was initially tested in the toad embryo test. In the case of sodium selenite, no effects were detected at the 0,1 mg/ℓ concentration while total inhibition/lethality occurred at the 100 mg/ℓ level. Only the 1,0 and 10 mg/ℓ concentrations were, therefore, used in further testing and are presented here (Table 48). Methyl viologen caused no effects at the 0,01 mg/ℓ concentration, while total inhibition/lethality was noticed at 10 mg/ℓ. Here, 0,1 and 1,0 mg/ℓ of the chemical was used for reference testing (Table 48). Results indicate that the deformation detected at the low test concentrations of the two chemicals was not sufficient to use for reference testing. In general, desirable effects were obtained at the upper concentrations. Of the two chemicals methyl viologen proved to be the most desirable to use because deformation was always ≥25% (at 1,0 mg/ℓ). In some instances lethality occurred, but effects were generally low in case of methyl viologen and thus did not interfere with deformation detection.

In general, the error and variability associated with statistical analysis when only one chemical concentration is tested, is large (Warren-Hicks, 1990). It is usually recommended to use a range of concentrations to enable the calculation of effective concentrations, *e.g.* LC_{50}'s or EC_{50}'s. For this reason additional tests were carried out with fish, water flea and algae using serial dilutions of cadmium and PCP. Linear regression was applied to calculate effective concentrations. The results are presented in Table 49. Fish were found to be the most sensitive to cadmium (LC_{50}: 0,03 mg/ℓ), followed by algae (EC_{50}: 0,078 mg/ℓ) and water flea (LC_{50}: 0,09 mg/ℓ). The sensitivity of fish and water flea towards PCP was almost similar (LC_{50}'s: 0,349 and 0,44 mg/ℓ, respectively). Algae were less sensitive with an EC_{50} of 7,78 mg/ℓ. Replicate tests showed some variation in results. However, these are within the ranges found in other laboratories (US EPA, 1991a). A comparison with the results obtained for individual concentrations (Table 47) showed that in general results were in agreement.

TABLE 47: Effect of pentachlorophenol and cadmium on test systems

Chemical		Fish test (96 h)	Water flea test (48 h)	Proto-zoan test	Algal test		Bac-terial test	Luciferase enzyme test		Urease enzyme test			Mammalian cell test	
					AAM	BG-11		Lum	Dec	0,5 mg/mℓ	1,0 mg/mℓ	2,0 mg/mℓ	BGM cells	V79 cells
Penta-chloro-phenol	Test concen-tration (mg/ℓ)	0,1	1,0	10	10	10	50	20	20	-	-	-	20	15
	Number of tests	4	11	1	4	4	13	4	4	-	-	-	5	5
	Average effect[1]	50	96	92	18	24	74	84	83	-	-	-	91	98
	Effect[1] range	20-80	65-100	-	4-35	2-46	50-93	81-92	79-90	-	-	-	63-100	95-100
Cadmium	Test concen-tration (mg/ℓ)	1,0	1,0	10	1,0	1,0	10	20	20	10	10	10	1,0	1,0
	Number of tests	4	10	1	5	5	13	4	4	25	25	25	5	5
	Average effect[1]	55	100	25	99	96	64	7	+24	77	55	11	72	93
	Effect[1] range	40-80	-	-	98-100	90-100	32-94	+23-34	+1-+48	37-98	28-80	+15-47	46-94	85-98

[1] % Lethality or inhibition
\- No results

TABLE 48: Effect of reference chemicals on the acetylcholinesterase and toad embryo test

Bioassay		Methyl viologen				Selenite				Carbofuran				Malathion			
		Test concentration (mg/ℓ)	Number of tests	Average effect[1]	Effect[1] range	Test concentration (mg/ℓ)	Number of tests	Average effect[1]	Effect[1] range	Test concentration (µg/ℓ)	Number of tests	Average effect[1]	Effect[1] range	Test concentration (µg/ℓ)	Number of tests	Average effect[1]	Effect[1] range
Acetyl-cholinesterase test		-	-	-	-	-	-	-	-	2	9	35	21-55	50	6	88	82-93
Toad embryo test	Lethality	1,0	7	17	0-61	10,0	3	42	0-83	-	-	-	-	-	-	-	-
		0,1	7	1	0-7	1,0	3	3	0-8	-	-	-	-	-	-	-	-
	Deformation	1,0	7	47	25-87	10,0	3	38	8-59	-	-	-	-	-	-	-	-
		0,1	7	19	10-43	1,0	3	18	9-30	-	-	-	-	-	-	-	-

[1] % Lethality/inhibition/deformation

\- No results

However, definitive testing resulted in improved results in the case of the algal test on PCP and the fish test on cadmium.

The fish, water flea, toad embryo and mammalian cell tests were exposed to the concentrations given in the tables. Bioassays like the algal, bacterial, urease and acetylcholinesterase tests involve some degree of dilution (80-90% of given concentration), while a 50% dilution takes place in the oxygen uptake and luciferase tests.

TABLE 49: Sensitivity of fish, water flea and algae to cadmium and pentachlorophenol[1]

Chemical	Criterium	96-h Fish lethality test	48-h Water flea lethality test	Algal growth inhibition test[2]
Cadmium	LC_{10} or EC_{10}[3] (mg/ℓ)	0,024 (0,018-0,032)	0,15 (0,13-0,17)	0,043 (0,03-0,055)
	LC_{50} or LC_{50}[4] (mg/ℓ)	0,08 (0,047-0,103)	0,23 (0,17-0,29)	0,154 (0,099-0,209)
	R[5]	0,9707-0,9965	0,9449-1,0	0,9510-0,9624
	Test concentration range (mg/ℓ)	0,016-1,0	0,03-0,5	0,016-0,5
PCP	LC_{10} or EC_{10}[3] (mg/ℓ)	0,20 (0,36-0,288)	0,19	3,3 (2,07-4,53)
	LC_{50} or LC_{50}[4] (mg/ℓ)	0,349 (0,192-0,50)	0,44	7,78 (5,46-10,1)
	R[5]	0,9600-1,0	0,9608	0,9887-0,9997
	Test concentration range (mg/ℓ)	0,016-1,0	0,03-1,0	3,1-50,0

1 Given as an average value and the range (in brackets)
2 Using BG-11 medium
3 Concentration causing 10% lethality or inhibition
4 Concentration causing 50% lethality or inhibition
5 Correlation coefficient

3.3.2.2 Control waters

The effects of moderately hard, hard and tap water on microbial systems are shown in Tables 50 to 52. Only one sample of each of the waters was tested with the protozoan oxygen uptake test. No significant changes in oxygen uptake in relation to deionized water were observed when using moderately hard and hard water (<5%). A slight stimulation occurred when exposed to tap water (7%). In most instances, moderately hard water (Table 50) resulted in reduced growth in the algal test. This was noted at low and high control growth. When hard water (2x the concentration of moderately hard water) was used (Table 51), algal

TABLE 50: Effect of moderately hard water on microbial test systems

Protozoan oxygen uptake test			Algal growth test						Bacterial growth test		
			AAM medium			BG-11 medium					
Control	Moderately hard water	% Effect [1]	Control	Moderately hard water	% Effect [1]	Control	Moderately hard water	% Effect [1]	Control	Moderately hard water	% Effect [1]
0,486	0,501	+3	0,052	0,038	**27**	0,050	0,068	**+36**	0,293	0,280	4
			0,090	0,062	**31**	0,063	0,068	**+8**	0,295	0,362	**+23**
			0,091	0,042	**54**	0,097	0,049	**50**	0,325	0,330	+2
			0,107	0,097	9	0,109	0,049	**55**	0,336	0,343	+2
			0,132	0,050	**62**	0,132	0,128	3	0,349	0,311	**11**
			0,166	0,044	**74**	0,146	0,078	**47**	0,351	0,335	5
			0,172	0,066	**62**	0,146	0,090	**38**	0,363	0,363	0
			0,172	0,082	**52**	0,167	0,117	**30**	0,371	0,334	**10**
						0,173	0,156	**10**	0,372	0,347	7
									0,407	0,384	6
									0,424	0,449	+6
									0,450	0,448	0
									0,510	0,534	+5
									0,551	0,546	1
									0,568	0,562	1
Effect range		-	Effect range		9-74	Effect range		+36-55	Effect range		+23-11

[1] Calculated in relation to control results
\+ Stimulation
\- No results
Results given in bold - effect above detection limit

TABLE 51: Effect of hard water on microbial test systems

Protozoan oxygen uptake test			Algal growth test						Bacterial growth test		
			AAM medium			BG-11 medium					
Control	Hard water	% Effect [1]	Control	Hard water	% Effect [1]	Control	Hard water	% Effect [1]	Control	Hard water	% Effect [1]
0,508	0,515	+2	0,052	0,063	**+21**	0,050	0,096	**+92**	0,293	0,269	8
			0,090	0,091	+1	0,063	0,143	**+127**	0,295	0,353	**+20**
			0,091	0,055	**40**	0,097	0,105	+8	0,336	0,348	+4
			0,132	0,122	8	0,109	0,099	9	0,363	0,474	**+31**
			0,166	0,143	**14**	0,167	0,143	**14**	0,407	0,430	+6
						0,173	0,260	**+50**	0,424	0,342	**19**
									0,510	0,518	+2
									0,568	0,629	**+11**
Effect range		-	**Effect range**		+21-40	**Effect range**		+127-14	**Effect range**		+31-19

[1] Calculated in relation to control results
\+ Stimulation
\- No results
Results given in bold - effect above detection limit

TABLE 52: Effect of laboratory tap water on microbial test systems

Protozoan oxygen uptake test			Algal growth test						Bacterial growth test		
			AAM medium			BG-11 medium					
Control	Tap water	% Effect [1]	Control	Tap water	% Effect [1]	Control	Tap water	% Effect [1]	Control	Tap water	% Effect [1]
0,508	0,542	+7	0,052	0,022	**58**	0,050	0,059	**+18**	0,293	0,270	8
			0,090	0,035	**61**	0,063	0,038	**40**	0,325	0,285	**12**
			0,091	0,032	**65**	0,097	0,057	**41**	0,336	0,350	+4
			0,107	0,073	**32**	0,106	0,095	**10**	0,357	0,337	+6
			0,112	0,048	**57**	0,132	0,116	**12**	0,363	0,341	6
			0,132	0,052	**61**	0,167	0,127	**24**	0,371	0,124	**67**
									0,372	0,351	3
									0,387	0,417	+8
									0,450	0,552	**+23**
									0,551	0,551	0
									0,568	0,367	**35**
Effect range		-	**Effect range**		32-65	**Effect range**		+18-41	**Effect range**		+23-67

[1] Calculated in relation to control results
\+ Stimulation
\- No results
Results given in bold - effect above detection limit

growth in AAM medium was either stimulated or reduced (effects ≥10%), or was similar to the control growth. With BG-11 medium, hard water mostly increased growth or showed results similar to that of the control. In general, tap water inhibited algal growth, and effects were more pronounced in the case of the AAM medium (Table 52). In most instances, bacterial growth in moderately hard water was the same as in the control (Table 50). One sample showed stimulation and two marginal reduced growth (effects ≥10%). Hard water samples either had no effect (50% of samples) or stimulated bacterial growth. Only one sample reduced bacterial growth (Table 51). Most of the tap water samples had no significant effect on bacterial growth (effects 10%) (Table 52). One sample stimulated growth and three samples showed inhibition.

The results of the effects of moderately hard, hard and tap water on enzyme systems are presented in Tables 53 to 55. A comparison between the control results of the luciferase test (tap water) and the synthetic hard waters showed that the luminescence was between 240 and 384% larger in moderately hard water, and between 111 and 236% larger in hard water (almost 50% less stimulation in hard water than in moderately hard water). It is because salt concentrations have such a large influence on the enzyme (the higher the salt concentration the lower the enzyme activity), and because water normally contains a variety of salts, that tap water was selected as the most representative control for the enzyme assay. While the majority of moderately hard water samples showed results similar to that of the controls in the urease enzyme test, stimulation and reduction in enzyme activity was also observed (effects ≥10%) (Table 53). The reduction in enzyme activity only occurred at the 0,5 and 1,0 mg/mℓ enzyme levels. The changes in activity were usually small, however in a few instances stimulation/reduction of between 20 and 32% was observed. About 50% of the results were similar to that of the control and the other 50% showed stimulation in the case of exposure to hard water (Table 54). Table 55 shows that some of the results obtained with tap water were in agreement with that of the control, while others showed stimulation or inhibition.

Inhibition was only detected at the 0,5 and 1,0 mg/mℓ enzyme concentrations. The acetylcholinesterase enzyme was only exposed to moderately hard and tap water (Tables 53 and 55). Results were similar to that of the controls (effects <10%).

Table 56 shows the effects of moderately hard, hard and tap water on mammalian cells. In general, the results obtained with the three test waters were in agreement with those of the controls (effects <20%). In some instances moderately hard and tap water had no effect on V79 cells (effect <10%), in others cloning efficiency was stimulated or inhibited. Hard water showed results similar to the control or stimulated cloning efficiency.

In a few instances moderately hard water was included in tests with fish and toad embryos. A good response was obtained indicating that moderately hard water is an ideal substitute for tap water as control.

3.3.3 Conclusions and recommendations

In general, the reference chemicals used with both the acute and chronic tests showed an appropriate response, indicating that the tests were successfully applied. A relatively large variation in the sensitivity of some of the tests was observed during the first year of study. These variations could have been due to changes in organism sensitivity, experimental error, or changes in the chemical composition of the stock solutions (*e.g.* precipitation). During the second year of study great care was taken that chemical solutions were freshly prepared, and

TABLE 53: Effect of moderately hard water on enzyme systems

Luciferase enzyme test						Urease enzyme test									Acetylcholinesterase enzyme test		
Immediate luminescence			Decay rate			0,5 mg/mℓ			1,0 mg/mℓ			2,0 mg/mℓ					
Control	Moderately hard water	% Effect [1]	Control	Moderately hard water	% Effect [1]	Control	Moderately hard water	% Effect [1]	Control	Moderately hard water	% Effect [1]	Control	Moderately hard water	% Effect [1]	Control	Moderately hard water	% Effect [1]
1582	6796	+330	9,879	43,253	+338	0,027	0,031	+15	0,045	0,049	+9	0,060	0,063	+5	0,279	0,276	1
1731	6364	+364	10,036	47,148	+370	0,035	0,039	+11	0,053	0,054	+2	0,063	0,075	+19	0,287	0,273	5
1904	6469	+240	11,218	45,891	+309	0,036	0,032	11	0,054	0,055	+2	0,066	0,068	+3			
1956	6976	+257	11,505	40,867	+255	0,038	0,041	+8	0,062	0,066	+7	0,068	0,082	+21			
						0,040	0,030	25	0,071	0,063	11	0,070	0,076	+9			
						0,056	0,038	32	0,080	0,090	+13	0,080	0,086	+8			
						0,069	0,068	2	0,084	0,061	27	0,088	0,097	+10			
						0,074	0,075	+1	0,084	0,085	+1	0,092	0,096	+4			
						0,074	0,082	+11	0,086	0,100	+16	0,093	0,091	2			
						0,077	0,083	+8	0,088	0,082	7	0,094	0,102	+9			
						0,078	0,081	+4	0,091	0,089	2	0,095	0,104	+10			
						0,080	0,072	10	0,092	0,084	9	0,096	0,117	+22			
						0,082	0,084	+2	0,095	0,100	+5	0,101	0,110	+9			
						0,083	0,085	+2	0,096	0,101	+5	0,101	0,106	+5			
						0,084	0,085	+1	0,102	0,108	+6	0,107	0,115	+8			
						0,084	0,073	13	0,104	0,085	18	0,111	0,120	+8			
Effect range		+240-+364	Effect range		+255-+370	Effect range		+15-32	Effect range		+16-27	Effect range		+22-2	Effect range		1-5

[1] Calculated in relation to control results

\+ Stimulation

Results given in bold - effect above detection limit

TABLE 54: Effect of hard water on enzyme systems

Luciferase enzyme test						Urease enzyme test									Acetylcholinesterase enzyme test		
Immediate luminescence			Decay rate			0,5 mg/mℓ			1,0 mg/mℓ			2,0 mg/mℓ					
Control	Hard water	% Effect [1]	Control	Hard water	% Effect [1]	Control	Hard water	% Effect [1]	Control	Hard water	% Effect [1]	Control	Hard water	% Effect [1]	Control	Hard water	% Effect [1]
1582	4451	**+181**	9,879	27,164	**+175**	0,027	0,023	15	0,045	0,043	4	0,060	0,062	+3	-	-	-
1731	5816	**+236**	10,036	37,360	**+272**	0,034	0,037	+9	0,052	0,059	**+14**	0,068	0,091	**+34**			
1904	4010	**+111**	11,218	31,717	**+183**	0,035	0,053	**+51**	0,053	0,063	**+19**	0,069	0,078	**+13**			
1956	4907	**+151**	11,505	25,548	**+122**	0,035	0,036	+3	0,053	0,067	**+26**	0,069	0,081	**+17**			
						0,038	0,051	**+34**	0,054	0,072	**+33**	0,070	0,087	**+24**			
						0,077	0,076	1	0,086	0,093	+8	0,096	0,095	1			
						0,078	0,070	10	0,091	0,085	7	0,101	0,111	**+10**			
						0,082	0,082	0	0,095	0,093	2	0,101	0,112	**+11**			
Effect range		+111-+236	**Effect range**		+122-+272	**Effect range**		+51-15	**Effect range**		+33-7	**Effect range**		+34-1	**Effect range**		-

[1] Calculated in relation to control results
\+ Stimulation
\- No results
Results given in bold - effect above detection limit

TABLE 55: Effect of laboratory tap water on enzyme systems

Luciferase enzyme test						Urease enzyme test									Acetylcholinesterase enzyme test		
Immediate luminescence			Decay rate			0,5 mg/mℓ			1,0 mg/mℓ			2,0 mg/mℓ					
Control	Tap water	% Effect [1]	Control	Tap water	% Effect [1]	Control	Tap water	% Effect [1]	Control	Tap water	% Effect [1]	Control	Tap water	% Effect [1]	Control	Tap water	% Effect [1]
-	-	-	-	-	-	0,034	0,042	+24	0,052	0,067	+29	0,063	0,070	+11	0,275	0,285	+4
						0,035	0,040	+14	0,053	0,060	+13	0,066	0,072	+9	0,279	0,277	1
						0,036	0,028	22	0,054	0,047	13	0,066	0,083	+26			
						0,040	0,034	15	0,057	0,065	+14	0,069	0,081	+17			
						0,046	0,041	11	0,062	0,056	10	0,069	0,079	+15			
						0,058	0,053	9	0,080	0,089	+11	0,088	0,100	+14			
						0,074	0,078	+5	0,080	0,083	+4	0,088	0,095	+8			
						0,080	0,035	56	0,088	0,092	+5	0,092	0,099	+8			
						0,083	0,081	2	0,096	0,100	+4	0,094	0,096	+2			
						0,084	0,086	+2	0,102	0,106	+4	0,107	0,116	+8			
						0,084	0,069	18	0,104	0,082	21	0,111	0,119	+7			
Effect range		-	Effect range		-	Effect range		+24-56	Effect range		+29-21	Effect range		+2-+26	Effect range		+4-1

[1] Calculated in relation to control results
\+ Stimulation
\- No results
Results given in bold - effect above detection limit

TABLE 56: Effect of moderately hard, hard and laboratory tap water on mammalian cells

BGM cells									V79 cells								
Control	Moderately hard water	% Effect [1]	Control	Hard water	% Effect [1]	Control	Tap water	% Effect [1]	Control	Moderately hard water	% Effect [1]	Control	Hard water	% Effect [1]	Control	Tap water	% Effect [1]
83	99	+19	83	89	+7	83	109	+31	109	137	+26	109	128	+17	109	122	+12
118	144	+22	118	112	5	106	105	1	129	137	+6	137	158	+15	129	165	+28
120	122	+2	120	116	3	118	114	3	137	143	+4	139	134	4	137	128	7
138	124	10	138	140	+2	120	102	15	139	116	17	143	139	3	139	120	14
						138	134	3	143	145	+1				143	138	4
Effect range		+19-10	**Effect range**		+7-5	**Effect range**		+31-15	**Effect range**		+26-17	**Effect range**		+17-4	**Effect range**		+28-14

[1] Calculated in relation to control results

\+ Stimulation

Results given in bold - effect above detection limit

were examined for possible changes in composition. The variation observed during this period was largely contributed to the natural variation in the sensitivities of the test organisms.

The chemicals selected for testing were found to be suitable as reference toxicants. Methyl viologen was found to be a better reference toxicant for the toad embryo teratogenicity test than sodium selenite.

Although the results obtained with individual solutions were satisfactory, the error and variability associated with the statistical analysis of such results is large. It is, therefore, recommended that definitive tests should be carried out with reference chemicals to establish effective concentrations which are more readily comparable. It is recommended that such tests should be carried out when new organism stock/material is obtained for testing. Furthermore, reference chemicals should be tested once a month (definitive test) with those tests used for routine analysis.

The evaluation of additional controls indicated that, in general, protozoan oxygen uptake, bacterial growth, and acetylcholinesterase enzyme activity and mammalian cell cloning efficiency in hard and moderately hard water were the same as in the standard control. In some instances increased function/activity was detected. The results obtained with algae and the urease enzyme varied, and showed increased as well as reduced function/activity. Tap water showed inhibition in several instances, indicating that this water is unsuitable as control. Since the use of moderately hard and hard water did not drastically change responses in the majority of tests, and since large variations occurred in some of the tests, it is recommended to continue to use deionized water as control in microbial, enzyme and mammalian cell tests. The results obtained with fish and toads indicate that moderately hard water is an ideal substitute for tap water as control.

4. GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE REFINEMENT

The study indicated that, with the exception of the luciferase enzyme test, all the biological toxicity tests employed have a viable role to play in water quality monitoring and control in the country. In general, tests showed good reproducibility, precision and consistency. The evaluation of water showed that there is no single method that can satisfy a comprehensive approach to aquatic life and human health protection. For this reason, toxicity tests should be applied in battery form, so that the tests can complement each other.

Not all the tests were well defined at the beginning of the study. As a result, time was spent to improve/optimize tests. Most of the recommendations suggested for improvement were followed through. Apart from the luciferase test, which was found to be unsuitable for water testing, the algal test still showed a low growth in certain instances. This was mainly due to deficient lighting, erratic subculturing, and insufficient nutrients as a result of medium precipitation. Problems were experienced with the reproduction of fish and toads, particularly in winter months. Furthermore, fish were very prone to disease.

In order to solve breeding and disease problems with fish, it is recommended that fish for toxicity testing purposes are bred and supplied from a central facility. Although satisfactory results are obtained with guppies, attention should be given to the development of procedures employing indigenous species. The algal test problems can be rectified by appropriate optimization. Algal growth was measured at 450 nm instead of at the standard wavelengths of 600-650 nm used in the USA and Europe. This wavelength was selected for density determinations because studies conducted by Slabbert and Hilner (1990) during technique development showed that higher and more acceptable readings can be obtained for microplate use. A wavelength of 450 nm is also used by Canada in their miniaturized algal test (Environment Canada, 1992a). Any interferences by organic or other chemicals at this wavelength should be detected in the blanks. In order to ensure that 450 nm is the most appropriate wavelength for future use, it is recommended that studies are carried out to establish the effect of wavelength on test results. In order to improve fertility of toads, culturing conditions could be revised and alternative hormone treatment could be investigated.

The battery of tests is not complete. Attention should be given to the development of other rapid acute toxicity bioassays. The array of tests should also include short-term chronic aquatic toxicity tests, particularly for effluent and ambient water monitoring. There is also a need for alternative rapid genotoxicity/mutagenicity tests, as the Ames test does not detect all potential carcinogens.

Many of the tests showed enhanced activity when exposed to the water samples. This is usually attributed to the presence of nutrients in the water. It is also possible that the stimulating effects were due to low levels of toxic chemicals. Stimulation in sublethal responses such as growth (Stebbing, 1982) and respiration (Slabbert and Morgan, 1982) has been reported when organisms and cellular systems were exposed to low levels of individual toxic chemicals. This phenomenon is known as hormesis. However, extensive research is required to prove that stimulation by water samples which contain complex mixtures of chemicals is due to toxic activity.

In order to ensure reliable toxicity results, proper quality assurance practices should be followed. Such practices include all aspects of the test that affect the accuracy and precision

of data, namely the test organism/material, test system, control/dilution water, test sample and results.

Reference chemicals are used to establish the validity of toxicity data generated by laboratories. It is recommended that all laboratories involved in toxicity testing should carry out tests with recommended reference chemicals so that inter- and intra-laboratory precision can be monitored.

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APPENDIX A

APPLICATION OF CHEMICAL EQUILIBRIUM MODELLING TO INTERPRET THE TOXIC EFFECTS OF BOREHOLE WATER

by

P J Pretorius

Water Quality Information Systems
Division of Water Technology
CSIR

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Introduction

Slabbert *et al.* (1993) investigated two classes of waters in their report **Development of guidelines for toxicity bioassaying of drinking and environmental waters in South Africa,** namely surface and borehole water. In general, the borehole water showed much higher toxicity than the surface water, and particularly the three standard aquatic tests (fish, *Daphnia*, algae) showed adverse activity. The questions arose: Why was the borehole water, in general, more toxic than surface water and which chemicals caused the effects? Although chemical data were available, effects could not be explained by simple comparison. As alternative, chemical equilibrium modelling (chemical speciation) was applied to interpret toxicity results. Since surface water was not analyzed for all the data required for modelling (particularly alkalinity), modelling was only applied to the chemical data of borehole water.

Approach followed

The approach which was adopted in the compilation of this report is the following. Only the samples which exhibited toxicity in a number of bioassays were considered. The chemical equilibrium modelling was used to simulate the speciation of the solutions, using the conditions listed in Table 39 of Slabbert *et al.* (1996). Because the exact pH of the different media - borehole water mixtures was not reported, the speciation of these tests could not be calculated. Therefore, explanations in terms of the chemical speciation of the solutions were only given in terms of the *Daphnia* test where no additional chemicals played a role. Where this was not possible, effects were explained in terms of known behaviour of certain chemical compounds. To enable this the total concentrations of chemical determinands in samples exhibiting toxicity were compared to values which were compiled by Dallas and Day (1993). Potential toxicants in samples were taken as those constituents which were close to critical values listed by Dallas and Day.

General explanation for toxicity

In general, the alkalinity of borehole water is low which results in a low buffering capacity. This means that, especially for metals, the formation of non-toxic complexes, which reduces toxicity, is limited.

Speciation calculations showed that very little interaction occurred in the specific samples used in this study and that in cases where chemicals were present at potentially toxic levels, adverse activity was likely to occur.

Samples 1, 2 and 5

In these samples no toxicity was observed, so they will not be discussed here.

Sample 3 (Winterveld 1)

Most likely toxicants:

The likely toxicant in this sample is zinc at 190 µg/ℓ. Dallas and Day lists a 48-h LC_{50} for *Daphnia magna* of 100 - 500 µg/ℓ Zn. The value is, however, dependent on zinc speciation in the water sample. Work carried out at CSIR (Pretorius *et al.*, 1994) indicates that a free zinc

(Zn^{2+}) concentration of 6.5 µg/ℓ (10^{-7} mol/ℓ) is detrimental to *Daphnia* in very soft waters with low alkalinity.

Sample 4 (Winterveld 2)

Most likely toxicants:

The most likely toxicants in this sample are nitrate at 44 mg/ℓ and zinc at 2380 µg/ℓ.

Sample 6 (Waterkloof)

Most likely toxicants:

The most likely toxicants in this sample are ammonia, iron, manganese, mercury and phenol. Using the total ammonium concentration of 11.31 mg/ℓ NH_4^+ (which is equivalent to 8.8 mg/ℓ ammonia expressed as N) for this sample in an equilibrium simulation, an NH_3 concentration of 0.009 mg/ℓ at pH 6.2 and 0.14 mg/ℓ at pH 7.4 is obtained. Dallas and Day cites evidence for a 48-h LC_{50} of 2.94 mg/ℓ NH_3 for *Daphnia magna* and a minimum acceptable tolerant concentration (MATC) of 0.41 - 0.87 mg/ℓ NH_3.

The iron and manganese concentrations are also high. Dallas and Day reports that reproductive impairment for *Daphnia magna* at 4.4 mg/ℓ iron has been observed, with a 48-h LC_{50} of 9.6 mg/ℓ. For manganese, they report a 21-day LC_{50} of 5.7 mg/ℓ and reproductive impairment at 4.1 mg/ℓ, using *Daphnia magna*. These values do not, however take the speciation of these elements into account.

The mercury concentration of 4 µg/ℓ is below the 21-day LC_{50} and 48-h EC_{50} values listed by Dallas and Day, which are 13 µg/ℓ and 5.2 µg/ℓ respectively. Reproductive impairment in *Daphnia magna* at 3.4 µg/ℓ has however, also been noted. Again, the effect of mercury on organisms will depend on mercury speciation.

The phenol concentration in this sample of 298 µg/ℓ is not of concern to *Daphnia*. According to the Canadian Water Quality Guidelines, the 48-h LC_{50} for *Daphnia magna* varies from 7.7 mg/ℓ to 19.8 mg/ℓ.

Observation:

When the sample was tested after aeration, the % mortality decreased from 40% to 0%.

Explanation:

Two possible causes for the decrease in *Daphnia* lethality may exist. First, it may be a consequence of ammonium (NH_4^+) being volatile. Thus, aeration of the solution will lead to a decrease in the ammonium concentration. This decrease will result in a lower ammonia (NH_3) concentration, which will decrease toxicity, since ammonia is the toxic species. Second, the solution is supersaturated with respect to a number of iron and manganese solids. It may be expected that after a period of 1 to 3 weeks, plus aeration of the sample for 4 hours, these solids will precipitate. The precipitation may have two effects: first, it will decrease the iron

and manganese concentration in solution and second, it may adsorb mercury from solution, which will decrease the concentration of mercury in solution.

Sample 7 (ISCOR)

Most likely toxicant:

The aluminium concentration of 272 µg/ℓ is approximately 10-times lower than the LC_{50} value for *Daphnia magna* listed by Dallas and Day. It is however, close to the 320 µg/ℓ which is reported to give rise to reproductive impairment in *Daphnia magna*.

The zinc concentration of 220 µg/ℓ is similar to that observed in Sample 3.

Observation:

When the sample was tested after aeration, *Daphnia* lethality decreased from 100% to 45%.

Explanation:

Equilibrium calculations indicated that this sample was supersaturated with respect to a number of solids belonging to the hydrous ferric oxide and aluminium hydroxide groups. The decrease in toxicity may be linked to this. If the sample was left to stand for a period of 1 to 3 weeks before the aerated tests were performed, these solids may have had time to form. They are strong adsorbents and may adsorb toxicants in the solution, effectively decreasing the toxic (zinc) concentration in solution. Aeration did not change zinc speciation at all. However, the pH of the aerated samples were not reported, so we do not have a very good understanding of the chemistry in the solution.

Sample 8 (Silverton 1)

Most likely toxicant:

Aluminium at 161 µg/ℓ, although it is half the concentration listed by Dallas and Day which has been noted to lead to reproductive impairment in *Daphnia magna*. Dissolved oxygen of below 5 mg/ℓ may play a role here.

Observation:

When the sample was tested after aeration, the *Daphnia* lethality decreased from 25 % to 5%.

Explanation:

Two possible explanations for this observation may be offered. First, it may simply be a case of an increase in dissolved oxygen which decreases the *Daphnia* lethality. Second, equilibrium calculations indicated that the sample was slightly supersaturated with respect to aluminium solids. Thus, depending on the time period between the two bioassays, the aluminium containing solids may have precipitated. Other changes in the solution, like an increase in pH with time, will favour this precipitation process. This precipitation process will lead to a reduction in concentration of aluminium in solution.

Sample 9 (Derdepoort)

Most likely toxicant:

The most likely toxicant is zinc at 620 µg/ℓ (*cf Sample 3*).

Observations:

(a) *Daphnia* mortality increased upon aeration
(b) *Daphnia* mortality was very similar to that observed for sample 7, even though the total zinc concentration was approximately 3 times lower in sample 7.

Explanation:

(a) The carbonate concentration in this sample was higher than would be expected for a system in equilibrium with the atmosphere. Such a system will tend towards equilibrium. The reaction describing the equilibrium between $CO_2(g)$ and $CO_3^{2-}(aq)$ is given by the following reaction:

$$CO_2(g) + H_2O <==> CO_3^{2-}(aq) + 2H^+ \quad \textbf{(A)}$$

In order to establish equilibrium, reaction A will tend to the left. Thus, carbonate will be removed from the system and liberated as $CO_2(g)$. During aeration, the solution will be agitated and that would facilitate removal of $CO_2(g)$ (and thus carbonate) from solution.

This will result in an equilibrium carbonate concentration less than the carbonate (alkalinity) of the solution upon sampling. Since the major ligand controlling zinc speciation in this solution is carbonate, the zinc speciation will change. A simulation carried out using the reported alkalinity value contained 43% of the total zinc as Zn^{2+}, which is the toxic zinc species. However, when the solution was equilibrated with air, this changed to 92% zinc as Zn^{2+}. Expressed in terms of µg/ℓ, the original solution contained 264 µg/ℓ Zn^{2+}, while the aerated solution contained 570 µg/ℓ Zn^{2+}. Thus, the increase in Zn^{2+} is the direct result of the decrease in carbonate concentration.

(b) The Zn^{2+} concentrations in the samples are remarkably similar. Sample 7 had a calculated Zn^{2+} concentration of 3.1×10^{-6} mol/ℓ (or 203 µg Zn^{2+}/ℓ), while sample 9 had a calculated Zn^{2+} of 4.05×10^{-6} mol/ℓ (or 264 µg Zn^{2+}/ℓ).

Sample 10 (Silverton 2)

Most likely toxicant:

Nitrate at 6.2 mg/ℓ.

Observation:

Aeration of the sample decreased *Daphnia* lethality from 15% to 0.

Explanation:

If the time period that elapsed between the initial and second test was sufficient, it is possible that nitrate was utilized in denitrification processes since the samples was not preserved with H_2SO_4. These processes will lead to a decrease in nitrate concentration and by-products which were formed may have been driven off during the aeration process.

Sample 11 (Fochville)

Most likely toxicant:

Nitrate at 5.4 mg/ℓ.

Observation:

Aeration decreased *Daphnia* lethality from 80% to 35%.

Explanation:

See explanation for Sample 10.

Sample 12 (Annlin)

Most likely toxicant:

Aluminium at 111 µg/ℓ and iron at 440 µg/ℓ. However, the aluminium concentration is more than 50% less than the concentration noted by Dallas and Day which would give rise to reproductive impairment to *Daphnia magna*. In the case of iron, the concentration in this sample is an order of magnitude less than the lowest concentration showing adverse effects to *Daphnia magna* (Dallas and Day, 1993).

The fact that the borehole is situated in a nursery, together with the reported DOC value, suggest that the toxicant may be a pesticide or herbicide in use in the nursery.

Observation:

Upon aeration, *Daphnia* mortality increased from 40% to 95%.

Explanation:

Based on the chemical analysis available, no explanation for this observation can be given.

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GUIDELINES FOR TOXICITY BIOASSAYING OF WATERS AND EFFLUENTS IN SOUTH AFRICA

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CONTRACT DOCUMENT FOR THE
WATER RESEARCH COMMISSION

Prepared by

J L Slabbert

Division of Water Technology
CSIR
P O Box 395
PRETORIA 0001

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TERMINOLOGY

Acclimation means to become physiologically adjusted to controlled laboratory conditions.

Acute means a stimulus severe enough to rapidly induce an effect in a short period of time. In aquatic toxicity tests an effect observed within 96 h or less is usually considered to be acute. An acute effect can be lethal or sub-lethal.

A **bioassay** is a biological test used to evaluate the relative potency of a chemical or a mixture of chemicals by comparing its effect on a living organism with the effect of a standard preparation on the same type of organism. Bioassays are frequently used in the pharmaceutical industry to evaluate the potency of drugs.

A **chemical** is any element, compound, formulation or mixture of a chemical substance that might be mixed with, deposited in or found in association with water/effluent.

Chronic refers to a stimulus that continues for a relatively long period of time (long-term effects of small doses and their cumulative effects over time). Chronic toxicity is measured in terms of sub-lethal effects. Endpoints include reproduction and growth. Most of the chronic aquatic tests in use today use a short exposure period of approximately 7 days and are called short-term chronic tests.

A **control** 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).

Control/dilution water is the water used for the sample control and for dilution. This may be deionized water, Milli-Q™ water, uncontaminated receiving water, dechlorinated water or reconstituted water.

A **culture** means the stock of organisms grown under defined and controlled conditions to produce health test organisms. **To culture** means to carry out the procedure of growing organisms.

Coefficient of variation (CV) is a standard statistical measure of relative variation of a distribution or set of data, defined as the standard deviation divided by the average (mean).

Dechlorinated water is chlorinated water (usually municipal drinking water) that has been treated to remove chlorine and chlorinated compounds from solution.

A **definitive test** estimates the concentrations at which a certain percentage or number of organisms/material exhibit a certain response. Organisms/material are exposed to various proportions of a chemical solution/effluent and dilution water (usually serial dilutions) for a predetermined period of time. At various times during the exposure period the response of the organisms/material in each concentration is observed and recorded, and the number of responses in relation to the test concentration analyzed.

Deionized water is fresh water from which the soluble ions has been removed.

The **EC** is the point estimate of the toxicant concentration at which a certain percentage of the test organisms/material would be affected (growth inhibition, respiration inhibition, immobility), *e.g.* the EC_{10} (10% effect) or EC_{50} (50% effect).

The **LC** (lethal concentration) is the point estimate of the toxicant concentration at which a certain percentage of the test organisms die, *e.g.* the LC_{10} (10% lethality) or LC_{50} (50% lethality). The exposure period is also included in the endpoint, *e.g.* 96-h LC_{50}.

Lethal means causing death by direct action.

Mutagenic is the ability of a chemical to damage/change an organism's genetic material.

Photoperiod is the duration of illumination and darkness within a 24-h period.

Precipitation means the formation of a solid (*i.e.* precipitate) from a solution.

Reconstituted fresh water is deionized, glass-distilled or Milli-Q water to which reagent-grade chemicals are added to obtain a desired hardness and pH.

A **reference chemical (toxicant)** is a standard chemical used to measure the sensitivity of test organisms to establish confidence in the toxicity data obtained for a test chemical/water/effluent. Furthermore, a reference chemical is used to determine the precision of results obtained by a laboratory for that chemical.

In a **screening test** organisms/material are directly exposed to water/effluent (100% concentration).

Static refers to a toxicity test in which test solutions are not renewed during the test.

Stock solution is a concentrated water solution of a substance to be tested. Test solutions are prepared by adding measured volumes of the stock solution to dilution water.

Sublethal means detrimental but not causing death.

A **teratogen** is defined as any agent capable of causing the formation of congenital anomalies or monstrosities. Thalidomide is a well-known teratogen.

Toxicity is the characteristic/inherent potential/capacity of a chemical (or a group of chemicals) to cause adverse effects on living organisms. Adverse effects include lethality or those effects limiting an organism's ability to survive in nature. Such effects could be acute or chronic.

A **toxicity test** is a technique that determines the effect of a chemical/water/effluent on a group of organisms or cellular/subcellular systems, using defined conditions. Such a 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 a chemical/water/effluent. In the toxicological field a toxicity test is often referred to as a toxicity bioassay/assay or a biological toxicity test.

1. INTRODUCTION

The use of biological toxicity tests has become an important approach to complement chemical analysis to monitor and control harmful chemicals in water. In South Africa, where industrial effluent and hazardous waste are manifesting a growing pollution problem, the demand for toxicity testing is also rapidly growing. Recently, the Department of Water Affairs and Forestry (DWA&F) followed the international trend and identified toxicity testing as an appropriate tool to manage toxic effluents (DWA&F, 1991). It is expected that this new approach of the DWA&F and international exposure will result in an increased use of bioassay techniques in the country. While the usefulness of biological toxicity tests has clearly been proven in local studies, it became necessary to establish guidelines for toxicity testing to ensure that standardized protocol and procedures will be introduced in South Africa.

During the past three years a range of locally developed/established toxicity tests were extensively evaluated in two Water Research Commission projects (Slabbert *et al.*, 1996a,b) in order to formulate guidelines for toxicity testing in the South African context. The information gained from these studies is compiled in this guideline document. The document provides guidance on test methodologies, quality control, sampling, and application, and has been prepared for use by decision makers (DWA&F, Department of National Health and Population Development, Department of Environmental Affairs) and water laboratories.

For additional information refer to the reports of Slabbert *et al.* (1996a,b) **Development of guidelines for toxicity bioassaying of drinking and environmental waters in South Africa** and **Development of procedures to assess whole effluent toxicity.**

2. TOXICITY TEST COMPONENTS

A toxicity test consists of several components including the test organisms/material, test system, test sample, control/dilution water and the test results. In order to ensure that a test is successfully employed it is essential that the test components meet certain general quality assurance requirements. Quality assurance practices include all aspects of the test that affect the accuracy and precision of the data. General guidance on good laboratory practices could be obtained from literature.

2.1 Test organisms/material

Organisms used for toxicity testing should be disease free, as indicated by minimal mortality in holding tanks/containers and in control tests, and be acclimatized to test conditions. In case of microbial tests proper microbial culturing and handling techniques should be followed to keep cultures axenic and contamination free. Cell culture procedures necessitate highly sterile facilities to avoid microbial contamination. Enzymes are sensitive to temperature changes and should be handled and stored as advised on the containers to avoid changes in activity. Test organisms/material should not have been exposed to pollutants or stressed prior to use. All organisms/cellular and subcellular material used for testing should have a known origin and history.

Care should be taken with handling and treatment of organisms to limit variation in their responses. To avoid unnecessary stress after collection, during transportation and acclimation, organisms should not be subjected to changes of more than 3°C in water temperature or 0,3% in salinity during any 12 h period (US EPA, 1985; 1991).

When organisms are obtained from a source known to have healthy stock, a minimum observation period of 48 h should be allowed. If organisms are obtained from unknown stock or from the wild, a period of 7 days should be allowed (US EPA, 1985).

Young organisms are often more sensitive to toxicants than adults, and for this reason early life stages are used, *e.g.* first instars of *Daphnia*, and juvenile fish. There may be special cases where the limited availability of organisms will require some deviation from the recommended life stage. In a given test all organisms should be approximately the same age and should be taken from the same source. The maximum recommended differences in age within a batch is approximately 3 days in case of fish and 12 h for *Daphnia* (US EPA, 1985).

Containers should not be crowded with animals to avoid the depletion of oxygen, the production of waste products and stress induced by crowding. For static tests it is recommended that loading in containers should not exceed 0,8 g/ℓ of test solution at temperatures ≤20°C and 0,4 g/ℓ at temperatures >20°C (US EPA, 1991).

2.2 Test system

Animal room/laboratory/incubator/waterbath temperature control systems should be adequate to ensure the maintenance of recommended test and culturing temperatures. Likewise, temperature control systems of cold storage rooms/refrigerators/freezers should be effective. Measuring devices, *e.g.* thermometers, pH meters, oxygen meters and monitors, and spectrophotometers should be calibrated by following the manufacturer's recommended methods before and at appropriate intervals during use. Instruments should also be serviced by the manufacturer/supplier at regular intervals to ensure optimum operation.

Air used for aeration should be free of oil and fumes. Test facilities should be well ventilated and free of fumes. It is very important to separate culture rooms from the test environment to ensure that organisms/cultures are not intoxicated when working with volatile chemicals/effluents. Ventilation systems should be designed and operated to prevent recirculation and leakage of air from other areas.

Materials that come into contact with water should not release, absorb or adsorb toxicants, and should be cleaned thoroughly before use. Tempered glass, perfluorocarbon plastics (*e.g.* TEFLON), and polystyrene are recommended for use to minimize sorption and leaching of toxic substances. These materials may be reused after decontamination.

Plastics such as polyethylene, polypropylene, polyvinyl chloride and TYGON, and fibreglass may be used as test chambers and to store effluents and dilution water or to convey them to a test system (US EPA, 1985). However, caution should be exercised when using untested plastic because it could be toxic.

Copper, galvanized material, rubber, brass and lead should not come in contact with holding water, dilution water and test samples. Information on the cleaning of containers and equipment can be obtained from literature (US EPA, 1985, 1991; Environment Canada, 1992).

2.3 Water used for dilution, control and culturing

The quality of water used for dilution, control and culturing is very important. It is recommended that water supplies are analyzed at regular intervals to ensure that the water does not contain harmful chemicals.

Water used for the rearing and holding of animals and for dilution and control testing may be natural waters (ground or surface water), dechlorinated tap water or synthetic water. When effluents are tested, receiving water is usually used for dilution and control testing (Slabbert, 1996). Tap water can be dechlorinated by aeration for 1 week or active aeration using air stones for 24 h, filtration through activated carbon, or the use of thiosulphate. However, halogenated organics formed during chlorination might not be removed by aeration and thiosulphate treatment, whereas carbon treatment will remove both the chlorine and the halogenated organics and is the preferred treatment.

Synthetic water has the advantage of having a standard quality. Such water is particularly important where there are large differences between the tap water quality of laboratories (*e.g.* hardness). The use of this water will contribute towards quality assurance practices. The recommended procedure for the preparation of synthetic moderately hard water is given in Table 1.

Dechlorinated tap water is usually used to rear larger organisms such as fish and toads where large volumes of water are required. Smaller animals like *Daphnia* can be easily and effectively cultured in synthetic water. Deionized water is usually used for media preparation, control testing and to prepare dilutions in the case of microbial, enzyme and mammalian cell tests.

Deionized water is prepared by means of standard equipment. However, the quality can vary. To ensure that the deionized process is effective, conductivity should be regularly measured. A very high quality water can be obtained when using a Milli-Q system (reverse osmosis and ion exchange). High quality water for use in toxicity tests can also be purchased from medical suppliers (*e.g.* Baxter water from Sabex).

Table 1: Moderately hard reconstituted water[1]

Reagent added[2] (mg/ℓ)	$NaHCO_3$	96,0
	$CaSO_4.2H_2O$	60,0
	$MgSO_4$	60,0
	KCl	4,0
Nominal water quality range[3]	pH	7,4 - 7,8 (8,2)
	Hardness[4]	80 - 100 (89)
	Alkalinity	60 - 70 (59)

1 US EPA (1985)
2 Prepared with deionized water
3 Measured value in parenthesis
4 As mg/ℓ $CaCO_3$

2.4 Test samples

Water and effluent should be sampled and stored in such a way that the sample is representative of the water/effluent. The toxicity of water/effluent can change because of volatilization of chemicals, precipitation or biological degradation. Samples should be kept at 4°C during transport and before testing. Samples should be tested as soon as possible (within 24 h) after sampling to limit changes in composition (Slabbert, 1996). Containers should be clean (free of chemicals and if necessary sterile). Containers should also seal well

to avoid the loss of volatiles. No preservatives are added to test samples.

Water/effluent variability will determine the sampling method and frequency (Slabbert, 1996; Slabbert *et al.*, 1996b). If there is a large variability in quality a composite sample can be taken. Grab samples are usually collected if variation in quality is small. The compositing process tends to dilute toxicity, and such samples are usually used for chronic tests. Grab samples collected during peaks of toxicity provide a measure of the maximum effect. Such samples taken at selected intervals are the most appropriate for acute tests.

Tests employing aquatic animals (*e.g.* fish, water flea) are usually carried out on the water/effluent without filtration. However, at times when water is very turbid it might be necessary to filter the sample (Whatman filter paper, glass wool). If it is the aim of a test to determine whether or not particulate matter is contributing to toxicity, tests could be carried out on filtered and unfiltered samples to compare results. Some toxicity tests are carried out using sterile conditions (microbial/mammalian cell tests). In these cases sterile filtration of the test samples are required. Water samples should not be autoclaved for sterilization, because the chemical composition of the water will be changed. Some tests might require filtration of samples because particulate matter interferes with measurements [*e.g.* optical density (OD) determination in enzyme tests].

Test organisms are sensitive to extreme pH's, low oxygen levels and chlorine. It is, therefore, important to measure these determinands at the time of sampling or alternatively when the sample is delivered. Usually, tests are carried out on water/effluent without correction of pH or oxygen. However, if it is the objective of the study to establish the contribution of pH/low oxygen, tests could be carried out on the samples as received and after adjustment. Waters containing chlorine should be neutralized by means of thiosulphate. When tests are conducted on drinking water the raw source, rather than the chlorinated water, should be sampled because neutralization is not always successful. The chlorinated water should be tested when mutagenicity tests are conducted on drinking water.

2.5 Test results

When single samples are tested (screening test) results are expressed in terms of a percentage effect, calculated in relation to the control test. In the case of effluents and chemicals, a range of test solutions are tested. This information can be used to calculate effective concentrations, *e.g.* LC_{50}'s or EC_{50}'s.

The sensitivity of toxicity tests depends in part on the number of replicates per concentration, the probability level selected and the statistical procedure used. The sensitivity of the test will increase as the number of replicates is increased. The minimum recommended number of replicates depends on the objectives of a test and the statistical procedures used.

Controls are the basis on which toxic effects are determined. Therefore, organisms/material in control tests should not exhibit any significant responses.

Reference chemicals/toxicants are used to establish the validity of toxicity data generated by toxicity test laboratories. Factors affecting the accuracy of data include the test organism's age, condition, sensitivity and test conditions (oxygen, temperature). The EPA (US EPA, 1985; 1991) recommends that if a laboratory does not have its own culturing programme and obtains organisms in large batches from an outside source, the sensitivity of each batch should be evaluated before use in toxicity tests, or the reference chemical could be tested

concurrently with the test. If laboratories do not conduct tests on a routine basis, and stock is obtained from an outside source, sensitivity tests could run concurrently with toxicity tests. Laboratories culturing their own stock should test sensitivity at regular intervals [*e.g.* once a month (US EPA, 1985; Environment Canada, 1992)].

Results for a reference toxicant are compared with historical test results to identify whether they fall within an acceptable range of variability. Results that do not fall within the acceptable range indicate a change in test organism health or genetic sensitivity, a procedural inconsistency, or a combination of these factors. A reference toxicant can, therefore, be used to confirm the acceptability of concurrent test results and demonstrate satisfactory laboratory performance. Chemicals which can be used as reference toxicants include: sodium chloride, zinc sulphate, phenol and potassium dichromate (Environment Canada, 1992), sodium dodecylsulfate, sodium pentachlorophenate and cadmium chloride (US EPA, 1985).

Record keeping is very important. Detailed records should be kept on test species, age, size, source, date of receipt, culture maintenance, disease treatment, information on calibration of equipment, test conditions, and test results.

3. TOXICITY TESTS

The toxicity test procedures which have been found suitable for local application are briefly outlined. The acute toxicity tests described include: traditional aquatic toxicity tests (*i.e.* fish and water flea lethality tests and an algal growth inhibition test), rapid microbial and enzyme tests (*i.e.* a protozoan oxygen uptake inhibition test; a bacterial growth inhibition test; and urease and acetylcholinesterase enzyme inhibition tests), and a sensitive mammalian cell cloning efficiency test. The fish and water flea tests are carried out according to standard procedures. The other tests have been locally developed (Slabbert *et al.*, 1996a). The sensitivities of the acute tests are shown in Table 2. The chronic tests include the standard Ames *Salmonella* mutagenicity test and a locally developed toad embryo teratogenicity test.

Several standard methods for fish, water flea and algal tests exist (Slabbert *et al.*, 1996a) which could be used for local application. New developments are also regularly published. Should new tests become available, such tests could be included into the local battery of tests. However, an important requirement for alternative/new tests is that they should be properly validated to ensure reliable data.

3.1 Fish lethality test

The fish test utilizes the guppy (*Poecilia reticulata*). This is an exotic fish, imported from the East or from Brazil. This fish is relatively easy to breed and maintain in the laboratory and its sensitivity compares well with those of other test fish species. The guppy is also used by some European countries and Brazil for toxicity testing.

Comment

Ideally, fish toxicity tests should be carried out with an indigenous species. This will, however, require an extensive evaluation to establish sensitivities and suitability for laboratory culturing.

TABLE 2: Sensitivities of bioassays to a number of toxic chemicals

Chemical	Fish lethality test[1]	Water flea lethality test[2]	Protozoan oxygen uptake assay[3]	Algal growth inhibition test[4]	Bacterial growth inhibition test[5]	Urease enzyme test[6]	Acetylcholin-esterase enzyme test[7]	BGM mammalian cell test[8]
	96-h LC_{50} (mg/ℓ)	48-h LC_{50} (mg/ℓ)	Minimum inhibiting concentration (mg/ℓ)	48-h EC_{50} (mg/ℓ) BG-11 medium	6-h EC_{50} (mg/ℓ)	Concentration inhibiting 0,5 mg/m t enzyme (mg/ℓ)	15-min EC_{50} (µg/ℓ)	Survival at concentration chemical (mg/ℓ (in parenthesis) (%)
Aldicarb	-	-	-	-	-	-	16,0	-
Azinphosmethyl	-	-	-	-	-	-	0,5	-
Cadmium	1,85	0,319	1,0	0,076	0,08	10,0	-	69,8 (0,065)
Carbofuran	-	-	-	-	-	-	0,049	-
Cobalt	-	-	-	-	-	10,0	-	-
Copper	0,55	0,031	0,5	0,081	0,1	1,0	-	30,2 (0,025)
Cyanide	0,13	-	0,014	0,362	0,018	-	-	-
Demeton-S-methyl	-	-	-	-	-	-	373	-
Lead	-	2,003	-	-	-	>30,0	-	37,7 (0,3)
Malathion	-	-	-	-	-	-	2,0	-
Manganese	-	-	-	-	-	>130	-	-
Mercury	0,2	0,004	0,5	0,303	0,025-0,05	0,05	-	57,0 (0,005)
Nickel	-	-	-	-	-	10,0	-	-
Phenol	15,0	-	90,0	>100	15,1	-	-	32,4 (0,5)
Propoxur	-	-	-	-	-	-	20,0	-
Silver	-	0,002	-	-	-	0,1	-	-
Zinc	-	-	0,5	0,015	0,15	5,0	-	-

1 Morgan (1982)
2 Elnabarawy *et al.* (1985)
3 Slabbert and Morgan (1982)
4 Slabbert and Hilner (1990)
5 Slabbert (1986)
6 Metelerkamp (1986)
7 Venter (1991)
8 Kfir (1981)

Guppies can be obtained from pet shops to start a laboratory culture. Alternatively, young fish for testing can be obtained from fish farms. It is important to make use of a known source to ensure that animals are disease free. When new stock is obtained fish should be kept separate from the existing stock to avoid the possible spread of disease.

Fish are maintained in dechlorinated tap water in glass containers, using continuous aeration. Temperature and light requirements are given in Table 3. Specific instructions for guppy culturing can be obtained from books on tropical fish (Wainwrite, 1983; Axelrod and Schultz, 1990), while general guidelines on fish culturing and maintenance can be obtained from the US EPA test methodologies (US EPA, 1985; 1991).

The toxicity test is carried out according to standard procedures (US EPA, 1985). The recommended test conditions for the fish test are summarized in Table 3.

TABLE 3: Fish bioassay test conditions[1]

Water temperature[1]	23 ± 2°C
Light quality[2]	Daylight
Photoperiod	9 - 14 h daylight (depending on season)
Oxygen concentration	As obtained (>40% of saturation)
pH	As obtained
Feeding regime	No food
Age of fish	2-3 weeks[5]
Size of test container	500 mℓ
Volume of test sample[3]	350 mℓ
Number of fish/vessel	5
Number of replicate vessels	2
Total number of fish/test[4]	10
Control/dilution water	Standard control water[6]
Test duration	96 h
Effect measured	Lethality
Detection limit	10%
Test acceptability	Control lethality <10%

1 Optimum temperature (Axelrod and Schultz, 1990)
2 Artificial light, set at the desired photoperiod can be used
3 A smaller volume can be used (*e.g.* 250 mℓ), provided that fish are not stressed
4 A better reproducibility is possible if more fish, *e.g.* 20, are used per test
5 When young test fish are obtained from an outside source, large losses can occur due to stress. Fish 3-6 weeks old can be used when fish are not cultured in the test laboratory. The age of the fish should, however, be reported in studies
6 Moderately hard reconstituted water (US EPA, 1985) (Table 1). Young fish should be acclimatized to this water before use in toxicity tests

Shortcomings:

* Maintenance and breeding is very time-consuming
* A very large stock is required to ensure enough fish for routine use
* Fungal diseases occur from time to time rendering fish unfit for use/breeding

3.2 Water flea lethality test

The recommended test species is *Daphnia pulex*. According to literature this test species is more sensitive than other *Daphnia* species (Elnabawary *et al.*, 1986). The organism has been locally isolated and identified by a taxonomist (Figure 1). A culture can be obtained from the Division of Water Technology (DWT), CSIR, Pretoria.

Culturing and testing is carried out according to US EPA procedures (US EPA, 1985; 1991). Stock cultures are maintained in glass beakers in moderately hard water (Table 1), without aeration. *Daphnia* are fed every second day, using a food suspension prepared from trout pellets, alfalfa and yeast (US EPA, 1985). Algae can also be used (US EPA, 1985) for feeding. Stock is subcultured at weekly intervals to control organism numbers in containers. [About 30 adults per 3 ℓ container will avoid overcrowding during a one week growth period (US EPA, 1985)]. Detailed information on organism distribution, life cycle, morphology and taxonomy, and culturing can be obtained from literature (US EPA, 1985; 1991).

Comments

* In order to ensure a healthy and homogeneous culture (only females) careful maintenance is essential. A proper feeding regime should be followed and containers should not be overcrowded.

* When a culture is stressed (high population densities, accumulation of excretory products, too much/little food) the culture is no longer homogeneous. Males and females will be present and ephippia (sexual eggs) will form. *Daphnia* used for toxicity tests should not be taken from cultures containing ephippia.

Young organisms (juveniles), 24 h or less in age, are used for toxicity testing. In order to obtain the necessary number of young for a test, adult females bearing embryos in their brood pouches are removed from the stock cultures 24 h preceding the initiation of a test, and placed in 100 mℓ beakers containing 50 mℓ of moderately hard water and food suspension. Test conditions are summarized in Table 4. Test organisms are transferred to a small intermediary holding beaker and from there to the test beakers.

Advantages:

* Maintenance is relatively easy and simple

* Ideal for routine use as they grow rapidly and produce large numbers of offspring

* Shows a high sensitivity to a wide range of chemicals (organic and inorganic), rendering water flea very suitable for the protection of aquatic life and human health

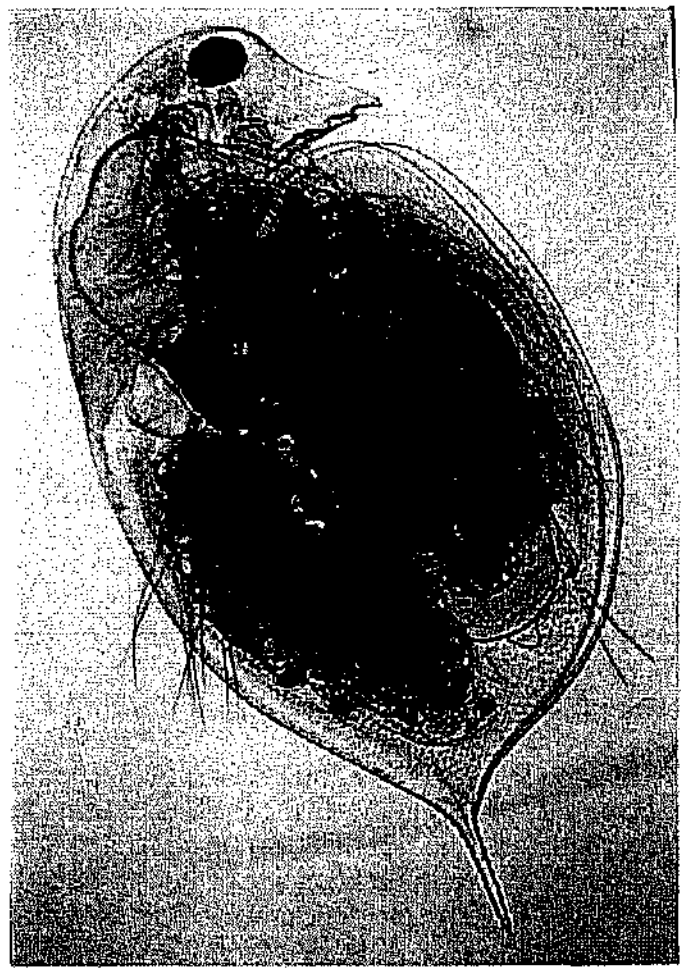

FIGURE 1: ***Daphnia pulex*** **(Maximum length: 39 mm)**

TABLE 4: Water flea bioassay test conditions[1]

Water temperature[1]	20 ± 1°C
Light quality[2]	Daylight
Photoperiod	9 - 14 h daylight (depending on season)
Oxygen concentration	As obtained (>40% of saturation)
pH	As obtained
Feeding regime	No food
Size of test beaker	50 mℓ
Volume of test sample	25 mℓ
Number of organisms/beaker	5
Number of replicate containers	4
Total number of organisms/test	20
Control/dilution water	Standard control water[3]
Test duration	48 h
Effect measured	Lethality (no movement of body or appendages on gentle prodding)
Detection limit	10%
Test acceptability	Control lethality <10%

[1] Optimum temperature (US EPA, 1985)
[2] Artificial light, set at a desired photoperiod can be used
[3] Moderately hard reconstituted water (US EPA, 1985) (Table 1)

3.3 Algal 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). Instead of using flasks, tests are carried out in 24-well tissue culture plates. The test has been miniaturized to enable rapid handling and processing of samples, to reduce incubation space and to eliminate washing of glassware. A miniaturized technique has also been developed in Canada (Blaise *et al.*, 1986), which has become one of their recommended tests (Environment Canada, 1992). In general, a good agreement has been found between the sensitivity of the miniaturized tests and the standard flask test (Slabbert *et al.*, 1996a).

The unicellular alga *Selenastrum capricornutum* is used as test organism. This alga has been proven to be more sensitive than other algal species (*e.g. Scenedesmus* and *Chlorella*) (Slabbert and Hilner, 1990). The algal culture was obtained from the Environmental Protection Service, Environment Canada (originally obtained from the US Environmental Protection Agency, Corvallis, Oregon). Cultures for local use are available from the DWT.

The alga is axenically maintained in Erlenmeyer flasks according to standard procedures (US EPA, 1978). Twenty percent algal solution to flask volume ratios are recommended to avoid growth inhibition due to carbon dioxide limitation. Culturing is carried out at constant temperature (25 ± 1°C) without shaking (swirled by hand, twice a day), using continuous illumination (cool white fluorescent light: approximately 95 $\mu E/m^2/s$). Algae are subcultured at weekly intervals to have a constant supply of logarithmic growth phase cells for bioassaying (*e.g.* 1 to 2 mℓ cell suspension/50 mℓ medium in a 250 mℓ flask).

A modified 10% BG-11 medium (Rippka *et al.*, 1979) has been found to be the most suitable for culture maintenance and toxicity testing (Slabbert and Hilner, 1990; Slabbert *et al.*, 1996a,b). One litre of growth medium is prepared by adding 1 mℓ of a range of stock

solutions (Table 5) (10 mℓ of $NaNO_3$) to deionized water. The medium is sterilized by means of membrane filtration (pore size: 0,22 μm). A 20-times concentrate of the growth medium, which is used for toxicity testing, is prepared by adding 1 mℓ stock solution (10 mℓ of $NaNO_3$) per 50 mℓ deionized water, followed by filter sterilization.

TABLE 5: Composition of modified 10% BG-11 medium[1]

Stock solution		Reagent	Stock solution concentration (g/ℓ)	Volume (mℓ) of stock solution added per litre of water	10% BG-11 nutrient concentration (mg/ℓ)[2]
Macro-nutrients	A	$CaCl_2.2H_2O$	3,6	1	3,6
	B	$NaNO_3$	15,0	10	150,0
	C	K_2HPO_4	4,0	1	4,0
	D	$MgSO_4.7H_2O$	7,5	1	7,5
	E	$NaHCO_3$	2,0	1	2,0
Micro-nutrients	F	H_3BO_3 $MnCl_2.4H_2O$ $CuSO_4.5H_2O$ $ZnSO_4.7H_2O$ $Co(NO_3)_2.6H_2O$ $Fe(NH_3)$-citrate $Na_2MoO_4.2H_2O$ Citric acid	0,286 0,181 0,0079 0,0222 0,00494 0,6 0,039 0,6	1	0,286 0,181 0,0079 0,0222 0,00494 0,6 0,039 0,6
	G	$Na_2EDTA.2H_2O$	0,1	1	0,1

[1] Rippka *et al.* (1979)
[2] pH 7,2
Deionized water used for the preparation of solutions

Toxicity tests are carried out in sterile 24-well tissue culture plates (well volume: 3,5 mℓ) (Slabbert and Hilner, 1990). When single samples are tested (screening test), each sample is distributed into a row of six wells per plate. One row of wells per plate receives the control sample. In case of definitive testing (*e.g.* testing dilutions of an effluent), a row of four wells per plate is used per sample/control. 1,8 mℓ of sterile sample/control is distributed into wells, using a micropipette. Water/effluent samples are sterilized by means of membrane filtration (pore size: 0,22 μm) and the control (deionized water) is autoclaved.

Algal cultures 4 to 6 days old are used for toxicity testing. 24 h before testing, the algae in flasks are allowed to settle (no agitation). On the day of testing the algal inoculum is prepared by removing the supernatant medium by means of a sterile pasteur pipette and vacuum pump. The cells are suspended in fresh culture medium (same volume as before) and the cell concentration determined by means of a haemacytometer and microscope.

The cell suspension is then diluted with fresh medium to a concentration of $4x10^6$ cells/mℓ. This suspension is added at a ratio of 1:1 (volume determined by number of wells to be inoculated) to a 20-times concentrate of the culture medium and used as 200 µℓ volumes for inoculation of the 1,8 mℓ sample in test/control wells (20-fold dilution in cell number and medium concentration). The final cell concentration at the start of a test is $2x10^5$ cells/mℓ (OD: 0,005 to 0,012 at 450 nm in microplate reader, using a volume of 280 µℓ per well). One well of each row of wells receives medium only (200 µℓ of a 1:1 dilution of fresh medium and the 20-times concentrated medium). These solutions are used as blanks during OD determinations.

Tissue culture plates are then covered with lids and sealed with parafilm strips along edge of lids. Incubation is carried out at 25 ± 1°C for 48 to 72 h (no agitation), using continuous illumination (cool white fluorescent light: approximately 95 $\mu E/m^2/s$). At the end of the incubation period the contents of each well is thoroughly mixed with a micropipette. 280 µℓ volumes (duplicate volumes from test/control wells) are then transferred to 96-well flat-bottomed microplates for OD determination on a microplate reader at 450 nm.

Test acceptability

The OD of the controls should be 0,15 ± 0,03 at the end of the incubation period and the variation between replicate control tests (CV) <10%

Factors influencing growth:

* Light plays an important role in test reproducibility. It has been found that growth varies largely from one position to another under the light. It is, therefore, important to position lights in such a way to have the best dispersion of light (white backgrounds are recommended). The use of the 24-well plates has the advantage that tests and controls are in close proximity, which reduces variation. However, it is important to use plates which are opaque around the margins to avoid a higher growth in the outer wells.

* The health of the algal culture is a very important factor influencing the test. Subcultures should be made regularly (at least once a week) to ensure steady log phase cultures.

* The medium tends to form a precipitate after standing for some time. For optimal growth, all the nutrients should be in solution. Therefore, fresh medium should be prepared at regular intervals. Solutions should be stored at 4°C to delay chemical changes.

Results are expressed as percentage inhibition (or stimulation), calculated as follows:

$$100\% - \left[\frac{ODT - OD_0}{ODC - OD_0} \times 100\%\right],$$

where

ODC = Average optical density of control
ODT = Average optical density of test
OD_0 = Optical density at time 0

Inhibition ≥10% (detection limit) is an indication of toxic activity.

Comments

* The inoculum is 20x larger than that used in the US EPA methodology. Results are, therefore, available within 2 to 3 days (as compared to 4 to 7 days). The use of this higher inoculum did not result in significant changes in sensitivity (Slabbert and Hilner, 1990).

* The subtraction of the OD at t=0 from final readings allows for improved sensitivity (Slabbert and Hilner, 1990).

* The use of a blank test ensures that increases in OD due to colour/precipitation are noted and subtracted from the actual growth readings.

* Most methodologies determine OD at wavelengths >600 nm. A wavelength of 450 nm has been selected for the miniaturized test because the OD is considerably higher than at wavelengths >600 (Figure 2). The higher readings are preferred to limit variation. Any interferences (which have been found to be minimal) at this lower wavelength will be detected with the blanks. The Canadian microplate method (Environment Canada, 1992) also uses a wavelength of 450 nm for OD determination.

* It appears to be generally agreed that algal toxicity tests should be terminated well before the growth of control cultures becomes severely depressed because of limiting factors and waste products (Nyholm, 1985). The test has been designed in such a way that control cultures grow exponentially (logarithmic growth phase) for the whole duration of the test (Figure 3).

* Cool white fluorescent light is the recommended light source (standard methods). Figure 4 shows that growth is slower when, for instance, Grow Lux lighting is applied.

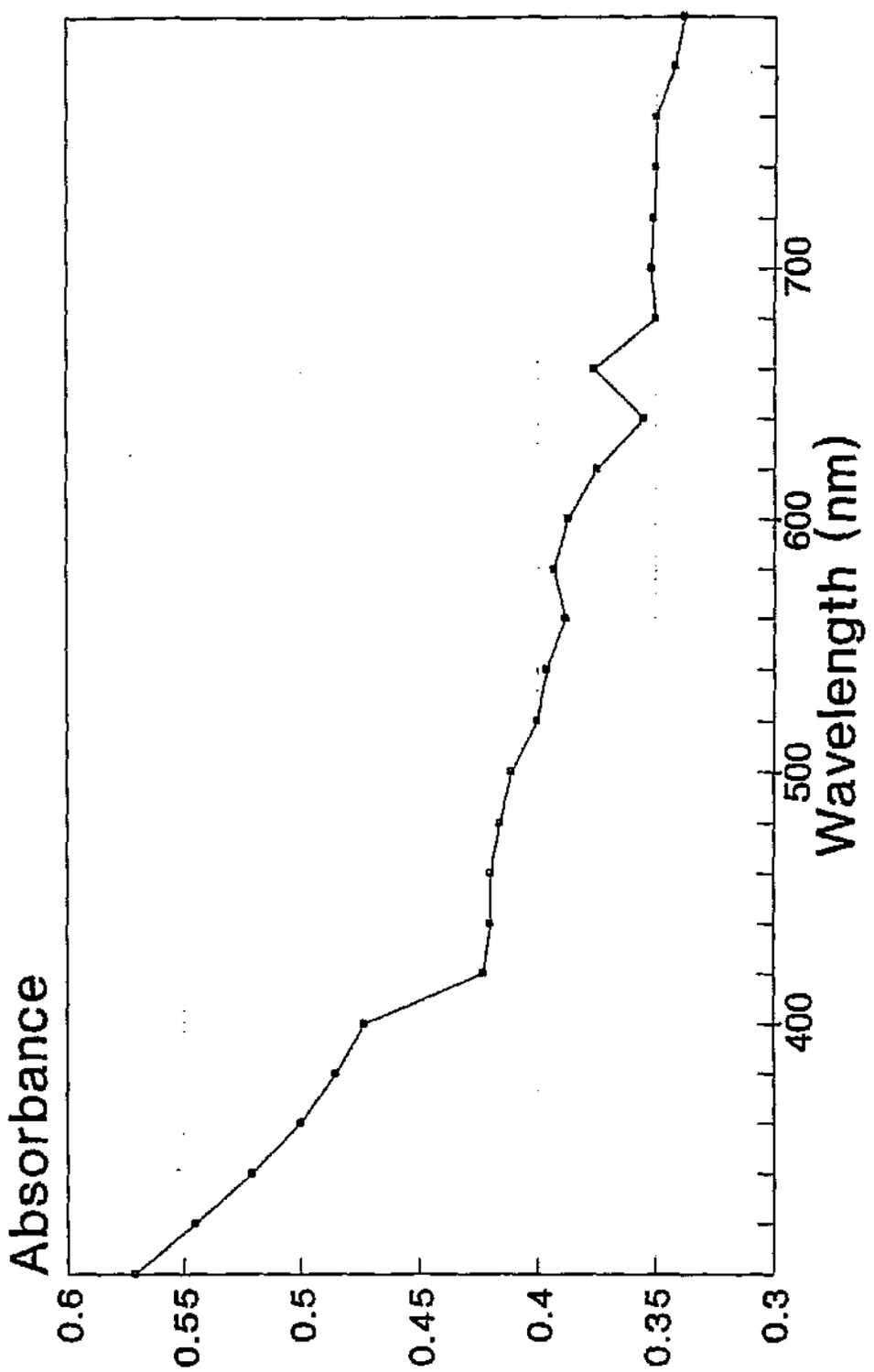

FIGURE 2: Absorbance of *Selenastrum capricornutum* at various wavelengths

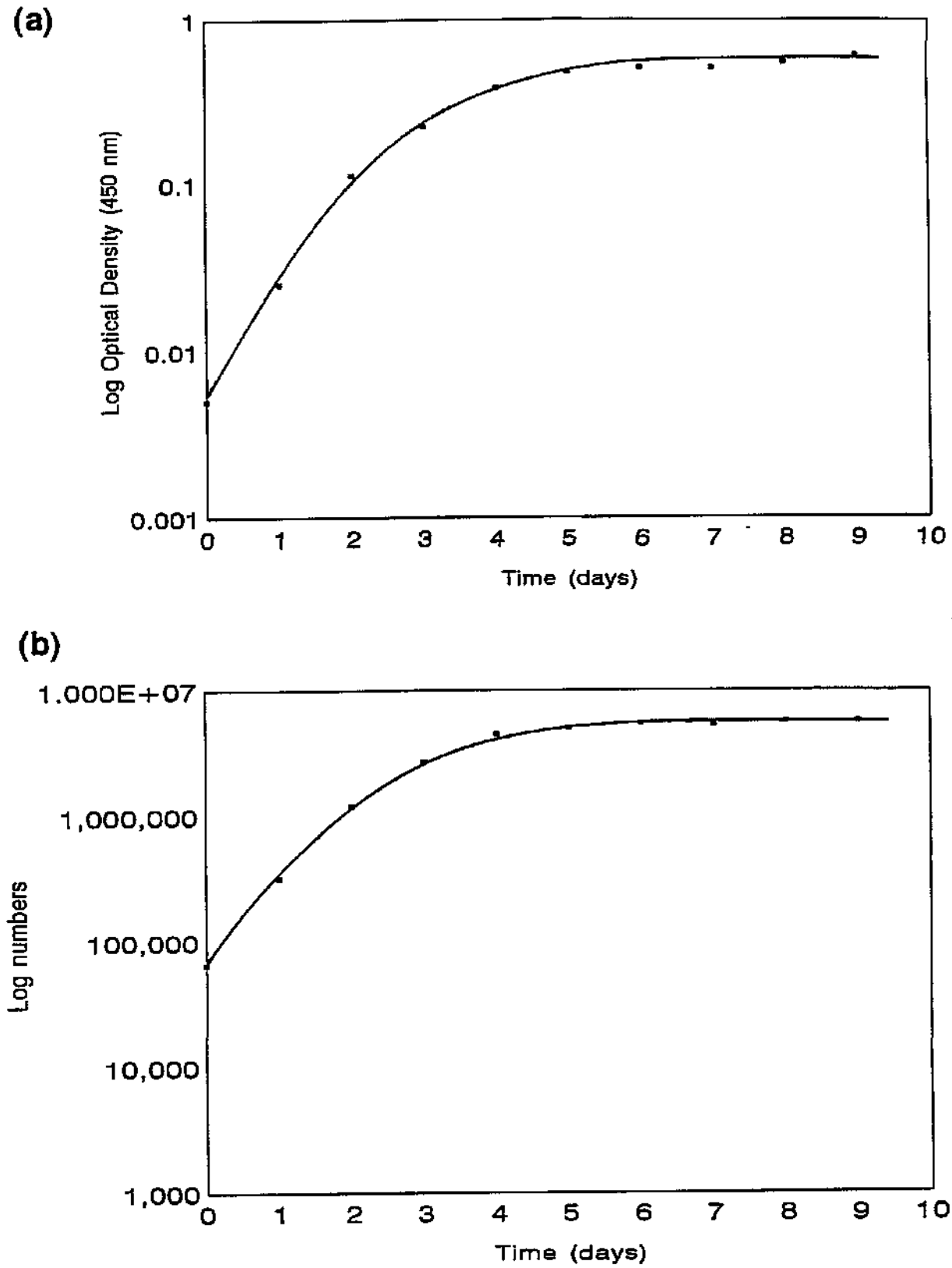

FIGURE 3: **Growth of *Selenastrum capricornutum* measured in terms of optical denisty (a) and numbers (b)**

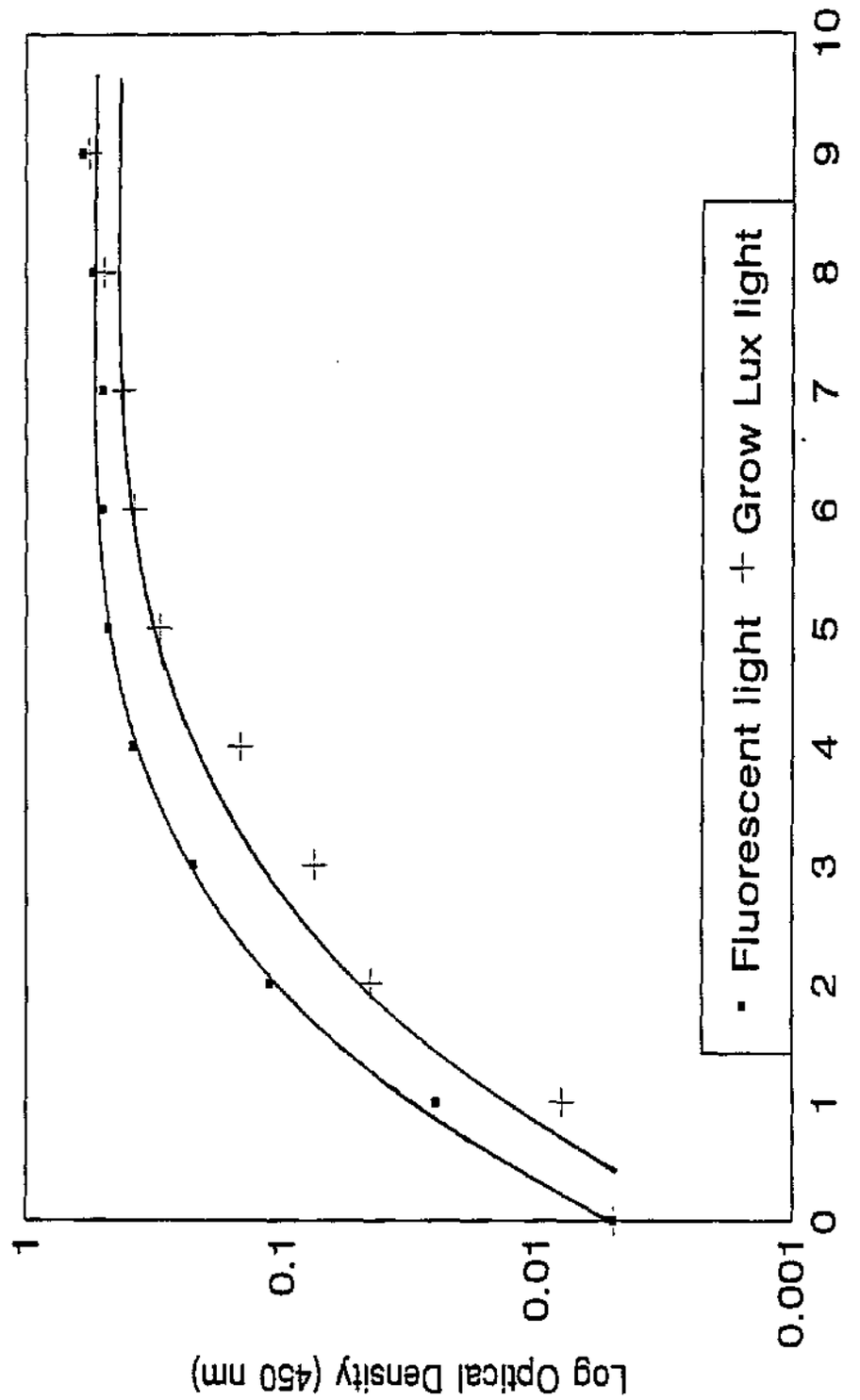

FIGURE 4: Growth of *Selenastrum capricornutum* using two light sources

Advantages:

* The test is particularly sensitive to heavy metals

Shortcomings:

* In some instances precipitates can form (*e.g.* waters containing high nutrient levels and paper mill effluents) (Pretorius, 1996) which interferes with OD determination. In such cases growth can be measured in terms of cell numbers (counting)

3.4 Protozoan oxygen uptake inhibition test

The protozoan oxygen uptake test was developed for rapid toxicity screening purposes and described in detail in a publication by Slabbert and Morgan (1982). This bioassay has the outstanding feature of providing results within the short period of 10 min, and has been found invaluable in emergency situations when rapid information on acute toxicity is needed.

The test utilizes a unicellular protozoan, *Tetrahymena pyriformis*, strain W (Figure 5). The cell culture was originally obtained from the Rand Afrikaans University, Johannesburg, and is available from the DWT for use. Cultures are maintained axenically in a broth comprising 10,0 g proteose peptone, 0,5 g yeast extract, 2,5 g glucose and 0,5 g sodium chloride per litre of deionized water (pH 6,9) (Slabbert and Morgan, 1982). Incubation is carried out at 27 ± 1°C in the dark. Logarithmic growth phase cells are used for bioassaying. An adequate cell concentration was obtained every 24 h using a 2% (v/v) inoculum for subculturing.

For bioassaying, cells are suspended in an osmotically balanced salt solution (Taylor and Strickland, 1935) using gravity filtration (filter pore size: 8 μm). The final cell concentration should yield an oxygen uptake rate of 6 - 8%/min (Slabbert and Morgan, 1982). Tests are carried out with a biological oxygen monitoring system (Yellow Springs Instrument Co, Yellow Springs, OH) which includes (Figure 6):

- An electronic unit (A) with meter readout of percentage dissolved oxygen;
- A standard bath assembly (B) consisting of a water bath with airtight test chambers and a built-in magnetic stirring device; and
- Oxygen probes (B) fitted in lucite plungers provided with slanting fronts and access slots along one side closely sliding into test chambers (Figure 7).

A recorder (D) is connected to the electronic unit to provide graphs of oxygen uptake rate (chart speed: 2 cm/min). The system as well as cell suspensions are maintained at constant temperature (27 ± 0,5°C) using a constant temperature water circulator (E). Air saturated deionized water is used to calibrate the apparatus.

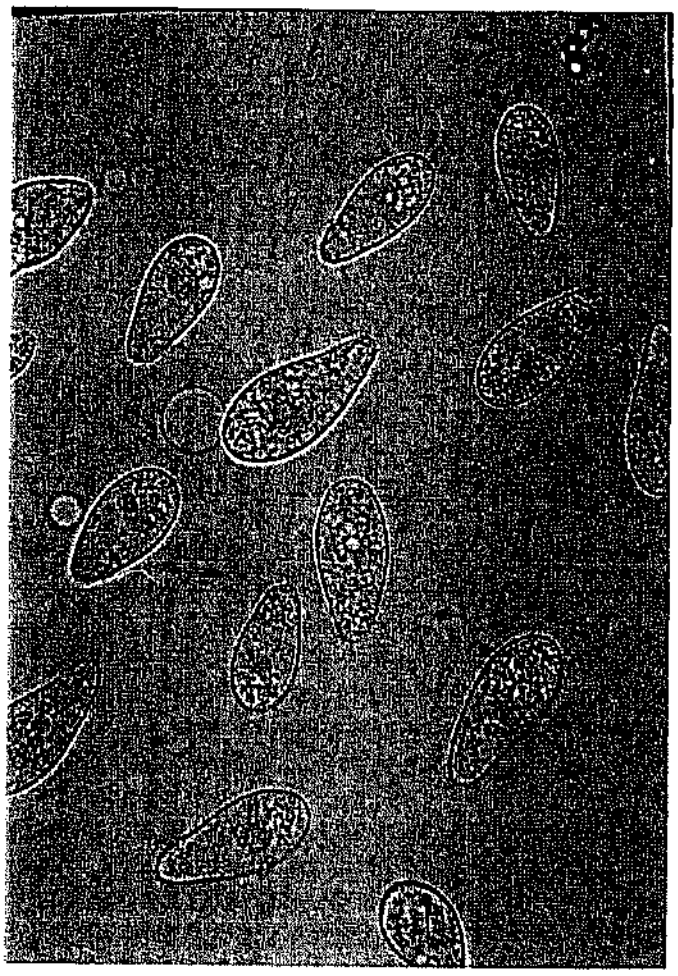

FIGURE 5: ***Tetrahymena pyriformis*** **(Size: 30 by 50 µm)**

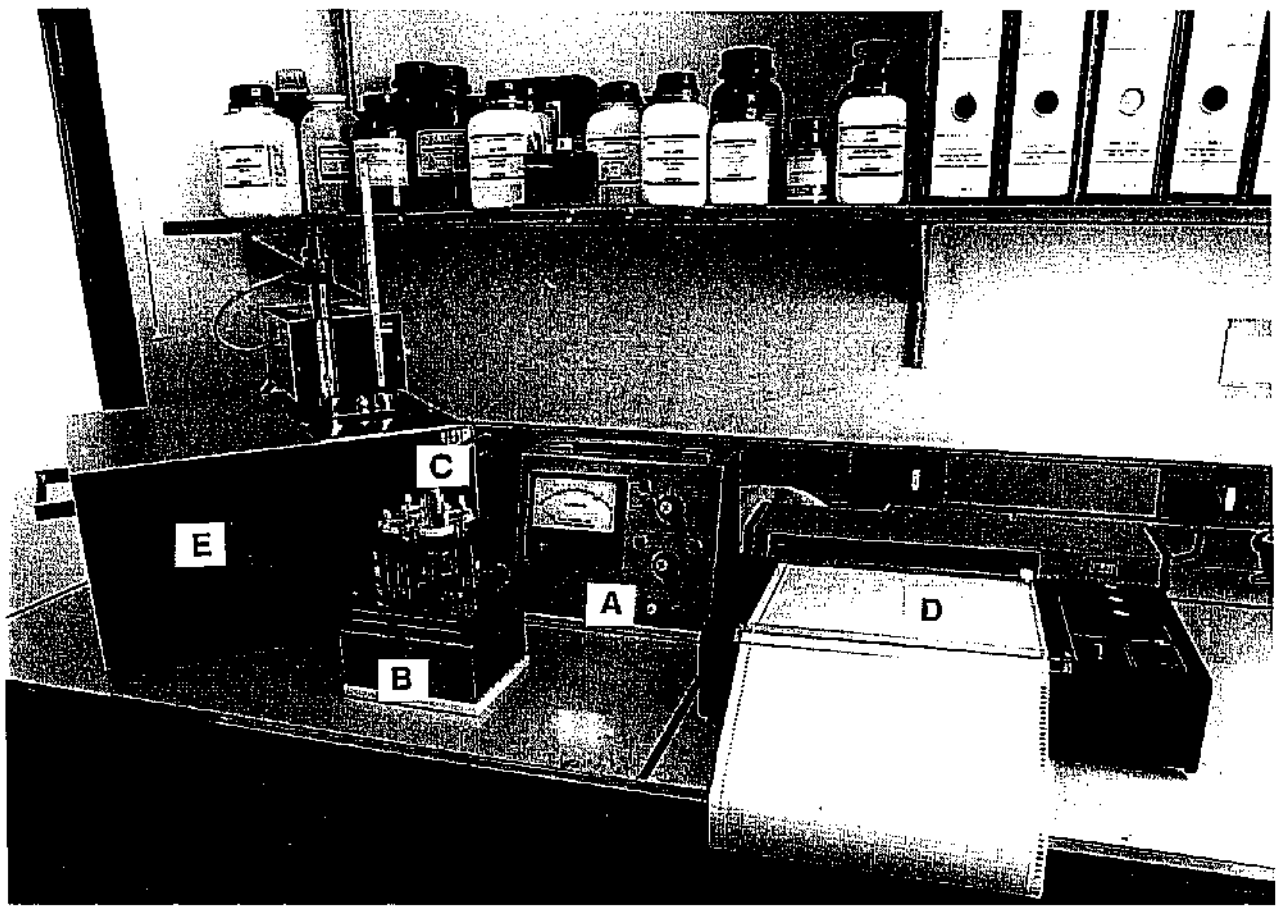

FIGURE 6: **Oxygen uptake monitoring system. (A) Electronic unit; (B) standard bath assembly; (C) oxygen probe; (D) recorder; (E) constant temperature circulator**

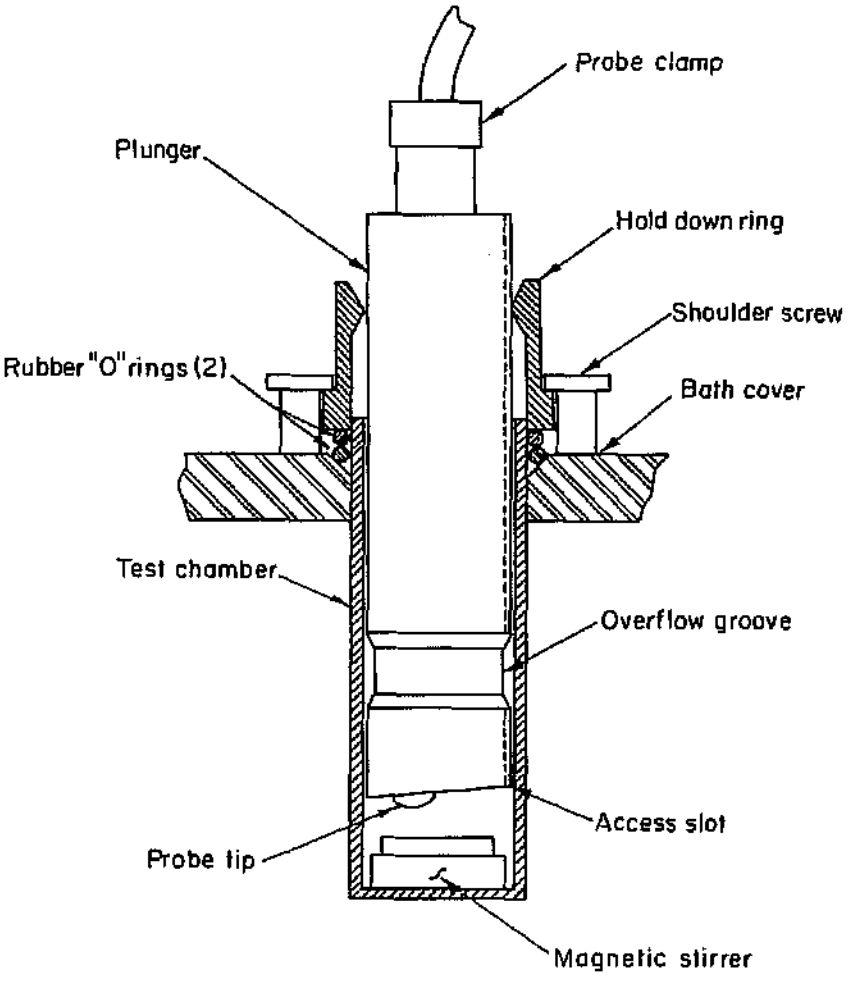

FIGURE 7: Probe, plunger and test chamber

Comment

A cell suspension should not be used for longer than 2 h to avoid stress. Filtration and testing is carried out under asterile conditions. Clean glassware and sterile saline/test samples should be used to avoid unnecessary contamination.

For each test 3 mℓ of the cell suspension is aerated for 5 min in a test chamber provided with a magnetic stirrer (Slabbert and Morgan, 1982; Slabbert, 1988). The oxygen probe is then inserted into the test chamber and dissolved oxygen is monitored continuously before (reference), during and after test sample addition, for a total period of approximately 10 min. Test samples (3 mℓ) (filtered through a 0,22 μm membrane) are introduced after a monitoring period of between 3 and 4 min. Deionized water is used for control testing. Each test and control is carried out in triplicate. Results are determined as a ratio of the oxygen uptake rate after sample addition (test slope) to that prior to sample addition (reference slope) (Figure 8) (Slabbert, 1988). Effects are expressed as percentage inhibition (or stimulation), calculated in relation to control tests. Inhibition ≥5% (detection limit) is an indication of toxic activity.

Test acceptability

The reference slope of the control should be between 6 and 8%/min, and the ratio of the oxygen uptake after sample addition to that prior to sample addition 0,49 ± 0,02. The variation between replicate control tests (CV) should be <5%

Advantages:

* The test provides results in a short period of time and is highly reproducible. Because of the good reproducibility (CV: <5%) single tests (no replication) are adequate in emergency situations

Shortcomings:

* The test is very time consuming and not recommended for routine testing as only one test can be carried out at a time

3.5 Bacterial growth inhibition test

Pseudomonas putida strain Berlin 33/2 is used as test bacterium. The organism has been obtained from Prof H Stolp, Deutsche Sammlung von Mikroorganismen, Teilsammlung, Bayreuth. Cultures for local use are available from the DWT laboratories. Stock cultures are made on nutrient agar slants in McCartney bottles. Cultures are kept at 4° C, after incubation at 27 ± 1° C for 18 h.

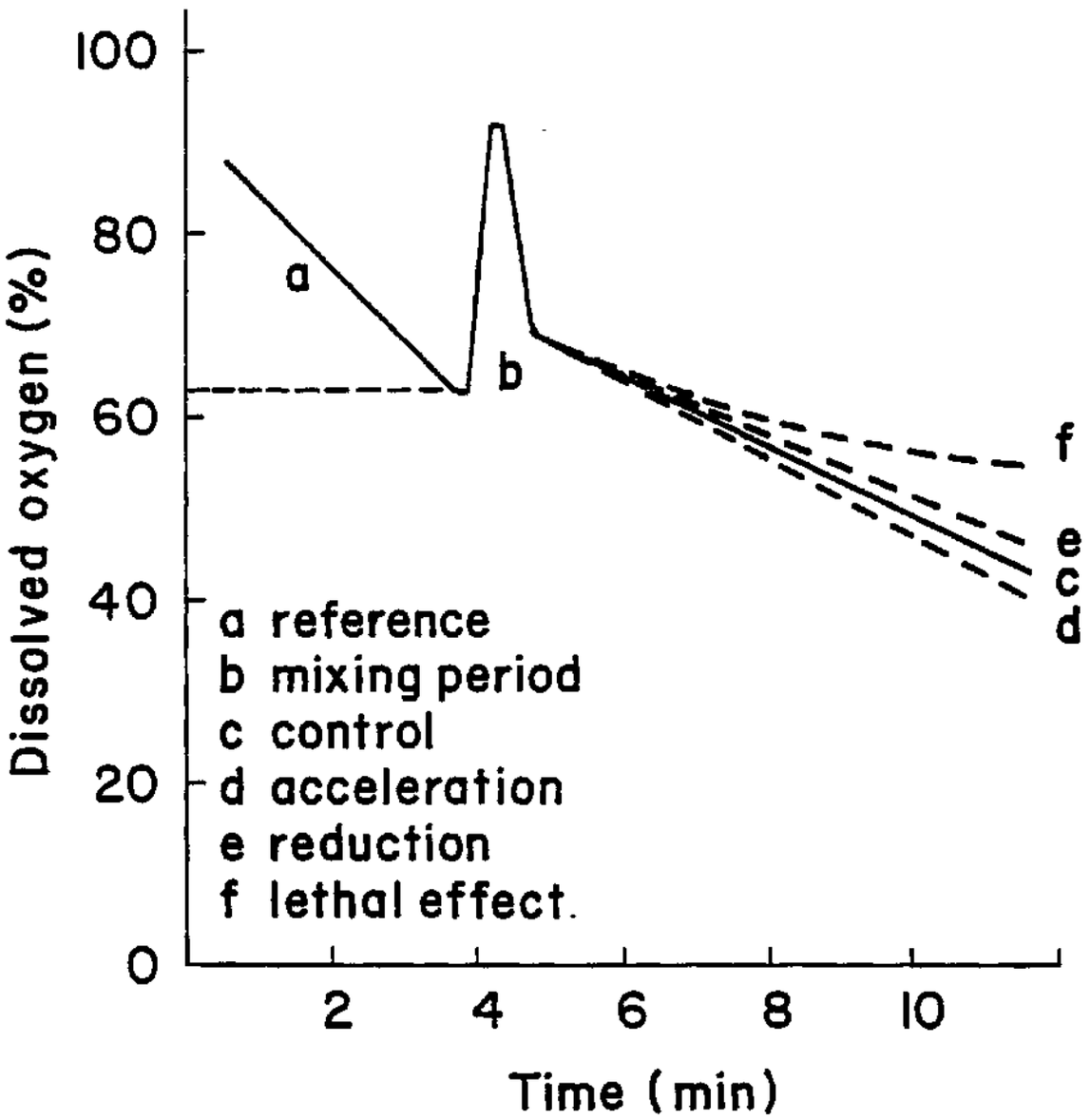

FIGURE 8: **Typical graphs obtained for *Tetrahymena pyriformis* oxygen uptake**

Toxicity tests are carried out in 25 mℓ minimal medium [1,05 g K_2HPO_4, 0,45 g KH_2PO_4, 0,047 g Na_3-citrate.$2H_2O$, 0,1 g $(NH_4)_2SO_4$,, 0,01 g $MgSO_4.7H_2O$ and 0,25 g glucose per litre of deionized water (pH: 7,15)] in 50 mℓ medical flats (Slabbert, 1986; 1988). A culture of *P. putida*, grown overnight in minimal medium, is diluted with fresh minimal medium to an OD of approximately 0,8, 30 min before inoculation of test samples (Slabbert, 1986). 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 concentrate of the minimal medium, and used as 2,5 mℓ volumes for inoculation of 22,5 mℓ test samples (filter sterilized using a 0,22 μm membrane filter). Solutions containing sample and medium only are prepared to blank the spectrophotometer. All preparations are carried out at a constant temperature (20±1°C). Each test and control is carried out in five-fold. Sterile (autoclaved) deionized water is used for control testing. Cultures are incubated at 27 ± 1°C for approximately 6 h after which growth is measured in terms of OD. Cells in controls are in the late logarithmic phase when the test is terminated (Figure 9).

Results are expressed as percentage inhibition (or stimulation), determined in relation to control results. A growth inhibition of ≥10% (detection limit) is considered as a toxic effect.

Test acceptability

The control growth should have an OD of 0,4 ± 0,05 at the end of the incubation period and the variation between replicate control tests (CV) should not exceed 10%

Advantages:

* The test is relatively rapid and can be completed in one day

Shortcomings:

* In some instances precipitates can form (*e.g.* waters containing high nutrient levels and paper mill effluents) (Pretorius, 1996) which interferes with the OD determination. In such cases growth can be measured in terms of cell numbers using a DNA stain (Slabbert and Hilner, 1991) or colony development using membrane filtration

3.6 Urease enzyme inhibition test

The urease enzyme test is specific for the detection of heavy metals (Metelerkamp, 1986). The test is carried out in microplates which allows for field testing. Microplates also allows for quantitative data calculation using a microplate reader at 450 nm. The test is rapid, providing results within 1 h. The enzyme reaction is as follows:

NH_2 - CO - NH_2 (urea) + H_2O (water) —> CO_2 (carbon dioxide) + $2NH_3$ (ammonia)

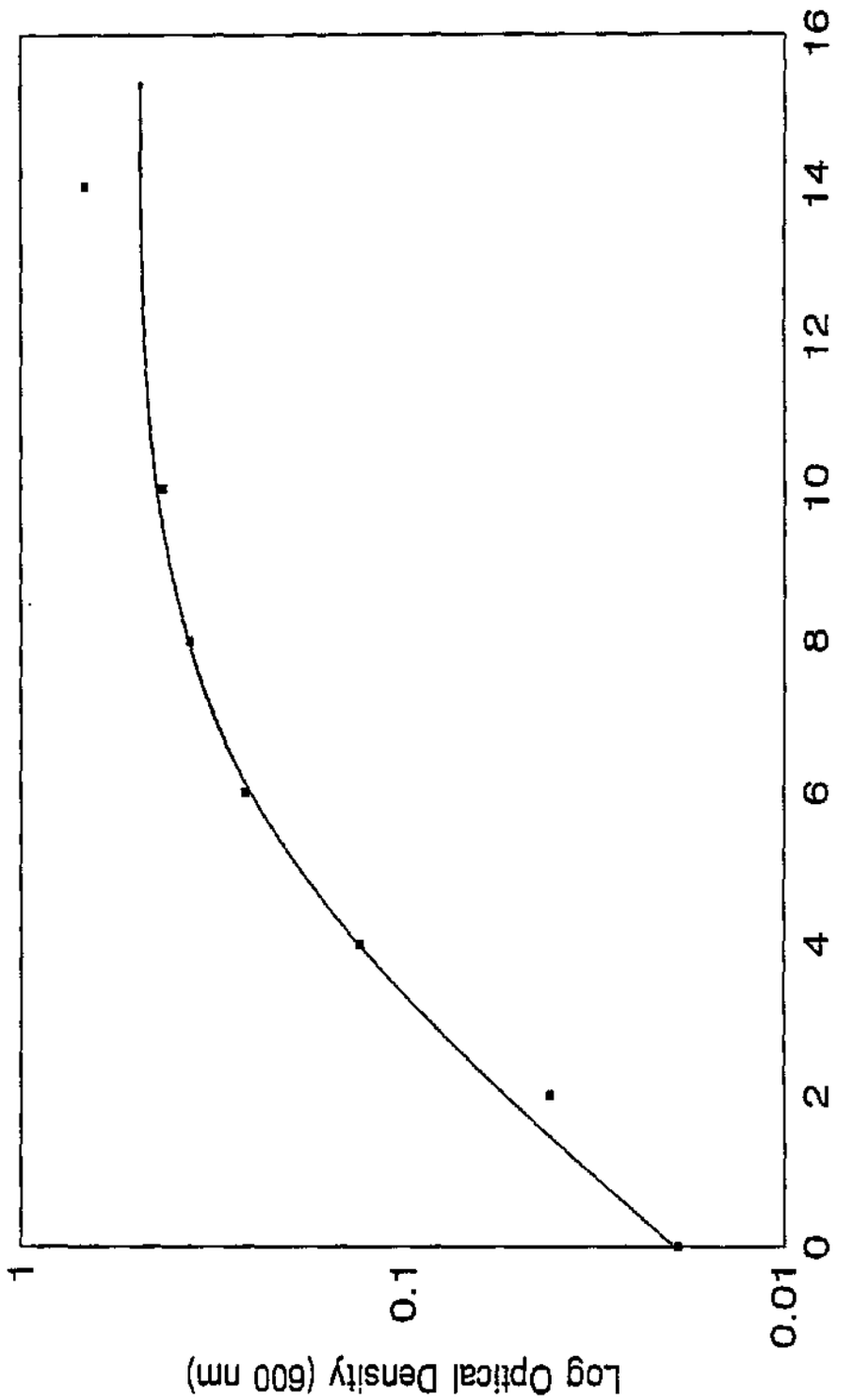

FIGURE 9: Growth of *Pseudomonas putida*

The ammonia causes an alkaline pH, resulting in a pink colour in the presence of the pH indicator phenolphthalein. When heavy metals are present, the above mentioned reaction is inhibited and the mixture remains colourless.

Urease type 3, isolated from Jack Beans, is used (33 000 units/g solid). This can be purchased from chemical suppliers. Three enzyme concentrations (0,5; 1,0; and 2,0 mg/mℓ) are used to detect different levels of heavy metal pollution (Metelerkamp, 1986). The test has been found to be the most sensitive at the 0,5 mg/mℓ enzyme concentration. The test has been designed to detect metals at the recommended limit for drinking water at this concentration (Kempster and Smith, 1985). The other concentrations detect metals at the maximum allowable and crisis limits.

Tests are carried out in 96-well microplates. Samples (filtered to remove particulate matter) are added in 160 µℓ volumes to 40 µℓ enzyme (prepared in buffer; pH: 7,7). An exposure period of 30 min at 25 ± 1°C is allowed after which 40 µℓ of substrate (urea) is added. After a 15 min enzyme-substrate interaction period, phenolphthalein (40 µℓ) is added. Enzyme activity is measured immediately with a microplate reader at 450 nm. Each test and control is carried out in triplicate. Deionized water serves as control. Effects are expressed as percentage inhibition (or increase), determined in relation to control results. Inhibition ≥10% (detection limit) indicates the presence of heavy metals.

(The concentrations of the buffer solution, substrate and phenolphthalein are not given as commercialization of the test in the form of a test kit is being investigated. Ms J.L. Slabbert, DWT, Pretoria, can be contacted for information).

Test acceptability

The control OD at the end of the test should be within the following limits:
0,5 mg/mℓ enzyme - 0,06 ± 0,02
1,0 mg/mℓ enzyme - 0,075 ± 0,02
2,0 mg/mℓ enzyme - 0,085 ± 0,02

The variation (CV) of controls should be <10%.

Comment

A visual observation in addition to the OD measurement is required. This is necessary to assure that high OD readings are not due to chemical interaction (*e.g.* precipitation). In the absence of a microplate reader effects can be established qualitatively by colour observation and reported as follows:

Total inhibition:	colourless	+++
Moderate inhibition:	very light pink	++
Slight inhibition:	light pink	+
No inhibition:	pink	-

Advantages:

* The test is rapid and a large number of samples can be tested simultaneously

* Because of its selectivity, a positive result usually indicates the presence of heavy metals

Shortcomings:

* Chemical interaction can occur between water/effluent samples and the enzyme preparations (density changes), which could interfere with OD determinations. In such instances results should be reported in terms of the colour development (see comment)

3.7 Acetylcholinesterase enzyme inhibition test

Acetylcholinesterase is an enzyme involved in the transmission of nerve impulses. This enzyme is selectively inhibited by organophosphates (direct or latent inhibitors) and carbamates (direct inhibitors) (Venter, 1990). Direct inhibition means that no change is required in the chemical structure of the compound to act as an inhibitor. The latent inhibitors are activated *in vivo* by the oxidation of the thio-groups (-P=S) to oxon-groups (-P=O) to become direct inhibitors. The acethylcholinesterase enzyme (isolated from the electric eel) can be obtained from chemical suppliers. The enzyme reaction is as follows:

$$\text{Acetylthiocholine} \xrightarrow{\text{Enzyme}} \text{thiocholine} + \text{acetic acid}$$

$$\text{Thiocholine} + \text{Ellman's Reagent (2,2'-dinitro-5-5'-dithiodibenzoic acid)} \longrightarrow \text{Thiocholine-2-nitro-5-thiobenzoic acid} + \text{2-nitro-5-thiobenzoic acid}$$

The reaction rate can be measured in terms of OD at 405 with a spectrophotometer:

For each test, 1,9 mℓ of test sample (filtered to remove particulate matter), 200 µℓ of potassium phosphate buffer (0,5 M), and 100 µℓ of enzyme solution [200 µg enzyme (1 enzyme unit)/mℓ 0,05 M potassium phosphate buffer) are added consecutively to a cuvette, mixed and kept at 37 ± 0,5° C (Venter, 1990). After an incubation period of 15 min, 100 µℓ of Ellman's reagent (10 mM) is added to the reaction mixture, followed by 100 µℓ of substrate (30 mM S-acetylthiocholiniodide). After a further incubation period of 1 min, the enzyme reaction rate is monitored by recording the OD of the mixtures (measured at 28 sec intervals) for a 2 min period.

When samples are suspected to contain latent inhibitors, a test is also conducted after oxidation of the sample. *In vitro* oxidation is carried out by adding 50 µℓ N-bromosuccinimide (NBSI) (0,2 g/ℓ) to 1,9 mℓ test sample. The sample is stirred for 20 min at room temperature after which the excess NBSI is reduced with 50 µℓ ascorbic acid (0,8 g/ℓ).

Each test and control is carried out in triplicate. Deionized water is used as control (deionized water plus NBSI when sample is oxidized). The enzyme reaction rate (slope) is used to calculate results. Results are expressed as percentage inhibition/stimulation, calculated in relation to control tests. Inhibition ≥10% (detection limit) indicates the presence of pesticides.

Test acceptability

The control OD at the end of the test should be within the following limits: 0,250 ± 0,05

The variation (CV) between replicate control tests should be <10%

Advantages:

* The test is rapid and sensitive to a range of pesticides
* Because of its selectivity, a positive result usually indicates the presence of pesticides

Shortcomings:

* Chemical interaction can occur between water/effluent samples and the enzyme preparations (density changes), which could interfere with OD determinations. In such instances, results should be reported in terms of the colour development
* The test is time consuming as only a few tests can be carried out simultaneously
* The sensitivity of the test can be improved by concentration of samples. This renders the test no longer rapid

3.8 Mammalian cell cloning efficiency test

This test has been developed primarily for drinking water toxicity testing (Kfir, 1981), and has proven to be highly sensitive to various chemicals (Table 2). The use of Buffalo Green monkey (BGM) kidney cells is recommended because of its high sensitivity (Kfir, 1981; Slabbert *et al.*, 1994a). The cell line can be obtained from Highveld Biological (Pty) Ltd, P O Box 488, Kelvin.

Cells are cultured in Dulbecco's modified Eagle medium (DME) with 10% foetal calf serum (FCS). Cultures are maintained in 250 mℓ flasks at 36 ± 1°C in a humidified incubator supplied with a constant flow of 7% carbon dioxide in air. Before reaching the confluent stage, cells are trypsinized and subcultured in fresh medium in culture flasks (Kfir, 1981). At this growth stage cells are also used for toxicity tests.

Toxicity tests are carried out in 6-plate culture dishes (diameter: 35 mm). Two hundred cells are seeded per plate in 4 mℓ medium. After approximately 18 h incubation at 36 ± 1°C, the medium is removed from each plate and replaced with fresh medium prepared with test samples (1,36 g DME, 0,37 g $NaHCO_3$ and 0,5 mℓ antibiotic-antimycotic per 100 mℓ water). The medium is decontaminated by filtration through a 0,22 µm membrane filter (fitted on a syringe). Control media are prepared in a similar way using deionized water. Each test and control is carried out in six-fold. Plates are incubated for a further 7 days. At the end of the incubation period the medium is removed. The plates are washed with phosphate buffer solution (PBS) and colonies are fixed with methanol and stained with Giemsa stain (Kfir, 1981). Colonies of cells are counted under a dissection microscope at a magnification of 25x. Results are expressed as percentage colony formation inhibition calculated according to the following formula:

$$100\% - \left[\frac{\text{Average number of colonies on treated plates}}{\text{Average number of colonies on control plates}} \times 100\%\right]$$

An inhibition ≥15% (detection limit) indicates toxic activity.

Test acceptability

The control plates should contain 100 ± 30 colonies [cloning efficiency (number of cells seeded/number of colonies developing x 100%): 50 ± 15%). The variation (CV) between replicate controls should be <15%

Advantages:

* The test is very sensitive and ideal for human health protection

Shortcomings:

* The test is time consuming and only suitable for use in specialized tissue culture laboratories

3.9 Ames *Salmonella* mutagenicity test

The Ames test is carried out according to US EPA (1983; 1985a) standard procedures using *Salmonella typhimurium* tester strains, TA98 and TA100. TA98 detects frame shift mutagens, whilst TA100 detects base-pair substitution mutagens. The cultures were originally obtained from Dr Bruce Ames (USA), the developer of the test. Stock cultures for local use can be obtained from the DWT or ordered from the US EPA.

Tests are carried out using a standard plate incorporation assay (US EPA, 1983; 1985a). In this test 100 $\mu\ell$ 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). Water (20 ℓ) is concentrated by means of XAD-7 resin extraction following standard procedures (US EPA, 1985a). The extracted organic material is dissolved in acetone to a final volume of 10 mℓ (after plate incorporation sample is 100x concentrated). Plates are incubated at 37 ± 1°C for 72 h whereafter the number of revertant colonies on plates are counted (Figure 10). Triplicate plates are used for each tester strain, with and without S9. Deionized water is used as standard control, while acetone is included as additional control. Results are expressed as mutation ratios, calculated as follows:

$$\text{Mutation ratio (MR)} = \left[\frac{\text{Number of mutants on test plates}}{\text{Number of mutants on control plates}}\right]$$

A MR ≥2,0 is considered to be a positive result.

Positive controls should be included in each set of tests to confirm the reversion properties and specificity of each strain and the efficacy of the S9 mix. Sterility controls are also carried out routinely (*e.g.* for the S9 mix) (Maron and Ames, 1983).

Test acceptability

The control plates should contain between 20 and 50 colonies in the case of TA98 and between 100 and 250 colonies in the case of TA100

The solvent controls should not be toxic, positive controls should show an appropriate response, and sterility controls should be negative

Advantages:

* 90% of all known carcinogens are also mutagens. The test has, therefore, been found to be a very useful means to protect human health against potential carcinogens

Shortcomings:

* The test is very time consuming and the reagents are expensive
* Concentration of water is time consuming
* The test does not detect metal containing mutagens

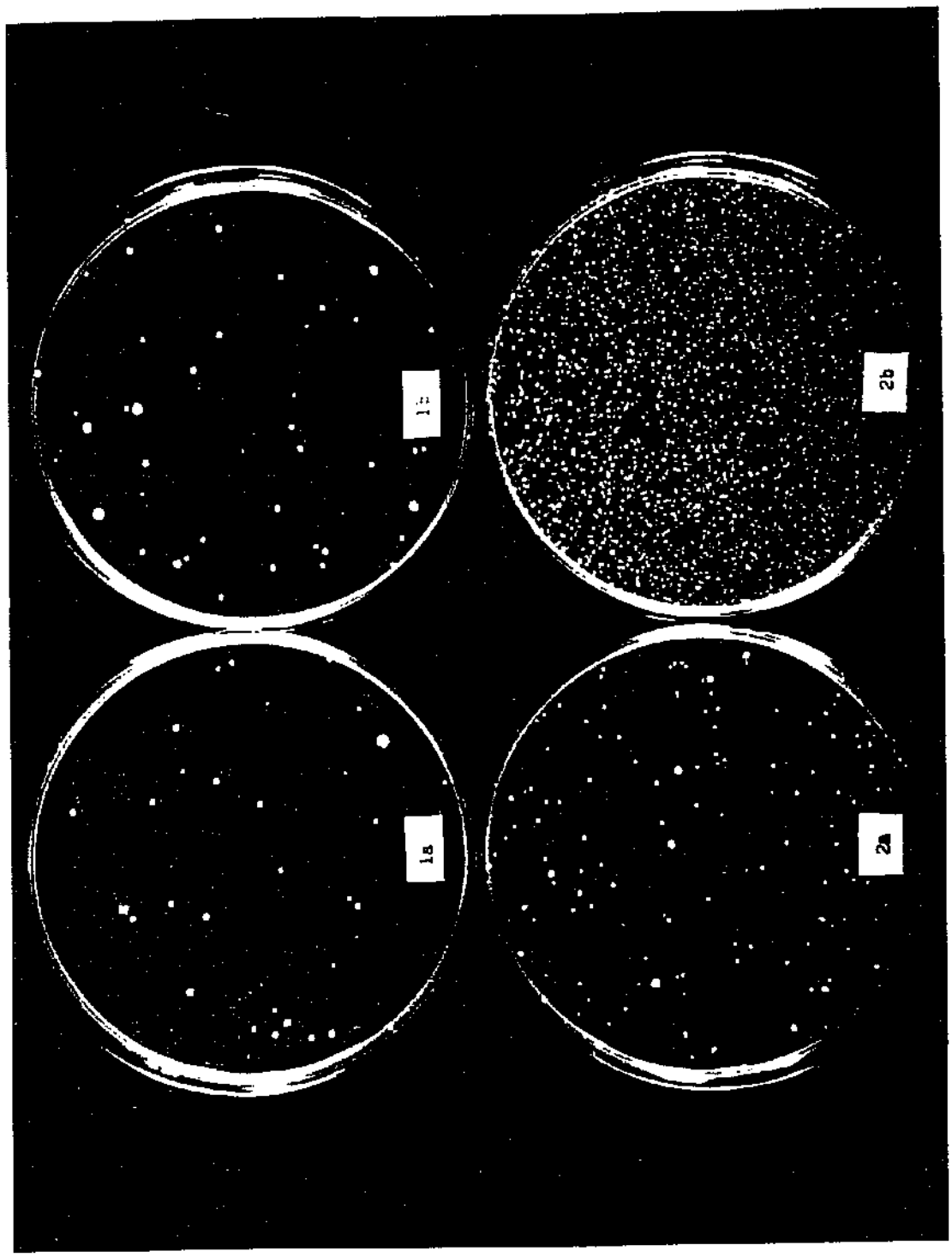

FIGURE 10: Control plates showing spontaneous revertant colonies in the Ames test. (1a) TA98-S9; (1b) TA98+S9; (2a) TA100-S9; (2b) TA100+S9

Comments

* Other extraction procedures and solvents (*e.g.* DMSO) can be used. Extraction (of organic pollutants) is particularly important when samples contain high nutrient/inorganic levels (*e.g.* surface water and effluents). Such samples could form precipitates with medium or be toxic.

* Drinking water can be concentrated by means of flash evaporation (2 and 4x concentrated). The water can then be tested by means of a modified plate incorporation test, using the water samples (directly, and 2 and 4x concentrated) to prepare the nutrient agar plates (Kfir *et al.*, 1982). However, the agar preparations are autoclaved to sterilize which could change the chemical composition/structures.

* Industrial effluents could be tested directly without concentration.

* The acceptable ranges of spontaneous reversion may be somewhat different in different laboratories, but they should be relatively consistent within a laboratory.

3.10 Toad embryo teratogenicity test

The African clawed toad, *Xenopus laevis*, is used as test animal. Toads can be obtained from African Xenopus Facility, P O Box 118, Noordhoek. The toads are maintained at 23 ± 2°C in plastic holding tanks in dechlorinated tap water, without aeration. Minced liver is used for feeding (twice a week). Detailed information on culturing can be obtained from the literature (Wu and Gerhart, 1991).

Three days before testing, three pairs of toads are given a primer injection (100 $\mu\ell$) of Human Chorionic Gonadotrophin (HGC) to stimulate fertility (Genthe and Edge, 1988). Pairs are placed in spawning tanks in dechlorinated tap water. Fourty eight hours later toads receive a HGC booster injection (females: 300 $\mu\ell$; males: 100 $\mu\ell$). After fertilization, eggs are removed from the spawning tanks and transferred to 500 mℓ glass containers with 200 mℓ test sample. Duplicate containers are used, each containing 50 eggs. If eggs from different pairs of toads are used, these should be mixed before use, or alternatively each batch of eggs should have its own control test. Tests are carried out at 23 ± 2 °C. Moderately hard water (Table 1) is used for control testing. After 48 h exposure the developing embryos are counted and examined under a dissection microscope for abnormalities. Features examined for malformation include embryo development (size and length), pigmentation, head shape, and form of spines and tails (Figure 11).

Comment

Eggs are often produced after the primer injection. If the eggs have been fertilized they can be used in tests.

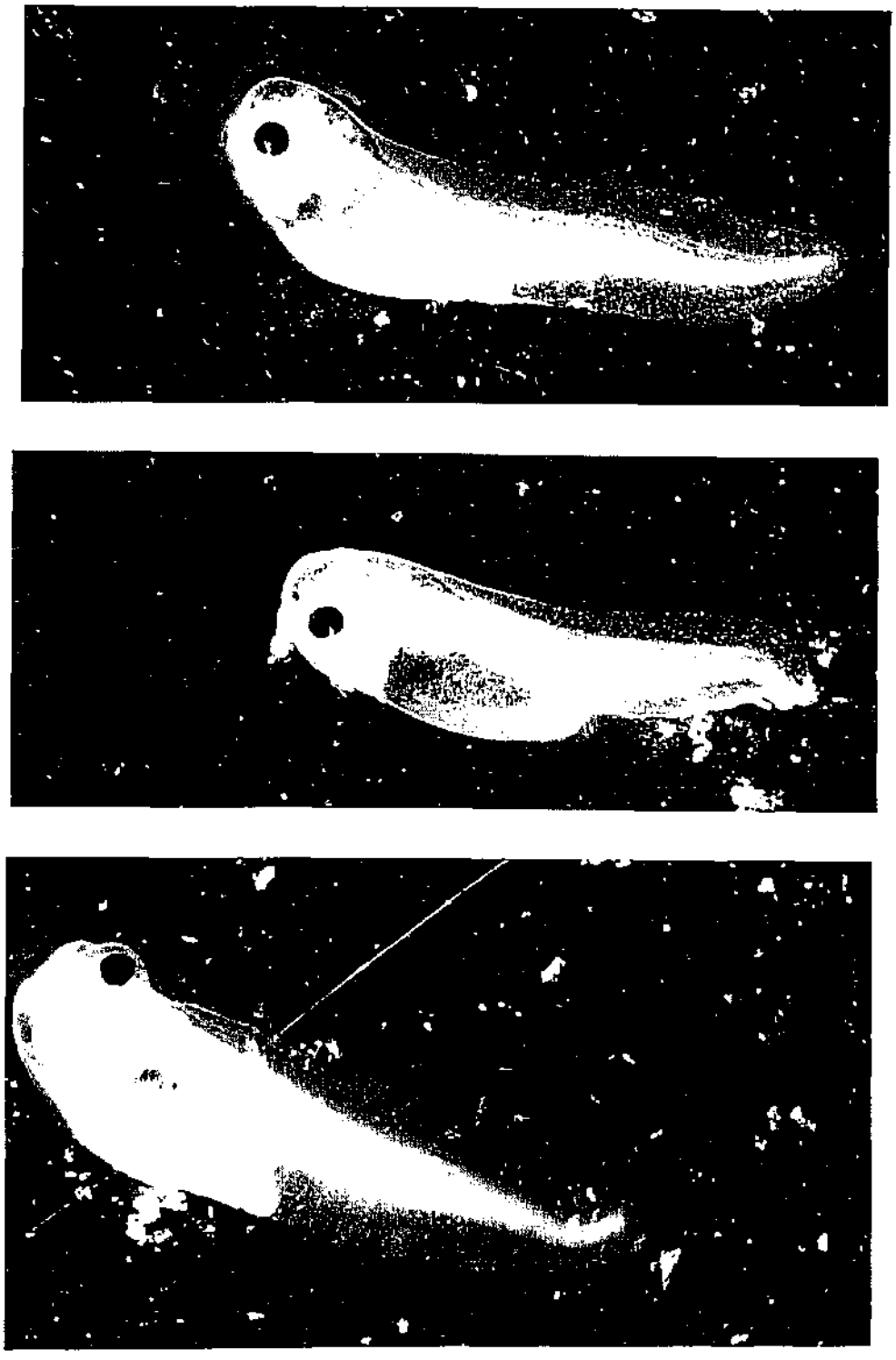

FIGURE 11: Toad embryo teratogenicity test - typical spinal deformities

Test embryos are compared to control embryos. Results are expressed in terms of percentage deformation and lethality. Deformation ≥20% and lethality ≥10% (detection limits) are considered as positive results.

Test acceptability

Controls should have a ≥80% hatching rate. Control deformation should be <20%

Advantages:

* Maintenance of animals is simple and easy

Shortcomings:

* Although hormone treatment is used to enhance fertility, problems are experienced in winter months (May to August) with the production of eggs

4. DATA ANALYSIS

The most convenient way to establish whether or not a sample is toxic, is to apply detection limits. The recommended detection limits for each of the biological toxicity tests have been given in Section 3. The detection limits given for the fish, water flea and mutagenicity tests are specified in the standard methodologies, while those of the locally developed tests were selected on the basis of the reproducibility of the tests (Slabbert *et al.*, 1996a). The reproducibility of the microbial, enzyme and mammalian cell tests was established by calculating the coefficient of variation (CV) of the controls of each set of tests. In the case of the toad embryo teratogenicity test the reproducibility was established by calculating the averages and standard deviations of the controls of different sets of tests.

As alternative to detection limits, Student's t-test (Clarke, 1969) can be applied to establish whether or not test results are significantly different from control results (P = 0,05).

When definitive tests (effluents and chemicals) are carried out, effective concentrations are derived from the data by means of statistical analysis (linear regression) using dose-response curves (% effect *versus* log concentration) (Slabbert, 1996). Endpoints which can be calculated are LC_{10}'s, EC_{10}'s, LC_{50}'s and EC_{50}'s. The minimum effect level (*e.g.* LC_{10}) depends on the selected detection limit of a test.

5. REFERENCE CHEMICALS

The following reference chemicals are recommended for use: cadmium chloride and pentachlorophenol (representative for inorganic and organic substances) for fish, water flea, protozoan, algal, *P. putida*, and mammalian cell tests; cadmium for the urease test; carbofuran (un-oxidized samples) and malathion (oxidized samples) for the acetylcholinesterase test; methyl viologen with the toad embryo test; and 2-amino-anthracene

TABLE 6: Effect of pentachlorophenol and cadmium on test systems

Chemical		Fish test (96 h)	Water flea test (48 h)	Protozoan test	Algal test	Bacterial test	Urease enzyme test			Mammalian cell BGM test
							0,5 mg/ml	1,0 mg/ml	2,0 mg/ml	
Penta-chloro-phenol	Test concen-tration (mg/l)	0,1	1,0	10	10	50	-	-	-	20
	Number of tests	4	11	1	4	13	-	-	-	5
	Average effect[1]	50	96	92	24	74	-	-	-	91
	Effect[1] range	20-80	65-100	-	2-46	50-93	-	-	-	63-100
Cadmium	Test concen-tration (mg/l)	1,0	1,0	10	1,0	10	10	10	10	1,0
	Number of tests	4	10	1	5	13	25	25	25	5
	Average effect[1]	55	100	25	96	64	77	55	11	72
	Effect[1] range	40-80	-	-	90-100	32-94	37-98	28-80	+15-47	46-94

1 % Lethality or inhibition

\- No results

TABLE 7: Effect of reference chemicals on the acetylcholinesterase and toad embryo test

Bioassay		Methyl viologen				Carbofuran				Malathion			
		Test concentration (mg/ℓ)	Number of tests	Average effect[1]	Effect[1] range	Test concentration (µg/ℓ)	Number of tests	Average effect[1]	Effect[1] range	Test concentration (µg/ℓ)	Number of tests	Average effect[1]	Effect[1] range
Acetylcholinesterase test		-	-	-	-	2	9	35	21-55	50	6	88	82-93
Toad embryo test	Lethality	1,0	7	17	0-61	-	-	-	-	-	-	-	-
		0,1	7	1	0-7	-	-	-	-	-	-	-	-
	Deformation	1,0	7	47	25-87	-	-	-	-	-	-	-	-
		0,1	7	19	10-43	-	-	-	-	-	-	-	-

[1] % Lethality/inhibition/deformation

\- No results

(2AA) (tested with TA98+S9 controls) and sodium azide (tested with TA100) for the Ames test using spot testing.

The Ames test should include the reference chemicals (also called positive controls) each time a test is conducted (see Section 3.9). When tests are not used on a regular basis (routine testing) one reference chemical concentration (Tables 6 and 7) can be included in every test. However, the error and variability associated with data analysis when single samples are tested is usually large. Therefore, definitive testing using a series of concentrations (*e.g.* 5 to 6) is recommended to establish effective concentrations. The obtained values should be compared over time to establish variation (CV) (intra-laboratory precision). Following international recommendations each of the tests used on a regular basis should be tested for sensitivity and precision once a month (using definitive testing). Values obtained for a limited number of tests on fish, water flea and algae are shown in Table 8.

Stock solutions of the reference chemicals should be prepared monthly and stored at 4° C to avoid changes in composition.

If the same test organisms and protocol is followed by a number of laboratories standard reference chemical preparations could be provided to each laboratory to establish inter-laboratory precision. Such steps will enhance quality assurance practices.

TABLE 8: Sensitivity of fish, water flea and algae to cadmium and pentachlorophenol[1,6]

Chemical	Criterium	96-h Fish lethality test	48-h Water flea lethality test	Algal growth inhibition test[2]
Cadmium	LC_{10} or EC_{10}[3] (mg/ℓ)	0,024 (0,018-0,032)	0,15 (0,13-0,17)	0,043 (0,03-0,055)
	LC_{50} or LC_{50}[4] (mg/ℓ)	0,08 (0,047-0,103)	0,23 (0,17-0,29)	0,154 (0,099-0,209)
	R[5]	0,9707-0,9965	0,9449-1,0	0,9510-0,9624
	Test concentration range (mg/ℓ)	0,016-1,0	0,03-0,5	0,016-0,5
PCP	LC_{10} or EC_{10}[3] (mg/ℓ)	0,20 (0,36-0,288)	0,19	3,3 (2,07-4,53)
	LC_{50} or LC_{50}[4] (mg/ℓ)	0,349 (0,192-0,50)	0,44	7,78 (5,46-10,1)
	R[5]	0,9600-1,0	0,9608	0,9887-0,9997
	Test concentration range (mg/ℓ)	0,016-1,0	0,03-1,0	3,1-50,0

1 Given as an average value and the range (in brackets)
2 Using BG-11 medium
3 Concentration causing 10% lethality or inhibition
4 Concentration causing 50% lethality of inhibition
5 Correlation coefficient
6 According to Slabbert *et al.* (1996a)

6. APPLICATION

Each of the toxicity bioassays described in this document has been developed/established to fulfil a particular need in the field of water testing. The fish, water flea and algal tests are primarily used to protect the aquatic environment and to some extent to predict environmental impacts. The rapid microbial and enzyme tests are invaluable in emergency situations when rapid answers on acute effects and impacts are needed. The highly sensitive mammalian cell culture test, and the Ames and toad embryo tests are aimed at human health protection.

Each toxicity test has its own sensitivity pattern, depending on the test organism and parameter used, and variables such as the complexity of the growth media or suspending fluids, exposure periods, and statistical interpretation. Since there is no single test which can satisfy a comprehensive approach aimed at aquatic life and human health protection, a battery approach should be followed so that the advantages of some tests can complement the limitations of others. A battery of tests may include different rapid tests or tests using organisms from various levels of the ecosystem. The tests used will depend on the situation and the objective of the evaluation.

6.1 Drinking water testing

The battery of tests applied for drinking water testing could consist of a fish/water flea test, the bacterial growth inhibition test, urease enzyme test, Ames test and toad teratogenicity test in cases of routine testing. The acetylcholinesterase test can be included if pesticide contamination is suspected. The mammalian cell test is too time consuming to be used for routine analysis.

6.2 Groundwater testing

Aquatic tests proved to be too sensitive to use to establish possible effects on human consumers. The most applicable tests for human health protection could be the fish test, urease enzyme test, acetylcholinesterase test, the mammalian cell test, and the two chronic tests.

If the groundwater is used to rear aquatic organisms, fish, water flea, algal and bacterial tests could be used.

6.3 Surface water testing

When surface water testing is aimed at human health protection, the same tests as mentioned under drinking water are recommended.

When the study is aimed at environmental protection, tests will be selected to include organisms from the different levels of the aquatic food chain, namely fish, water flea, algal, protozoan, bacterial and toad embryo tests.

6.4 Effluent testing

It was found that fish, water flea and algae are the most applicable to protect aquatic life against acute toxicity of effluents (Slabbert *et al.*, b). If the receiving water is used for human consumption the two chronic tests should also be included.

6.5 Emergency analysis

In emergency situations where drinking water is tested all the rapid tests, namely the protozoan oxygen uptake test, urease and acetylcholinesterase enzyme tests, and the bacterial growth inhibition test are applied (Slabbert, 1989). The fish and water flea tests are included to confirm the results of rapid tests.

The same tests will be used in case of environmental pollution, but the algal test will also be included.

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