Development of Procedures to Assess Whole Effluent Toxicity

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Report to the Water Research Commission
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AND

GUIDELINES FOR WHOLE EFFLUENT TOXICITY TESTING

by

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EXECUTIVE SUMMARY

Biological toxicity testing has become a valuable component of effluent monitoring and control in many countries. The important role of toxicity tests has recently been acknowledged by the Department of Water Affairs and Forestry (DWA&F) when Whole Effluent Toxicity (WET) testing was identified as an appropriate tool to evaluate the suitability of hazardous effluents for discharge into receiving waters. The decision of the DWA&F to include WET testing into its toxic effluent management policy has necessitated the establishment of suitable procedures for use in the South African context. This project was aimed at the development of methodology for WET testing that would meet the requirements of the DWA&F's policy.

The specific objectives for the project were:

- To establish a set of toxicity testing procedures with which to quantify whole effluent toxicity;
- To develop procedures to evaluate and interpret the results of toxicity tests; and
- To determine the dilution of an effluent that would not be toxic after discharge to receiving water.

Locally available bioassays were applied to different effluents in order to select the most appropriate bioassays for WET testing. Range finding (testing ten-fold dilutions) as well as definitive tests (testing serial dilutions to establish effective concentrations) were carried out. Selected tests were used to evaluate different effluent and receiving water conditions (*e.g.* seasonal changes, effluent variability), to establish the effect of holding time on effluent quality, and to investigate sampling procedures (grab/composite). Acute as well as chronic tests were used. Acute tests included: fish and water flea lethality tests; a protozoan oxygen uptake test; algal and bacterial growth inhibition tests; and urease and acetylcholinesterase enzyme inhibition tests. The Ames *Salmonella* mutagenicity assay and a toad embryo teratogenicity test were used for chronic toxicity detection (human health protection). Tests were carried out on secondary treated effluent from a sewage works receiving both domestic and industrial effluent, paper mill effluent, and effluent from a metal refinery.

All the effluents evaluated in this study exhibited toxicity. In general, the paper mill effluent after secondary clarification was the least toxic, followed in order of magnitude by secondary treated sewage effluent, paper mill effluent before primary clarification, and metal plating effluent. Paper mill effluent after secondary clarification and treated sewage effluent were mostly only toxic at the 100% concentration. Paper mill effluent before primary clarification showed toxicity at concentrations ranging from 10 to 100%. The metal plating effluent was highly toxic and effective concentrations ranged between 0,01 and 10%.

The effluents mostly exhibited acute toxicity, causing lethal and/or sub-lethal effects (*e.g.* growth inhibition). However, chronic effects were also detected. On occasion, all the effluents showed some degree of teratogenicity (deformation of toad embryos). Mutagenicity was exhibited only once, namely by paper mill effluent after secondary clarification. The mutation ratios were similar to those occasionally detected in environmental waters.

Range finding tests showed that the fish, water flea, algal, bacterial and toad embryo tests were the most sensitive for the detection of adverse chemical activity in secondary treated sewage effluent and paper mill effluent 1. Algal and bacterial growth inhibition tests, the toad

embryo test, and the mutagenicity test detected adverse chemical activity in paper mill effluent 2. All the tests, except the mutagenicity test, were sufficiently sensitive to screen metal plating effluent for adverse chemical activity. The algal test generally appeared to be the most sensitive to metal plating effluent.

Based on the results of the range finding tests the fish and water flea lethality tests and the algal growth inhibition test were selected for extensive toxicity testing of effluents. During extensive toxicity testing effective concentrations could not be established for treated sewage effluent and paper mill effluents because effluents were either not toxic or toxicity was only detected at the 50 or 100% concentrations. For these effluents results were expressed as a percentage effect. It is possible to present such results as follows: EC_{50}>100%. For metal plating effluent effective concentrations were established by means of definitive tests. Inherent toxicity (using standard dilution water) was usually larger than the relative toxicity (using receiving water for dilution). In general, the algal test was found to be the most sensitive to the effluent. Based on the minimum effective concentration (EC_{10}/LC_{10}/EC_{20}/LC_{20}) of the most sensitive test, dilutions were established for the effluent to avoid acute effects on aquatic organisms. These dilutions ranged from 250 to 500 times.

In some instances the receiving water used for dilution of effluents showed toxicity, *e.g.* the Small Blesbok Spruit. Acute as well as chronic toxicity (teratogenicity) was detected. Usually, the dilution water should be free of toxicants. This implies that when toxicity is present, stream water should not be used for dilution purposes. However, this water should be used if it is the objective to establish the contribution of the effluent to the receiving water.

The negative results obtained with the acetylcholinesterase enzyme test indicated that organophosphate and carbamate pesticides were absent from effluents and river/stream water. The urease test showed positive results with metal plating effluent and the Small Blesbok Spruit water, indicating the presence of heavy metals.

Many of the tests showed enhanced activity when exposed to the effluent/water samples. This is usually attributed to nutrients in the water. In the case of the urease test higher density readings could have been due to precipitation rather than increased activity.

Treated sewage effluent and paper mill effluent caused precipitation in the algal and bacterial growth inhibition tests. Paper mill effluent 1 generally showed the largest background reading, which interfered with the interpretation of results. Because of the possibility of precipitate formation, these tests are not compatible with all effluents.

The study on effluent variability showed that treated sewage effluent was toxic during the period August to October 1992, but no toxicity was detected from February to June 1993, implying that seasonal changes could have influenced the effluent quality. With the paper mill effluent no clear pattern due to seasonal changes could be observed. The Small Blesbok Spruit water was toxic during the August to October 1992 sampling period until February 1993. Hereafter, no toxicity was detected, indicating that the quality possibly improved due to rain. The metal plating effluent was not influenced by seasonal changes.

No conclusions could be made on the effect of holding time on the quality of sewage effluent and paper mill effluent 2 because no toxicity was detected on the first day of testing. The results obtained with paper mill effluent 2 showed that on one occasion there was a quality

change during the first 48 h of holding, rendering the sample not toxic. A second sample showed that toxicity remained the same for at least 48 h. The toxicity of the first sample was probably caused by a volatile chemical. Toxicity tests with water flea showed that the toxicity of metal plating effluent started to decrease after a holding period of 4 days. The findings indicate that it is important that toxicity tests should be carried out as soon as possible, preferably within 24 h, after sampling.

No short-term variation in acute toxicity could be established in treated sewage effluent and paper mill effluent 2, when grab and composite sampling methods were used. Grab sampling will probably be the most suitable for these effluents (for acute testing) because there is already a retention period involved. As short-term variation is improbable, the testing frequency could have a wider spread (once per week or once per month). Paper mill effluent 2 and metal plating effluent showed a large variation in quality. Grab samples showed high peaks of toxicity at certain times of the day. Some composite samples showed low toxicity, indicating that dilution by less toxic effluent took place. In some instances, composite samples were very toxic, suggesting that high levels of toxicants were discharged at a continuous rate. These results showed that effluents with high variability should preferably be subjected to retention facilities. For these type of samples grab sampling will be the most suitable for acute testing. For chronic testing a composite sample taken over for example a 24 h period will be most suitable.

On one occasion, all the tests showing toxic responses during range finding tests were applied to establish effective concentrations and to compare sensitivity. As was previously found with treated sewage effluent, effective concentrations could not be established by means of linear regression because toxicity was absent or very low. Only the toad embryo test showed adverse chemical activity in 100% treated sewage effluent (lethality and teratogenicity). Paper mill effluent 1 affected bacterial growth, killed water flea and fish, and showed teratogenicity. Paper mill effluent 2 inhibited bacterial growth and caused teratogenicity. None of the samples were mutagenic. Metal plating effluent showed the highest toxicity to algae, followed in order of magnitude by water flea, urease enzyme, protozoa, bacteria and toad embryos. Fish were not used in this evaluation. Teratogenicity was also detected. Based on algal sensitivity, a dilution of 5 000 times was necessary in this instance to avoid acute toxicity in receiving water.

Tests were also applied to receiving water and water sampled downstream of discharge (ambient water). As was previously, found receiving water showed some degree of toxicity. The ambient water downstream of paper mill discharge showed toxicity with the bacterial growth inhibition test. Because of low toxicity in case of ambient waters, chronic aquatic tests would be more applicable.

Chemical analyses of the effluents indicated that, in general, potentially toxic chemicals in treated sewage effluent and paper mill effluents were low, indicating that effects were probably due to combined toxicity. The paper mill effluents occasionally showed low pH levels and oxygen content which could have contributed to adverse activity. The metal plating effluent contained high levels of a variety of metals and cyanide which individually or in combination could have caused the adverse effects. The Small Blesbok Spruit water showed a presence of manganese, cyanide and phenol, and occasionally low oxygen levels, which could have been the result of toxicity.

Most of the biological tests used in the study are well established and optimized. Problems were, however, occasionally experienced with the algal test. In many instances algal growth was insufficient, particularly when using AAM medium. The large variation in algal growth was probably due to variation in illumination and attention should be given to standardization. Although most of the fish tests were carried out with 10 fish, there were occasions when enough fish were not available. In some instances control fish showed lethality, indicating that the stock was not healthy. In order to ensure that the fish test is optimally used for WET testing proper facilities for maintenance and culturing will be required.

Although the algal AAM medium test was occasionally more sensitive than the BG-11 medium test, the BG-11 medium test appeared to be a wiser choice for future work because it gives better growth and shows less variation. A good growth was obtained with moderately hard water when using BG-11 medium. However, results showed some variation. Because the test using deionized water was not properly standardized it was difficult to compare the efficiency of the two waters for inherent toxicity determination. It appears that it will be best to use deionized water, because this is the international approach.

Based on sensitivity, the fish and water flea lethality tests, and algal growth inhibition test are recommended for regulatory and management purposes of effluents. These tests are also reproducible, simple and relatively cost effective. These test also have the advantage that they are similar to standard effluent tests carried out in other countries. Some of the rapid microbial and enzyme tests could be valuable screening tools to identify and categorize toxic effluents. Most of the tests are, however, not sufficiently sensitive to test effluents with low toxicity.

In addition to the recommended battery of acute tests, mutagenicity and teratogenicity tests should also be used if water downstream of discharge is used for drinking water purposes.

The study has indicated that there is a great need for short-term aquatic chronic tests. These are particularly needed in case of effluents with low toxicity and for ambient water testing. The establishment of tests using water flea are under way. However, urgent attention should be given to the development of chronic tests using fish. Additional chronic tests for human health protection are also needed, particularly because the Ames test is not sensitive to metal containing effluents.

The recommended procedures for WET testing in South Africa (toxicity tests, data evaluation and interpretation, dilution determination) are outlined in a separate document **Guidelines for Whole Effluent Toxicity Testing** (Slabbert, 1996).

During the study several questions on toxicity test responses arose. These included possible interaction between growth media and effluents, algal growth in general, precipitation, and growth stimulation. These questions have been addressed by means of chemical equilibrium modelling and the findings are presented in a report entitled **Chemical equilibrium modelling investigation to explain biological toxicity test responses** (Pretorius, 1996), which is attached as Appendix A.

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Development of procedures to assess whole effluent toxicity.

The Steering Committee responsible for this project consisted of the following persons:

Dr S A Mitchell	Water Research Commission (Chairman)
Mr P W Weideman	Water Research Commission (Secretary for 1992)
Mrs A M du Toit	Water Research Commission (Secretary after 1992)
Dr M J Pieterse	Water Research Commission
Prof J U Grobbelaar	University of the Orange Free State
Prof J J Alexander	University of the Witwatersrand
Mr M C Steynberg	Rand Water Board
Dr P L Kempster	Department of Water Affairs
Dr A Kühn	Department of Water Affairs
Dr P Ashton	Division of Water Technology, CSIR

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1. INTRODUCTION AND LITERATURE STUDY

1.1 Introduction

The Department of Water Affairs and Forestry's (DWA&F) approach towards water quality management in South Africa has undergone considerable change in the last few years. The Uniform Effluent Standards (UES) approach for water pollution control, enforcing compliance with the physical-chemical based General and Special Effluent Standards, was followed for many years. In order to provide additional protection for surface waters the DWA&F has recently adopted the Receiving Water Quality Objectives (RWQO) approach (DWA&F, 1991). This focuses on the fundamental water quality management goal of maintenance of fitness for use of water resources. In addition, the DWA&F has proposed the Pollution Prevention (PP) approach (Van der Merwe and Grobler, 1990) to manage hazardous effluents.

The PP approach entails total prohibition of the discharge of hazardous effluents. However, it has been realized that the complexity of effluent quality prevents such a simple one-dimensional approach. Enforcement of the approach will be very expensive. There is also a lack of existing technology for complete neutralization of hazardous wastes and a necessity to provide alternative disposal routes. In order to address the hazardous waste problem in a more realistic manner the DWA&F has identified Whole Effluent Toxicity (WET) testing as a tool to evaluate the suitability of hazardous effluents for discharge into receiving waters.

WET testing involves the setting of limits using effluent toxicity as a control parameter. This is the alternative to the pollutant-specific approach which involves setting limits for single chemicals based on laboratory derived no-effect levels (Mount *et al.*, 1984; US EPA, 1991a). Toxicity testing evaluates the response of living organisms or biological material to the combined effect of all constituents of complex effluents and, therefore, has the advantage over chemical-specific analyses which cannot identify unknown pollutants in an effluent and cannot detect toxicity due to chemical interaction, *e.g.* synergism, antagonism and addition. Toxicity tests/bioassays provide invaluable information on the type of hazardous chemical activity in an effluent, *i.e.* toxicity, mutagenicity and teratogenicity, the degree of effect on exposed test organisms, and the impact on particular groups of target organisms. Toxicity information can indicate the need for additional characterization of an effluent and can also provide a basis for permit limits based on water quality standards for toxicity or technology-based requirements (Mount *et al.*, 1984).

Various countries are currently applying biological toxicity tests to monitor and control the discharge of harmful effluents into the aquatic environment. For example, in the USA the Environmental Protection Agency (EPA) uses an integrated hazard assessment scheme in which biological toxicity tests play a key role (US EPA, 1989). The use of bioassays to control toxicants in industrial effluents and related receiving waters is also advocated by international organizations such as the Organization for Economic Co-operation and Development (OECD, 1987). In South Africa toxicity bioassays have been in use for water and wastewater testing since the early 1980's (Grabow *et al.*, 1985; Slabbert, 1988). The locally available bioassays, developed and evaluated by CSIR's Division of Water Technology, are similar to those applied in other countries. They measure both acute and chronic toxicity with their corresponding lethal and sublethal effects, and utilize organisms from different levels of the aquatic food chain (*e.g.* fish, water flea, algae, bacteria) as well as mammalian cell culture and enzyme systems.

While the usefulness of these toxicity tests has clearly been proven, the DWA&F's decision to incorporate WET testing into its toxic effluent management policy has necessitated the establishment of appropriate procedures for use in the South African context. This project was aimed at the development of methodology for WET testing that would meet the requirements of the DWA&F's policy and was carried out in collaboration with staff of the Department's Institute for Water Quality Studies (IWQS).

The specific objectives for the project were:

- To establish a set of toxicity testing procedures with which to quantify whole effluent toxicity;
- To develop procedures to evaluate and interpret the results of toxicity tests; and
- To determine the dilution of an effluent that would not be toxic after discharge to receiving water.

In order to achieve the objectives, a comprehensive literature review on the use of bioassays for effluent testing was carried out to serve as a basis for the project. In addition, the different bioassays currently available in the country were extensively evaluated for WET testing by applying them to selected effluents.

A large part of the literature study *i.e.* policies and strategies of other countries and international organizations on the use of toxicity tests, and information on bioassays used elsewhere and in South Africa for water quality testing, is covered in the review carried out for another Water Research Commission project **Development of guidelines for toxicity bioassaying of drinking and environmental waters in South Africa** (Slabbert *et al.*, 1996). This report focuses on literature related to WET testing.

Bioassays were evaluated in terms of sensitivity, reproducibility, and compatibility with complex chemical wastes. Other criteria of consideration included simplicity, cost-efficiency and applicability for routine testing. Range finding (testing ten-fold dilutions) as well as definitive tests (testing serial dilutions to establish effective concentrations) were carried out. Selected tests were used to evaluate different effluent and receiving water conditions (*e.g.* seasonal changes, effluent variability), to establish the influence of holding time on effluent quality and to investigate sampling procedures (grab *versus* composite samples).

This report presents the findings of the study. The recommended procedures for WET testing in South Africa (toxicity tests, data evaluation and interpretation, dilution determination) are outlined in a separate document **Guidelines for Whole Effluent Toxicity Testing** (Slabbert, 1996). During the study several questions on toxicity test responses arose. These are addressed in the document **Chemical equilibrium modelling investigation to explain biological toxicity test responses** (Pretorius, 1996), attached as Appendix A.

1.2 Literature study

1.2.1 Current experience with WET testing

The WET testing approach is followed by various countries. The USA took the lead by being the first country to incorporate toxicity testing into its toxic pollutant control policy. The Environmental Protection Agency's (EPA) *surface toxics control* regulation 54 FR 23868,

promulgated on 2 June 1989, established specific requirements for the use of an integrated water quality-based approach for controlling toxic discharges, which includes the use of whole effluent toxicity testing (US EPA, 1989). Toxicity testing is carried out with at least three acute and three chronic toxicity tests using fish, water flea and algae. Acute and chronic toxicity units are used as a mechanism to quantify instream toxicity.

Canada is expected to follow suit with legislation on toxicity testing. 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). *Initially, the 96-h rainbow trout and the Daphnia* lethality tests are the most likely regulatory tests. The minimum requirement states that industrial and municipal discharges must be non-acutely lethal to the test organisms before discharge. Other acute and chronic tests will follow after thorough evaluation.

European countries follow the OECD's guidelines for toxic effluent testing (OECD, 1987). However, each country has its own discharge criteria and some countries have developed their own toxicity tests. The current pollution control legislation of Germany relies strictly on emission parameters which conforms to the strict standards laid down under European Community (EC) directives. The General Administrative Regulation 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 G_F value (the dilution factor for which all the fish survive under the conditions specified in the standard method) (DIN, 1989). Recent literature (German Technology Report, 1992) indicates that water authorities will in future be relying more extensively on a range of biological tests, which will move Germany away from its current strict reliance on emission parameters towards a quality standards for receiving waters approach. It is envisaged that fish, *Daphnia*, algal and light bacterial tests will be used in three years time to monitor all waters (German Technology Report, 1992).

Some of the regulatory measures for effluents in France include biological toxicity tests (OECD, 1987). Standardized fish and *Daphnia* lethality tests are usually used. In Ireland, guidelines for restrictions on the discharge of toxic effluents are given in terms of toxic units, and are developed on an industry-specific basis. For each toxic unit discharged a certain dilution factor is stipulated. The United Kingdom (UK) has proposed an approach similar to that of the USA, with a few adaptations to satisfy UK circumstances (Hunt *et al.*, 1992). It recommends the use of a rapid luminescent bacterial test for preliminary screening purposes to categorize and prioritize effluents. Three standard acute toxicity tests using species from three taxonomic groups, namely fish, invertebrates and algae will be used to derive toxic based consents. It is proposed that the bacterial test should be calibrated against the most sensitive of the test species to provide a simple and relatively inexpensive test for routine monitoring.

Detailed information on the policies and strategies of other countries can be found in the literature study conducted for the project **Development of guidelines for toxicity bioassaying of drinking and environmental waters in South Africa** (Slabbert *et al.*, 1996).

1.2.2 Role of toxicity testing in water/effluent quality management

Toxicity testing is used by regulatory authorities to (OECD, 1987):

* Establish and set limits of permissible effluent discharge, using WET as the control parameter;
* Monitor effluents to assure compliance with discharge limits;
* Assist a discharger to reduce effluent toxicity;
* Identify toxic discharges and affected receiving waters, and establish priorities for pollution control action, and
* Monitor receiving water (ambient toxicity testing) to evaluate long-term effects of water pollution and control measures.

1.2.3 General information regarding toxicity testing

1.2.3.1 Concept of toxicity

Toxicity is a characteristic of a chemical (or a group of chemicals) that causes adverse effects in organisms. Adverse effects are mortality or those effects limiting an organism's ability to survive in nature, and can be acute or chronic (US EPA, 1990; 1991a). **Acute** means a stimulus severe enough to rapidly induce an effect (short-term effects). In aquatic toxicity tests an effect observed within 96 h or less is usually considered acute. An acute effect is usually but not always measured in terms of lethality. **Chronic** means 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 such as reduced growth, reduced reproduction, etc. in addition to lethality. Traditionally chronic tests are full-cycle tests or a shortened test of approximately 30 days and known as an early life stage test. Most of the EPA's tests have been shortened to 7 days, and called short-term chronic tests.

Toxicity is measured by observing the responses of organisms to increasing concentrations of a chemical substance. One substance is more toxic than another when the same adverse effect is caused at a lower concentration. For any given substance, toxic effects are alleviated when concentrations are reduced. Thus, when the toxicity of a discharge is reduced (concentrations of toxic constituents reduced) the toxic effect of that discharge on receiving waters is also reduced. Similarly, greater dilution of a toxic discharge will result in lower toxicity in receiving waters (US EPA, 1990).

The **inherent toxicity** of an effluent is established by using standard dilution water, prepared from distilled or deionized water and known chemicals, for dilution. **Relative toxicity** is the toxicity of an effluent when it is mixed with the receiving water, to test for interaction after discharge. If receiving waters are not available for dilution other water of similar composition (standard test water, other surface water, or ground water) should be used. Usually the dilution water should be free of toxic chemicals. Dechlorinated tap water is generally not recommended. If the objective is to establish the contribution of the effluent to receiving waters, receiving water is appropriate as dilution water, whether it is toxic or not. **Absolute toxicity** is the toxicity of the effluent without considering dilution (US EPA, 1990; 1991a).

1.2.3.2 Toxicity test

A toxicity test is a technique which determines the toxicity of a chemical or an effluent using living organisms. A toxicity test measures the degree of effect on exposed test organisms (US EPA, 1991a).

Three general types of tests can be carried out (US EPA, 1990):

* **Screening test** - In this type of test organisms are directly exposed to the effluent (no dilution) or to one dilution of the effluent. Ambient water (instream water) is also tested without dilution. It is not recommended to use one effluent concentration only (*e.g.* receiving water concentration - concentration of the parameter toxicity in the water after mixing) for compliance monitoring, as the error and variability associated with this type of statistical analysis is large (Warren-Hicks, 1990). For compliance monitoring the EPA recommends the use of five effluent concentrations that bracket the receiving water quality (two above and two below). In this way the test endpoints can be compared to the permit limit, and if measured over time the variability of the effluent can be determined.

* **Range finding test** - This is usually a 24-h test conducted on a wide range of ten-fold dilutions of an effluent to determine the approximate level of toxicity, and the concentration range to be used for definitive testing.

* **Definitive test** - This test estimates the concentrations at which a certain percentage of organisms exhibit a certain response. Organisms are exposed to various proportions of 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 in each test concentration is observed and recorded, and the number of responses in relation to test concentration analyzed.

The various acute and chronic toxicity tests used by different countries have been reviewed for the project **Development of guidelines for toxicity bioassaying of drinking and environmental waters in South Africa** (Slabbert *et al.*, 1996).

1.2.3.3 Test results

In toxicity tests the measured effect is brought into relationship with the corresponding pollutant concentrations or percentages of effluent using computational or graphic methods (DIN, 1982; ISO, 1989; US EPA, 1991a,b,c).

1.2.3.3.1 Toxicity endpoints

Acute toxicity endpoints commonly include lethal concentrations (LC's). The LC 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 duration is also included in the endpoint, namely 24, 48, 72 or 96 h (*e.g.* 96-h LC_{50}) (US EPA, 1991a).

Chronic toxicity endpoints normally include the no observed effect concentration (NOEC), the lowest observed effect concentration (LOEC), the effect concentration (EC), and the inhibition concentration (IC) based on observations of reduced reproduction, growth, and/or survival from life cycle, partial life cycle, and early life stage tests with aquatic organisms (US EPA, 1990; 1991a).

* The NOEC is the highest **tested** concentration where no adverse effects are observed on the test organisms at a specific time of observation and the LOEC is the lowest

tested concentration where an adverse effect on test organisms is observed at a specific time of observation. These values are determined using hypothesis testing and are not point estimates (US EPA, 1991b). The values are dependent upon the concentrations initially selected for testing. The calculated geometric mean of the NOEC and LOEC (square root of the product of NOEC and LOEC) provides the ChV (chronic value) (US EPA, 1990).

* The EC is the point estimate of the toxicant concentration at which a certain percentage of the test organisms would be affected, *e.g.* the EC_{10} (10% effect) or EC_{50} (50% effect). For some species like *Daphnia* where the point of death is not certain, immobility is often used as a surrogate for death (ISO, 1989). Results for responses like immobility may be reported as an EC_{50}. However, usually no distinction is made between EC_{50}'s and LC_{50}'s when the response is a surrogate for death.

* The IC is a point estimate of the toxicant concentration that would cause a given percent reduction (*e.g.* IC_{25}).

Point estimates such as LC's, EC's and IC's are statistically derived from dose-response curves, assuming a linear relationship (US EPA, 1991a,b,c). The IC_{25} has lately become the preferred method for determining the NOEC. Comparisons of data indicated that a NOEC derived using the IC_{25} is approximately the analogue of a NOEC derived from hypothesis testing, and the IC_{25} is, therefore, the preferred statistical method to determine the NOEC. The new method of calculation has the advantage that estimates of test precision, namely CV's (coefficient of variation) can be calculated. A CV is a standard statistical measure of the relative variation of a distribution or set of data, defined as the standard deviation divided by the mean (US EPA, 1991a).

1.2.3.3.2 Toxic units

Since toxicity involves an inverse relationship to effect concentrations (the lower the effect concentration, the higher the toxicity of an effluent), it is more understandable to translate concentration-based toxicity measurements into toxic units (TU's). The major advantage of using toxic units to express toxicity test results is that toxic units increase linearly as the toxicity of the effluent increases. Toxic units also make it easy to specify water quality criteria based on toxicity. One common method of deriving toxic units is to divide 100 (full strength effluent expressed as %) by the concentration (in %) that causes acute or chronic toxicity (OECD, 1987; US EPA, 1991a), *e.g.*:

(TU_a) (acute toxicity unit) = $100/LC_{50}$
(TU_c) (chronic toxicity unit) = 100/NOEC

For example, an effluent with an LC_{50} or EC_{50} of 1,25% has a value of 80TU, and an effluent with an LC_{50} or EC_{50} of 20%, a value of 5TU. The first effluent is, therefore, 16 times more toxic than the latter.

1.2.3.3.3 Acute-to-chronic ratio

The acute-to-chronic ratio (ACR) expresses the relationship between acute (LC_{50}) and chronic toxicity (NOEC) (obtained with the same organism). It is used as a factor for estimating

chronic toxicity on the basis of acute toxicity data, or for estimating acute toxicity on the basis of chronic toxicity data. The ACR may be used in developing chronic toxicity limits where chronic toxicity is not directly measured in order to minimize costs. If the toxicity of an effluent is mostly manifested in chronic endpoints an acute test may not be appropriate for compliance monitoring. If both acute and chronic data is available for an effluent the ACR is calculated directly for that effluent.

The ACR varies between species for a given toxicant, and for any one species between different toxicants, and is, therefore, a source of uncertainty (OECD, 1987). This is also a reason why the ACR for a complex effluent may not be constant. Regardless of this variability, regulators apply some ACR when converting wasteload allocations to common terms in the permit limit derivation process (US EPA, 1991a).

Data on acute and chronic toxicity for complex effluents showed that ACR's range from 1.0 to 50.0, with the majority of ACR's falling below 20. The EPA recommends that an ACR of 10 could be used in the absence of data. This represents the upper 90% of all the ACR data (US EPA, 1991a).

1.2.3.4 Toxicity test method precision

Like all measurements toxicity tests exhibit variability. Factors such as the test organism age, condition, sensitivity, temperature control, salinity, pH control, etc. can effect precision. Quality assurance practices should, therefore, be established. The use of a standard control water and the inclusion of a reference toxicant with test procedures is recommended. Minimum criteria of test acceptability specific for each endpoint that is measured in the controls should also be established (US EPA, 1991a).

Interlaboratory precision is the ability to obtain consistent results repeatedly when doing a specific test with the same reference toxicant while intralaboratory precision (round robin tests) indicates how reproducible a method is when carried out by different laboratories using the same test and reference toxicant.

1.2.4 Considerations involved when implementing WET testing

1.2.4.1 Sampling

An effluent or instream sampling programme is important to obtain a sample from which a representative measure of the parameter of interest can be made. Effluent variability is an important factor to consider when selecting the method of sampling and the frequency. Sampling must be tailored to measure the type of toxicity of concern for a particular discharge, for example long-term effects which are more constant or acute effects which are more variable and subject to peaks of intensity.

1.2.4.1.1 On-site *versus* off-site testing

In general off-site testing will be acceptable for most effluents except when volatiles are present. When flow-through tests are carried out, on-site testing is strongly recommended (US EPA, 1991a).

1.2.4.1.2 Flow-through *versus* static and renewal toxicity testing

Two basic types of systems are available for toxicity testing: flow-through and static systems. A diluter system with a continuous feed of effluent and dilution water is used for a flow-though system. A static test is conducted in test chambers into which effluent and diluent is added manually. The renewal test is a variation of the static test. In this test the solutions are changed on a predetermined basis (*i.e.* 24 h intervals). Fresh effluent samples are usually collected for renewal (US EPA, 1991a).

A flow-through exposure is not a continuous analysis as only one result is obtained at the end of the test. The flow-through system can, however, provide a measure of time-varying effects and indicate peaks. The test organisms would be exposed for periods proportional to the flow rate, the duration of the peak and the length of the test. Peaks can also be measured with the static and renewal tests depending on the type of sampling used and if the sampling occurred at the time of peak toxicity.

A flow-through or renewal test is best applicable if an effluent is continuously discharged and is highly variable. The same systems will be applicable if the effluent is highly variable with intermittent discharge. Composite samples collected over the period of discharge should be collected for the renewal test. If the effluent is not variable, *e.g.* a discharge from a 30 day retention dam, a static or renewal test would be the appropriate systems, testing grab or 24 h composite samples. A 24 h composite sample is usually recommended for a chronic test. For an acute test grab samples taken at selected intervals are most appropriate to establish peaks of toxicity.

1.2.4.1.3 Grab sampling *versus* composite sampling

Grab samples collected during peaks of toxicity provide a measure of maximum toxicity if the toxicity of the effluent is highly variable. Grab samples may be necessary if there is little mixing of effluent with the receiving water. A grab sample will only reveal the toxicity peak in an effluent if the sample has been collected at the time of the toxicity peak. A 24 h composite sample may catch the toxicity peaks, but the compositing process may tend to dilute the toxicity resulting in misleading measures of the maximum toxicity of the effluent. Composite samples are recommended for chronic tests where peak toxicity of short duration is of lesser concern (US EPA, 1991a,b,c).

The EPA permit requirements for sampling generally involve a single grab or 24 h composite sample. Such samples could, however, conceal daily extreme values. To optimize sampling cost and effectiveness, it may be desirable to reduce long term frequency in order to increase daily frequency. For example, instead of collecting samples on a weekly basis, samples could be collected and tested on a monthly or bimonthly basis and several grab samples can be collected daily (*e.g.* four taken 6 h apart, six taken 4 h apart). If an effluent does not exhibit a large variation, it could be more desirable to collect composite samples, each composited over a three-hour interval (US EPA, 1985b).

1.2.4.2 Variability

1.2.4.2.1 Effluent variability

Effluent variability is caused by changes in composition. Studies conducted by the EPA (US EPA, 1991a) showed that the toxicity of effluents vary and that any one effluent can exhibit significantly varying toxicity to different test organisms over time. The variability can be handled by proper sampling and testing procedures. Effluents were found to be rarely toxic below 10 percent concentrations and not toxic below 0,1 percent concentrations.

1.2.4.2.2 Exposure variability

Exposure variability is caused by changes in flow rates of the effluent as well as the receiving water. Receiving water parameters, *e.g.* background toxicant levels, pH, suspended solids, hardness and temperature, also play a role. Exposure variability can be addressed in two ways. The easier approach is to assume a steady state exposure (estimate of a worst case situation with a low receiving water flow and a typical effluent flow). The second approach is to estimate/measure the variable exposure state at the discharge site. This requires statistical analysis and dynamic modelling.

1.2.4.2.3 Species sensitivity variability

Organisms differ in their sensitivity to toxicants. For example, trout are considered to be very sensitive, requiring high quality water. This generalization is almost universally accepted. However, when dealing with toxicity, this generalization may be inaccurate. Trout are sensitive to low oxygen content, but may be relatively insensitive to certain toxicants (US EPA, 1985b). Since the measured toxicity of an effluent will be caused by unknown chemical constituents, the relative sensitivities of test species will also be unknown. Therefore, proper effluent toxicity evaluation requires an assessment of a range of sensitivities of different test species to that effluent. The indigenous community in the receiving water is likely to be less sensitive to the effluent than the sensitive test species and will thus be protected if the NOEC of the test species is not exceeded.

The EPA (US EPA, 1991a) recommends the use of a minimum number of three test species representing different phyla (*e.g.* a fish, an invertebrate, and an algae) for effluent testing to provide sufficient information to make permit decisions. Fewer species might be used in certain instances, depending on such factors as how thoroughly the effluent has been characterized, dilution, consistency of effluent, variation in treatment processes, raw materials in use, and treatment efficiency. For example, if the effluent is very consistent and little variation occurs in treatment processes, raw materials and treatment efficiency, the use of the two most sensitive or even the one most sensitive species may be appropriate as determined on a case-by case basis.

The use of resident species toxicity testing is not recommended unless required for some or other important reason. Such testing is more expensive, more difficult to do and subject to more variability (disease, age) than standardized testing (US EPA, 1985b). Organisms collected directly from the receiving water should never be used because existing effects may mask toxicity.

1.2.4.3 Magnitude, duration and frequency of exposure to toxic effects

1.2.4.3.1 Magnitude

Criteria for toxicity range from the narrative prohibition (no discharge of toxic chemicals in toxic amounts) to detailed requirements that specify the test organism and the allowable toxicity level. The EPA uses two expressions of allowable magnitude, namely a criterion maximum concentration (CMC) to protect against acute (short-term) effects, and a criterion continuous concentration (CCC) to protect against chronic (long-term) effects. For example, the recommended instream acute toxicity criterium provides that acute toxicity should not exceed a factor of 0,3 times one acute toxicity unit (TU_a) within the mixing zone, as measured by the most sensitive species of at least three tests (US EPA, 1991a). A factor of 0,3 is used to adjust the typical LC_{50} end-point to an LC_1 value (concentration causing 1% lethality). The factor of 0,3 was found to include 91% of the observed LC_{50} to LC_1 ratios in 496 effluent (municipal and industrial) toxicity tests (OECD, 1987). In order to protect aquatic life against chronic effects the ambient (instream) toxicity should not exceed 1,0 chronic toxic unit TU_c at the edge of the mixing zone, as measured by the most sensitive of at least three test species.

For both acute and chronic data, if fewer than three test species are tested, the test results are divided by 10 to account for species sensitivity differences. For example if only two species are used the chronic toxicity would be $0,1TU_c$.

1.2.4.3.2 Duration

The effect of an effluent on the aquatic environment also depends on the duration of exposure to toxic levels. The time factor can be addressed by careful selection of acute and chronic toxicity test procedures. When defining a criterium for water quality it is necessary to set a time period over which the exposure should be compared with the criterium.

Many toxicants exhibit acute toxic effects very quickly. It is, therefore, necessary to limit periods of high chemical concentrations to short time spans. For acute toxicity criteria the EPA recommends an averaging period of 1 h (US EPA, 1991a). Thus, the 1 h average exposure must be smaller than acute toxicity criterium to protect against acute effects. This period was derived from data on response time for toxicity to ammonia, a very fast-acting toxicant. This period is thus expected to fully protect against fast-acting toxicants. Different averaging periods can be derived from information relating toxic effect to exposure time or from models of toxicant uptake and action. In practice it is difficult to model exposure for periods shorter than one day and it is difficult to monitor effluent concentrations or toxicity on a continuous basis. Therefore, it is recommended that efforts should be made to reduce short term variability in the effluent discharge (*e.g.* installation of stabilization ponds).

Chronic criteria should protect against effects caused by long-term exposure to effluents. It is difficult to determine under fluctuating conditions what duration of exposure is acceptable because toxicity information is mostly derived from tests in which exposure is constant. The EPA recommends an averaging period of 4 days for chronic criteria (US EPA, 1991a). That is, the 4 day average exposure should be smaller than the chronic toxicity criterium. The 4 day averaging period was selected because this is the shortest duration in which chronic effects are sometimes observed for certain species and toxicants, and thus should be fully protective, even for fast-acting toxicants. Different chronic averaging periods could be derived

depending on the nature of the pollutant and the toxic endpoint (*e.g.* rate of uptake and accumulation, mode of action).

1.2.4.3.3 Frequency

To predict or ascertain the attainment of criteria it is necessary to specify the allowable frequency for exceeding the criteria. This is because it is statistically impossible to project that criteria will never be exceeded, and because ecological communities are able to recover from stresses (US EPA, 1985b; 1991a).

Criteria may be exceeded as a result of predictable impacts (low flow, high toxicity) or because of spills. Individual organisms may recover from severe but non-lethal exposures, provided that the intervals between such exposures are longer than the time needed to repair damage. The same applies to ecosystems, although the response of ecosystems is much more complex. Communities that are not under stress can withstand an event better than a community under stress. If the level and duration of toxicity is severe enough to damage major trophic levels or communities, the recovery may take years. A repetition of similar high levels of toxicity prior to full recovery will prolong the recovery period or even prevent recovery (OECD, 1987).

The EPA recommends a once in 3 year average frequency for excursions of both acute and chronic criteria. The allowable frequency depends on site-specific factors. To implement alternative frequencies, site-specific factors or other data should be taken into account (US EPA, 1991a).

1.2.4.4 Mixing zones

A conservative approach to protect the aquatic environment against toxic effects is to require that an effluent has no observable toxicity prior to entering a receiving water. In practice it is often necessary to allow the receiving water to dilute a toxic effluent so that non-toxic levels occur in most of the receiving water. This means that the area of immediate discharge (mixing zone) will experience an effluent concentration which is toxic, and organisms in this area will be more or less severely affected.

One reason for designating a mixing zone would be to reduce treatment costs of a discharger in situations where rapid and complete mixing will adequately reduce the effluent's toxicity. In suitable locations this approach will prevent affects on the major part of a watercourse and its organisms. Another reason for allowing such an area could be as an interim measure while control procedures are being developed (OECD, 1987).

The dimensions of a mixing zone should be defined in terms of space, duration and toxicity of the toxic effluent's plume. Allowing a mixing zone should be conditioned on the absence of toxic effects as defined by the NOEC or TU_c outside the mixing zone's boundary (Fetterolt, 1973). In some instances, a mixing zone may be divided into two regions. In the immediate area of discharge, acute toxicity (>maximum allowable acute criterium) might be permitted. In the remainder of the mixing zone and in accordance with time or toxicity limits, chronic toxicity (>maximum allowable chronic criterium) is permitted. Outside this area the usual toxicity criteria for receiving water should apply (NOEC or TU_c). The EPA specifies that acute toxicity should be prevented within the mixing zone (US EPA, 1991a).

Important considerations in determining the size of a mixing zone are the volume of dilution water available, and the speed and uniformity of dilution (dilution is most effective when using a high-velocity diffuser). In the US most States specify that the zone should not be as wide as the stream in order to allow a zone of passage for fish. In very few instances the allowable length is given. The size of a zone is determined on a case by case basis taking into account the critical resource areas that need to be protected (US EPA, 1991a). It is recommended that a mixing zone should be limited to a small area of receiving water located away from valuable fisheries or other sensitive water uses.

The US recommends that mixing zones should be evaluated and used for regulation in cases where complete mixing does not occur within a short distance of the outfall (discharges into large rivers, lakes, estuaries) (US EPA, 1985b). If mixing is assumed to be rapid and complete when it is not, a toxic discharge that appears to meet criteria may cause zones of chronic toxicity that can extend for kilometres.

1.2.4.5 Ambient toxicity

Factors such as toxicity persistence/degradation, toxicant interaction (additivity, antagonism, synergism), and physical/chemical interferences (pH, temperature, hardness, salinity) play an important role when evaluating the receiving water behaviour of toxicants and toxicity. A toxicity test carried out with water upstream of discharge or water from an uncontaminated area is an analogue of the mixing and fate processes taking place in the receiving water. The different chemical reactions taking place in the mixing zone can also be expected to take place when effluents and receiving waters are mixed for toxicity tests. Effects on chemical persistence in the receiving water or in the effluent, however, cannot be accurately predicted from such results. Ambient toxicity tests measure the ambient interactions of effluent and receiving water and can be used to assess persistence, interaction and interferences.

Ambient toxicity testing is particulary important where impact is caused by multiple point sources. Since each effluent consists of individual chemicals, mixing with the receiving water produces a mixture of these individual pollutants. The ambient toxicity could be equal to the sum of each discharge's toxicity (additive), less than the sum (antagonistic) or more than the sum (synergistic). Although synergism can theoretically occur, it has not been observed during field and on-site effluent toxicity studies and is not considered an important factor in the toxicity assessment of effluents. From all the data available it appears that, on average, the combined effect is additive. This is true for acute toxicity, but not for chronic toxicity.

Ideally, sampling should be done under conditions of low flow in the receiving water so that worst case toxicity conditions become apparent. It is very important to closely replicate the worst case receiving water conditions in toxicity tests, because of the influence of environmental conditions. There may be situations when the test conditions differ from the worst case conditions, which may result in an under or over prediction of the toxicity in the receiving water. For example, ammonia is present in water, and the highest expected ambient temperature is 20° C. The toxicity test is conducted at 25° C, which causes a larger ammonia toxicity. The temperature requirements of the test, therefore, induces toxicity not found in the ambient water. It is thus very important for the regulatory body to look carefully at test protocols and all the chemical data. If it is found that a toxicity test result is artificial due to environmental parameters, the test should be overridden by subsequent valid toxicity tests (US EPA, 1991a).

Simultaneous testing of water collected from various points may generate a better picture of instream toxicity and interactive effects. However, it should be kept in mind that variations in the nature, volume and timing of effluent discharge at each source may make tests useful as an investigative tool and limit their usefulness for regulatory purposes.

The basic ambient toxicity testing procedure consists of exposing organisms to water samples taken from selected sampling points above, at and below the point of discharge. With such testing the actual receiving water is directly tested. No extrapolation from exposure or ACR is needed. Since the concentration after discharge is usually very low, chronic toxicity tests should be conducted. The location of sampling points should be determined on the basis of known mixing characteristics of the receiving water and knowledge of important sensitive areas within the receiving water.

1.2.5 Correlation between toxicity test data and actual receiving water impact

The EPA evaluated the results of several studies to determine the validity of toxicity tests to predict receiving water community impact (US EPA, 1991a). It was found that a strong correlation exists between toxicity and biological impact. The results were in agreement (toxicity test positive/impact observed; toxicity test negative/impact not observed) for between 81 and 90% of the observations. False positives (toxicity test positive/impact not observed) and negatives (toxicity test negative/impact observed) ranged from 10 to 19%.

The EPA cautions that toxicity as measured by toxicity tests is only one of the many parameters affecting aquatic community health. Substrate differences and physical conditions (*e.g.* dissolved oxygen, temperature) can also adversely affect the community.

1.2.6 Human health protection

Two types of human health effects can occur, namely non-threshold effects (carcinogenicity) and threshold effects (acute, subacute or chronic toxicity). The effects are divided into two groups because of different mechanisms of action. In the case of carcinogens a small number of molecular events can lead to changes in a single cell which can result in uncontrolled cellular proliferation. Carcinogenesis is referred to as a non-threshold effect because there is no level of exposure that does not pose a small, finite, probability of causing uncontrolled growth. For these pollutants incremental risk levels can be determined based on the carcinogenic potency.

Threshold chemicals have an identifiable exposure level below which no effects are observable and which are presumed to be safe. Such chemicals exhibit effects other than cancer, affecting the function of organ systems.

Currently the regulation of human health impacts is based on the control of individual chemicals. Water quality criteria for human health protection guard against the consumption of contaminated water and aquatic organisms. There is no mechanism like the aquatic toxicity test to determine the effect of an effluent on human health. There are, however, tests that can establish a wastewater's potential to cause carcinogenicity or mutagenicity (*e.g.* Ames test) and acute or chronic toxicity (Slabbert *et al.*, 1996). Such data will indicate the presence of harmful chemicals, but are difficult to use to establish cause and effect relationships between exposure to effluents and human health problems. The EPA (US EPA, 1991a) determines the

bioaccumulation potential of whole effluent by means of a bioconcentration analysis followed by the identification of such pollutants. The chemicals are controlled by limiting them on an individual basis.

1.2.7 Development of permit limits for WET

The fundamental principle in setting water quality based limits/controls/criteria, is that the receiving water concentration (RWC) must be less than the water quality criterium. For protection of aquatic life a simple equation can be used to illustrate this (US EPA, 1991a):

RWC < chronic toxicity criterium (NOEC)

The RWC is the measured or projected exposure concentration in the receiving water (after mixing). The RWC is calculated at the edge of a mixing zone if such a zone is allowed.

The described relationship is used for both effluent characterization and wasteload allocation (WLA) (US EPA, 1985b; 1991a). For effluent characterization the objective is to project receiving water concentrations based on existing effluent quality to determine whether or not an excursion above ambient criteria occurs. For WLA the objective is to fix the RWC at the desired criteria level and determine an allowable effluent loading that will not cause excursions.

The first step in the process of effluent permit limit development is to establish whether or not an impact is possible (is there a need for a permit limit). This can be done directly without generating effluent data, or a characterization of the effluent can be carried out to obtain data to determine whether or not discharge will cause an impact. Once the permitting authority determines that an effluent causes, has the reasonable potential to cause or contributes to an excursion of water quality criteria, the authority will develop permit limits that will control the discharge (US EPA, 1991a). This determination is made at each re-issuance of a permit and also for new permits. Permit limits for effluent toxicity are usually set based on data generated during the characterization process. However, limits for effluent toxicity can also be set directly without using toxicity tests or generating toxicity data (US EPA, 1985b). Once a permit is issued containing a limit for toxicity, the permittee is responsible for attaining, monitoring and maintaining compliance with the permit.

1.2.7.1 Effluent characterization to establish the need for permit limits

1.2.7.1.1 Characterization in the absence of effluent monitoring data

When determining the need for a permit limit for whole effluent toxicity the regulator is required to consider some pertinent factors (Hunt *et al.*, 1991; OECD, 1987; US EPA, 1985b; 1991a):

Existing controls on point and nonpoint sources of pollution

* Industry type: primary or secondary (primary industries have a larger potential for toxicity than secondary industries; within primary industries no generalizations should be made; toxicity problems associated with types of industries are site specific), raw materials used, products produced, best management practices, control equipment, treatment efficiency
* Privately owned treatment type: pretreatment, industrial loadings (works with

large loadings from indirect dischargers, particularly primary industries, are high priority candidates for toxicity testing; absence of industrial input is no guarantee of absence of toxicity problems)

Variability of the pollutant/s in the effluent

* Compliance history
* Existing data from monitoring reports and applications

Sensitivity of the involved species to toxicity testing

* Stream survey data
* Receiving water type and designated or existing uses

Dilution of the effluent with receiving water

* Dilution calculations [toxic impact is directly related to dilution. The lower dilution, the higher the potential for toxic effect; a recommendation in this connection is if concentrations of instream effluents reach 1% and higher during critical low flow periods (dilution ≤100) those effluents should be given high priority for toxicity assessment]

Given these factors, priorities for toxicity testing can be set. The presence of a combination of these factors, such as low dilution, high quality receiving water, poor compliance record, and clustered industrial or municipal discharges could constitute a high priority for effluent limits. If, after evaluating the information it is not clear whether violation is possible, the authority should require whole effluent toxicity chemical-specific testing (chemical characterization) to gather further information. In such a case the regulator can require the testing prior to permit issuance, if sufficient time exists (to allow adequate time for toxicity tests data collection should begin 12 to 18 months in advance of permit development), or it may require the testing as a condition of the issued/reissued permit (direct setting of limits). The EPA recommends that monitoring data should be generated on effluents before permit limits are developed because of the following: a) the presence or absence of effluent toxicity can be more clearly established or refuted and b) where toxicity is shown, effluent variability can be more clearly defined.

If the regulator decides to impose an effluent limit after conducting an effluent assessment without generating data (direct setting of limits) (2.8.1.2), adequate justification for the limit will be required.

1.2.7.1.2 Characterization using effluent monitoring data

1.2.7.1.2.1 *EPA's approach until 1990 and OECD recommendations*

Until 1990 the EPA followed a tiered testing approach to generate data to establish toxic impact (US EPA, 1985b). This approach was also recommended by the OECD (1987). The purpose of tiered testing was to collect information in an orderly, cost-effective manner. Testing began with short, inexpensive screening tests (first tier) and progressed to more definitive data generation techniques (second tier). The tiered approach integrated effluent

effect measurements and simplified exposure assumptions into various stages and arrived at one of three decisions: a) apparently safe because of a large margin between exposure (RWC) and NOEC, b) unacceptable hazard because the exposure concentration exceeds the NOEC, or c) additional data are needed to more clearly resolve the marginal difference between exposure and effects. The first tier, *i.e.* initial screening, was aimed at establishing the probability of adverse impact. The aim of the second tier testing was to generate sufficient data on effluents to support appropriate regulatory decisions (technical measures needed or further testing), to better quantify the environmental impact of a discharge, and to require the necessary controls.

All effects testing and exposure assessment parameters have some degree of uncertainty associated with them. The more limited the data, the larger the uncertainty. To establish which effluent needs additional testing, each tier of data should be judged in the light of the uncertainty of data through the use of uncertainty factors. There are three sources for which uncertainty factors are given, namely effluent variability, species sensitivity and ACR (US EPA, 1985b). (The ACR is actually a conversion factor, but for simplicity it is seen as an uncertainty factor). Examples of uncertainty factors applied by the EPA until 1990 (US EPA, 1985b):

* For species sensitivity -
  10x, where two species are used,
  1x, where three species are used.

* For effluent variability -
  100x, where testing is limited to one sample per test species quarterly,
  10x, where testing is limited to two samples per test species monthly,
  1x, where testing is carried out on four samples per test species monthly.

* For ACR, 10x

For example, a limited data base of a few acute toxicity tests on one species could require a level of uncertainty of 1 000 (10x species, 10x variability of effluent, 10x ACR).

First tier testing

Several procedures could be applied for first tiered testing. One option followed by the EPA was to carry out acute toxicity tests (fish and water flea), calculate the lowest LC_{50}, divide the value by the available dilution (IWC) and compare this with the appropriate level of uncertainty. The formula used is:

LC_{50} (%)/IWC >level of uncertainty, where

IWC = the instream waste concentration. Where the receiving water is the facility's source water, IWC = Q_w/Q_r, where Q_w = wastestream flow and Q_r = receiving water flow.

Level of uncertainty = uncertainty factors multiplied

If the ratio of LC_{50}/IWC was not >level of uncertainty, the second tier of testing was required.

Another option for first tier testing was to use simple, fast screening tests conducted in the absence of serial dilutions at 100% effluent together with simple dilution models or calculations. If a pre-specified toxicity level was observed, the potential for toxic impact exists and further testing was conducted. Uncertainty factors were not applied in this option because LC_{50}'s were not generated. The following scheme could be applied (OECD, 1987):

- If the dilution in the receiving water at the edge of the regulatory mixing zone exceeds 10 000:1, then effluent should be given low priority for further attention;
- If the dilution is between 10 000:1 and 1 000:1, short term acute toxicity screening tests (24 h) (fish and water flea) should be conducted. If the lethality in the 100% effluent is <50%, then the effluent should be given low priority. If the lethality is >50%, continue with the second tier of testing.
- If the dilution is between 1 000:1 and 100:1, conduct short term chronic tests (7 days or less) (fish and water flea) on the 100% effluent. If the effect is <50%, then the effluent should be given low priority, but if it is >50%, continue with second tier of testing. Acute tests could be conducted, but there will be cases where no acute toxicity is measured while the effluent is chronically toxic.
- If the dilution is less than 100:1, it is recommended that screening is skipped and that the second tier of testing should be carried out. Screening has already been done through dilution analysis. Even in situations where no toxicity is observed in screening tests, the narrow margin between effect concentration and available dilution suggests that more complete effluent toxicity characterization is necessary. If uncertainty factors are applied in a 100:1 discharge situation, dilution alone will indicate that further testing is necessary.

Second tier testing

Once screening has indicated the potential for toxic impact, definitive toxicity data generation is necessary. Second tier testing is normally conducted using an increased number of tests and more extensive or advanced techniques in order to reduce uncertainty.

The EPA applies the same simple formula as used for screening testing to determine whether more data is necessary, whether to stop testing and start establishing permit conditions, or whether to stop testing because of a wide margin between toxicity and the IWC's:

LC_{50} or NOEC (%)/IWC >level of uncertainty

The following steps are followed by the EPA to eliminate uncertainty. Baseline or initial testing will include two acute toxicity tests (fish and water flea). Tests will be conducted monthly on grab or composite samples. The level of uncertainty = 1 000 to 10 000 (10-100x for effluent variability, 10x for species variability and 10x for ACR), where chronic toxicity is of concern but no chronic data is available.

To eliminate uncertainty factors the following is done:

Effluent variability - Conduct the same tests as already mentioned. Estimate the variability of toxicity to establish sampling frequency. If effluents have short term variability, measure the highest toxicity during a short period of duration, *e.g.* examine four to six samples (grab/composite) taken over a 24 h period, and conduct tests

monthly for at least one year. Another option is to conduct flow-through acute tests with each test species. If effluents show long term variability, schedule testing frequency to conform to expected changes in composition. For long term variability, a year-long monitoring programme may be required. The level of uncertainty = 100 (10x for species sensitivity and 10x for ACR).

Species sensitivity - Conduct acute toxicity tests on a total of three to five species (fish, water flea, algae). Use the same sampling frequency as mentioned above. The level of uncertainty = 10 (10x for ACR).

ACR factor - Conduct short term chronic toxicity tests using three species (*Ceriodaphnia*, fish growth test, or other short term tests available). Use the same sampling frequency as mentioned above. The level of uncertainty = 1.

Dye studies are strongly recommended for effluent characterization, unless mixing is known to be rapid and complete.

The use of toxicity tests in multiple-source discharge situations

Two options exist (US EPA, 1985b; 1991a). The regulator may decide to handle each source separately and then the already mentioned procedures will be followed. The other option is to treat each discharge as an interactive component of the whole. The following procedures can then be followed:

Where effluents make up more than 1% of the flow of the receiving water, conduct chronic toxicity tests as described. Where effluents make up less than 1% of the receiving water flow use acute toxicity tests as already mentioned. Conduct the tests at appropriate frequencies as described. If possible all testing should be conducted simultaneously by each discharger. Tests should be started concurrently, within a short period of one to two days.

An additional data requirement is the assessment of relative and absolute toxicity of each source so that appropriate permit conditions can be set for individual dischargers. In order to obtain data on absolute toxicity conduct one set of toxicity tests on the effluents using uncontaminated receiving water upstream of the points of discharge or reconstituted water as control (dilution). In order to obtain information on relative toxicity run a parallel set of tests on the effluents using a dilution water taken directly upstream from the point of discharge. This dilution water may be contaminated with upstream effluents and can result in a change in the standard concentration-response curve. If there are adverse effects, this does not invalidate the test. Instead, analysis of toxicity trends can be used to assess the effluent's toxicity in relation to other sources and ambient receiving water conditions. A control with no toxicity is always required for quality assurance and determination of absolute toxicity. Ambient toxicity tests should be carried out to a) determine whether or not the effluent has a measurable toxicity after mixing, b) measure instream persistence of toxicity from all sources contributing to instream toxicity, c) determine combined instream toxicity resulting from the mixing. These tests should be conducted during low flow. Repeated ambient toxicity analysis will be desirable if effluents are showing variability.

Here again, dye studies of effluent dispersion are strongly recommended.

Confirmatory tests after the second tier

As a further step after the second tier a number of advanced techniques for measuring and assessing effluent are available or emerging. These advanced methods include chemical fate modelling, microcosm tests for prolonged exposure at reduced concentrations, and physiological investigations with fish or bivalve molluscs in combination with advanced field monitoring (OECD, 1987).

New discharges

The application of toxicity tests may differ when the potential impact of a new plant is evaluated, including a new discharge at an existing site. A careful hazard assessment which includes analysis of potential chemical, physical-chemical and biological effects should be conducted prior to the commencement of discharge. Once a new discharge has commenced a tiered testing approach should be followed as described to ensure that the receiving water is sufficiently protected from toxic impacts (OECD, 1987).

1.2.7.1.2.2 *EPA's approach since 1991*

Since the production of the first **Technical Support Document for Water Quality-based Toxics Control** (US EPA, 1985b) significant additional experience was gained by the EPA in generating effluent toxicity data upon which to make decisions as to whether an effluent can cause a toxicity impact or not. In the 1991 document (US EPA, 1991a) the EPA has revised its previous effluent toxicity generation recommendations based on three observations:

* Effluents rarely have LC_{50}'s <1,0% or NOEC's <0,1%. However, there is always a possibility that an effluent could be toxic at low concentrations.
* In general, ACR's above 20 were not found. Most of the ACR's were seldom >10%.
* The three commonly found freshwater species (fish, water flea and algae) have generally been sufficient to establish effluent toxicity impact and to make regulatory decisions.

The changes in the EPA's data generation recommendations eliminate the application of multiple sets of uncertainty factors as proposed in the 1985 document. The new approach makes use of three dilution scenarios (>1 000:1; 100:1 to 1 000:1; <100:1), eliminates species sensitivity uncertainty factors (three test species used), and target LC_{50}'s of 1,0% and NOEC's of 0,1% as the most extreme toxicity measurements. Because the new data generation requirements are much less expensive than the previous requirements, tiered testing is unnecessary.

The basic principle used by the EPA in making decisions is to compare available dilution to known or projected toxic effect concentration in order to place an effluent into one of three categories (criterium exceeded - toxicity limit needed for permit; reasonable potential of exceeding criterium - limit needed; low potential - no limit needed for permit). The process is divided into three steps, namely 1) determining dilution, 2) toxicity testing, and 3) developing decision criteria for the permit limit.

Dilution determination

Dilution of the effluent is determined at the edge of the mixing zone assuming mixing zones are allowed. If no initial mixing is allowed to control acute toxicity, acute toxicity is limited at the end of the pipe. Permit limits derived to enforce this requirement would be based on an ambient criterium (NOEC). Regardless, the use of both acute and chronic toxicity tests is recommended.

Toxicity testing

The EPA recommends the use of three species (fish, water flea and algae) applied quarterly for one year. Conducting tests quarterly for one year enables adequate assessment of variability of effluent toxicity. The result for the most sensitive species is considered to be the measured toxicity of a particular effluent sample. If required, this test frequency can be increased to improve the assessment of effluent variability. If a permit requires less frequent testing, it is preferable to use three species less frequently than to test the effluent more frequently with only a single species whose sensitivity to the effluent is not well characterized.

Acute tests are recommended at effluent concentrations >1 000:1. The rationale for this is that the effluent concentration would be below 0,1% at the end of the mixing zone and would thus not cause an excursion above the chronic toxicity criterium. The use of acute or chronic tests are recommended if dilution is between 100:1 and 1 000:1. This is because acute and chronic toxicity have been found within this dilution range. Acute tests will be more appropriate at the higher end of the dilution range and chronic tests at the lower end. If only one of the two types of tests is used, an ACR can be used for conversion. Should the dilution of the effluent fall below 100:1, chronic tests are recommended. This is because chronic toxicity has been detected in some effluents at the 1% concentration. Because there is a potential for acute toxicity in this range, an ACR may be applied to calculate acute toxicity.

Decision criteria for permit limit development

Once the toxicity data are generated, a decision must be made on possible impact. The data are used to project receiving water concentrations which are compared to the acute and chronic criteria. In order to establish whether there is a reasonable potential for an excursion above the toxicity criteria the EPA recommends the use of a statistical analysis of effluent data which accounts for limited sample size and effluent variability. This procedure is as follows:

- Determine the number of total observations (n) for a particular set of effluent data (concentrations or TU's), and the highest value for the data set. *E.g.* there are four samples with the highest toxicity at $9TU_c$;

- Determine the coefficient of variation (CV) for the data set. This can be obtained from the data set (mean/standard deviation) or by using an estimate (where n<10). *E.g.* the estimate of the CV is 0,6;

- Obtain the multiplying factor for your data from a table drawn up by the EPA (99% confidence interval and 95/99% probability), using the number of observations (n) and the CV. *E.g.* the multiplying factor is 4,7 (99% confidence interval and 99% probability);

- Establish the projected receiving water concentration (RWC) by multiplying the highest value by the multiplying factor and the expected dilution. *E.g.* the dilution 2% (0,02) -

$$RWC = 9TU_c \times 4,7 \times 0,02$$
$$= 0,85TU_c$$

- Use the relationship RWC <criterium (acute/chronic) to establish possible excursion -

$0,85TU_c < 1,0\ TU_c$. This indicates no potential for an excursion.

An alternative approach may be followed using a stochastic dilution model that incorporates ambient dilution and effluent variability. In some instances it might not be clear from data analysis whether an excursion above the criteria is certain. Under such conditions an element of judgement by the regulator will be necessary.

In cases of inadequate information to determine a reasonable potential for an excursion the permit should contain whole effluent toxicity monitoring requirements and a reopener clause. This would require reopening of the permit and establishing of a limit based on data or other factors which might substantiate that there is an impact. If no excursion above the criteria is found, the EPA recommends that toxicity tests are carried out once every five years as a part of the permit application.

Multiple source discharges are handled the same way as described under 2.8.1.1.1.

1.2.7.1.2.3 *Proposed UK approach*

The UK uses a simple, rapid acute luminescent bacterial test (Microtox test - based on light inhibition) to screen effluents for potential toxicity. The Microtox test results are reported as EC_{50}'s (concentration causing 50% reduction in luminescence after 5-30 min exposure, relative to the control). Microtox EC_{50}'s are used to calculate the effluent Microtox acute toxicity (EMAT), namely EMAT = $100/EC_{50}$ (in TU's). Using certain factors the estimated chronic in-stream toxicity (ECIST) in TU is derived by using the following formula (Hunt *et al.*, 1991):

$$\mathbf{ECIST = EMAT \times ACR \times AF/WCDF}$$

where AF = application factor; WCDF = worst case dilution factor in receiving water.

On the basis of the ECIST values effluents can be divided into one of four categories, namely high (>1,0TU), intermediate (0,1-1,0TU), low (0,01-0,1TU) and nil (<0,01TU). Those effluents falling in the high and intermediate categories are subjected to a full toxicity based consent (TBC). A Microtox-based consent will be established for the effluent showing low toxicity but whose toxicity is likely to vary while the effluent showing little or no toxicity will be controlled by chemical-specific measures.

TBC's for the effluents from the high and intermediate categories are set based on acute tests using at least three representative species (fish, invertebrate and algae) and well-established standard protocols. The tests should be applied to four samples taken over a minimum period of three months. It is recommended that the Microtox test be calibrated against the most sensitive of the three species and a consented EMAT set, equivalent to an ECIST value

of 1,0TU. This will provide a simple and relatively inexpensive test for routine monitoring purposes. However, from time to time it will be necessary to retest the effluent with the three test species and to adjust the consented EMAT by recalibration.

There will be instances that the Microtox test will lack sensitivity and that another test will be needed. It is also suggested that for important discharges a sub-lethal test using water flea reproduction may be an appropriate test to include with the other tests.

1.2.7.2 Permit limit derivation

1.2.7.2.1 Setting a permit limit without effluent monitoring data

If the regulator so chooses or if the circumstances dictate permit limits can be developed and imposed for whole effluent toxicity without conducting toxicity tests or generating effluent toxicity data (US EPA, 1985b). Toxicity based permit limits can be established by using the available dilution and the water quality criterion for toxicity. The relationship is expressed as:

allowable effluent toxicity x 1/dilution factor
≤ criterium concentration

Since both the criterium and the dilution factor are known the value for allowable effluent toxicity (toxicity based limit) can be calculated as follows:

allowable effluent toxicity ≤criterium x dilution factor

For example, assume that one particular effluent is the only source of toxicity to a receiving water and that the available dilution is 30 to 1 (dilution factor = 30) (US EPA, 1985b). The relation between criteria concentrations (0,3TU_a or 1,0TU_c), dilution and allowable effluent toxicity is as follows (acute and chronic toxicity):

allowable acute effluent toxicity
≤0,3TU x 30
≤9TU_a (or LC_{50} ≥11%, using 100%/9)

and

allowable chronic effluent toxicity
≤1,0TU_c x 30
≤30TU_c (or NOEC ≥3,3%, using 100%/30)

In this case the regulatory body can set a limit of 30 TU_c without requiring effluent toxicity testing. However, toxicity testing will be required to ensure compliance with the limit.

In case of multiple source discharge situations (where impact zones overlap) additional data may be needed. Testing that provides information to separate the relative impact of each source can be conducted. Factors such as addition, antagonism, and persistence of toxicity may become more important, and detailed testing to measure these factors would be needed. Flows must be summed up and allowable toxicity allotted to the individual sources using some option for establishing toxicity WLA's (US EPA, 1985b). Two options for assessment exist, namely each source can be handled separately or each discharge can be treated as an

interactive component of the whole system. The relationship for multiple sources is expressed as:

$$\Sigma(Q_e T_e)/Q_s \leq \text{criterium}$$

where Q_e = effluent flow
T_e = effluent toxicity
Q_s = stream flow at the point of reference (below the last source)

1.2.7.2.2 Derivation of permit limits using effluent monitoring data

The various assumptions used in the permit limit development process should be consistent with the assumptions and principles inherent in the effluent characterization and exposure assessment steps already described.

The EPA states that the permit limit derivation procedure used should be fully enforceable and should adequately account for effluent variability, consider available receiving water dilution, protect against acute and chronic toxicity, account for compliance monitoring sampling frequency, and protect water quality standards (US EPA, 1991a). The EPA recommends the use of a statistical permit limit derivation procedure, applying steady state or dynamic wasteload allocation modelling (US EPA, 1991a).

The toxicity tests (test species), dilutions for testing, the specific end-points, the statistical procedures for analyzing data, quality assurance, and any other important factors should be clearly stated as permit conditions. In some cases methodologies allow significant choice in how the method is actually conducted and a simple reference to the methodology may result in the test being done differently than intended.

Either acute or chronic tests might apply to a given situation depending on the test detection levels or sensitivity. For example, a limit of $5TU_c$ (NOEC: 20%) would require chronic toxicity testing where the ACR is 20 for that effluent. An acute test would not be enough to measure toxicity because the $5TU_c$ would be equivalent to $0{,}25TU_a$ (LC_{50}: 400%). If the ACR was 2, then an acute test could be used because the $5TU_c$ will be $2{,}5TU_a$. An acute toxicity test when using LC_{50} as endpoint, has an upper sensitivity level of 100% effluent or $1{,}0TU_a$. If less than 50% of the test organisms die at the 100% effluent an LC_{50} cannot be determined from the data set, and the true LC_{50} cannot be measured. In this case an acute toxicity test could still be used for compliance monitoring but the endpoint would be changed to a greater sensitivity. The endpoint could be specified in terms of no statistically significant difference in acute toxicity between 100% effluent sample and the control. This is the most sensitive application for an acute test and could be used for compliance with a limit that, because of a lack of available dilution applies the acute criterium to the end of the pipe. Such tests would not accurately quantify any level of chronic toxicity present (*e.g.* applying an ACR). For chronic testing an effluent with an NOEC of greater than 100% presents a similar test sensitivity problem. Such an effluent contains less than $1{,}0TU_c$, and would meet the chronic criterium at the edge of the mixing zone, if dilution was available, and the chronic criterium at the end of the pipe if no dilution was possible.

There is no fixed guidance on the establishment of monitoring frequencies. These will depend on factors such as:

- Type of treatment process, including retention time;
- Environmental significance and nature of the pollutant/s;
- Cost of monitoring relative to the discharger's capabilities and benefit obtained;
- Compliance history;
- Number of monthly samples used in developing the permit limit; and
- Effluent variability.

Where monitoring indicates unacceptable effluent toxicity, one principle mechanism for bringing the discharger to compliance is a toxicity reduction evaluation (TRE). The purpose of a TRE is to investigate the causes of toxicity and to identify corrective actions for difficult effluent toxicity problems. The requirement to conduct a TRE may be written into the special conditions part of a permit (US EPA, 1991a).

1.2.7.2.2.1 *Ireland's approach in setting limits*

Ireland's guidelines recognize the importance of mixing conditions by stipulating that at least 20 dilutions be available in the immediate vicinity of a discharge for each toxic unit discharged. Such guidelines are incorporated on a case-by-case basis in individual permits issued for dischargers. Effluents are divided into categories using fish 96-h LC_{50}'s and the derived TU's:

Priority group	Category	96-h LC_{50}	TU
A	Chemical/pharmaceutical industry	4%	25
B	Metal plating	10%	10
C	Textiles, tanning, paper and glass making	20%	5,0
D	Agricultural and food, untreated municipal sewage	70%	1,4
E	Treated sewage (secondary)	100%	1,0

1.2.7.2.3 Deriving limits with and without effluent monitoring data - advantages and disadvantages

There are advantages and disadvantages to the two ways of setting limits (US EPA, 1985b). For setting limits directly the advantages and disadvantages are:

Advantages -

* Cost of data generation is eliminated;
* Permit limit is quickly derived and permit issuance is not delayed.

Disadvantages -

* Because no real data exists there is an uncertainty as to whether or not a real toxicity problem exists;
* Exposure must be simplified so that a steady state exposure condition is used. This can be overprotective or underprotective, depending on the design flow used;
* Permittee may object to the limit since no data are available to show toxicity is an actual problem.

Where chemical characterization is used to derive limits the advantages and disadvantages are as follows:

Advantages -

* Will indicate whether or not a real toxicity problem exists;
* Can generate the data needed to assess exposure in a more sophisticated manner. A sensitive test species is identified and variability is assessed;
* Because requirement is established on a sound basis the permittee is less likely to object to limits set.

Disadvantages -

* High cost;
* Testing and analysis could delay permit issuance.

1.2.7.3 Compliance monitoring

Once a permit containing limitations and conditions to control effluent has been issued, the permittee is responsible for attaining, monitoring, and maintaining compliance with the requirements of the permit (US EPA, 1985b; 1991a). All records must be maintained by the facility and be available for a certain inspection period. The EPA has three ways of determining compliance with a permit, namely self-monitoring reports, monthly discharge monitoring and quality assurance reports and inspections. Poor quality assurance is a violation if the permit explicitly specifies adequate quality assurance.

2. MATERIALS AND METHODS

2.1 Bioassays

The following bioassays were evaluated for WET testing: fish and water flea lethality tests; a protozoan oxygen uptake inhibition test; algal and bacterial growth inhibition tests; a urease (detection of heavy metals) and acetylcholinesterase (detection of organophosphate and carbamate pesticides) enzyme inhibition test; the Ames *Salmonella* mutagenicity assay, and a toad embryo teratogenicity test. The mutagenicity and teratogenicity tests detect chronic toxicity (human health protection). All the other tests are aimed at acute toxicity detection. Fish, water flea, and mutagenicity tests were carried out according to standard procedures (US EPA, 1985a; Maron and Ames, 1983) and microbial, enzyme, and teratogenicity tests according to procedures developed by the Division of Water Technology. The bioassays are described in the final report of the project **Development of guidelines for toxicity bioassaying of drinking and environmental waters in South Africa** (Slabbert *et al.*, 1996).

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

Tests were carried out in a constant temperature room at 22°C using 1 - 2 weeks old fish. Ten fish were used per test (5 per container), unless otherwise indicated. Fish were exposed for a period of 96 h, and effects were observed at daily intervals. Lethality >10% was taken as an indication of toxicity (provided that the control lethality ≤10%).

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 beakers containing moderately hard water (Table 1) and food suspension (trout chow, alfalfa and yeast). Tests were carried out for 48 h at 20°C, using 20 organisms per test (5 water flea in 4 replicate beakers). Effects were recorded after 24 and 48 h of exposure. Results are expressed in terms of % lethality (no movement of body or appendages on gentle prodding). Lethality ≥10% indicates toxicity (provided that the control lethality <10%).

2.1.3 Protozoan (*Tetrahymena pyriformis*) oxygen uptake assay

Tests were carried out with a standard biological oxygen monitoring system (Yellow Springs Instrument Co, Yellow Springs, OH) consisting of an electronic unit, a water bath assembly fitted with test chambers, and an oxygen probe. The electronic unit was connected to a recorder for the production of graphs. The monitoring system, cell suspensions and test samples were maintained at 27°C using a constant temperature water circulator. For each test, the dissolved oxygen of a protozoan cell suspension (3 mℓ) was 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. Only one test was carried out per sample. Controls were conducted 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 and Morgan, 1982; Slabbert, 1988). Effects are expressed as percentage inhibition (or stimulation), calculated in relation to control tests. Inhibition ≥5% indicates toxicity.

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	pH	7,4 - 7,8
	Hardness[3]	80 - 100
	Alkalinity	60 - 70

[1] US EPA (1985a)
[2] Prepared with deionized water
[3] As mg/ℓ $CaCO_3$

2.1.4 Algal (*Selenastrum capricornutum*) growth inhibition test

Toxicity tests were carried out in sterile 24-well tissue culture plates (Slabbert and Hilner, 1990). Test samples were inoculated with 200 000 cells/mℓ, yielding an initial optical density (OD) of between 0,005 and 0,012. 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 µℓ volumes for inoculation of 1,8 mℓ sample in test wells (well volume: 3,5 mℓ). Two different media, namely algal assay medium (AAM 30%) (US EPA, 1971; 1978) and 10% modified BG-11 (Rippka *et al.*, 1979) were used. 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 to 72 h at 25°C, using continuous illumination. At the end of the incubation period cells were suspended, and transferred in 280 µl quantities to 96-well plates for OD reading. Individual samples were tested in five-fold, and serial dilutions in triplicate. Results are expressed as percentage inhibition (or stimulation), calculated in relation to control results. Initially, inhibition ≥20% was used as an indication of toxicity (the variation between control results were considerable because of problems with low growth and because only three replicates were used). In later experiments 10% growth inhibition was taken as the detection limit (improved experimental conditions and five replicates).

2.1.5 Bacterial (*Pseudomonas putida*) growth inhibition assay

Tests were carried out in minimal medium in 50 mℓ medical flats (Slabbert, 1986; 1988). A culture of *P. putida*, grown overnight at 28°C, was diluted with fresh minimal medium to an OD of between 0,8 and 0,95, 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. Cultures were incubated at 28 °C for 6 h after which growth was measured in terms of optical density. Effects are expressed as percentage inhibition (or stimulation), determined in relation to control results. Inhibition ≥10% indicates toxicity.

2.1.6 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). Tests were carried out in 96-well microplates. Samples were added in 160 $\mu\ell$ volumes to 40 $\mu\ell$ enzyme. An exposure period of 30 min at 25°C was allowed. Urea (substrate) and phenolphthalein (pH indicator) were then added consecutively, (40 $\mu\ell$ each) and 15 min were allowed for enzyme-substrate interaction. The enzyme interacts with the substrate to form ammonia, which results in a dark pink colour development. In the presence of heavy metals the reaction mixture remains colourless or shows gradients of pink. Enzyme activity was measured by means of OD (450 nm) and also examined visually. Each test and control was carried out in triplicate. Effects are expressed as percentage inhibition (stimulation), calculated in relation to control results. Inhibition ≥10% is an indication of toxicity.

2.1.7 Acetylcholinesterase enzyme inhibition test

The bioassay is selectively sensitive to organophosphate and carbamate pesticides. 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/mℓ 0,05 M potassium phosphate buffer) was added consecutively to a cuvette, mixed and kept at 37°C (Venter, 1990; 1991). After an incubation period of 15 min, 100 $\mu\ell$ of Ellman's reagent were added to the reaction mixture, followed by 100 $\mu\ell$ of substrate (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. Inhibition ≥10% indicates toxic activity.

2.1.8 Ames *Salmonella* mutagenicity assay

Salmonella typhimurium tester strains, TA98 and TA100, were used for mutagenicity testing. 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 tested directly and after concentration by means of flash evaporation (2 and 4 x concentrated) and/or XAD resin extraction (20 ℓ sample concentrated; extracts dissolved in 10 mℓ acetone). A modified plate incorporation assay was used to test the unconcentrated and flash evaporated samples (Kfir *et al.*, 1982). In this method nutrient agar plates were prepared with the test sample. Media were either autoclaved or filter sterilized (0,22 μm filter) (Slabbert *et al.*, 1996). XAD extracts were incorporated into the top-agar (100 $\mu\ell$ per 2 mℓ agar) (Maron and Ames, 1983). Sterile deionized water or acetone was used for control testing. Each test was carried out in triplicate. Results are expressed as mutation ratios which are calculated:

Number of colonies on test plate/number of colonies on control plate

Mutation ratios ≥2 indicate mutagenic activity.

2.1.9 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 toads received a HGC booster injection (females: 300 $\mu\ell$; males: 100 $\mu\ell$) and transferred in pairs to spawning tanks. After fertilization (18 h later), eggs were transferred to test containers. One hundred eggs were used per test (50 eggs in duplicate containers). Tests were carried out at 22°C. After 2 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. Results are expressed as % deformation and lethality. Deformation ≥20% and lethality ≥10% are considered as positive results.

2.2 Control and dilution water

The standard control water used in the different bioassays were:

- Dechlorinated tap water (aeration) - fish and toad embryo tests;
- Sterile deionized water - microbial and enzyme tests; and
- Moderately hard water (Table 1) - water flea test.

Since the beginning of 1993 the dechlorinated tap water used in fish tests was replaced by moderately hard water. Standard control water was used for dilution to establish the inherent toxicity of effluents. Moderately hard water (sterile) was later included in algal and bacterial growth inhibition tests for control and dilution purposes. The relative toxicity of the effluents (to simulate effluent/receiving water interactions) was established by using the receiving river/stream water for dilution and control testing. A standard control test was always included in all evaluations.

2.3 Reference chemicals

From time to time tests were carried out on reference chemicals (positive controls) to assess the precision of methods. Cadmium ($CdCl_2$) and pentachlorophenol (PCP) were used in fish, water flea, protozoan, algal and bacterial toxicity tests. Cadmium was included in every urease enzyme test. Methyl viologen was used in the toad embryo test, and carbofuran in the acetylcholinesterase enzyme test. Solutions were prepared with standard control waters. The reference chemicals 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).

2.4 Effluent and river/stream water samples

Three types of effluents have been identified for testing in consultation with the DWA&F's IWQS, namely secondary treated effluent from a sewage works receiving both domestic and industrial effluent, paper mill effluent, and effluent from a metal refinery. The sewage effluent was collected from Kempton Park Sewage Works, the paper mill effluent from Sappi Enstra in Springs, and the metal plating effluent from a plant in the Pretoria area. The Kempton Park Sewage Works receives effluent from some 70 major and approximately 400 small industrial dischargers. The treated, chlorinated effluent is discharged into the Hennops River where very little, if any dilution, takes place for the largest part of the year. River water some 50 metres upstream of the point of the effluent discharge was collected for dilution purposes. A single sample was collected from a point some 3 kilometres downstream, to evaluate ambient

toxicity. The paper mill effluent (combined factory effluent) was sampled at two points, namely before primary clarification and after secondary clarification. After primary clarification effluent is aerated in an aeration lagoon for a period of 7 days. The secondary clarifier effluent is discharged into the Cowles Dam which receives water from the Small Blesbok Spruit. The dam has a retention time of 30 to 40 days. The Small Blesbok continues at the overflow of the dam and joins the Blesbok Spruit after passing through reed beds. The Small Blesbok Spruit water was used for dilution of the effluents and was collected a few kilometres upstream of the dam. A single sample was taken from the overflow of the Cowles Dam to examine ambient toxicity. The metal plating effluent is discharged directly (intermittently) into the sewer after lime treatment. Water from a nearby stream was used for dilution purposes.

The pH, dissolved oxygen and temperature of the effluents and river/stream water were measured on site. Extensive chemical analyses were carried out when required. Immediately after receipt, the samples used for the enzyme and microbial tests (except Ames test) were sterilized by means of filtration through a 0,22 μm membrane filter (samples in this form could be stored for an extended period of time). Samples were used to prepare the nutrient medium for the modified plate incorporation Ames assay. The medium was then sterilized by autoclaving/filtration. The acetone extracts were used directly. All samples were stored at 4°C before testing.

3. RESULTS AND DISCUSSION

The positive controls of both the acute and chronic tests showed an appropriate response (Slabbert *et al.*, 1996), indicating that tests were successfully applied and that test precision was good. The reproducibility of tests was good and coefficients of variation were mostly within the limits given for specific tests (Slabbert *et al.*, 1996). In general, the control results obtained during the study were within the ranges given in the final report of the Water Research Commission project **Development of guidelines for toxicity bioassaying of drinking and environmental waters in South Africa** (Slabbert *et al.*, 1996). Although the algal test performed much better than in previous studies by Slabbert *et al.* (1996) as a result of optimization, there were still occasions when the growth was not satisfactory (OD: <0,05). This was mainly because of problems experienced with uniform illumination. Problems occasionally arose in obtaining a sufficient number of fish for tests. In some instances control fish also showed lethality, rendering test results invalid.

3.1 Screening of toxicity tests for WET testing

All the bioassays were applied to the selected effluents during this phase of the study. The sensitivities of the bioassays were compared by conducting range finding tests (testing 10-fold dilutions). Range finding tests provide information on the degree of toxicity and the effective concentration range of an effluent. Each effluent was tested on three consecutive dates between August and October 1992. The receiving water was also tested. Standard control water was used for dilution and control testing. The results of the evaluation are presented in Tables 2 to 4. Only those results obtained at the end of the exposure period are presented for fish and water flea tests. When possible, ten fish were applied in tests. However, only five fish were used when testing the sewage effluent and paper mill effluent samples collected on 19-10-1992, and the metal plating effluent samples of 15-09-1992 and 12-10-1992. A 20% lethality occurred in the control test when the sewage effluent and paper mill effluent samples of 19-10-1992 were tested. The positive results obtained in these tests (Tables 2 and 3) are, therefore, not valid. On the first sampling dates, each of the effluents tested with the Ames test was concentrated by means of flash evaporation and XAD resin extraction. The nutrient medium for plates was prepared using autoclaving and filtration. The results given in Tables 2 to 4 for these dates present the findings of all three tests (using both tester strains, with and without S9). The effluent sampled on the other sampling occasions was concentrated by XAD resin extraction only. The metal plating effluent sample of 12-10-1992 was tested directly by incorporation into the top-agar.

Table 2 shows that the sewage effluent was toxic to some of the test systems at the 100% concentration. The sample of 04-08-1992 inhibited algal and bacterial growth, and showed slight lethality and 100% deformation in the toad embryo test. The effluent collected on 21-09-1992 was lethal to fish, water flea and toad embryos, caused bacterial growth inhibition, and was teratogenic. This sample also showed some degree of toxicity with the bacterial growth inhibition test after dilution (10%). The sample of 19-10-1992 only affected toad embryos, causing 26% lethality and 100% deformation. The river water was not toxic except on 19-10-1992 when slight teratogenicity was detected. None of the samples showed mutagenicity. The effluent, and in some instances the river water, caused slight precipitation with the algal and bacterial growth tests (OD ≤0,02). Some of the effluent and river water samples stimulated protozoan oxygen uptake, algal growth and enzyme activity. The negative results

TABLE 2: Effect of secondary treated sewage effluent and receiving water on biological test systems

Sample	Concentration (%)	Fish test	Water flea lethality test	Protozoan oxygen uptake test	Algal growth test		Bacterial growth test	Urease enzyme test			Acetylcholinesterase enzyme test	Toad embryo teratogenicity test		Mutagenicity test[3]
					AAM medium	BG-11 medium		0,5 mg/ml	1,0 mg/ml	2,0 mg/ml				
		% Lethality after 96 h	% Lethality after 48 h	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Lethality	% Deformation	Mutation ratio
Effluent 04-08-1992	100	0	0	+6	51*	49*	35	+22	+9	+18	+8	12	100	<2,0[5]
	10	nt	0	nt	+17	+74	nt	nt	nt	nt	nt	nt	nt	nt
	1	nt	0	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
Effluent 21-09-1992	100	50	50	1	+39*	+120*	43*	+7	+15	+10	+17	100	100	<2,0[4]
	10	10	0	nt	9	+69	29	4	5	+2	nt	2	8	nt
	1	nt	0	nt	16	10	+1	0	+1	2	nt	0	16	nt
River water 21-09-1992	100	0	0	+2	+5*	+104*	+2	+3	+24	+21	+11	0	5	<2,0[4]
Effluent 19-10-1992	100	40[1,2]	0	+6	+52*	+108*	+7*	+23	+13	+20	+14	26	100	<2,0[4]
	10	0[1,2]	0	nt	+43	+56	+1	+4	+2	+5	nt	nt	nt	nt
	1	nt	0	nt	nt	nt	7	nt	nt	nt	nt	nt	nt	nt
River water 19-10-1992	100	20[1,2]	0	+5	+2*	4*	3*	+11	4	0	+18	0	21	nt

* Precipitation - background reading subtracted
\+ Stimulation
Bold Positive results
[1] Control lethality: 20%
[2] Only 5 fish used for testing
[3] Results of all four tester strains
[4] XAD resin extract tested
[5] Samples concentrated by means of flash evaporation and XAD extraction
nt Not tested

Detection limits: oxygen uptake test - 5%; water flea and toad embryo lethality, bacterial growth inhibition and urease and acetylcholinesterase enzyme inhibition tests - 10%; fish lethality test - >10%; algal growth inhibition and toad embryo deformation tests - 20%; mutagenicity test - MR 2,0

TABLE 3: Effect of paper mill effluent and receiving water on biological test systems

Sample	Concentration (%)	Fish test	Water flea test	Protozoan oxygen uptake test	Algal growth test		Bacterial growth test	Urease enzyme test			Acetylcholinesterase enzyme test	Toad embryo teratogenicity test		Mutagenicity test[1]
					AAM medium	BG-11 medium		0,5 mg/mℓ	1,0 mg/mℓ	2,0 mg/mℓ				
		% Lethality after 96 h	% Lethality after 48 h	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Lethality	% Deformation	Mutation ratio
Effluent 1 18-08-1992	100	0	100	0	60*	*93	*	+6	+23	+59	+3	38	18	<2,0[2]
	10	0	100	nt	7*	11*	60*	nt	nt	nt	nt	0	11	nt
	1	nt	0	nt	+9	1	17	nt	nt	nt	nt	nt	nt	nt
Effluent 2 18-08-1992	100	0	0	+3	24*	2*	+16*	+28	+30	+164	+22	0	19	<2,0[2]
	10	nt	nt	nt	+17*	10*	nt	nt	nt	nt	nt	0	4	nt
Stream water 18-08-1992	100	0	0	+2	56	51	71*	35	9	+19	+17	0	54	nt
Effluent 1 07-10-1992	100	80	100	0	76*	+37*	23	6	5	+26	+14	100	nt	<2,0[2]
	10	0	0	nt	+58*	+120*	3	8	9	+12	nt	17	19	nt
Effluent 07-10-1992	100	0	0	1	+128*	+176*	27*	+20	+44	+39	+17	27	15	<2,0[2]
Stream water 07-10-1992	100	0	0	+3	78*	52*	69*	5	+34	+29	0	19	25	nt

* Precipitation - background reading subtracted
\+ Stimulation
Bold Positive results
[1] Results of all four tester strains
[2] XAD resin extract tested
nt Not tested
[3] Samples concentrated by means of flash evaporation and XAD resin extraction

Detection limits: oxygen uptake test - 5%; water flea and toad embryo lethality, bacterial growth inhibition and urease and acetylcholinesterase enzyme inhibition tests - 10%; fish lethality test - >10%; algal growth inhibition and toad embryo deformation tests - 20%; mutagenicity test - MR 2,0

TABLE 3: Effect of paper mill effluent and receiving water on biological test systems (continue)

Sample	Concentration (%)	Fish test	Water flea test	Protozoan oxygen uptake test	Algal growth test		Bacterial growth test	Urease enzyme test			Acetylcholinesterase enzyme test	Toad embryo teratogenicity test		Mutagenicity test[3]
					AAM medium	BG-11 medium		0,5 mg/ml	1,0 mg/ml	2,0 mg/ml				
		% Lethality after 96 h	% Lethality after 48 h	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Lethality	% Deformation	Mutation ratio
Effluent 1 19-10-1992	100	20[1,2]	20	+10	+16*	46*	+6*	+46	+135	+159	+15	100	nt	<2*
	10	20[1,2]	0	nt	1*	2*	8	0	+5	5	nt	nt	nt	nt
Effluent 2 19-10-1992	100	20[1,2]	0	+1	+4*	18*	67*	+9	+22	+74	+46	14	14	98-:<2,0* 98+: 2,0 100-: 3,8 100+: 3,6
Stream water 19-10-1992	100	0[1,2]	0	9	+6*	15*	47*	+4	+33	+89	+23	36	26	nt

* Precipitation - background reading subtracted
\+ Stimulation
Bold Positive results
[1] Control lethality: 20%
[2] Only 5 fish used for testing
[3] Results of all four tester strains
[4] XAD resin extract tested
nt Not tested

Detection limits: oxygen uptake test - 5%; water flea and toad embryo lethality, bacterial growth inhibition and urease and acetylcholinesterase enzyme inhibition tests - 10%; fish lethality test - >10%; algal growth inhibition and toad embryo deformation tests - 20%; mutagenicity test - MR 2,0

TABLE 4: Effect of metal plating effluent on biological test systems

Sample	Concentration (%)	Fish test	Water flea test	Protozoan oxygen uptake test	Algal growth test		Bacterial growth test	Urease enzyme test			Toad embryo teratogenicity test		Mutagenicity test[2]
					AAM medium	BG-11 medium		0,5 mg/ml	1,0 mg/ml	2,0 mg/ml			
		% Lethality after 96 h	% Lethality after 48 h	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Lethality	% Deformation	Mutation ratio
15-09-1992	100	**100**[1]	**100**	**35**	**83***	**91***	**+27**	**17**	**4**	**+8**	**100**	nt	<2,0[4]
	10	**40**[1]	**100**	**21**	**31**	**36**	**0**	**7**	**0**	**+27**	**0**	**13**	nt
	1,0	**20**[1]	**0**	**2**	**0**	**11**	**+6**	**6**	**+14**	**+15**	**0**	**16**	nt
	0,1	**0**[1]	**0**	nt	+5	+23	+6	1	+12	+15	nt	nt	nt
21-09-1992	100	**100**	**100**	**44**	**95***	**98***	**82***	**63**	**64**	**38**	**100**	nt	nt
	10	**100**	**100**	**30**	**100**	**96**	**31**	**80**	**78**	**26**	**18**	**30**	nt
	1,0	**60**	**100**	**2**	**86**	**74**	**5**	**10**	**8**	**6**	**10**	**32**	nt
	0,1	nt	**0**	nt	**38**	**58**	**3**	**6**	**9**	**8**	**5**	**15**	nt
	0,01	nt	nt	nt	**24**	**47**	nt	nt	nt	nt	nt	nt	nt
	0,001	nt	nt	nt	4	6	nt	nt	nt	nt	nt	nt	nt
12-10-1992	100	**100**[1]	**100**	**65**	**76***	**94***	**100***	**34**	**47**	**6**	**100**	nt	<2,0[3]
	10	**100**[1]	**90**	**28**	**76**	**100**	**54**	**63**	**56**	**8**	**40**	**100**	nt
	1,0	**0**[1]	**50**	**3**	**40**	**56**	**26**	**18**	**9**	+5	**4**	**33**	nt
	0,1	**0**[1]	**0**	nt	**36**	**37**	**11**	nt	nt	nt	**9**	**19**	nt
	0,01	nt	**0**	nt	1	+34	nt	nt	nt	nt	nt	nt	nt

* Precipitation - background reading subtracted
\+ Stimulation
Bold Positive results
[1] Only 5 fish used for testing
[2] Results of all four tester strains
[4] Tested directly
[4] Samples concentrated by means of flash evaporation and XAD resin extraction
nt Not tested
Detection limits: oxygen uptake test - 5%; water flea and toad embryo lethality, bacterial growth inhibition and urease enzyme inhibition tests - 10%; fish lethality test - >10%; algal growth inhibition and toad embryo deformation tests - 20%; mutagenicity test - MR 2,0

obtained with the urease and acetylcholinesterase enzyme tests indicated the absence of heavy metals and organophosphate/carbamate pesticides, respectively.

The paper mill effluent before primary clarification (effluent 1) was toxic to some of the test systems on all three sampling occasions (Table 3). Toxicity was generally detected at the 100% concentration, but on occasion the 10 and 1% concentrations were also toxic. On 18-08-1992 the effluent was lethal to water flea and toad embryos, and inhibited algal and bacterial growth. The 10% concentration still caused 100% lethality to water flea, while the 1% concentration slightly inhibited bacterial growth. On 07-10-1992 the 100% concentration of effluent 1 caused lethality to fish, water flea and toad embryos, and inhibited algal and bacterial growth. Slight lethality also occurred at the 10% concentration using the toad embryo test. On 19-10-1992 effluent 1 was toxic to water flea, algae and toad embryos (lethal), but only at the 100% concentration. This sample did not cause teratogenicity or mutagenicity. Deformation was not detectable at the 100% concentrations of the last two samples because embryos/eggs disintegrated. The effluent after secondary clarification (effluent 2) affected very few tests and showed low toxicity. The 100% concentration of 18-08-1992 slightly inhibited algal growth. The samples of 07-10-1992 and 19-10-1992 inhibited bacterial growth and were lethal to toad embryos. Effluent 2 did not exhibit teratogenic activity. However, mutagenicity was detected in the sample of 19-10-1992 (mutation ratio: TA98+S9 - 2,0; TA100-S9 - 3,8; TA100+S9 - 3,8). The effects are similar to those occasionally detected in environmental samples. The negative results obtained with the urease and acetylcholinesterase enzyme assays indicated the absence of heavy metals and organophosphate/carbamate pesticides, respectively. On all three sampling dates the Small Blesbok Spruit water showed toxicity. The first sample inhibited algal and bacterial growth, inhibited the urease enzyme (indicating the presence of heavy metals) and caused teratogenicity. The second sample was toxic to algae and bacteria, and affected toad embryos causing 19% lethality and 25% deformation. The final sample collected on 19-10-1992 inhibited protozoan oxygen uptake and bacterial growth, killed toad embryos and resulted in embryo deformation. Usually, the dilution water should be free of toxicants, which implies that the stream water will not be suitable for dilution purposes. However, this water can be used if it is the objective to establish the contribution of the effluent to the receiving water. Effluent and receiving water caused precipitation with the algal and bacterial growth inhibition assays. The background OD readings due to precipitation ranged from low (algae: 0,01-0,03; bacteria: 0,030-0,050) to very high (algae: 0,08-0,12; bacteria: 0,5-0,7), and interfered with growth measurement, particularly in case of effluent 1 at high concentrations. A few samples stimulated protozoan oxygen uptake, and algal and bacterial growth, indicating the presence of nutrients. In general, enzyme activity was increased. The larger OD readings in case of the urease test might be the result of chemical interaction causing some degree of precipitation.

Metal plating effluent was highly toxic (Table 4). All the test systems, except the mutagenicity test, showed positive results. In one instance toxicity was detected at a concentration as low as 0,01%. The effluent of 15-09-1992 was the least toxic. Bacterial growth was not inhibited by this sample. The 100% concentration only caused a slight effect on urease enzyme activity, but killed 100% of the toad embryos. Fish, water flea, protozoan and algal tests were the most sensitive detecting toxicity at 1,0 and 10% concentrations. The effluent of 21-09-1992 was very toxic. The algal test detected toxicity at the 0,01% concentration, fish, water flea, urease enzyme and toad embryo tests at the 1,0% concentration, and the protozoan oxygen uptake and bacterial growth inhibition tests at the 10% concentration. The

effluent showed teratogenicity at the 1,0 and 10% concentrations. With the effluent of 12-10-1992 the algal and bacterial growth inhibition tests showed the highest sensitivity, detecting toxicity at 0,1% of the effluent. The water flea, urease enzyme and toad embryo test showed positive responses at the 1,0% concentration and the fish and protozoan test at the 10% concentration. The effluent caused some degree of precipitation with the algal and bacterial growth tests, but only at the 100% concentration (algae: 0,02-0,05; bacteria: 0,02-0,1). Very few tests showed increased activity. No observations about deformation could be made at the 100% concentrations because the embryos/eggs disintegrated. In two instances the inhibiting effects on the urease enzyme test appeared to be lower at the 100% effluent concentration than at the 10% concentration. This could be due to precipitation which increased the OD reading and falsely indicated lower effects. The acetylcholinesterase enzyme was not used in the evaluation because pesticides were not expected to be present in the effluent. The Ames *Salmonella* mutagenicity test is known to be insensitive to metal containing mutagens. The negative results obtained with the Ames test, therefore, do not rule out the presence of such mutagens.

Table 5 summarizes the effective concentration ranges of the effluents as established by the different bioassays. The results indicate that in general paper mill effluent 2 was the least toxic, followed in order of magnitude by secondary treated effluent, paper mill effluent 1 and the metal plating effluent. The fish, water flea, algal, bacterial and toad embryo tests were the most sensitive for the detection of adverse chemical activity in the secondary treated sewage effluent and paper mill effluent 1. Algal and bacterial growth inhibition tests, the toad embryo test, and the mutagenicity test detected adverse chemical activity in paper mill effluent 2. All the tests, except the mutagenicity test, were sufficiently sensitive to screen metal plating effluent for adverse chemical activity. In general, the algal test appeared to be the most sensitive to this effluent.

Table 6 presents the results of the chemical analyses on the effluent and water samples. Most of the potentially toxic chemicals in the secondary treated sewage effluent were low (below chemical detection limits), indicating that effects were probably due to combined toxicity rather than to individual chemicals. Ammonia levels were relatively high on 21-09-1992 and 19-10-1992, which could have contributed to toxicity to fish, water flea and toad embryos. Low pH and low oxygen content in paper mill effluent 1 and 2 could have caused adverse activity in some of the bioassays. In general, these effluents showed a high organic content, but how this contributed to toxicity is not known. The effluents contained relatively high levels of aluminium (*e.g.* effluent 2: 18-08-1992 - 1015 $\mu g/m\ell$), iron and manganese, which in combination could have resulted in toxicity. These samples were very hard, which probably caused the precipitation in the algal and bacterial growth tests. Table 6 shows that the metal plating effluent contained very high levels of cadmium, chromium, copper, iron (12-10-1992), nickel, and zinc, which individually or in combination could have caused the large toxic effects on test systems. The sample of 12-10-1992 also had a low pH and high concentration of cyanide. The ammonia level in the sample of 21-09-1992 was very high. Most of the potentially toxic chemicals in the Hennops River water were low, confirming the absence of toxicity. On 19-10-1992 the oxygen content of the Small Blesbok Spruit was very low, possibly causing the adverse effects in some of the tests. On this occasion dead fish were found in the stream. The water also showed high levels of manganese and relatively high levels of cyanide and phenol which could have been the cause of toxicity. As with the paper mill effluents, the hardness of the water was high, and possibly the result of the precipitate formation in algal and bacterial growth tests.

TABLE 5 : Effective concentration range (%) of effluents as established by means of different biological toxicity tests

Sample	Date	Fish lethality test	Water flea lethality test	Protozoan oxygen uptake inhibition test	Algal growth inhibition tests	Bacterial growth inhibition test	Urease enzyme inhibition test	Acetyl-cholin-esterase inhibition test	Toad embryo teratogenicity test		Mutagenicity test
									Lethality	Defor-mation	
Secondary treated sewage effluent	04-08-1992	>100	>100	>100	10 - >100	10 - >100	>100	>100	100 - >100	10 - 100	neg
	21-09-1992	10 - >100	10 - >100	>100	>100	1,0 - >100	>100	>100	10 - 100	10 - 100	neg
	19-10-1992	>100	>100	>100	>100	>100	>100	>100	10 - >100	10 - 100	neg
Paper mill effluent before primary clarification	18-08-1992	>100	1,0 - 10	>100	10 - 100	0,1 - 100	>100	>100	10 - >100	neg	neg
	07-10-1992	10 - 100	10 - 100	>100	10 - 100	10 - >100	>100	>100	10 - 100	neg	neg
	19-10-1992	>100	10 - >100	>100	10 - >100	10 - >100	>100	>100	10 - 100	neg	neg
Paper mill effluent after secondary clarification	18-08-1992	>100	>100	>100	100 - >100	>100	>100	>100	>100	neg	neg
	07-10-1992	>100	>100	>100	>100	10 - >100	>100	>100	10 - >100	neg	neg
	19-10-1992	>100	>100	>100	>100	10 - >100	>100	>100	>100	neg	pos
Metal plating effluent	15-09-1992	1,0 - 100	0,1 - 10	1,0 - >100	1,0 - 100	>100	100 - >100	nt	10 - 100	neg	neg
	21-09-1992	0,1 - 10	0,1 - 1,0	1,0 - >100	0,001 - 10	1,0 - 100	1,0 - 10	nt	1,0 - 100	0,1 - 100	neg
	12-10-1992	1,0 - 10	0,1 - 10	1,0 - >100	0,01 - 10	0,1 - 100	1,0 - 10	nt	1,0 - 100	0,1 - 10	neg

Neg Negative
Pos Positive
nt Not tested

TABLE 6: Chemical quality of effluents and receiving water

Determinand (mg/l except for *)	Secondary treated sewage effluent			Hennops River water			Paper Mill effluent - before primary clarification			Paper Mill effluent - after secondary clarification			Small Blesbok Spruit water			Metal plating effluent		
	04-08-1992	21-09-1992	19-10-1992	04-08-1992	21-09-1992	19-10-1992	18-08-1992	07-10-1992	19-10-1992	18-08-1992	07-10-1992	19-10-1992	18-08-1992	07-10-1992	19-10-1992	15-09-1992	21-09-1992	12-10-1992
pH*	7,7	7,56	7,64	8,7	9,43	8,24	8,1	4,7	6,31	8,1	7,8	7,49	7,4	7,5	7,55	9,5	6,5	3,01
Oxygen	7,4	7,3	7,2	11,3	16,1	9,8	7,8	1,7	4,8	4,2	2,9	1,6	8,1	8,1	0,8	8,6	6,8	6,8
Temperature (°C)*	16,9	21,8	24	8,6	20,5	26,5	26	43,6	36,8	21	25,2	25,5	14	17,5	25,4	21,3	23,8	20,9
Electrical con. (mS/m)*	nd	83,3	79,6	nd	80,1	96,0	nd	199,0	221,0	nd	187,0	198,0	nd	115,6	220	nd	175,0	155,4
Hardness ($CaCO_3$)	nd	108,5	94,9	nd	172,6	187,3	298,6	317,0	383,6	354,5	289,9	263,2	244,1	416,3	335,9	nd	161,8	237,7
Calcium (Ca)	nd	27	23	nd	42	44	90	76	93	92	76	67	66	127	82	nd	29	57
Magnesium (Mg)	nd	10	9	nd	17	19	18	31	37	30	24	23	20	24	32	nd	22	23
Ammonia-N	nd	17,4	13,9	nd	2,7	8,5	nd	0,6	1,5	nd	3,5	1,2	nd	2,3	6,0	nd	66,9	18,9
Nitrate-nitrite-N	nd	6,4	8,9	nd	1,0	3,0	nd	1,8	4,1	nd	<0,2	<0,2	nd	0,9	<0,2	nd	6,6	5,6
Total phosphate	nd	3,2	4,1	nd	0,3	3,4	nd	1,4	1,3	nd	1,6	1,2	nd	0,3	1,5	nd	3,3	1,5
Ortho-phosphate	nd	2,4	4,0	nd	0,2	3,2	nd	0,9	1,2	nd	1,2	0,8	nd	<0,2	1,0	nd	<0,2	<0,2
Chloride (Cl)	nd	nd	nd	nd	nd	nd	2	311	286	250	220	256	87	99	285	147	204	341
COD	102	70	74	nd	45	44	306	631	440	290	146	221	24	23	146	137	142	109
DOC	25	17,3	15,9	nd	17,5	13,4	9,8	164,3	119,0	6,5	33,6	25,0	0,6	6,2	22,5	nd	nd	nd

nd Not determined

TABLE 6: Chemical quality of effluents and receiving water (continue)

Determinand (µg/ℓ)	Secondary treated sewage effluent			Hennops River water			Paper Mill effluent - before primary clarification			Paper Mill effluent - after secondary clarification			Small Blesbok Spruit water			Metal plating effluent		
	04-08-1992	21-09-1992	19-10-1992	04-08-1992	21-09-1992	19-10-1992	18-08-1992	07-10-1992	19-10-1992	18-08-1992	07-10-1992	19-10-1992	18-08-1992	07-10-1992	19-10-1992	15-09-1992	21-09-1992	12-10-1992
Silver (Ag)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd-	nd	nd	nd	nd	nd	<25	<25	<25
Aluminium (Al)	<100	137	<100	nd	173	<100	179	320	279	1015	271	467	118	<100	257	153	212	245
Arsenic (As)	<5	<5	<5	nd	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	nd	nd	nd
Cadmium (Cd)	<5	6	<5	nd	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	237	1500	1380
Chromium (Cr)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3170	1210	3500
Chromium [6]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	<30	40
Copper (Cu)	30	<25	<25	nd	<25	<25	<25	<25	30	<25	<25	<25	<25	30	<25	1290	2990	1100
Iron (Fe)	80	50	30	nd	90	90	30	280	230	190	50	140	50	80	380	780	220	3400
Mercury (Hg)	<1	<1	<1	nd	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	8	nd	nd	nd
Manganese (Mn)	40	40	<25	nd	60	540	230	740	750	830	510	630	2110	620	1480	<25	180	280
Nickel (Ni)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3700	4800	2930
Lead (Pb)	<50	<50	<50	nd	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	nd	nd	nd
Selenium (Se)	<5	5	<5	nd	<5	<5	<5	<5	<5	<5	<5	<5	<5	7	<5	nd	nd	nd
Zinc (Zn)	100	<25	<25	nd	<25	<25	<25	60	<25	<25	<25	<25	120	<25	<25	950	53000	16000
Cyanide (Free) (CN)	<50	<50	<50	nd	<50	<50	<50	<50	<50	<50	<50	<50	56	178	<50	nd	nd	4784
Phenol	<10	<10	<10	nd	<10	<10	65	<10	<10	<10	<10	<10	233	<10	11	nd	nd	nd

nd Not determined

The range finding tests indicated that, in general, the fish, water flea and algal test were the most sensitive to use for effluent testing. These tests are relatively easy and simple to carry out and are not as labour intensive as some of the rapid tests. In some instances it could be useful to include the bacterial growth inhibition test in the battery of tests. However, it has been found that algal and bacterial growth inhibition tests are not compatible with some effluents (forms precipitate). Although the teratogenicity test also showed a high sensitivity in terms of acute toxicity, this test is time-consuming and not suitable for routine testing.

3.2 Extensive toxicity testing

During this phase of the study the sensitivities of selected bioassays were compared by conducting definitive tests where applicable (testing serial dilutions). Such tests estimate the concentrations at which a certain percentage of organisms exhibit a certain response (paragraph 1.2.3). Point estimates/effective concentrations, such as LC_{50}'s, EC_{50}'s and EC_{50}'s were statistically derived (linear regression) using dose-response curves (% effect versus log concentration). In the case of microbial and enzyme tests which involve a dilution of test sample (paragraph 2.1), test concentrations were multiplied with the dilution value before the calculation of point estimates to reflect the inherent dilution (*e.g.* protozoan test - 0.5; algal and bacterial growth tests - 0.9; urease test - 0.8).

Range finding tests were carried out with water flea (24 h exposure) to select the required test concentrations for all the tests. Because low toxicity was experienced with treated sewage effluent and paper mill effluents, tests were later carried out directly without pre-screening to eliminate delay in testing. In case of metal plating effluent the water flea test was replaced by the rapid urease enzyme test to select the effective concentration range.

3.2.1 Effluent variability and seasonal changes

Fish, water flea and algal (AAM and BG-11 growth medium) toxicity tests were used to evaluate effluent variability. The bacterial growth inhibition test was included in one of the tests on metal plating effluent. Each of the effluents were tested on four occasions during a five month period from February to June 1993. This allowed examination of variation in effluent quality as well as the effect of seasonal changes. Algal growth (and bacterial growth where applicable) was determined in terms of deionized water, moderately hard and river water (control and dilution). Moderately hard and river water were used for dilution and control testing in fish and water flea tests.

Ten fish were applied in all the tests, except for the treated sewage effluent sample of 05-04-1993 where 20 fish were used, and the metal plating effluent of 13-04-1993 where 5 fish were used. For most of the tests algal growth was lower in AAM medium than in BG-11 medium (Table 7). In the majority of cases the control growth in AAM medium using deionized and moderately water as controls, was <0,050 OD, and therefore not very satisfactory. When growth in deionized and moderately hard water was compared it was found that for 50% of the time growth was better in deionized water and for the other 50% it was better in moderately hard water, with AAM medium. When BG-11 medium was used a better control growth was obtained in moderately hard water for 75% of the time.

TABLE 7: Control growth recorded for algal tests during extensive testing of the different effluents

Sample	Sampling date	OD					
		AAM medium			BG-11 medium		
		Deionized water	Moderately hard water	Stream water	Deionized water	Moderately hard water	Stream water
Treated sewage effluent	01-02-1993	**0,038**[1]	0,049	0,121	**0,087**	0,125	0,104
	15-02-1993	**0,042**	0,047	0,048	**0,103**	0,144	0,125
	05-04-1993	0,064	**0,037**	0,135	**0,131**	0,157	0,164
	16-06-1993	0,034	**0,026**	0,089	0,038	**0,030**	0,080
Paper mill effluent	15-02-1993	**0,044**	0,045	<u>0,018</u>[2]	**0,113**	0,136	<u>0,047</u>
	10-05-1993	0,038	**0,033**	0,037	0,189	**0,161**	<u>0,061</u>
	12-05-1993	0,054	**0,053**	0,045	**0,044**	0,074	0,061
	28-06-1993	**0,035**	0,062	0,045	**0,037**	0,050	0,053
Metal plating effluent	09-02-1993	**0,034**	0,047	0,096	**0,090**	0,153	0,160
	13-04-1993	0,065	**0,045**	0,153	**0,135**	0,156	0,193
	06-05-1993	0,065	**0,045**	0,093	0,113	**0,085**	0,155
	10-06-1993	**0,040**	0,048	0,119	**0,053**	0,084	0,161

[1] The results in bold indicate in which of the two waters, deionized or moderately hard water, the lowest control growth was obtained

[2] The underlined results indicate toxicity in stream water

3.2.1.1 <u>Secondary treated sewage effluent</u>

The effect of secondary treated sewage effluent on the selected biological test systems is shown in Tables 8 and 9. The treated sewage effluent was not toxic to algae on any of the sampling occasions. Algal growth was mostly stimulated by the effluent. The stimulation obtained with AAM medium was high, probably because of the low control growth (Table 7). Effects in both media were usually less pronounced when using moderately hard/river water as control. The river water samples mostly stimulated algal growth (compared to deionized and moderately hard water) when using AAM medium. When BG-11 medium was used the stimulation by river water was usually small or effects were below detection limits (<20%). In general, similar effects were obtained with river and moderately hard water (control and dilution) in case of BG-11 medium. The control growth in these waters was mostly also similar (Table 7). Some of the effluent samples caused precipitation in the algal test (Tables 8 and 9). The background OD was usually small, ranging between 0,01 and 0,02 (450 nm). No adverse chemical activity was detected in any of the samples when using water flea. The sample of 15-02-1993 was toxic to fish, causing 60% lethality at the end of the exposure

TABLE 8: Effect of secondary treated sewage effluent on selected biological toxicity tests

Sample	Concentration (%)	Dilution water/ control	Algal growth test		Water flea test		Fish test			
			AAM medium	BG-11 medium	% Lethality after:		% Lethality after:			
			% Inhibition	% Inhibition	24 h	48 h	24 h	48 h	72 h	96 h
Effluent 01-02-1993	100	Deionized water	+345*	+119*	nt	nt	nt	nt	nt	nt
	50		+293	+99	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	+263*	+56*	0	0	0	0	0	0
	50		+247	+44	0	0	nt	nt	nt	nt
	100	River water	+47*	+88*	nt	nt	nt	nt	nt	nt
	50		+21*	+5*	0	0	nt	nt	nt	nt
River water 01-02-1993	100	Deionized water	+203*	+17*	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	+147*	17*	0	0	0	0	0	0
Effluent 15-02-1993	100	Deionized water	+212*	+63*	nt	nt	nt	nt	nt	nt
	50		+205*	+55	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	+196*	+14*	0	0	30	30	60	60
	50		+215*	+10	0	0	nt	nt	nt	nt
	100	River water	+173*	+30*	nt	nt	nt	nt	nt	nt
	50		+148*	+17*	0	0	nt	nt	nt	nt
River water 15-02-1993	100	Deionized water	+14*	+21	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	+14*	13	0	0	0	0	0	0

* Precipitation - background reading subtracted + Stimulation **Bold** Toxicity nt Not tested

Detection limits: algal growth inhibition test - 20%; water flea lethality test - 10%; fish lethality test - >10%

TABLE 9: Effect of secondary treated sewage effluent on selected biological toxicity tests

Sample	Concentration (%)	Dilution water/ control	Algal growth test		Water flea test		Fish test			
			AAM medium	BG-11 medium	% Lethality after:		% Lethality after:			
			% Inhibition	% Inhibition	24 h	48 h	24 h	48 h	72 h	96 h
Effluent 05-04-1993	100	Deionized water	+151*	+56*	nt	nt	nt	nt	nt	nt
	50		+132	+35	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	+353*	+25*	0	0	0	0	**15**	**15**
	50		+258	+1	0	0	0	0	5	5
	100	River water	+21*	+17*	nt	nt	nt	nt	nt	nt
	50		+8	4	0	0	0	0	5	10
River water 05-04-1993	100	Deionized water	+108	+33	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	+275	+6	0	0	0	5	5	5
Effluent 16-06-1993	100	Deionized water	+175*	+97*	nt	nt	nt	nt	nt	nt
	50		+154	+94	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	+287*	+133*	0	0	0	0	0	10
	50		+294	+173	0	0	0	0	0	0
	100	River water	+16*	+18*	nt	nt	nt	nt	nt	nt
	50		+10	+15	0	0	0	0	0	0
River water 16-06-1993	100	Deionized water	+139	+82	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	+207	+167	0	0	0	0	0	0

* Precipitation - background reading subtracted + Stimulation **Bold** Toxicity nt Not tested
Detection limits: algal growth inhibition test - 20%; water flea lethality test - 10%; fish lethality test - >10%

period. Unfortunately, dilutions were not tested because of limited numbers of fish. A very slight effect was observed with the sample of 05-04-1993, but only at the 100% concentration. Control lethality was always <10%. The receiving water was not toxic to water flea or fish on any of the sampling dates.

The pH of the treated sewage effluent ranged between 7,5 and 7,7, and the dissolved oxygen from 4,7 (16-06-1993) to 13,1 mg/ℓ (15-02-1993). During the summer months temperatures around 27°C were recorded. On 16-06-1993 the temperature was 19,8°C. The river water showed similar temperatures, except on 16-06-1993 when the water was much colder at 10,5°C. The pH of the river water ranged between 7,8 and 8,5, and the dissolved oxygen between 6,3 (01-02-1993) and 16,3 mg/ℓ (15-02-1993). The Hennops River was flowing at a rapid pace on 01-02-93. On the other sampling dates no flow was noticed upstream of the discharge point or the river was only trickling. Extensive chemical analyses were not carried out on the treated sewage effluent because samples were generally not toxic.

3.2.1.2 Paper mill effluent

The effect of paper mill effluent on the different test systems is shown in Tables 10 to 13. The paper mill effluent 1 collected on 15-02-1993 (Table 10) was slightly toxic to all the test organisms. A positive response with the algal test was obtained with the AAM growth medium, but only at the 50% concentration using deionized water for dilution. Toxicity was probably not detected at the 100% concentration because of interferences due to precipitation. Background readings of between 0,01 and 0,025 were recorded. A lethality of 40% was observed with the water flea test at the 100% effluent concentration. Upon dilution no toxicity was detected. The fish test showed 30% lethality with the 100% effluent. No toxicity was detected in the 50% dilution when moderately hard water was used. However, a slight effect was noted in the case of dilution with stream water. This effect was probably due to toxicants in the stream water. The results show that the stream water was highly toxic to all the test systems on this sampling date. In general, the effluent was not toxic after secondary clarification. However, marginal inhibition (20%) was observed with the algal test using BG-11 medium, when moderately hard water was used as control. This effluent also caused precipitation with the algal growth medium which could have interfered with the interpretation of results (OD: 0,01-0,02). Since the stream water was toxic, a very low algal growth was obtained in this water (Tables 7 and 10). This explains the large stimulation observed with effluents 1 and 2 when stream water was used as control.

The effluent samples collected on 10-05-1993 were not toxic (Table 11). In general, the effluents stimulated algal growth. The effects were more pronounced in case of AAM medium, because the control growth was generally low (considerably lower than in BG-11 medium) (Table 7). Precipitation was again observed (OD: 0,01-0,025). Compared to deionized and moderately hard water as controls the algal test using BG-11 medium showed that the stream water sample was toxic (Tables 7 and 11). No toxicity was, however, detected with the water flea and fish tests. Because of the toxicity problems experienced with the stream water, making it unsuitable for dilution, an additional stream water sample was taken on this sampling date for comparison purposes. This sample was taken several kilometres upstream from the normal sampling point in the Small Blesbok Spruit. The results show that this water was extremely toxic (100% lethality with fish and water flea). Similar effects were obtained on water flea and fish when this water was used for dilution (results not shown).

TABLE 10: Effect of paper mill effluent on selected biological toxicity tests

Sample	Concen-tration (%)	Dilution water/ control	Algal growth test		Water flea test		Fish test			
			AAM medium	BG-11 medium	% Lethality after:		% Lethality after:			
			% Inhibition	% Inhibition	24 h	48 h	24 h	48 h	72 h	96 h
Effluent before primary clarification 15-02-1993	100	Deionized water	9*	+17*	nt	nt	nt	nt	nt	nt
	50		31*	+89*	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	+31*	3*	15	40	0	0	10	30
	50		14	+37*	0	0	0	0	0	0
	100	Stream water	+517*	+405*	nt	nt	nt	nt	nt	nt
	50		+233*,[1]	+255[1]	0	0	0	0	10	20
Effluent after secondary clarification 15-02-1993	100	Deionized water	+44*	+4*	nt	nt	nt	nt	nt	nt
	50		+132*	+27	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	+70*	20*	0	0	0	0	0	0
	50		+126*	17*	0	0	nt	nt	nt	nt
	100	Stream water	+278*	+273*	nt	nt	nt	nt	nt	nt
	50		17*,[1]	+93[1]	0	0	nt	nt	nt	nt
Stream water 15-02-1993	100	Deionized water	56	73	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	64	77	75	100	40	40	50	50

* Precipitation - background reading subtracted + Stimulation **Bold** - Toxicity nt Not tested

[1] Stream water toxic

Detection limits: algal growth inhibition test - 20%; water flea lethality test - 10%; fish lethality test - >10%

TABLE 11: Effect of paper mill effluent on selected biological toxicity tests

Sample	Concentration (%)	Dilution water/ control	Algal growth test		Water flea test		Fish test			
			AAM medium	BG-11 medium	% Lethality after:		% Lethality after:			
			% Inhibition	% Inhibition	24 h	48 h	24 h	48 h	72 h	96 h
Effluent before primary clarification 10-05-1993	100	Deionized water	+445*	+34*	nt	nt	nt	nt	nt	nt
	50		+397*	+3	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	+212*	+37*	0	0	0	0	0	0
	50		+374	+25*	0	0	0	0	0	0
	100	Stream water	+485*	+378*	nt	nt	nt	nt	nt	nt
	50		+393*	+150	0	0	0	0	0	0
Effluent after secondary clarification 10-05-1993	100	Deionized water	+323	6	nt	nt	nt	nt	nt	nt
	50		+260	16	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	+313*	7	0	0	0	0	0	0
	50		+254	7	0	0	nt	nt	nt	nt
	100	Stream water	+245*	+231*	nt	nt	nt	nt	nt	nt
	50		+151	+159	0	0	nt	nt	nt	nt
Stream water 10-05-1993	100	Deionized water	+18	**67**	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	11	**70**	0	0	0	0	0	0
Stream water (additional)	100	Moderately hard water	nt	nt	**100**	**100**	**100**	**100**	**100**	**100**

* Precipitation - background reading subtracted + Stimulation **Bold** - Toxicity nt Not tested

Detection limits: algal growth inhibition test - 20%; water flea lethality test - 10%; fish lethality test - >10%

TABLE 12: Effect of paper mill effluent on selected biological toxicity tests

Sample	Concentration (%)	Dilution water/ control	Algal growth test		Water flea test		Fish test			
			AAM medium	BG-11 medium	% Lethality after:		% Lethality after:			
			% Inhibition	% Inhibition	24 h	48 h	24 h	48 h	72 h	96 h
Effluent before primary clarification 12-05-1993	100	Deionized water	+237	+415	nt	nt	nt	nt	nt	nt
	50		+104*	+246*	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	+222	+144	0	0	0	0	0	0
	50		+142*	+228*	0	0	0	0	0	0
	100	*Stream water*	+250	+292	nt	nt	nt	nt	nt	nt
	50		+45*	+104*	0	0	0	0	0	0
Effluent after secondary clarification 12-05-1993	100	Deionized water	+159*	+203*	nt	nt	nt	nt	nt	nt
	50		+103*	+114*	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	+160*	+74*	0	0	0	0	0	0
	50		+92*	+67*	0	0	nt	nt	nt	nt
	100	Stream water	+132*	+118*	nt	nt	nt	nt	nt	nt
	50		+35*	+76*	0	0	nt	nt	nt	nt
Stream water 12-05-1993	100	Deionized water	17	+32	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	22	13	0	0	0	0	0	0

* Precipitation - background reading subtracted + Stimulation **Bold** - Toxicity nt Not tested

Detection limits: algal growth inhibition test - 20%; water flea lethality test - 10%; fish lethality test - >10%

TABLE 13: Effect of paper mill effluent on selected biological toxicity tests

Sample	Concentration (%)	Dilution water/ control	Algal growth test		Water flea test		Fish test			
			AAM medium	BG-11 medium	% Lethality after:		% Lethality after:			
			% inhibition	% inhibition	24 h	48 h	24 h	48 h	72 h	96 h
Effluent before primary clarification 28-06-1993	100	Deionized water	19*	2*	nt	nt	nt	nt	nt	nt
	50		+84*	+87*	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	**46***	**36***	**60**	**65**	**20**	**20**	**20**	**50**
	50		+38*	0	0	0	10	10	10	20
	100	Stream water	**51***	16*	nt	nt	nt	nt	nt	nt
	50		+19	+52	0	0	0	10	20	30
Effluent after secondary clarification 28-06-1993	100	Deionized water	+222*	+189*	nt	nt	nt	nt	nt	nt
	50		+165*	+160*	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	+126*	+77*	0	0	0	0	10	20
	50		+118	+42	0	0	0	10	20	30
	100	Stream water	+130*	+157*	nt	nt	nt	nt	nt	nt
	50		+98	+100	0	0	0	10	10	20
Stream water 28-06-1993	100	Deionized water	+38	+40	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	19	17	0	0	0	30	40	40

* Precipitation - background reading subtracted + Stimulation **Bold** - Toxicity nt Not tested

Detection limits: algal growth inhibition test - 20%; water flea lethality test - 10%; fish lethality test - >10%

The paper mill effluents sampled two days later (12-05-1993) (Table 12) again showed an absence of toxicity. In general, a large stimulation was detected with algal growth tests. Background readings due to precipitation was also increased (OD: 0,02-0,06). When tested directly (100%), effluent 1 did not cause precipitation. Precipitation only occurred when the sample was diluted. The stream water was not toxic to water flea and fish. A slight inhibition in algal growth was detected when using AAM medium and applying moderately hard water as control.

The effluent sample collected before primary clarification 28-06-1993 (Table 13) was toxic. Algal growth inhibition was detected with both growth media, but only at the 100% concentration. A 65% lethality was observed with the water flea test at the 100% effluent concentration. Lethal effects were also obtained with the fish test. These results are, however, not valid because 40% of the fish in the standard control water (moderately hard water) died. The algal and water flea tests showed an absence of toxicity in the effluent after secondary clarification and in stream water. The 50% dilution of effluent 1, and 50 and 100% of effluent 2 stimulated algal growth. The stream water also showed growth stimulation when deionized water was used as control. Background densities of between 0,01 and 0,025 were recorded for the effluents with the algal tests.

The pH of the effluent before primary clarification ranged from 6,8 to 7,3, that of the effluent after secondary clarification from 7,2 to 7,8, and that of the river water from 6,0 to 7,8. The oxygen in both effluents was usually low (effluent 1: 1,3-4,3 mg/ℓ; effluent 2: 2,0-5,3), and could have contributed to adverse effects on the test systems. Oxygen concentrations >8,0 mg/ℓ were generally recorded for the stream water. The temperature of effluent 1 was always >30° C. The additional stream water sample collected on 10-05-1993 showed a low pH of 2,65. At the usual sampling point the Blesbok Spruit showed a strong flow. The additional sample was taken from standing water. Extensive chemical analyses were carried out on the samples of 20-05-1993 and 28-06-1993. The results are presented in Table 14. In general, concentrations of potentially toxic chemicals in the effluents and stream water (usual sample) of 10-05-1993 were low. A relatively high concentration of aluminium was present in effluent 1. The additional stream water sample showed high levels of aluminium, arsenic, copper, iron, manganese, nickel and zinc, explaining the extreme toxic effects on test systems. There is a lot of industrial activity in the area of this steam, particularly mining, and these chemicals probably leached into the water. The effluent 1 sample of 28-06-1993 which was toxic to algae and water flea contained a high level of dissolved organic material, aluminium and copper.

3.2.1.3 Metal plating effluent

Rapid range finding tests were particularly important in the case of the metal plating effluent in order to reduce the holding time and possible changes in effluent quality. The urease enzyme test was applied to select the effective concentration range for the different tests. This was carried out immediately after arrival of samples in the laboratory and ensured that samples were not standing for the usual 24 h while the water flea screening test was carried out. The decision was taken because the quality of the effluent appeared to change when the first sample (09-02-1993) was evaluated. For effective interpretation of the urease test results, a few range finding tests were carried out using the enzyme and water flea tests. The results (Table 15) indicated that when an inhibition of between 40 and 60% was found with the enzyme test with the 10% effluent concentration, and no effect was observed at the 1,0%

TABLE 14: Chemical quality of effluent

Determinand (mg/ℓ except for *)	Paper mill effluent					Metal plating effluent						
	10-05-1993				28-06-1993	09-02-1993	13-04-1993		06-05-1993		10-06-1993	
	Effluent 1	Effluent 2	Stream 1	Stream 2	Effluent 1	Effluent	Effluent	River	Effluent	River	Effluent	River
Electrical con. (mS/m)*	nd	nd	nd	nd	215	2464	199	24	nd	nd	176	45
Hardness ($CaCO_3$)	nd	nd	nd	nd	nd	167	198	86	nd	nd	202	197
Alkalinity ($CaCO_3$)	152	209	7	<5	nd	18	70	74	80	142	57	151
Calcium (Ca)	88	93	45	190	84	32	38	20	61	36	44	35
Magnesium (Mg)	28	20	13	58	<1	21	25	9	30	16	22	26
Ammonia (N)	0,8	2,8	<0,2	5,5	2,1	15	26	<0,2	nd	nd	16	<0,2
Nitrate-nitrite (N)	2,3	0,3	0,3	6,2	3,7	6,6	8,8	0,2	5,9	0,3	5,8	0,4
Sulphate (SO_4)	138	215	223	1192	nd	104	272	20	nd	nd	227	29
Total phosphate (PO_4)	2,7	1,3	0,2	0,4	1,8	8,1	4,7	0,9	1,5	0,2	3,6	0,2
Ortho-phosphate (PO_4)	2,0	1,0	<0,2	0,3	5,7	6,9	<0,2	0,8	<0,2	<0,2	<0,2	<0,2
Chloride (Cl)	190	84	70	72	206	190	185	11	83	24	178	20
COD	285	140	12	49	550	146	70	16	67	11	84	<10
DOC	39	11	<0,5	<0,5	159	nd	nd	6,1	nd	nd	<0,5	22

nd Not determined

TABLE 14: Chemical quality of effluent (continue)

Determinand (µg/ℓ)	Paper mill effluent					Metal plating effluent						
	10-05-1993				28-06-1993	09-02-1993	13-04-1993		06-05-1993		10-06-1993	
	Effluent 1	Effluent 2	Stream 1	Stream 2	Effluent 1	Effluent	Effluent	River	Effluent	River	Effluent	River
Silver (Ag)	<25	<25	<25	30	nd	<25	60	nd	30	<25	<25	<25
Aluminium (Al)	1138	453	<100	16611	1880	507	405	<100	189	<100	218	<100
Arsenic (As)	<5	<5	<5	240	<5	nd	nd	<5	nd	nd	<5	<5
Cadmium (Cd)	<5	<5	<5	10	<5	1155	3380	<5	2630	<5	4910	<5
Chromium (Cr)	<30	<30	<30	240	nd	3350	12600	nd	9000	<30	7300	<30
Chromium [6]	nd	nd	nd	nd	nd	50	70	nd	nd	nd	<30	nd
Copper (Cu)	50	40	40	1330	100	3440	2160	30	nd	nd	2600	30
Iron (Fe)	220	120	310	138000	760	21300	24100	370	21700	140	13600	360
Mercury (Hg)	nd	nd	150	7	50	nd	nd	<1	nd	<1	nd	<1
Manganese (Mn)	230	400	620	9400	280	240	460	50	590	<25	420	30
Nickel (Ni)	40	<25	210	5800	nd	11500	5020	nd	9250	<25	10300	<25
Lead (Pb)	<50	<50	<50	<50	<50	nd	nd	<50	nd	nd	60	<50
Selenium (Se)	<5	<5	<5	<5	<5	nd	nd	<5	nd	nd	<5	<5
Zinc (Zn)	90	90	160	4520	110	36600	55400	100	31400	<25	32900	60
Cyanide (free) (CN)	<50	<50	<50	<50	<50	9835	8642	<50	nd	nd	5032	<50
Phenol	x	x	10	13	<10	nd	nd	<10	nd	nd	<10	<10

x Interference
nd Not determined

TABLE 15: Effect of metal plating effluent on the urease enzyme and water flea tests

Sample	Concentration (%)[1]	Urease enzyme test			Water flea test
		% Inhibition			% Lethality after:
		0,5 mg/mℓ enzyme	1,0 mg/mℓ enzyme	2,0 mg/mℓ enzyme	24 h
05-04-1993	10	46	26	+5	100
	1,0	4	1	+11	30
	0,1	4	7	1	0
	0,01	+4	3	+5	0
05-04-1993	10	61	64	0	100
	1,0	+20	+7	+12	55
	0,1	+22	+7	+10	0

[1] Dilution water: enzyme test - deionized water; water flea test - moderately hard water

concentration, concentrations ranging between 0,1 and 10% should be selected for definitive tests with algae, water flea and fish.

Tables 16 to 19 present the results of the definitive tests on metal plating effluent. The tables also indicate the correlation coefficients and effective concentrations calculated by means of regression analysis. In most instances the correlation between % effect and concentration was very good (R: >0,9). Although $LC_{10}/LC_{20}/EC_{20}$'s were reported in all cases where linear regression was applied, some of these values are not considered valid (indicated in tables) because the lowest effect used for calculation was above the detection limits (for example: Table 16, BG-11 medium, deionized water - the lowest effect 32%). Table 16 shows that the bacterial growth inhibition test was not very sensitive to the effluent. The onset of toxicity (EC_{10}) was at approximately 22.5% of the effluent (25% x 0.9). EC_{50}'s could not be calculated (EC_{50}>100%). Initial tests showed that the selected test concentrations for the test (0,3-10%) were too low. Tests were repeated one week later, but the effluent quality appeared to have changed during the holding period, resulting in unsatisfactory results. The highest sensitivity with the algal test was obtained when BG-11 medium was used. A higher sensitivity was obtained with deionized water than with moderately hard water (control and dilution). The effluent caused precipitation with algal growth media at the upper concentrations (OD: 0,01-0,02). A comparison of the results showed that the algal (BG-11 medium) test was the most sensitive, followed by the water flea and fish tests. As expected, the inherent toxicity (using a standard dilution water) was higher than the relative toxicity (using receiving water for dilution) (algal test with AAM medium excluded). The lower sensitivity of the algal AAM medium test when using deionized and moderately hard water for control testing and dilution could be due to low growth in the controls (Table 7). The stream water showed an absence of toxicity. Based on the results of the most sensitive test (algal test - EC_{20}: 0,4%) a dilution

TABLE 16: Effect of metal plating effluent on selected biological toxicity tests (09-02-1993)

Concentration[1] (%)	Bacterial growth test			Algal growth test						Water flea test				Fish test							
				AAM medium			BG-11 medium			% Lethality after:				% Lethality after:							
	% Inhibition			% Inhibition			% Inhibition			24 h	48 h	24 h	48 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
	DW*	MW[2]	SW*	DW	MW	SW	DW	MW	SW	MW		SW		MW				SW			
100	42	37	35	40*	62*	54*	35*	59*	69*	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
50	18	19	30	72*	71*	71	62*	81*	83	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
25	9	7	13	86	96	79	88	91	88	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
12,5	9	11	+4	81	83	80	97	93	88	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
10	8	22	2	73	66	80	93	89	84	100	100	65	100	nt	nt	nt	nt	nt	nt	nt	nt
5	13	17	0	50	64	77	86	85	79	70	100	50	100	nt	nt	nt	nt	nt	nt	nt	nt
2,5	0	20	+14	42	16	65	83	63	60	35	85	40	60	0	10	40	60	20	20	20	40
1,25	+18	15	+10	15	+16	47	79	46	43	0	80	0	5	0	0	20	20	20	20	20	20
0,63	+18	13	+7	+84	+71	17	61	20	17	5	10	0	0	nt	nt	nt	nt	nt	nt	nt	nt
0,31	5	5	12	+184	+151	+11	32	6	13	0	0	0	0	nt	nt	nt	nt	nt	nt	nt	nt
R[5]	nd	nd	nd	0,98	0,93	0,95	0,92	1,00	0,98	nd	0,94	nd	0,96	nd	nd	nd	nd	nd	nd	nd	nd
LC50/ EC50[6]	nd	nd	nd	3,9	4,6	1,6	0,4	1,4	1,6	nd	1,1	nd	2,1	nd	nd	nd	1,3-2,5	nd	nd	nd	>2,5
LC10/ LC20/ EC20[7]	nd	nd	nd	1,1	2,0	0,4	0,1	0,5	0,5	nd	0,4	nd	1,0	nd	nd	nd	nd	nd	nd	nd	nd
SW	+3	+8	nt	+61	+54	nt	+40	+30	nt	0	0	nt	nt	0	0	0	0	nt	nt	nt	nt

* Precipitation - background reading subtracted
+ Stimulation
Bold Values used for linear regression
Shaded block - result not valid
nt Not tested
nd Not determined
[1] Concentrations adjusted for dilution in bacterial and algal tests
[2] Deionized water
[3] Moderately hard water
[4] Stream water
[5] Correlation coefficient
[6] Concentration causing 50% lethality/inhibition
[7] Minimum effective concentrations

Detection limits: bacterial growth test - 10%; algal growth test - 20%; *water flea lethality test* - 10%; fish lethality test: >10%

TABLE 17: Effect of metal plating effluent on selected biological toxicity tests (13-04-1993)

Concentration[1] (%)	Algal growth test						Water flea test				Fish test							
	AAM medium			BG-11 medium			% Lethality after:				% Lethality after:							
	% Inhibition			% Inhibition			24 h	48 h	24 h	48 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
	DW[2]	MW[3]	SW[4]	DW	MW	SW	MW		SW		MW				SW			
5	90	79	90	88	80	80	60	90	35	90	nt	nt	nt	nt	0	60	60	80
2,5	84	62	87	87	75	70	50	95	50	75	nt	nt	nt	nt	0	20	40	40
1,25	74	41	73	86	59	41	50	95	0	10	0	20	60	100	0	20	20	40
0,63	43	12	44	76	31	19	35	90	0	0	0	0	40	60	0	0	0	0
0,31	+25	12	29	57	21	16	35	85	0	0	0	0	0	20	0	0	0	0
0,16	+36	+20	7	27	5	+7	0	45	0	0	nt	nt	nt	nt	nt	nt	nt	nt
0,08	+68	6	7	18	7	+12	0	0	0	0	nt	nt	nt	nt	nt	nt	nt	nt
0,04	+12	+17	6	8	8	+4	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
R[5]	0,97	0,99	0,99	0,99	0,99	0,97	nd	0,96	nd	0,94	nd	nd	nd	1,0	nd	nd	nd	0,95
LC50/EC50[6]	0,8	1,7	0,6	0,3	1,0	1,4	nd	0,2	nd	2,2	nd	nd	nd	0,5	nd	nd	nd	2,4
LC10/LC20/ EC20[7]	0,4	0,7	0,2	0,1	0,3	0,4	nd	0,1	nd	1,1	nd	nd	nd	0,3	nd	nd	nd	1,0
SW	+100	+206	nt	+39	+29	nt	0	0	nt	nt	0	0	0	0	nt	nt	nt	nt

\+ Stimulation

Shaded block - result not valid

Bold Values used for linear regression

nt Not tested

nd Not determined

[1] Concentrations adjusted for dilution in algal test

[2] Deionized water

[3] Moderately hard water

[4] Stream water

[5] Correlation coefficient

[6] Concentration causing 50% lethality/inhibition

[7] Minimum effective concentrations

Detection limits: algal growth test - 20%; water flea lethality test - 10%; fish lethality test: >10%

TABLE 18: Effect of metal plating effluent on selected biological toxicity tests (06-05-1993)

Concentration[1] (%)	Algal growth test						Water flea test		Fish test							
	AAM medium			BG-11 medium			% Lethality after:		% Lethality after:							
	% Inhibition			% Inhibition			24 h	48 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
	DW[2]	MW[3]	SW[4]	DW	MW	SW	SW		MW				SW			
10	nt	nt	nt	nt	nt	nt	100	100	nt	nt	nt	nt	nt	nt	nt	nt
5	100	96	97	99	77	85	100	100	40	50	80	100	20	60	90	100
2,5	100	66	88	97	53	74	50	100	0	40	90	100	20	40	80	100
1,25	100	34	60	86	20	57	15	100	0	0	50	90	0	0	40	70
0,63	88	5	43	81	9	45	0	25	0	0	40	90	0	0	10	40
0,31	65	9	34	70	4	38	0	0	0	0	40	90	0	0	20	40
0,16	27	+45	11	46	+15	8	nt	nt	0	0	40	70	0	0	0	0
0,08	25	+29	16	44	+12	21	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
0,04	16	+8	24	37	+7	9	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
R[5]	0,95	1,0	0,99	0,97	0,99	0,98	nd	0,96	nd	nd	nd	nd	nd	nd	nd	0,97
LC50/EC50[6]	0,2	1,6	0,6	0,1	2,1	0,8	nd	0,7	nd	nd	nd	<0,2	nd	nd	nd	0,6
LC10/LC20/ EC20[7]	0,1	0,8	0,2	0,02	0,9	0,2	nd	0,4	nd	nd	nd	<0,2	nd	nd	nd	0,3
SW	+94	+200	nt	+66	+80	nt	0	0	0	0	0	0	nt	nt	nt	nt

\+ Stimulation
Bold Values used for linear regression
Shaded block - result not valid
nt Not tested
nd Not determined
[1] Concentrations adjusted for algal tests
[2] Deionized water
[3] Moderately hard water
[4] Stream water
[5] Correlation coefficient
[6] Concentration causing 50% lethality/inhibition
[7] Minimum effective concentrations
Detection limits: algal growth test - 20%; water flea lethality test - 10%; fish lethality test: >10%

TABLE 19: Effect of metal plating effluent on selected biological toxicity tests (10-06-1993)

Concentration[1] (%)	Algal growth test						Water flea test		Fish test							
	AAM medium			BG-11 medium			% Lethality after:		% Lethality after:							
	% Inhibition			% Inhibition			24 h	48 h	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
	DW[2]	MW[3]	SW[4]	DW	MW	SW	SW		MW				SW			
10	nt	nt	nt	nt	nt	nt	100	100	nt	nt	nt	nt	nt	nt	nt	nt
5	98	86	85	71	58	63	100	100	30	80	100	100	0	50	100	100
2,5	96	71	69	67	41	51	50	95	30	90	100	100	0	40	80	100
1,25	91	49	54	60	26	33	10	55	0	90	90	100	0	20	30	50
0,63	68	29	36	64	15	17	0	10	0	40	100	100	0	0	0	40
0,31	39	9	19	50	6	26	0	0	0	10	5	80	0	1	10	20
0,16	+6	+45	10	31	+10	11	nt	nt	0	0	0	0	0	0	10	10
R[5]	0,99	1,00	1,00	0,90	0,99	1,00	nd	1,00	nd	nd	nd	0,95	nd	nd	nd	0,95
LC50/EC50[6]	0,4	1,2	1,0	0,4	3,4	2,4	nd	1,2	nd	nd	nd	0,3	nd	nd	nd	0,6
LC10/LC20/ EC20[7]	0,2	0,4	0,3	0,02	0,7	0,6	nd	0,6	nd	nd	nd	0,2	nd	nd	nd	0,3
SW	+94	+200	nt	+66	+80	nt	0	0	0	0	0	0	nt	nt	nt	nt

\+ Stimulation

Bold Values used for linear regression

Shaded block - result not valid

nt Not tested

nd Not determined

[1] Concentrations adjusted for algal tests

[2] Deionized water

[3] Moderately hard water

[4] Stream water

[5] Correlation coefficient

[6] Concentration causing 50% lethality/inhibition

[7] Minimum effective concentrations

Detection limits: algal growth test - 20%; water flea lethality test - 10%; fish lethality test: >10%

of >250x (100%/0,4%) with the receiving water will be required to avoid acute effects on aquatic life.

With the sample of 13-04-1993 (Table 17), the algal test using BG-11 medium showed a higher sensitivity than the test using AAM medium, in case of deionized and moderately hard water. A higher sensitivity was, however, obtained with AAM medium with stream water as control (EC_{50}: 0,6%; EC_{20}: 0,2%). Algal sensitivity was lower with moderately hard water than with deionized water. The inherent toxicity of the effluent was slightly higher when using water flea (moderately hard water) than when using algae (BG-11 medium; deionized water) and considerably higher than when using fish (moderately hard water). A comparison between the results obtained for the relative toxicity indicated that the algal test (AAM medium) was the most sensitive (EC_{50}: 0,6%; EC_{20}: 0,2%), followed by the fish test (LC_{50}: 2,4%; LC_{20}: 1,0%) and the water flea test (LC_{50}: 2,2%; LC_{10}: 1,1%). No toxicity was detected in the receiving water. Based on the results of the algal test (EC_{20}: 0,2%) this effluent should be diluted by >500x to avoid acute toxicity.

On 06-05-1993 (Table 18) the algal test, using BG-11 medium, was more sensitive than the test using AAM medium when deionized water was used for control testing and dilution. However, when moderately hard water was used the test using AAM medium showed the highest sensitivity. Results obtained with stream water were very much the same in both media, with the AAM medium test showing the slightly lower EC_{50}. With both media the sensitivity was the lowest when using moderately hard water. It was not possible to calculate the effective concentrations for the fish test when using moderately hard water because the effects recorded were above 50%. Water flea tests were only carried out with stream water (control and dilution). A comparison of the results (LC_{50}'s/EC_{50}) obtained for relative toxicity showed that the algal test using AAM medium and the fish test were the most sensitive. These were followed in order of magnitude by the water flea test (LC_{50}: 0,7%) and the algal test using BG-11 medium (EC_{50}: 0,8%). The EC_{20} of the algal test (AAM: 0,2%; BG-11: 0,2%) was lower than the LC_{20} of the fish test (0,3%) and the LC_{10} of the water flea test (0,4%). The stream water was not toxic to any of the test systems. The findings of the algal test (EC_{20}: 0,2%) showed that a dilution of >500x is required with river water to avoid acute toxicity.

The results of the effluent tested on 10-06-1993 are presented in Table 19. In general, the sensitivity of the algal test was the best when using AAM medium. In case of BG-11 medium the EC_{20} obtained with deionized water is not valid because the lowest recorded effect was >20%. The algal test was the least sensitive when moderately hard water was used for control and dilution purposes (less sensitive than when using stream water). When comparing the results obtained for inherent toxicity the fish test showed a higher sensitivity than the algal test. Based on relative toxicity the fish test was found to be the most sensitive to this particular effluent sample (LC_{50}: 0,8%; LC_{20}: 0,3%), followed in order of magnitude by the algal test (AAM medium) (EC_{50}: 1,0%; EC_{20}: 0,3%) and the water flea test (LC_{50}: 1,1%; LC_{10}: 0,6%). A dilution of approximately 333x (water flea LC_{10}: 0,3%; algal EC_{20}: 0,3%) with stream water will be adequate to avoid acute toxicity with this sample composition. The stream water showed an absence of toxicity.

The pH of the effluent ranged between 5,7 and 6,7, and that of the stream water between 5,3 and 7,7. The oxygen levels in both the effluent and stream water were always >7,0 mg/ℓ. During the summer months the temperature of the effluent and water was between 20 and 24° C. On 10-06-1993 the effluent had a temperature of 3° C and the water a temperature of

13°C. The colour of the effluent ranged from yellow to rusty. A strong flow was observed in the stream during the summer sampling period but the flow was low on the last two sampling dates. The results of extensive chemical analyses are presented in Table 14. In general, potentially toxic chemicals in the river water were low. The effluent contained high levels of cadmium, chromium, copper, iron, nickel, zinc and cyanide, which individually or in combination could have caused the toxicity at high dilutions.

3.2.2 Sensitivity of a range of selected toxicity tests

A range of bioassays was applied to each of the effluents to compare their sensitivities. The selection of tests was based on the findings of the range finding tests (Section 3.1). Secondary treated sewage effluent and paper mill effluent were evaluated by means of fish and water flea lethality tests, algal and bacterial growth inhibition tests, the toad embryo teratogenicity test and the mutagenicity test. Metal plating effluent was tested using the water flea lethality test, algal and bacterial growth inhibition tests, the oxygen uptake inhibition test, the urease enzyme inhibition test and the toad embryo teratogenicity test. The fish test was omitted in the latter case because of limited fish numbers. Ten fish were used for each test preparation. The algal test was carried out with BG-11 medium only, using a detection limit of 10% instead of 20% [because of stabilized growth (OD of control growth approximately 0,1) and improved reproducibility]. Algal growth was determined in terms of deionized water, moderately hard and river water and bacterial growth in terms of deionized water and river water (control and dilution), in case of the sewage effluent and paper mill effluents. Moderately hard and river water were used for dilution and control testing in fish, water flea and toad embryo tests. In the case of the metal plating effluent standard dilution water (deionized/moderately hard water) was used for dilution and control testing.

Each of the effluents was sampled on one occasion only, during the period November 1993 to January 1994. In the case of the sewage and paper mill effluents, samples downstream of the point of discharge were collected to establish ambient toxicity.

3.2.2.1 Secondary treated sewage effluent

The results of the effect of secondary treated sewage effluent on selected bioassays are presented in Table 20. The effluent did not show toxicity in the bacterial and algal growth inhibition tests and the water flea test. The Ames test also showed an absence of mutagenicity. Algal growth was stimulated by the effluent, indicating a presence of nutrients. When compared to moderately hard water the 100% effluent caused a marginal lethality (10%) to toad embryos. No lethality was observed when compared to river water. The 100% effluent also exhibited a low level of teratogenicity (24%). While the 100% effluent had no effect on fish, the 50% dilution with river water resulted in a 20% lethality after 96 h exposure. A similar effect was observed when testing river water (lethality: 20%), indicating that the effect was due to toxicants in the river water. The river water collected upstream of the point of discharge also showed a slight inhibition in algal growth. None of the other tests were adversely affected by the river water upstream of the point of discharge. The river water downstream of the point of discharge (ambient water) showed an absence of toxicity in all the tests. This indicates that effluent diluted the river water and so reduced toxicity. Algal growth was stimulated, indicating that the effluent was introducing nutrients into the river. A low level of precipitation was observed in case of the algal test, when evaluating the 100% effluent and the river water downstream of discharge (OD approximately 0,01). The results of the evaluation indicated that

TABLE 20: Effect of secondary treated sewage effluent on selected biological toxicity tests (22-11-1993)

Sample	Concentration (%)	Dilution water/ control	Bacterial growth test	Algal growth test	Water flea test		Fish test				Toad embryo teratogenicity test		Ames *Salmonella* mutagenicity test[1]			
			% Inhibition	% Inhibition	% Lethality after:		% Lethality after:				% Lethality	% Deformation	TA98-S9	TA98+S9	TA100-S9	TA100+S9
					24 h	48 h	24 h	48 h	72 h	96 h			Mutation ratio			
Effluent	100	Deionized water	5	+59*	nt	nt	nt	nt	nt	nt	nt	nt	1,4	1,3	1,3	1,2
	50		3	+29	nt	nt	nt	nt	nt	nt	nt	nt				
	100	Moderately hard water	nt	+51*	0	0	0	0	0	0	10	24				
	50		nt	+21	0	0	0	0	0	0	5	7				
	100	River water	4	+77*	nt	nt	nt	nt	nt	nt	4	24				
	50		3	+45	0	0	0	0	10	20	+5	7				
River above	100	Deionized water	1	10	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	nt	15	0	0	0	0	0	20	7	10				
River below	100	Deionized water	9	+29*	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	nt	+23*	0	0	0	0	0	0	2	11				
	100	River water	9	+42*	nt	nt	nt	nt	nt	nt	+5	11				

* Precipitation - background reading subtracted
Bold Toxicity
[1] XAD resin extract tested
\+ Stimulation in growth/test survival >control survival
TA *Salmonella* tester strains
nt Not tested

Detection limits: bacterial growth inhibition, algal growth inhibition, water flea and toad embryo lethality tests - 10%; fish lethality test - >10%; toad embryo deformation test - 20%; mutagenicity test - MR 2,0

in general the effluent was not acutely toxic. However, teratogenicity was detected, indicating chronic toxicity.

Table 21 shows that the pH, dissolved oxygen levels and temperature of the effluent and river water were within the ranges required for the sustenance of aquatic life. However, the pH of the effluent was relatively low at 5,67. The effluent was clear with no odour while the river water was muddy and the flow was rapid.

3.2.2.2 Paper mill effluent

Table 22 shows that effluent 1 was acutely toxic, inhibiting bacterial growth and causing lethality to water flea. A slight degree of teratogenicity (chronic toxicity) was also observed. Both the 100 and 50% concentrations affected bacterial growth. When applying regression analysis to the test results (R>0,9), the EC_{50} was found to be 94% when using deionized water as dilution/control water and >100% when using stream water. The EC_{10}'s were 33% and 22%, respectively, for deionized and stream water, indicating a slightly higher toxicity in the latter case. Water flea (lethality: 40%) and toad embryos (deformation: 21%) were only affected at the 100% concentration. The results implied that the bacterial growth test was the most sensitive acute test followed by the water flea test. Algal growth showed stimulation at some concentrations, indicating the presence of nutrients. The effluent caused precipitation with both the algal (OD: 0,03-0,05) and bacterial growth (OD: 0,021-0,331) tests. The large background readings obtained with the bacterial growth test could have interfered with the interpretation of results.

Effluent 2 (Table 22) inhibited bacterial growth and caused teratogenicity. Bacterial growth was affected at the 100 and 50% concentrations and toad embryos only at the 100% level. When applying linear regression (R>0,9), the EC_{50} was established at 81% in case of deionized water as control/dilution water and at >100% in case of stream water. EC_{10}'s were 29 and 32%, respectively, for deionized water and stream water as control/dilution water, indicating that the sensitivity of the test was slightly larger when using deionized water as control/dilution water. Deformation was large at 33%. In some instances algal growth was stimulated by effluent 2. Both the bacterial growth and algal tests showed precipitation with the effluent, which was considerably larger than in the case of effluent 1 (bacteria - OD: 0,026-0,665; algae - OD: 0,035-0,06). As was mentioned in the case of effluent 1, the large background density experienced with the bacterial growth test could have interfered with the interpretation of the results of effluent 2. The results indicated that the bacterial growth test was the most sensitive acute toxicity test. Apart from acute toxicity the effluent also exhibited chronic toxicity.

Both the stream water samples inhibited bacterial growth, while no adverse activity was detected with the other tests (Table 22). Algal growth was stimulated by the downstream water sample. The downstream water caused precipitation with both the bacterial (OD: 0,277) and algal tests (OD: 0,067), which could have interfered with the interpretation of results.

The pH and oxygen content of effluent 1 were low (pH: 5,98; oxygen: 2,3 mg/ℓ), which could have contributed to adverse activity (Table 20). The temperature was, as previously observed, >30°C. Effluent 2 has a pH of 3,3, and contained foam. The pH, oxygen content and temperature of the river water were within the limits for sustenance of aquatic life. The Small

TABLE 21: Quality of water and effluent samples

Determinand	Secondary treated effluent			Paper mill effluent				Metal plating effluent
	22-11-1993			15-11-1993				24-01-1994
	Effluent	River above	River below	Effluent 1	Effluent 2	Stream above	Stream below	Effluent
pH	5,67	6,93	7,45	5,98	6,81	7,83	6,21	3,23
Dissolved oxygen (mg/ℓ)	6,4	8,3	5,8	2,3	3,3	6,6	6,1	6,2
Temperature (°C)	23,5	22,4	23,0	37,3	28,3	22,9	23,8	22,8
Aesthetic observation	Clear, no smell	Muddy, rapid flow	Muddy	Greyish, no smell	Rusty, foam	Clear, strong flow	Clear, animal and plant life	Blue-green, turbid, rotten fish smell

TABLE 22: Effect of paper mill effluent on selected biological toxicity tests (15-11-1993)

Sample	Concentration (%)	Dilution water/ control	Bacterial growth test	Algal growth test	Water flea test		Fish test				Toad embryo teratogenicity test		Ames *Salmonella* mutagenicity test[1]			
			% Inhibition	% Inhibition	% Lethality after:		% Lethality after:				% Lethality	% Deformation	TA98-S9	TA98+S9	TA100-S9	TA100+S9
					24 h	48 h	24 h	48 h	72 h	96 h			Mutation ratio			
Effluent 1	100	Deionized water	**52***	**+57***	nt	nt	nt	nt	nt	nt	nt	nt	1,2	1,0	1,4	1,3
	50		**14***	**+24***	nt	nt	nt	nt	nt	nt	nt	nt				
	25		+2	**+4***	nt	nt	nt	nt	nt	nt	nt	nt				
	100	Moderately hard water	nt	**4***	**40**	**40**	**0**	**0**	**0**	**0**	**7**	**21**				
	50		nt	**+12***	**0**	**0**	**0**	**0**	**0**	**0**	**+1**	**5**				
	25		nt	**8***	**0**	**0**	**0**	**0**	**0**	**0**	**+3**	**2**				
	100	Stream water	**40***	**+42***	nt	nt	nt	nt	nt	nt	**9**	**21**				
	50		**34***	**+28***	**0**	**0**	**0**	**0**	**0**	**0**	**5**	**18**				
	25		7	**+19***	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**9**				
Stream water above	100	Deionized water	17	5	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	nt	9	**0**	**0**	**0**	**0**	**0**	**0**	**+2**	**7**				

* Precipitation - background reading subtracted
+ Stimulation in growth/test survival >control survival
Bold Toxicity
TA *Salmonella* tester strains
[1] XAD resin extract tested
nt Not tested
Detection limits: bacterial growth inhibition, algal growth inhibition, water flea and toad embryo lethality tests - 10%; fish lethality test - >10%; toad embryo deformation test - 20%; mutagenicity test - MR 2,0

TABLE 22: Effect of paper mill effluent on selected biological toxicity tests (15-11-1993) (continue)

Sample	Concentration (%)	Dilution water/ control	Bacterial growth test	Algal growth test	Water flea test		Fish test				Toad embryo teratogenicity test		Ames *Salmonella* mutagenicity test[1]			
			% Inhibition	% Inhibition	% Lethality after:		% Lethality after:				% Lethality	% Deformation	TA98-S9	TA98+S9	TA100-S9	TA100+S9
					24 h	48 h	24 h	48 h	72 h	96 h			Mutation ratio			
Effluent 2	100	Deionized water	**54***	**+48***	nt	nt	nt	nt	nt	nt	nt	nt	1,3	1,4	1,4	1,3
	50		**28***	**+52***	nt	nt	nt	nt	nt	nt	nt	nt				
	25		+1	nt	nt	nt	nt	nt	nt	nt	nt	nt				
	100	Moderately hard water	nt	**+7***	0	0	0	0	0	0	4	**33**				
	50		nt	**+2***	0	0	0	0	0	0	+1	6				
	100	Stream water	**39***	**+77***	nt	nt	nt	nt	nt	nt	6	**33**				
	50		**19***	**+20***	0	0	0	0	10	10	4	13				
	25		+6	nt	nt	nt	nt	nt	nt	nt	nt	nt				
Stream below	100	Deionized water	**56***	**+130***	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
	100	Moderately hard water	nt	**+67***	0	0	0	0	0	10	4	**12**				
	100	Stream water	**43***	**+174***	nt	nt	nt	nt	nt	nt	6	**12**				

* Precipitation - background reading subtracted
Bold Toxicity
[1] XAD resin extract tested
\+ Stimulation in growth/test survival >control survival
TA *Salmonella* tester strains
nt Not tested

Detection limits: bacterial growth inhibition, algal growth inhibition, water flea and toad embryo lethality tests - 10%; fish lethality test - >10%; toad embryo deformation test - 20%; mutagenicity test - MR 2,0

Blesbok Spruit had a strong flow and the water was clear. The water downstream of discharge, collected at the outflow of the Cowles Dam was clear and contained animal and plant life.

3.2.2.3 Metal plating effluent

The results of definitive tests on metal plating effluent are presented in Table 23. In general, the correlation between % effect and concentration was very good (R: 0,96-0,99). Linear regression could not be applied to the results of the urease test using 2,0 mg/mℓ enzyme and the toad embryo test, because toxicity was either undetectable or only one positive result was obtained. Although the EC_{10} was calculated for the algal test, the result is not considered valid because the lowest recorded effect (23%) was above the detection limit of 10%. As previously experienced (section 3.1), the 0,5 mg/mℓ enzyme concentration showed the highest sensitivity to the effluent. Furthermore, the water flea test was more sensitive after 48 h exposure. A comparison of EC_{50}/LC_{50} values showed that the algal and water flea tests were the most sensitive (EC/LC_{50}: 0,1%), followed in order of magnitude by the urease enzyme test (0,5 mg/mℓ enzyme EC_{50}: 0,2%; 1,0 mg/mℓ enzyme EC_{50}: 0,5%), the protozoan oxygen uptake test (EC_{50}: 2,1%) and the bacterial growth test (EC_{50}: 10,7%). Unfortunately a comparison with toad embryo lethality was not possible because no adverse effects exhibited at the selected concentration range (0,02-5,0%). Chronic toxicity (deformation) only occurred at the 5% effluent concentration. The effluent caused precipitation in the bacterial growth test at the 25 and 50% concentrations (OD: 0,076-0,306). In order to eliminate interferences in the results due to the large background readings, the bacterial suspensions at the high effluent concentrations and that of the control were also plated onto nutrient agar plates and the number of colonies counted after 24 h incubation at 37° C. The results showed that there was indeed a high percentage inhibition at the high effluent concentrations (100% effluent: 100% inhibition; 50% effluent: 100% inhibition; 25% effluent: 98% inhibition; 12,5% effluent; 17% inhibition). The effluent also interacted with the urease test at the upper concentrations which resulted in a milky suspension giving high density readings. Only concentrations where interaction did not occur were selected for data analysis.

Based on the results of the most sensitive test (algal EC_{10} and water flea LC_{10}: 0,02%) a dilution of 5 000x will be necessary to avoid acute effects on aquatic life. This dilution might be somewhat extreme because the results are expressed in terms of absolute toxicity (standard dilution water) and not in terms of relative toxicity (stream water).

Table 21 shows that the effluent had a low pH of 3,23. The effluent was blue-green and turbid, and had a rotten-fish smell. Table 24 shows an analysis of the chemical quality of the effluent. The effluent contained high levels of aluminium, cadmium, chromium, copper, iron, manganese, nickel, zinc and cyanide, which individually or in combination could have caused the high toxicity.

3.2.3 Effect of holding time on the toxicity of effluents

The effect of storage/holding time on the quality of effluent was investigated by testing effluent directly after sampling and at various times thereafter for a period of one week. The algal BG-11 medium and water flea tests were used to evaluate the effect of holding/storage time on the toxicity of effluents. This algal test was selected because, in general, growth in BG-11 medium was better than in AAM medium. The control results obtained during the evaluation

TABLE 23: Effect of metal plating effluent on selected biological toxicity tests (24-01-1994)

Concentration[1] (%)	Urease enzyme test			Concentration (%)	Bacterial growth test	Protozoan oxygen uptake test	Concentration (%)	Algal growth test	Water flea test		Toad embryo teratogenicity test	
	0,5 mg/ml	1,0 mg/l	2,0 mg/l						% Lethality after:			
	% Inhibition				% Inhibition	% Inhibition		% Inhibition	24 h	48 h	% Lethality	% Deformation
1,0	57	68	23	100	nt	100	5,0	100	100	100	8	98
0,5	81	46	0	50	74*	100	2,5	97	100	100	9	6
0,25	52	11	1	25	48*	96	1,25	100	90	100	0	12
0,13	26	5	1	12,5	46	90	0,63	100	75	95	0	9
0,06	14	0	4	6,25	45	47	0,31	95	25	80	4	5
nt	nt	nt	nt	3,13	42	31	0,16	71	15	70	+1	4
nt	nt	nt	nt	1,56	26	25	0,08	50	0	45	3	2
nt	nt	nt	nt	0,78	18	12	0,04	31	0	25	3	4
nt	nt	nt	nt	0,39	13	4	0,02	23	0	0	0	4
nt	nt	nt	nt	0,20	0	nt	nt	nt	nt	nt	nt	nt
R	0,99	0,97	nd	R	0,97	0,96	R	0,99	0,97	0,98	nd	nd
EC50	0,2	0,5	nd	EC50	10,7	2,1	EC50/LC50	0,1	0,4	0,1	nd	nd
EC10	0,1	0,1	nd	EC5/EC10	0,3	0,3	LC10/EC10/ EC20	0,02	0,1	0,02	nd	nd

* Precipitation - background reading subtracted
\+ Test survival >control survival
Bold Values used for linear regression
Shaded block - result not valid
nt Not tested
nd Not determined
R Correlation coefficient
EC Effective concentration
LC Lethal concentration
[1] Concentrations adjusted for dilution in enzyme, bacterial, protozoan and algal tests
Detection limits: oxygen uptake test - 5%; urease enzyme test, bacterial growth inhibition test, algal growth inhibition test, water flea lethality test and toad embryo lethality test - 10%; toad embryo deformation test - 20%

TABLE 24: Chemical quality of metal plating effluent

Determinand (mg/ℓ, except for *)	Effluent	Determinand (µg/ℓ)	Effluent
Electrical con. (mS/m)*	382	Silver (Ag)	50
Hardness ($CaCO_3$)	273	Aluminium (Al)	1136
Alkalinity ($CaCO_3$)	<5	Cadmium (Cd)	13778
Calcium (Ca)	58	Chromium (Cr)	11440
Magnesium (Mg)	31	Chromium [6]	<30
Nitrate-nitrite (N)	6,9	Copper (Cu)	7333
Sulphate (SO_4)	647	Iron (Fe)	94440
Total phosphate (PO_4)	1,7	Manganese (Mn)	1789
Ortho-phosphate (PO_4)	1,2	Nickel (Ni)	2311
Chloride (Cl)	380	Zinc (Zn)	205554
COD	170	Cyanide (free) (CN)	6704

TABLE 25: Control values of algal BG-11 medium test

Sample	Time delay before testing (days)	OD		
		Deionized water	Moderately hard water	Stream water
Treated sewage effluent	0	0,038	0,030	0,080
	2	0,075	0,093	0,162
	4	0,077	0,090	0,172
	7	0,116	0,128	0,244
Paper mill effluent	0	0,037	0,050	0,053
	2	0,049	0,053	0,056
	4	0,076	0,092	0,082
	7	0,074	0,069	0,055
Metal plating effluent	0	0,053	0,084	0,161
	2	0,030	0,049	0,063
	4	0,021	0,049	0,063
	7	0,058	0,063	0,157

TABLE 26: Influence of holding time on the effect of secondary treated sewage effluent and paper mill effluents on selected biological toxicity tests

Sample	Concentration (%)	Dilution water/ control	t=0 days			t=2 days			t=4 days			t=7 days		
			Algal growth test	Water flea test		Algal growth test	Water flea test		Algal growth test	Water flea test		Algal growth test	Water flea test	
			BG-11 medium	% Lethality after:		BG-11 medium	% Lethality after:		BG-11 medium	% Lethality after:		BG-11 medium	% Lethality after:	
			% Inhibition	24 h	48 h	% Inhibition	24 h	48 h	% Inhibition	24 h	48 h	% Inhibition	24 h	48 h
Secondary treated sewage effluent	100	Deionized water	+97*	nt	nt	+163*	nt	nt	+178*	nt	nt	+152*	nt	nt
	50		+94	nt	nt	+145	nt	nt	+145	nt	nt	+127	nt	nt
	100	Moderately hard water	+133*	0	0	+120*	0	0	+128*	0	0	+124*	0	0
	50		+173	0	0	+52	0	0	+107	0	0	+68	0	0
	100	River water[1]	+18[a]	nt	nt	+27*	nt	nt	+21*	nt	nt	+22*	nt	nt
	50		+15	0	0	+23	0	0	+20	0	0	+4	0	0
Effluent before primary clarification	100	Deionized water	19*	nt	nt	+105*	nt	nt	+67*	nt	nt	+80*	nt	nt
	50		+64*	nt	nt	+109*	nt	nt	+75*	nt	nt	+97*	nt	nt
	100	Moderately hard water	46*	60	65	+87*	0	0	+50*	0	0	+200*	0	0
	50		+38	0	0	+149	0	0	+78*	0	0	+126*	0	0
	100	Sprull water[2]	51*	nt	nt	+99*	nt	nt	+92*	nt	nt	+160*	nt	nt
	50		+19	0	0	+95	0	0	+101*	0	0	+169	0	0
Effluent after secondary clarification	100	Deionized water	+222*	nt	nt	+175*	nt	nt	+98*	nt	nt	+106*	nt	nt
	50		+165*	nt	nt	+168*	nt	nt	+76*	nt	nt	+104	nt	nt
	100	Moderately hard water	+126*	0	0	+155*	0	0	+91*	0	0	+116*	0	0
	50		+118	0	0	+149	0	0	+82	0	0	+117	0	0
	100	Sprull water[2]	+130	nt	nt	+163*	nt	nt	+147*	nt	nt	+206*	nt	nt
	50		+98	0	0	+134*	0	0	+105	0	0	+163	0	0

[a] Precipitation - background reading subtracted
[z] Water slightly stimulated algal growth or had no effect

+ Stimulation
Bold Toxicity
nt Not tested
* Water stimulated algal growth

Detection limits: algal growth inhibition test - 20%; water flea lethality test - >10%

are presented in Table 25. On a few occasions the growth in BG-11 medium was below the OD of 0,05, and therefore not satisfactory.

The treated sewage effluent sample collected for this study was not toxic to start with (Table 26). During the holding period the effluent remained this way, *e.g.* no toxicity was detected with algae or water flea. Algal growth was stimulated by the effluent. In general, stimulation was small when river water was used for dilution and control testing, or no effects were observed (<20%). Growth stimulation varied during the 7 day test period, probably because of different levels of growth in the controls (Table 25). After this evaluation several attempts were made to examine the effect of holding time on the quality of the treated sewage effluent, but on all occasions the effluent was not toxic.

Table 26 shows that the toxicity detected at the 100% concentration of paper mill effluent 1 on the first day of testing by the algal and water flea tests had disappeared by the time when the next tests (t=2 days) were conducted. The effluent stimulated algal growth on the following test dates and had no effect on water flea. Effluent 2, and the 50% dilutions of the two effluents, stimulated algal growth on all the occasions and showed no effect on water flea. The variation noted in the stimulation of algal growth was probably due to variation in control growth (Table 25). One more sample of paper mill effluent 1 was tested to examine the effect of holding time. Only the water flea test was applied and moderately hard water was used for control and dilution (Table 27). Effects were only exhibited by the 100% concentration. The results show that effects were very much the same until 2 days of storage. Thereafter, the toxicity decreased. However, even after 7 days storage a slight effect was still noticeable. It is possible that the effect in the first study (Table 26) was caused by a volatile chemical, therefore the quick reduction in toxicity.

TABLE 27: Influence of holding time on the effect of paper mill effluent 1 on water flea

Concentration[1]	t=0 days		t=2 days		t=4 days		t=7 days	
	% Lethality after:		% Lethality after:		% Lethality after:		% Lethality after:	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
100	40	40	35	60	10	20	10	20
50	0	0	0	0	0	0	0	0

[1] Moderately hard water used for control and dilution
Detection limit: water flea - 10%

The results obtained with metal plating effluent during the 7 day test period are shown in Table 28. For each set of results the correlation coefficient, LC_{50}/EC_{50} and LC_{10}/EC_{20} were calculated using regression analysis. The correlation between results was generally good (>0,9). Because of variation in responses, regression analysis could not be applied to the results of the algal test of t=4 days where deionized water was used for dilution and control testing.

TABLE 28: Influence of holding time on the effect of metal plating effluent on selected biological toxicity tests

Concentration[1] (%)	t=0 days					t=2 days					t=4 days					t=7 days				
	Algal growth test			Water flea test		Algal growth test			Water flea test		Algal growth test			Water flea test		Algal growth test			Water flea test	
	BG-11 medium			% Lethality after:		BG-11 medium			% Lethality after:		BG-11 medium			% Lethality after:		BG-11 medium			% Lethality after:	
	% inhibition			24 h	48 h	% inhibition			24 h	48 h	% inhibition[4]			24 h	48 h	% inhibition[7]			24 h	48 h
	DW[2]	MW[3]	SW[4]	SW		DW	MW	SW	SW		DW	MW	SW	SW		DW	MW	SW	SW	
10	nt	nt	nt	100	100	nt	nt	nt	100	100	nt	nt	nt	100	100	nt	nt	nt	70	95
5	71	58	63	100	100	76	85	80	100	100	100	100	83	50	85	80	83	80	20	40
2,5	67	41	51	50	95	45	79	69	60	100	95	90	85	0	50	75	79	77	0	0
1,25	60	26	33	10	55	45	54	66	40	85	81	57	67	0	0	64	49	65	0	0
0,63	64	15	17	0	10	38	35	26	0	10	71	+10	49	0	0	64	43	44	0	0
0,31	50	6	26	0	0	38	25	23	0	0	75	20	39	0	0	59	37	29	0	0
0,16	31	+10	11	nt	nt	23	20	+3	nt	nt	80	20	33	nt	nt	51	12	21	nt	nt
0,08	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	75	nt	nt	nt	nt	51	nt	nt	nt	nt
0,04	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	40	nt	nt	nt	nt	34	nt	nt	nt	nt
R[5]	0,90	1,00	1,00	nd	1,00	0,89	0,98	0,97	nd	0,93	nd	0,99	0,98	nd	1,00	0,97	0,97	0,98	nd	1,00
LC50/EC 50[6]	0,4	3,3	2,4	nd	1,2	1,3	0,8	1,0	nd	1,0	0,1-1,0	1,4	0,6	nd	2,7	0,1	0,8	0,7	nd	5,4
LC10/LC 20/EC20[7]	0,02	0,8	0,6	nd	0,6	0,1	0,2	0,3	nd	0,5	<0,1	0,9	0,1	nd	1,4	<0,1	0,2	0,1	nd	3,0
SW	+186	+94	nt	nt	nt	+178	+49	nt	nt	nt	+136	+86	nt	nt	nt	+245	+130	nt	nt	nt

\+ Stimulation
nt Not tested
[3] Moderately hard water
[6] Concentration causing 50% lethality/inhibition
Detection limits: algal growth test - 20%; water flea lethality test - 10%

Bold Values used for linear regression
nd Not determined
[4] Stream water
[7] Minimum effective concentrations

Shaded block - result not valid
[1] Concentrations adjusted for dilution in algal test
[5] Correlation coefficient
[2] Deionized water
[8] Test concentration ranged from 6,25 to 0,05%

In general, the dose-response curve for algae stretched over two log concentrations when deionized water was used for control testing and dilution, and in most cases effects below 30% were not recorded. A similar trend was observed with river water at t=4 days. The EC_{20} results obtained in these cases were, therefore, not valid. The results of the algal test showed relatively large variation during the 7 day test period (Table 28). For example, the EC_{50} when using deionized water was 0,4% when exposed immediately, 1,3% on t=2 days, and 0,1% on t=7 days (result of t=4 days omitted because of too much variation in effects). With moderately hard water as control and dilution water the EC_{50} was 3,3% on t=0 days, 0,8% on t=2 days, 1,4% on t=4 days and 0,8% on t=7 days. EC_{50}'s recorded for river water were 2,4% at t=0, 1,0% after 2 days of storage, 0,6% after 4 days and 0,7% after 7 days. No specific trend, *e.g.* a reduction in toxicity over time, could therefore be established with the algal test. The variation in control results during the 7 day test period is suspected to be the cause for the variation in toxicity (Table 25). The results of the water flea test indicated that until day 2 the toxicity of metal plating effluent remained very much the same (LC_{50}'s: 1,0-1,2%; LC_{10}'s: 0,5-0,6%). However, after storage of 4 days a reduction in toxicity of about 2-fold was noted (LC_{50}: 2,7%; LC_{10}: 1,4%), and after 7 days the reduction was even more pronounced (LC_{50}: 5,5%; LC_{10}: 3,0%).

3.2.4 Sampling procedure (grab/composite) and effluent toxicity

The quality of effluents varies, therefore, the correct sampling method is very important to obtain a complete picture of the degree and extend of toxicity. The water flea test was used to evaluate grab and composite samples taken from secondary treated sewage effluent and paper mill effluents, while the urease enzyme test was applied to metal plating effluent samples. The urease test was used in the latter case because the evaluation involved a massive amount of dilutions which could be tested more quickly by means of the enzyme test. Strictly spoken, sampling should be carried out over a 24 h period (*e.g.* in case of acute tests four grab taken 6 h apart or six samples taken 4 h apart). Because of problems in entering the facilities outside working hours sampling was restricted to a maximum period of 8 h. Grab samples were collected every 2 h in case of sewage effluent and paper mill effluent grab samples. Composite samples were prepared by combining four grab samples (fixed volume) taken every 30 min (total period: 1,5 h). Grab samples of metal plating effluent were taken every 2 or 3 h, while composite samples were continuously collected by means of a peristaltic pump over 2 or 3 h periods.

Table 29 presents the results of the evaluation of treated sewage effluent. Sampling started at 09h30 and was terminated at 15h00. Three of each of the samples was tested. The oxygen levels of the samples ranged from 4,2 to 4,8 mg/ℓ. Temperature and pH were not measured. The results show that none of the samples exhibited toxicity indicating that there was very little variation in effluent quality with reference to acute toxicity. Although the effluent is discharged directly into the receiving stream sewage works have a certain retention period within the system. It is, therefore, expected that the normal variations in quality will be small.

Paper mill effluent samples (Table 30) were collected from 09h30 to 15h30. Four grab samples and three composite samples were taken. The results show that most of the effluent 1 samples were toxic. Toxicity was primarily detected at the 100% concentration, but in some instances the samples were also toxic after dilution (50%). Grab as well as composite samples showed variation in composition. The first grab sample of the day (09h30) showed a low toxicity, and at the 100% concentration only (lethality after 48 h: 25%). The sample

TABLE 29: Effect of grab and composite secondary treated sewage effluent samples on water flea

Grab sample	Concentration (%)	t=0 h		t=2 h		t=4 h	
		% Lethality after:		% Lethality after:		% Lethality after:	
		24 h	48 h	24 h	48 h	24 h	48 h
	100	0	0	0	0	0	0
	50	0	0	0	0	0	0
Composite sample[1]	**Concentration (%)**	**t=0-1,5 h**		**t=2-3,5 h**		**t=4-5,5 h**	
		% Lethality after:		**% Lethality after:**		**% Lethality after:**	
		24 h	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**
	100	0	0	0	0	0	0
	50	0	0	0	0	0	0

[1] Sampled every 30 min (4 times)
Detection limit: water flea lethality test - 10%

TABLE 30: Effect of grab and composite paper mill effluent samples on water flea

Effluent 1	Grab sample	Concentration (%)	t=0 h		t=2 h		t=4 h		t=6 h	
			% Lethality after:		% Lethality after:		% Lethality after:		% Lethality after;	
			24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
		100	5	25	35	45	0	10	85	85
		50	0	0	25	25	0	0	45	60
	Composite sample[1]	Concentration (%)	t=0-1,5 h		t=2-3,5 h		t=4-5,5 h		-	
			% Lethality after:		% Lethality after:		% Lethality after:		-	
			24 h	48 h	24 h	48 h	24 h	48 h	-	-
		100	85	85	10	10	35	55	-	-
		50	5	5	0	0	10	10	-	-
Effluent 2	Grab sample	Concentration (%)	t=0 h		t=2 h		t=4 h		t=6 h	
			% Lethality after:		% Lethality after:		% Lethality after:		% Lethality after:	
			24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
		100	0	0	0	0	0	0	0	0

[1] Sampled every 30 min (4 samples)
Detection limit: water flea lethality test - 10%

collected two hours later at 11h30 was slightly more toxic showing 45% and 25% lethality at the 100% and 50% concentrations, respectively, after 48 h exposure. The sample collected at 13h30 had no effect after 24 h exposure, and only a marginal effect after 48 h exposure (lethality: 10%) at the 100% concentration and no effect at the 50% concentration. The last sample of the day (15h30) showed a very high toxicity at both the 100 and 50% concentrations (85 and 60% lethality after 48 h exposure, respectively). The composite sample collected during the first 1,5 h (09h30-11h00) was highly toxic, but at the 100% concentration only (lethality after 48 h: 85%). Since this effect is considerably larger than those detected in grab samples at the beginning and end of the sampling period and since compositing of samples tends to reduce toxicity as a result of dilution, the result indicates high toxic peaks during the observation period. The composite sample collected during the period 11h30 to 13h30 showed a low toxicity (10% lethality after 48 h exposure to 100% effluent), suggesting the discharge of low concentrations of toxicants or very few peaks with a considerable amount of dilution. The last composite sample (14h00-15h30) showed a 55% lethality to water flea after 48 h exposure to 100% effluent. A marginal effect (10%) was observed at the 50% level. These results indicate the discharge of peak levels during the observation period. Figure 1 shows the variation in toxicity of the different samples. This effluent comes directly from the factory without any retention time, and results show just how much the variation in toxicity can be in such a situation.

Since effluent 2 has a 7 day retention period (Table 30), only grab samples were taken every 2 h. As was experienced with the treated sewage effluent, the samples were not toxic, indicating very little variation in quality as far as acute toxicity is concerned.

The pH of effluent 1 ranged between 6,17 and 6,46 and the temperature between 37,1 and 39,5° C. The oxygen content was always <2,0 mg/ℓ and could have contributed to the adverse effects on water flea. The pH of effluent 2 ranged between 6,53 and 7,08, the oxygen level between 5,7 and 5,9 mg/ℓ, and the temperature between 27,8 and 29,4° C.

Metal plating effluent samples were collected during the period 09h00 to 17h00. Four grab samples and three composite samples were taken. Definitive tests were carried out using concentrations ranging from 0,78 to 100%. Linear regression could not be applied to all the data sets because of too low/no toxicity in some instances. Where low effect levels were not observed (>10% inhibition), EC_{10}'s were not reported. Likewise, EC_{50}'s were not reported if effects were sufficiently above 50% were not detected. Tables 31 and 32 present the results. In general, the correlation between % effect and concentration was very good (R: 0,93-1,0). In all instances the best sensitivity was obtained with the 0,5 mg/mℓ enzyme concentration.

Table 31 and Figure 2 show that the first grab sample of the day (09h00) had a very low toxicity towards the urease enzyme. The second sample, collected at 12h00 was extremely toxic with an EC_{50} of 1,6% and an EC_{10} of 0,5%, when using 0,5 mg/mℓ enzyme. Although there was a slight decrease in the toxicity of the third and fourth grab samples, toxicity was still very high (14h00: EC_{50} - 3,7%, EC_{10} - 1,0%; 17h00: EC_{50} - 4,5%, EC_{10} - 1,6%, using 0,5 mg/mℓ enzyme), indicating a relatively constant load of toxicants throughout the afternoon. The first composite sample (09h00-12h00) (Table 32 and Figure 2) showed low toxicity with an EC_{10} of 16,6% with the 0,5 mg/mℓ enzyme concentration. This result implies that the load of toxicants was fairly low during the 3 h morning discharge period with few high peaks, and that discharge of toxicants probably started late in the morning. The composite sample collected during the next 2 h period (12h00-14h00) was three to four times more toxic than

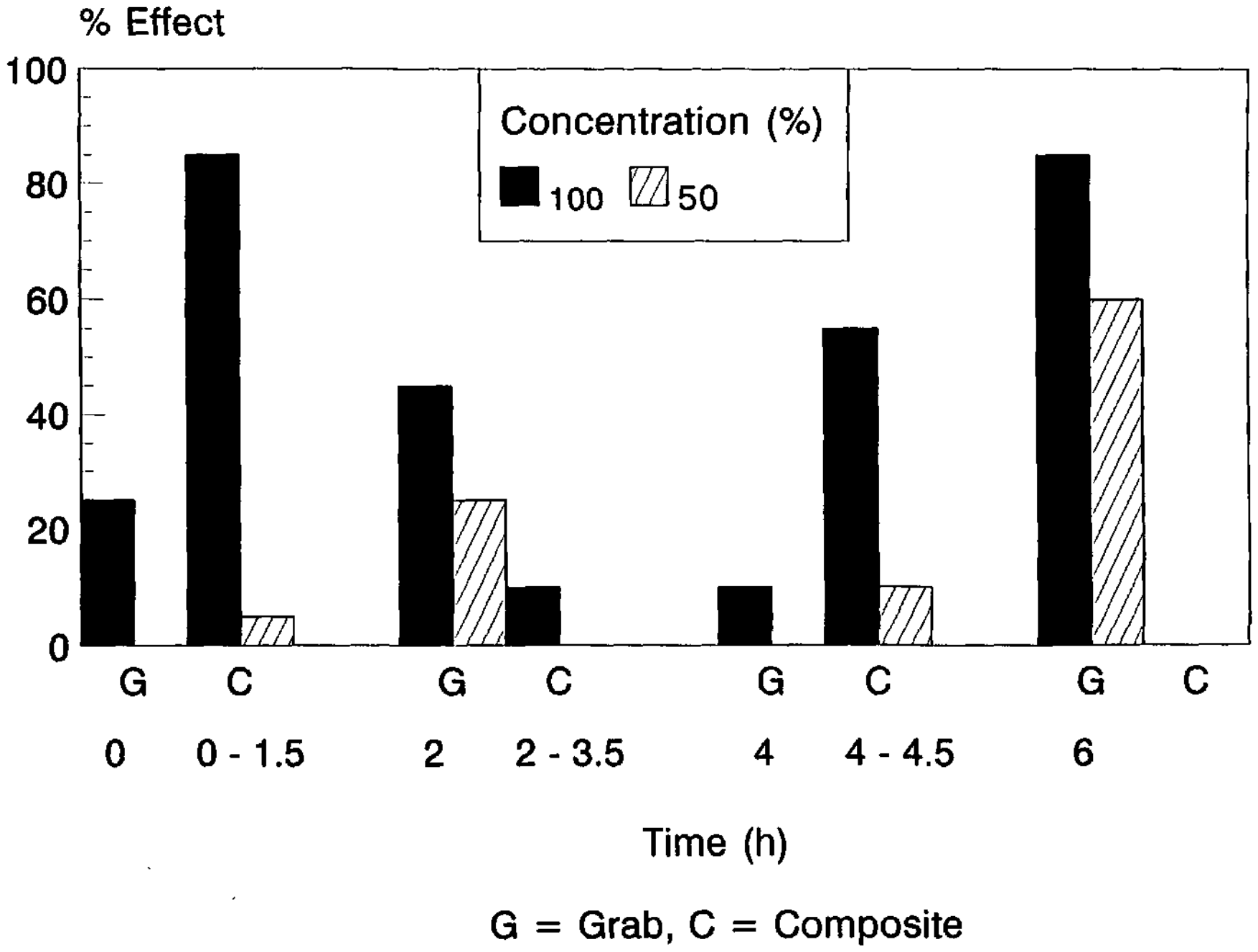

FIGURE 1: Effect of grab and composite paper mill samples on water flea

TABLE 31: Effect of grab samples of metal plating effluent on the urease enzyme test

Concentration[1] (%)	t=0 h			t=3 h			t=5 h			t=8 h		
	0,5 mg/mℓ	1,0 mg/mℓ	2,0 mg/mℓ	0,5 mg/mℓ	1,0 mg/mℓ	2,0 mg/mℓ	0,5 mg/mℓ	1,0 mg/mℓ	2,0 mg/mℓ	0,5 mg/mℓ	1,0 mg/mℓ	2,0 mg/mℓ
	% Inhibition			%Inhibition			% Inhibition			% Inhibition		
100	17	+10	0	61	30	+14	36	35	24	51	54	2
50	20	+8	+12	57	26	+20	65	59	**36**	61	42	+20
25	12	+8	6	84	**84**	**56**	77	65	**19**	51	25	+1
12,5	7	+3	11	84	**80**	12	**83**	**75**	**9**	**77**	**71**	14
6,25	26	0	2	**89**	**52**	**4**	**63**	**6**	+10	**66**	**25**	3
3,13	13	10	9	**60**	**16**	3	**29**	+2	8	**18**	**0**	6
1,56	0	3	0	**38**	**9**	2	**13**	4	8	**4**	+22	0
0,78	10	10	2	**24**	8	3	**2**	+1	0	3	+23	+1
R	nd	nd	nd	0,99	0,97	0,93	0,98	nd	0,99	0,97	0,99	nd
EC50	nd	nd	nd	1,6	5,3	20,0	3,7	7,8	86,5	4,5	7,1	nd
EC10	nd	nd	nd	0,5	1,5	6,9	1,0	5,2	11,2	1,6	3,3	nd

\+ Stimulation
Shaded block - result not valid
R Correlation coefficient
[1] Concentrations adjusted for dilution in enzyme test

Bold Values used for linear regression
nd Not determined
EC Effective concentration
Detection limits: urease enzyme test - 10%

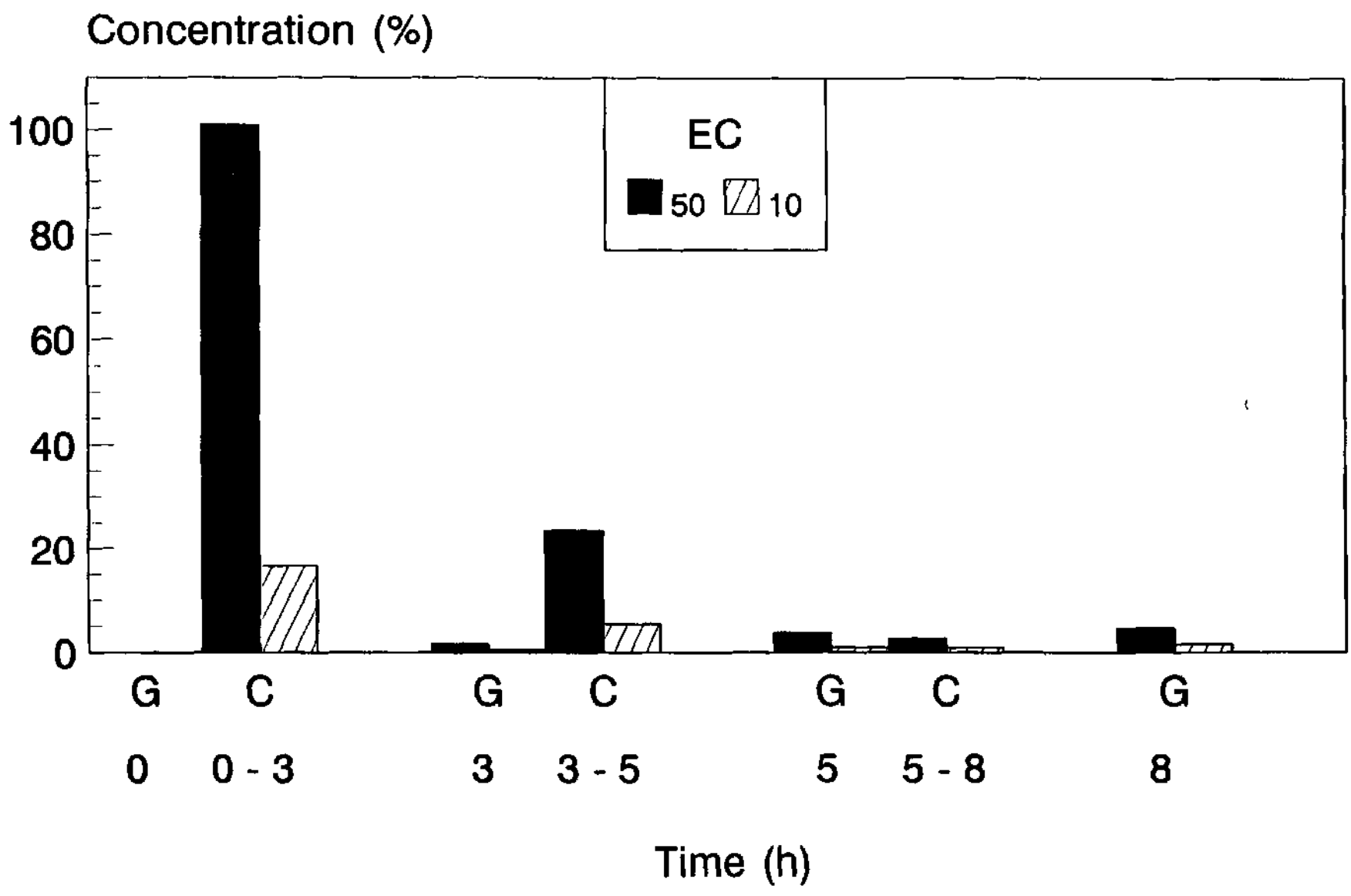

FIGURE 2: Effect of grab and composite metal plating effluent samples on 0.5 mg/ml urease

TABLE 32: Effect of composite samples of metal plating effluent on the urease enzyme test[1]

Concentration[2] (%)	t=0-3 h			t=3-5 h			t=5-8 h		
	0,5 mg/mℓ	1,0 mg/mℓ	2,0 mg/mℓ	0,5 mg/mℓ	1,0 mg/mℓ	2,0 mg/mℓ	0,5 mg/mℓ	1,0 mg/mℓ	2,0 mg/mℓ
	% Inhibition			%Inhibition			% Inhibition		
100	**46**	20	+29	40	**63**	**43**	+96	+36	31
50	**28**	2	+26	**59**	**55**	**10**	+41	53	+2
25	**13**	+2	+11	**52**	**25**	**3**	74	73	**61**
12,5	**0**	+16	+18	**29**	**0**	+12	81	**77**	**4**
6,25	+16	+20	+22	**4**	+16	+12	**80**	**30**	+28
3,13	16	2	+18	2	0	+18	**43**	**10**	+7
1,56	13	+9	+19	+5	+6	+8	**16**	10	+4
0,8	2	+11	+16	+9	+4	+19	**0**	2	+14
R	1,0	nd	nd	0,98	0,98	0,94	0,99	0,97	nd
EC50	100,9	nd	nd	23,6	44,2	nd	2,6	6,3	17,5
EC10	16,6	nd	nd	5,4	12,5	29,5	0,9	2,8	10,8

\+ Stimulation
nd Not determined
EC Effective concentration
[1] Continuous sampling
Detection limits: urease enzyme test - 10%

Bold Values used for linear regression
R Correlation coefficient

[2] Concentrations adjusted for dilution in enzyme test

the first sample with a 0,5 mg/mℓ enzyme EC_{50} of 23,6% and an EC_{10} of 5,4%. The observation that the composite sample was not as toxic as the grab samples collected during this period indicates the intermittent discharge of high toxic peaks. The third sample of the day, collected over a 3 h period from 12h00 to 17h00, was highly toxic with a 0,5 mg/mℓ EC_{50} of 2,6% and an EC_{10} of 0,9%. These values are similar to those of the grab samples collected at 14h00 and 17h00 and imply that the concentrations of toxicants were fairly constant during the afternoon discharge period with very few peaks.

The pH's of the grab samples ranged from 7,56 to 9,93, indicating that pH could have played a role in toxicity. The composite samples had pH's ranging between 7,92 and 8,7. The oxygen content of the samples ranged from 6,8 to 8,8 mg/ℓ, with the oxygen in composite samples at the upper concentration range.

4. CONCLUSIONS AND RECOMMENDATIONS

All the effluents evaluated in this study exhibited toxicity. In general, the paper mill effluent after secondary clarification was the least toxic, followed in order of magnitude by secondary treated sewage effluent, paper mill effluent before primary clarification, and metal plating effluent. Paper mill effluent after secondary clarification and treated sewage effluent were mostly only toxic at the 100% concentration. Paper mill effluent before primary clarification showed toxicity at concentrations ranging from 10 to 100%. The metal plating effluent was highly toxic and effective concentrations ranged between 0,01 and 10%.

The effluents were mostly acutely toxic, causing lethal and/or sub-lethal effects (*e.g.* growth inhibition). However, chronic effects were also detected. On occasion, all the effluents showed some degree of teratogenicity (deformation of toad embryos). Mutagenicity was exhibited only once, namely by paper mill effluent after secondary clarification. The mutation ratios were similar to those occasionally detected in environmental waters.

Range finding tests showed that the fish, water flea, algal, bacterial and toad embryo tests were the most sensitive for the detection of adverse chemical activity in secondary treated sewage effluent and paper mill effluent 1. Algal and bacterial growth inhibition tests, the toad embryo test, and the mutagenicity test detected adverse chemical activity in paper mill effluent 2. All the tests, except the mutagenicity test, were sufficiently sensitive to screen metal plating effluent for adverse chemical activity. The algal test generally appeared to be the most sensitive to metal plating effluent. Based on the results of the range finding tests the fish and water flea lethality tests and the algal growth inhibition test were selected for extensive toxicity testing of effluents.

In some instances the receiving water used for dilution of effluents showed toxicity, *e.g.* the Small Blesbok Spruit. Acute as well as chronic toxicity (teratogenicity) was detected. Toxicity was exhibited during range finding and extensive toxicity testing. Usually, the dilution water should be free of toxicants. This implies that when toxicity is present, stream water should not be used for dilution purposes. However, this water should be used if it is the objective to establish the contribution of the effluent to the receiving water.

The negative results obtained with the acetylcholinesterase enzyme test indicated that organophosphate and carbamate pesticides were absent from effluents and river/stream water. The urease test showed positive results with metal plating effluent and the Small Blesbok Spruit water, indicating the presence of heavy metals.

During extensive toxicity testing effective concentrations could not be established for treated sewage effluent and paper mill effluents because effluents were either not toxic or toxicity was only detected at the 50 or 100% concentrations. For these effluents results were expressed as a percentage effect. It is possible to present such results as follows: EC_{50} >100%.

Effective concentrations for metal plating effluent were established by means of definitive tests. Inherent toxicity (using standard dilution water) was usually larger than the relative toxicity (using receiving water for dilution). In general, the algal test was found to be the most sensitive to the effluent. Based on the minimum effective concentration (EC_{10}/LC_{10}/EC_{20}/LC_{20}) of the most sensitive test, dilutions were established for the effluent to avoid acute effects on aquatic organisms. These dilutions ranged from 250 to 500 times.

Many of the tests showed enhanced activity when exposed to the effluent/water samples. This is usually attributed to nutrients in the water. In the case of the urease test higher density readings could have been due to precipitation rather than increased activity.

Treated sewage effluent and paper mill effluent caused precipitation in the algal and bacterial growth inhibition tests. Paper mill effluent 1 generally showed the largest background reading, which interfered with the interpretation of results. Because of the possibility of precipitate formation, these tests are not compatible with all effluents.

The study on effluent variability showed that treated sewage effluent was toxic during the period August to October 1992, but no toxicity was detected from February to June 1993, implying that seasonal changes could have influenced the effluent quality. With the paper mill effluent no clear pattern due to seasonal changes could be observed. The paper mill effluent showed toxicity during the period August to October 1992 and also in February 1993. The samples collected during May 1993 were not toxic. Toxicity was again detected in paper mill effluent 1 during June 1993. The Small Blesbok Spruit water was also toxic during the August-October 1992 sampling period until February 1993. Hereafter, no toxicity was detected, indicating that the quality possibly improved due to rain. The metal plating effluent was not influenced by seasonal changes. The effluent showed considerable variation.

No conclusions could be made on the effect of holding time on the quality of sewage effluent and paper mill effluent 2 because no toxicity was detected on the first day of testing. The results obtained with paper mill effluent 2 showed that on one occasion there was a quality change during the first 48 h of holding, rendering the sample not toxic. A second sample showed that toxicity remained the same for at least 48 h. The toxicity of the first sample was probably caused by a volatile chemical. Toxicity tests with water flea showed that the toxicity of metal plating effluent started to decrease after a holding period of 4 days. The findings indicate that it is important that toxicity tests should be carried out as soon as possible, preferably within 24 h, after sampling.

No short-term variation in acute toxicity could be established in treated sewage effluent and paper mill effluent 2, when grab and composite sampling methods were used (over a 6 h period) and all samples tested negative for toxicity. Grab sampling will probably be the most suitable for these effluents (for acute testing) because there is already a retention period involved. As short-term variation is improbable, the testing frequency could have a wider spread (once per week or once per month). Paper mill effluent 2 and metal plating effluent showed a large variation in quality (6 to 8 h observation period). Grab samples showed high peaks of toxicity at certain times of the day. Some composite samples showed low toxicity, indicating that dilution by less toxic effluent took place. In some instances, composite samples were very toxic, suggesting that high levels of toxicants were discharged at a continuous rate. These results showed that effluents with high variability should preferably be subjected to retention facilities. For these type of samples grab sampling will be the most suitable for acute testing. For chronic testing a composite sample taken over for example a 24 h period will be most suitable.

On one occasion, all the tests showing toxic responses during range finding tests were applied to establish effective concentrations and to compare sensitivity. As was previously found with treated sewage effluent, effective concentrations could not be established by means of linear regression because toxicity was absent or very low. Only the toad embryo

test showed adverse chemical activity in 100% treated sewage effluent (lethality and teratogenicity). Paper mill effluent 1 affected bacterial growth, killed water flea and fish, and showed teratogenicity (50 and 100% effluent). Paper mill effluent 2 inhibited bacterial growth and caused teratogenicity (50 and 100% effluent). None of the samples were mutagenic. Metal plating effluent showed the highest toxicity to algae, followed in order of magnitude by water flea, urease enzyme, protozoa, bacteria and toad embryos. Fish were not used in this evaluation. Teratogenicity was also detected. Based on algal sensitivity, a dilution of 5 000 times was necessary in this instance to avoid acute toxicity in receiving water.

Tests were also applied to receiving water and water sampled downstream of discharge (ambient water). As was previously found, receiving water showed some degree of toxicity. The ambient water downstream of paper mill discharge showed toxicity with the bacterial growth inhibition test. Because of low toxicity in case of ambient waters, chronic aquatic tests would be more applicable.

Chemical analyses of the effluents indicated that, in general, potentially toxic chemicals in treated sewage effluent and paper mill effluents were low, indicating that effects were probably due to combined toxicity. The paper mill effluents occasionally showed low pH levels and oxygen content which could have contributed to adverse activity. The metal plating effluent contained high levels of a variety of metals and cyanide which individually or in combination could have caused the adverse effects. The Small Blesbok Spruit water showed a presence of manganese, cyanide and phenol, and occasionally low oxygen levels, which could have been the result of toxicity.

Most of the biological tests used in the study are well established and optimized. Problems were, however, occasionally experienced with the algal test. In many instances algal growth was insufficient, particularly when using AAM medium. The large variation in algal growth was probably due to variation in illumination and attention should be given to standardization. Although most of the fish tests were carried out with 10 fish, there were occasions when enough fish were not available. In some instances control fish showed lethality, indicating that the stock was not healthy. In order to ensure that the fish test is optimally used for WET testing proper facilities for maintenance and culturing will be required.

Although the algal AAM medium test was occasionally more sensitive than the BG-11 medium test, the BG-11 medium test appeared to be a wiser choice for future work because it gives better growth and shows less variation.

A good growth was obtained with moderately hard water when using BG-11 medium. However, results showed some variation. Because the test using deionized water was not properly standardized it was difficult to compare the efficiency of the two waters for inherent toxicity determination. It appears that it will be best to use deionized water, because this is the international approach.

Based on sensitivity, the fish and water flea lethality tests, and algal growth inhibition test are recommended for regulatory and management purposes of effluents. These tests are also reproducible, simple and relatively cost effective. These test also have the advantage that they are similar to standard effluent tests carried out in other countries.

Some of the rapid microbial and enzyme tests could be valuable screening tools to identify and categorize toxic effluents. Most of the tests are, however, not sufficiently sensitive to test effluents with low toxicity.

In addition to the recommended battery of acute tests, mutagenicity and teratogenicity tests should also be used if water downstream of discharge is used for drinking water purposes.

The study has indicated that there is a great need for short-term aquatic chronic tests. These are particularly needed in case of effluents with low toxicity and for ambient water testing. The establishment of tests using water flea are under way. However, urgent attention should be given to the development of chronic tests using fish.

Additional chronic tests for human health protection are also needed, particularly because the Ames test is not sensitive to metal containing effluents.

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APPENDIX A

CHEMICAL EQUILIBRIUM MODELLING INVESTIGATION TO EXPLAIN BIOLOGICAL TOXICITY TEST RESPONSES

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March 1996

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Summary of results

The chemical speciation of growth media

(a) The protozoan and bacterial growth media did not exhibit any interesting chemical behaviour.

(b) The DME and MEM media are supersaturated with respect to several solids. The DME medium is supersaturated with respect to carbonate, phosphate and hydrous ferric oxide solids. The MEM medium is supersaturated with respect to carbonate and phosphate solids.

(c) Both algal growth media are supersaturated with respect to several solids. Of these, hydrous ferric oxides are the most highly supersaturated. Precipitation of the hydrous ferric oxide solids will lead to a loss of iron from solution. A secondary effect may be that copper, zinc and other essential trace elements will adsorb onto the solids, which will effectively decrease their bioavailability to the test organisms.

(d) The speciation of copper and zinc is dominated by metal - EDTA complexes in the AAM medium. The effect of this may be that these essential trace elements are no longer available to the test organisms. This may explain the higher control growth observed in the BG-11 growth medium.

The chemical speciation of the effluents

(a) Both effluents investigated are supersaturated with respect to numerous solids. In general, hydrous ferric oxides are the most highly supersaturated, but other hydroxide, carbonate and phosphate solids are also supersaturated.

(b) Precipitation of the supersaturated solids may explain changes observed in effluent toxicity after prolonged storage periods. Toxic metals may be removed by the precipitation process or may adsorb onto hydrous ferric oxides. Both of these mechanisms will decrease the bioavailable/toxic metal fraction in the effluent.

The chemical speciation of effluent - growth media mixtures

(a) The differences in sensitivity observed between the two algal growth media may be explained by noting that the BG-11 - metal plating effluent mixture had a higher free metal abundance than the AAM - metal plating effluent mixture.

(d) The differences in sensitivity in the algal tests caused by the use of various dilution waters may be linked to the resultant free metal concentrations of the mixtures. Deionized water as dilution water gave rise to higher free metal concentrations than the other dilution waters investigated.

(c) The stimulation of algal growth in the AAM medium by paper mill effluent results from (i) the contribution of the paper mill effluent to the total concentrations of essential trace elements in the mixture and (ii) by causing an increase in the percentage (and thus concentration) free zinc and copper in the effluent - AAM mixture.

(d) The speciation of the protozoan and bacterial growth media - effluent mixtures will be dominated by effluent speciation. Thus, precipitates observed in these mixtures will be similar to that in the effluent.

(e) No chemical explanation can be found to explain the insensitivity of the bacterial test to the metal plating effluent.

Introduction

This document aims to shed more light on the chemical behaviour of bioassay test solutions. The overall question which is being addressed by this work is how does the chemical behaviour of the test media influence toxicity test results. Throughout, results obtained by Slabbert *et al.* (1996a,b) will be used. Two industrial effluents will be considered:

(a) a metal plating effluent and
(b) a paper mill effluent after secondary treatment.

The following specific questions are addressed:

(a) Why was the BG-11 medium more sensitive than the AAM medium in the metal plating tests?
(b) Why did the use of deionized water as dilution water give rise to higher sensitivity than the use of moderately hard (or *Daphnia*) water in the algal tests?
(c) Why did the BG-11 medium have higher control growth than the AAM medium?
(d) The metal plating effluent appeared to change with time. Why?
(e) Why was the bacterial growth test not very sensitive in the metal plating effluent?
(f) What precipitate forms in the algal and bacterial growth media?
(g) Why did the paper mill effluent stimulate algal growth compared to the AAM medium?

In order to answer these questions, chemical equilibrium modelling of the test systems were performed. Simulations were carried out using MINTEQ version 3.00 (Allison *et al.*, 1991). The structure of this documents is as follows: first, a general description of the chemical speciation of various growth media employed in bioassays is given. Second, the chemical speciation of the industrial effluents will be described, using one sample of each. Third, the chemical speciation of the effluent - bioassay media will be described. The questions listed above will be discussed in the course of this process.

The chemical speciation of growth media

This section describes the chemical speciation of selected bioassay media, as used in Slabbert *et al.* (1996a,b). The test systems described here are the protozoa test medium, the bacterial growth medium, the AAM and BG-11 algal growth media and the mammalian cell test medium.

Assumptions made in simulations:

* temperature of 25° C was specified.
* no solids were allowed to precipitate.
* no redox potential was specified and no redox reactions were allowed to occur.
* no pH was specified, so pH was calculated.

Chemical speciation of the protozoan test medium

The component concentrations used to simulate the chemistry of this test medium are shown in Table 1.

Table 1: Component concentrations used in simulating the protozoan cell test medium

Component	Concentration (mol/ℓ)
Ca^{2+}	6.8E-06
Mg^{2+}	5.78E-05
K^{+}	3.08E-05
Na^{+}	1.8E-03
Cl^{-}	1.9E-05
SO_4^{2-}	1.6E-05

Results

The results obtained from this simulation indicate that all the components are present in their free (or unbound) form in solution. No supersaturated solids are present.

Chemical speciation of the bacterial growth medium

Table 2 shows the component concentrations used in this simulation.

Results and discussion

The % distribution of the chemical species present in the bacterial growth medium are shown in Table 3. No supersaturated solids were present.

Table 2: Component concentrations used in simulating the bacterial growth medium

Component	Concentration (mol/ℓ)
H^+	1.264E-05
K^+	1.536E-05
PO_4^{3-}	9.335E-06
Na^+	5.464E-08
Citrate	1.821E-07
NH_4^+	1.515E-07
SO_4^{2-}	1.163E-07
Mg^{2+}	4.059E-08

Table 3: Relative species abundance in the bacterial growth medium at equilibrium

Component	% Distribution	
K^+	K^+	[100]
PO_4^{3-}	HPO_4^{2-} $H_2PO_4^-$	[62] [38]
Na^+	Na^+	[100]
Citrate	Citrate HCitrate	[93] [7]
NH_4^+	NH_4^+ NH_3	[99] [1]
SO_4^{2-}	SO_4^{2-}	[100]
Mg^{2+}	Mg^{2+}	[100]

Chemical speciation of the mammalian cell growth medium

Table 4 shows the component concentrations used in the simulations of the two media used in this test *viz.* Dulbecco's Modified Eagle's (DME) and the Minimum Essential Medium (MEM).

Table 4: Component concentrations used in simulating the DME and MEM media

Component	DME	MEM
H^+	4.61E-02	2.82E-02
Ca^{2+}	1.80E-03	1.80E-03
Cl^-	1.18E-01	1.25E-01
Fe^{3+}	2.48E-07	**
NO_3^-	7.40E-07	**
K^+	5.40E-03	5.36E-03
Mg^{2+}	8.10E-04	8.12E-04
Na^+	1.50E-01	1.43E-01
CO_3^{2-}	4.40E-02	2.62E-02
PO_4^{3-}	1.03E-03	1.01E-03

Results and discussion

The relative abundance of species are shown in Table 5.

The calculated pH's for the two media are 7.7 (DME) and 7.6 (MEM) respectively. The aqueous speciation of the two media are similar. The MEM medium does not have iron and nitrate as components.

There is, however, a marked difference in the degree of supersaturation of the two media. The MEM medium is supersaturated mainly with respect to carbonate and phosphate solids. The DME medium is supersaturated with respect to carbonate, phosphate and hydrous ferric oxide solids. This implies that problems with precipitation are more likely to be experienced with the DME medium than with the MEM medium. A simulation in which the solids were allowed to precipitate indicated that hydroxyapatite will precipitate in the MEM medium, while dolomite, hydroxyapatite and hematite will precipitate from the DME medium.

The effect of the precipitation of these solids are that, in the DME medium, all iron is removed from solution. In the MEM medium, 89% of the calcium and 95% of the phosphate is removed. In both cases pH was also lowered by approximately 0.4 pH units.

Table 5: Relative species abundance in the DME and MEM media at equilibrium

Component	Species	% Species distribution	
		DME	*MEM*
Ca^{2+}	Ca^{2+}	83	88
	$CaHCO_3^+$	11	7
	$CaCO_3$	2	1
	$CaHPO_4$	3	3
Cl	Cl	100	100
CO_3^{2-}	$NaHCO_3$	4	4
	HCO_3^-	91	91
	H_2CO_3	3	4
NO_3^-	NO_3^-	100	**
K^+	K^+	100	100
Na^+	Na^+	99	100
	$NaHCO_3$	1	**
Fe^{3+}	$Fe(OH)_2^+$	61	**
	$Fe(OH)_3$	24	**
	$Fe(OH)_4^-$	15	**
PO_4^{3-}	$MgHPO_4$	3	4
	$NaHPO_4^-$	7	6
	HPO_4^{2-}	72	70
	$H_2PO_4^-$	10	13
	$CaHPO_4$	6	6
Mg^{2+}	Mg^{2+}	82	87
	$MgHCO_3^+$	12	8
	$MgCO_3$	2	**
	$MgHPO_4$	4	4

Chemical speciation of the algal growth media

The component concentrations used in these simulations are shown in Table 6. Although both media contain cobalt and molybdenum, these metals were not included in the simulations because of data unavailability. However, this is not expected to influence the dominating chemical processes to a large extent since these metals are present at very low

concentrations. Nevertheless, should data become available, they should be included in the simulations. pH for these media were not reported. Calculated pH's are 8 (AAM) and 7.2 (BG-11).

Table 6: Component concentrations used in simulating the AAM and BG-11 algal growth media

Component	Concentration (M)	
	AAM	BG-11
H^+	1.9E-04	5.6E-05
Ca^{2+}	3.0E-05	2.5E-05
Cl^-	1.6E-04	5.1E-05
Na^+	4.8E-04	1.8E-03
NO_3^-	3.0E-04	1.8E-03
K^+	1.2E-05	4.6E-05
PO_4^{3-}	6.0E-06	2.3E-05
Mg^{2+}	1.1E-04	3.0E-05
SO_4^{2-}	5.9E-05	3.1E-05
CO_3^{2-}	1.8E-04	2.4E-05
H_3BO_3	3.4E-07	4.6E-06
Mn^{2+}	2.1E-06	9.1E-07
Cu^{2+}	8.9E-11	3.2E-08
Zn^{2+}	2.4E-09	7.7E-08
Fe^{3+}	5.7E-07	2.3E-06
$EDTA^{4-}$	8.1E-07	2.7E-07
Citrate	**	5.1E-06
NH_4^+	**	2.3E-06

Results and discussion

Tables 7, 8 and 9 summarize the results from these simulations. Table 7 compares free (or uncomplexed) component concentrations in the two growth media. Table 8 compares the % distribution of species present in these solutions while Table 9 compares supersaturated solids present in the solutions.

The main points to note from Tables 7 and 8 are, first, the higher concentrations of free metal in the BG-11 medium and second, the domination of zinc and copper speciation by EDTA in the AAM medium. In this medium, copper and zinc occurs exclusively as EDTA complexes. This would limit their availability to the test organisms as essential elements (Luoma, 1983; Fleming and Trevors, 1989). Copper and zinc speciation in the BG-11 medium are not dominated by EDTA. In this medium, uncomplexed (or free) metal species together with the metal citrate complexes predominates. Thus, the higher growth rates reported by Slabbert may be explained by the higher abundance of bioavailable species to algae in the BG-11 medium.

Table 7: Equilibrium concentrations of components in the two algal growth media

Component	Concentration (M)	
	AAM	BG-11
H^+	9.821E-09	6.653E-05
Ca^{2+}	2.925E-05	2.251E-05
Cl^-	1.642E-04	5.078E-05
Na^+	4.800E-04	1.789E-03
NO_3^-	3.000E-04	1.765E-03
K^+	1.199E-05	4.591E-05
PO_4^{3-}	2.764E-10	1.105E-10
Mg^{2+}	1.074E-04	3.003E-05
SO_4^{2-}	5.842E-05	3.012E-05
CO_3^{2-}	9.536E-07	1.802E-08
H_3BO_3	3.186E-07	4.583E-06
Mn^{2+}	2.071E-06	7.126E-07
Cu^{2+}	9.330E-17	8.676E-10
Zn^{2+}	3.991E-13	2.655E-08
Fe^{3+}	2.009E-20	4.596E-15
$EDTA^{4-}$	5.298E-15	3.117E-20
NH_4^+	**	2.262E-06
Citrate	**	2.675E-06

The AAM medium is supersaturated with respect to nine solids, while the BG-11 medium is supersaturated with respect to 11 solids. An indicator for the degree of supersaturation of a solid is the logarithm of the saturation index, denoted by log SI. A log SI greater than zero

Table 8: Comparison of relative species abundance in AAM and BG-11 algal growth media

Component	Species	% Species distribution	
		AAM	BG-11
Ca^{2+}	Ca^{+2} CaCitrate	98 **	92 8
Cl^-	Cl^-	100	100
Na^+	Na^+	100	100
$NO3^-$	NO_3^-	100	100
K^+	K^+	100	100
PO_4^{3-}	$MgPO_4^-$ $MgHPO_4AQ$ HPO_4^{2-} $H_2PO_4^-$	1 5 81 11	** ** 53 46
Mg^{2+}	Mg^{2+}	99	99
$SO4^{2-}$	SO_4^{2-} $MgSO_4$ AQ	98 1	99 **
CO_3^{2-}	HCO_3^- H_2CO_3 AQ	97 2	88 12
H_3BO_3	H_3BO_3 $H_2BO_3^-$	94 6	99 **
Mn^{2+}	Mn^{2+} MnCitrate	99 **	78 22
Cu^{2+}	Cu^{2+} $Cu(OH)_2$ CuEDTA CuCitrate	** ** 100 **	3 12 12 73
Zn^{2+}	Zn^{2+} ZnEDTA ZnCitrate	** 100 **	34 ** 63

Component	Species	% Species distribution	
		AAM	BG-11
Fe^{3+}	$Fe(OH)_2^+$	**	72
	$Fe(OH)_3$	**	13
	$Fe(OH)_4^-$	**	2
	FeEDTA	40	9
	FeOHEDTA	59	2
$EDTA^{4-}$	CaEDTA	27	**
	MgEDTA	2	**
	CuEDTA	**	1
	FeEDTA	28	19
	FeOHEDTA	42	19
Citrate	Citrate	**	52
	HCitrate	**	6
	CaCitrate	**	34
	MnCitrate	**	4
	MgCitrate	**	2
NH_4^+	NH_4^+	**	100

Table 8 continued

for solid X indicates that the solution is supersaturated with respect to solid X. The degree of supersaturation in the BG-11 medium is higher than the AAM medium since the log SI's calculated for the BG-11 medium is greater than those for the AAM medium.

Most of the supersaturated solids present are hydrous ferric oxides (HFO's). The only exceptions are the phosphate minerals hydroxyapatite, $MnHPO_4$ and strengite. The phosphate minerals are, however, not very highly supersaturated (log SI close to 0). The hydrous ferric oxides are highly supersaturated (high log SI). A characteristic of the supersaturated HFO's are their reddish brown-yellow colour. This may be used as an indication of their presence in solution.

The effect of these solids on toxicity test results will depend on the methodology followed for test sample preparation. If, for instance, the growth media is made up and then filtered through a 0.45μm (or smaller) filter, most of the precipitates will be removed. Otherwise, precipitates which form may influence metal toxicity through adsorption mechanisms. Adsorption of the toxic metals on the HFO's will effectively reduce the toxicity of a sample simply because adsorbed metal is not available to the test organism.

Recommendations

Based on the simulation results, it may be recommended that the use of lower concentrations of the various constituents in the test media should be investigated. At the present concentrations used, precipitation of various solids may be expected, which would effectively remove elements from solution. These removed elements will not be available to the test organisms and although no adverse effects may be caused by this, no benefit will arise either. At the very least, using less concentrated solutions will save reagents.

Table 9: Supersaturated solids present at equilibrium in the two algal growth media

Solid	Log SI	
	AAM	BG-11
Ferrihydrite	**	2.2
$Fe(OH)_{2.7}Cl_{0.3}$	3.728	6.6
Hydroxyapatite	0.156	**
Hematite	12.477	18.1
Maghemite	2.083	7.7
Goethite	3.735	6.6
Cupriferrite	2.544	13.4
$MnHPO_4(C)$	1.935	1.8
Magnesiumferrite	3.720	7.1
Lepidocrocite	2.864	5.7
Strengite	**	1.7

The chemical speciation of the effluents

This section describes the chemical speciation of the industrial effluents tested by Slabbert *et al.* (1996a). The effluent speciation is discussed in terms of aqueous speciation and potential solids which may form.

Chemical speciation of the metal plating effluent

This effluent was sampled on 7 occasions. Results for the samples of 92/10/12 and 93/06/10 were used for equilibrium simulations. Both of these samples had very high metal concentrations. The analyses of both these samples were reasonably complete, although alkalinity was not measured in the first sample. It was thus not possible to obtain an estimate of carbonate content in that sample. The simulations were carried out with pH fixed at the values measured on site.

Results and discussion

Table 10 shows the predominant dissolved species present in the two metal plating effluent samples. Supersaturated solids are shown in Table 11.

The major difference between the speciation of the two metal plating effluents lies in the free metal and free cyanide abundance. The effluent sample (92/10/12) with lower pH has a higher abundance of free (or uncomplexed) metal ions. Examples of this are nickel, zinc, iron, copper and aluminium. Comparing cyanide speciation for the two samples, it is clear that the low pH sample contains cyanide as HCN exclusively.

Slabbert noted a change in effluent quality with time, *i.e* toxicity changed as the effluent stood for a prolonged period. This may be explained by noting that in both of the simulations carried out with the metal plating effluent, a substantial amount of supersaturated solids were present. These are shown in Table 11. The most highly supersaturated solids were hydrous ferric oxides. Thus, if the effluent sample was allowed to stand, these solids would have time to form. This would lead to toxic metals adsorbing onto these solids. The effect will thus be an effective decrease in toxicity, since the adsorbed metals will not be available to test organisms.

Chemical speciation of the paper mill effluent after secondary clarification

Only the paper mill effluent after secondary clarification will be examined since this is the final effluent discharged into the environment. The analytical results for the sample taken on 93/05/10 will be used in this simulation. In this simulation, the relatively high degree of dissolved organic carbon will introduce some uncertainty. This follows from the fact that an estimation of the metal binding capacity of the DOC was not determined as part of the current study. If an estimate of the metal binding capacity was available, the effect of DOC on the effluent speciation could be determined.

The simulation was carried out at a pH of 7.7, which was measured upon sampling.

Table 10: Relative species abundance in metal plating effluent samples at equilibrium

Component	% Species distribution		
	Species	(92/10/12)	(93/06/10)
Ca^{2+}	Ca^{2+}	99	86
	$CaSO_4$	**	14
Mg^{2+}	Mg^{2+}	99	87
	$MgSO_4$	**	12
NH_4^+	NH_4^+	100	98
	$NH_4SO_4^-$	**	2
NO_3^-	NO_3^-	100	100
PO_4^{3-}	$FeH_2PO_4^{2+}$	56	**
	HPO_4^{2-}	**	4
	$H_2PO_4^-$	32	91
	H_3PO_4	11	**
	$MgH_2PO_4^+$	**	2
	$CaH_2PO_4^+$	**	1
Cl	Cl	100	100
Al^{3+}	Al^{3+}	100	7
	$AlOH^{2+}$	**	19
	$Al(OH)_2^+$	**	46
	$Al(OH)_3$	**	23
	$Al(OH)_4^-$	**	1
	$AlSO_4^+$	**	4
Cd^{2+}	Cd^{2+}	65	62
	$CdCl^+$	36	19
	$CdCl_2$	1	**
	$CdHCO_3^+$	**	5
	$CdSO_4$	**	14
$Cr(OH)_2^+$	Cr^{3+}	97	2
	$Cr(OH)^{2+}$	2	46
	$Cr(OH)_2^+$	**	35
	$Cr(OH)_3$	**	1
	$CrOHSO_4$	**	16
Cu^{2+}	Cu^{2+}	98	65
	$CuCl^+$	2	**
	$CuCO_3$	**	4
	$CuHCO_3^+$	**	20
	$CuSO_4$	**	10

Component	% Species distribution		
	Species	(92/10/12)	(93/06/10)
Fe^{3+}	Fe^{3+}	22	**
	$FeOH^{2+}$	90	90
	$Fe(OH)_2^+$	2	**
	$FeCl_2^+$	3	**
	$FeH_2PO_4^{2+}$	44	**
	$CaFe(CN)_6^-$	**	1
	$Fe(CN)_6^{3-}$	**	7
Ni^{2+}	Ni^{2+}	99	70
	$NiCl^+$	1	**
	$Ni(CN)_2$	**	1
	$Ni(CN)_3^-$	**	1
	$NiH(CN)_4^-$	**	3
	$NiCO_3$	**	6
	$NiHCO_3^+$	**	6
	$NiSO_4$	**	11
Zn^{2+}	Zn^{2+}	98	78
	$ZnCl^+$	2	**
	$ZnHCO_3^+$	**	6
	$ZnSO_4$	**	14
CN^-	$Ni(CN)_2$	**	2
	$CaFe(CN)_6^-$	**	8
	$Fe(CN)_6^{3-}$	**	52
	HCN	100	16
	$MgFe(CN)_6^-$	**	6
	$Ni(CN)_4^{2-}$	**	2
	$Ni(CN)_3^-$	**	3
	$NiH(CN)_4^-$	**	10
	NiH_2CN_4	**	1
Mn^{2+}	Mn^{2+}	98	86
	$MnCl^+$	2	1
	$MnHCO_3^+$	**	1
	$MnSO_4$	**	12
SO_4^{2-}	SO_4^{2-}	**	83
	$MgSO_4$	**	5
	$CaSO_4$	**	6
	$ZnSO_4$	**	3
CO_3^{2-}	HCO_3^-	**	18
	H_2CO_3	**	81

Table 10 continued

Table 11: Supersaturated solids present at equilibrium in the metal plating effluent

Solid	Log SI (92/10/12)	Log SI (93/06/10)
$Al_4(OH)_{10}SO_4$	**	4.16
Boehmite	**	1.69
$Fe(OH)_{2.7}Cl_{0.3}$	3.99	8.26
Goethite	1.83	7.12
Hematite	8.68	19.24
Strengite	1.76	4.79
Lepidocrocite	0.96	6.25
Aragonite	**	1.72
Ferrihydrite	**	2.73
Gibbsite	**	1.53
Maghemite	**	8.85
$MnHPO_4$ (crystalline)	**	2.16
Mag-Ferrite	**	6.54
Otavite	**	1.26
$Cr(OH)_3$ amorphous	**	2.13
Cr_2O_3	**	6.16

Results and discussion

Table 12 shows the predominant dissolved species in the effluent. Supersaturated solids present at equilibrium are shown in Table 13.

The species distribution shown in Table 12 will be altered by the presence of dissolved organic carbon. Although the change can not be quantified, it may be stated that the abundance of the species listed will decrease because DOC will compete with the inorganic ligands for the available heavy metals.

The paper mill effluent is supersaturated with respect to 19 solids. Again, most of the solids belong to the hydrous ferric oxide group. There are, however, also a few aluminium hydroxides, phosphate and carbonate solids present.

Table 12: Relative species abundance at equilibrium in the paper mill effluent after secondary clarification sampled on 93/05/10

Component	% Species distribution	
	Species	Paper Mill
Ca^{2+}	Ca^{2+} $CaHCO_3$ $CaSO_4$	84 2 13
Cl^-	Cl^-	100
Na^+	Na^+	100
NO_3^-	NO_3^-	100
K^+	K^+	100
PO_4^{3-}	$MgPO_4^-$ $MgHPO_4$ $CaHPO_4$ $CaPO_4$ HPO_4^{2-} $H_2PO_4^-$	1 11 22 3 51 12
Mg^{2+}	Mg^{+2} $MgHCO_3^+$ $MgSO_4$	86 3 11
SO_4^{2-}	SO_4^{-2} $MgSO_4$ $CaSO_4$	83 4 13
CO_3^{2-}	HCO_3^- H_2CO_3 $CaHCO_3^+$	94 4 1
Mn^{2+}	Mn^{2+} $MnSO_4$ $MnHCO_3^+$	84 11 4
Cu^{2+}	Cu^{2+} $Cu(OH)_2$ $CuHCO_3^+$ $CuCO_3$	2 51 2 44
Zn^{2+}	Zn^{2+} $Zn(OH)^+$ $ZnSO_4$ $ZnHCO_3$ $ZnCO_3$ $Zn(CO_3)_2^{2-}$	34 1 6 10 37 11
Fe^{3+}	$Fe(OH)_2^+$ $Fe(OH)_3$ $Fe(OH)_4^-$	55 29 16

Component	% Species distribution	
	Species	Paper Mill
Al^{3+}	$Al(OH)_4^-$ $Al(OH)_3$	85 15
NH_4^+	NH_4^+ NH_3 $NH_4SO_4^-$	96 2 1

Table 12 continued

Table 13: Supersaturated solids present at equilibrium in the paper mill effluent

Solid	Log SI (93/02/10)
$Al(OH)_3$	0.02
$Al_4(OH)_{10}SO_4$	0.58
Boehmite	1.83
$Fe(OH)_{2.7}Cl_{0.3}$	7.32
Goethite	6.89
Hematite	18.6
Hydroxyapatite	7.36
Lepidocrocite	6.02
Aragonite	0.36
Ferrihydrite	2.50
Gibbsite	1.63
Maghemite	8.40
$MnHPO_4$ (crystalline)	2.29
Magnesiumferrite	10.1
Calcite	0.50
Diaspore	3.53
Cupriferrite	16.1
Strengite	0.69
Dolomite	0.60

Chemical speciation of the growth media - effluent mixtures

This section describes the chemical speciation of the resulting solutions in the whole effluent toxicity test work carried out by Slabbert *et al.* (1996a). First, the speciation of the effluents used will be discussed. This will be followed by a discussion of the speciation of the resultant solutions when effluents and growth media are mixed for toxicity testing. The effects of different dilution waters will also be investigated.

Chemical speciation of the metal plating - algal growth media mixtures

In the toxicity tests using algae, 0.2 m*l* of 20x concentrated algal medium is added to 1.8 ml of effluent. The component concentrations were calculated using equation 1:

$$\frac{(c_j V_1 + c_j V_2)}{(V_1 + V_2)} = c_{final,j} \qquad (1)$$

where

c_j = concentration of component j
V_i = volume i added to mixture, i = 1.2
$c_{final,j}$ = final concentration of component j.

Only the metal plating sample taken on 93/06/10 will be used to describe the effect of the dilution water/growth media on effluent speciation.

Results and discussion

Table 14 shows the % Distribution of species in an undiluted effluent sample in the two algal growth media employed by Slabbert *et al.* (1996a). The speciation of the two resulting solutions are similar. There are, however, minor differences, particularly with respect to the free metal abundance for manganese, nickel, copper, zinc and cadmium. Of these metals, nickel, copper, zinc and cadmium are the most toxic. Thus, in the following discussion, only these metals will be considered. It should also be noted that the assumption that the free metal species is responsible for toxic effects is made throughout. This assumption is necessary since the identities of all species contributing to toxicity is not known. Available literature evidence (Sunda *et al.*, 1978; Luoma, 1983; Fleming and Trevors, 1989) as well as work done at CSIR (Pretorius *et al.*, 1994) does, however, make this assumption defensible.

Effects of using different dilution waters

The speciation of the metal plating effluent - Algal growth media using three different dilution waters are shown in Tables 15 (AAM) and 16 (BG-11). It was noted that tests carried out with deionized water as dilution water exhibited a higher sensitivity than tests where moderately hard water or receiving water was used. This question will thus be addressed here.

Table 14: Comparison of the speciation of the metal plating effluent with AAM and BG-11 medium added

Component	% Species distribution		
	Species	AAM	BG-11
Ca^{2+}	Ca^{2+} $CaSO_4$	87 13	88 11
Cl^-	Cl^-	100	100
Na^+	Na^+	100	100
NO_3^-	NO_3^-	100	100
K^+	K^+	100	100
PO_4^{3-}	$MgH_2PO_4^+$ HPO_4^{2-} $H_2PO_4^-$ $CaH_2PO_4^+$	2 4 90 1	1 4 91 1
Mg^{2+}	Mg^{2+} $MgSO_4$	88 11	90 10
SO_4^{2-}	SO_4^{2-} $MgSO_4$ $CaSO_4$ $ZnSO_4$	83 5 6 3	83 1 5 3
H_3BO_3	H_3BO_3	100	100
Mn^{2+}	Mn^{2+} $MnSO_4$ $MnCl^+$	87 12 1	89 10 **
Cu^{2+}	Cu^{2+} $CuHCO_3$ $CuSO_4$	84 2 13	87 ** 11
Zn^{2+}	Zn^{2+} $ZnSO_4$	84 14	86 13

Component	% Species distribution		
	Species	AAM	BG-11
Fe^{3+}	$Fe(OH)_2^+$	90	86
	$Fe(CN)_6^{3-}$	6	7
	$CaFe(CN)_6^-$	1	**
	FeHCitrate	**	4
$EDTA^{4-}$	NiEDTA	1	1
	FeEDTA	98	98
Al^{3+}	Al^{3+}	7	8
	$AlOH^{2+}$	19	20
	$Al(OH)_2^+$	46	45
	$Al(OH)_3$	23	22
	$Al(OH)_4^-$	1	1
	$AlSO_4^+$	4	3
Cd^{2+}	Cd^{2+}	66	69
	$CdCl^+$	19	18
	$CdSO_4$	14	12
$Cr(OH)_2^+$	Cr^{3+}	2	2
	$Cr(OH)^{2+}$	46	48
	$Cr(OH)_2^+$	35	35
	$Cr(OH)_3$	1	1
	$CrOHSO_4$	15	14
Ni^{2+}	Ni^{2+}	81	83
	$NiSO_4$	12	10
	$Ni(CN)_2$	1	1
	$Ni(CN)_3^-$	1	1
	$NiH(CN)_4^-$	2	3
CN^-	$CaFe(CN)_6^-$	8	7
	$Fe(CN)_6^{3-}$	48	52
	HCN	18	18
	$MgFe(CN)_6^-$	6	5
	$Ni(CN)_4^-$	2	2
	$Ni(CN)_3^-$	4	3
	$NiH(CN)_4^-$	11	11
	NiH_2CN_4	1	1
	$Ni(CN)_2$	2	2
NH_4^+	NH_4^+	99	99
	$NH_4SO_4^-$	1	1
CO_3^{2-}	HCO_3^-	20	20
	H_2CO_3	80	80

Table 14 continued

AAM medium: Table 15 lists the percentage distribution calculated in the test system using the AAM algal medium with the metal plating effluent diluted to 50% using deionized water, moderately hard water and receiving water.

It is evident that for the metals copper, zinc, cadmium and nickel, the use of deionized water as dilution water leads to more free metal being present. The average percentage free copper, nickel, zinc and cadmium is 77% (deionized water), 67% (moderately hard water) and 66% (receiving water). For comparison, simulations were also performed using chemical data for the 93/04/13 sample. Results obtained using this data exhibited the same trend, but the percentages were lower than those obtained for the 93/06/10 sample (57% for deionized water, 45% for moderately hard water and 42% for receiving water). This decrease in free metal content is due to the higher pH of the 93/04/13 sample (6.4 vs 5.6 on 93/06/10).

BG-11 medium: Table 16 lists the percentage distribution calculated for the BG-11 growth media with a 50% dilution of metal plating effluent collected on 93/06/10. It is interesting to note that for this medium, the nature of the dilution water influences metal speciation only slightly. For the results shown in Table 16, the average percentage free metal is 84% (deionized water), 83% (moderately hard water) and 84% (receiving water). Simulations which were carried out using chemical data for the 93/04/13 sample exhibited a slightly different trend. The results obtained here were 67% (deionized water), 74% (moderately hard water) and 61% (receiving water). Again, the lower percentages are caused by the higher pH of the 93/04/13 sample.

The results obtained explain two observed phenomena: First, tests carried out in the BG-11 growth medium was more sensitive than tests carried out in the AAM medium. Second, the AAM test was more sensitive with deionized water as dilution water. In each instance, higher sensitivity was observed for test systems which had the highest calculated percentage free metal. Simulations performed with a 1% effluent - AAM medium mixture exhibited the same trends as those described above. These results are shown in Table 17.

Table 15: Comparison of dilution water effects on the speciation of the AAM - metal plating effluent system at 50% dilution

Component	% Species distribution			
	Species	Deionized H_2O	Moderately hard water	River water
Ca^{2+}	Ca^{2+} $CaSO_4$ $CaHCO_3^+$	90 9 **	88 11 1	90 9 1
Cl^-	Cl^-	100	100	100
Na^+	Na^+	100	100	100
NO_3^-	NO_3^-	100	100	100
K^+	K^+	100	100	100
PO_4^{3-}	$MgH_2PO_4^+$ HPO_4^{2-} $H_2PO_4^-$ $CaH_2PO_4^+$ $MgHPO_4$	1 3 93 ** **	2 4 92 ** **	2 4 91 1 1
Mg^{2+}	Mg^{2+} $MgSO_4$ $MgHCO_3^+$	91 8 **	89 9 1	91 8 1
SO_4^{2-}	SO_4^{2-} $MgSO_4$ $CaSO_4$ $ZnSO_4$	88 4 4 2	87 5 5 2	84 7 6 2
H_3BO_3	H_3BO_3	100	100	100
Mn^{2+}	Mn^{2+} $MnSO_4$ $MnHCO_3^+$	91 8 **	88 10 2	89 8 2
Cu^{2+}	Cu^{2+} $CuHCO_3$ $CuSO_4$ $CuCO_3$	73 16 7 4	56 30 7 6	54 33 5 7
Zn^{2+}	Zn^{2+} $ZnSO_4$ $ZnHCO_3^+$	85 10 10	78 11 10	78 9 12
Fe^{3+}	$Fe(OH)_2^+$ $Fe(CN)_6^{3-}$ FeEDTA	91 5 1	91 5 1	91 5 1
$EDTA^{4-}$	NiEDTA FeEDTA	1 98	** 98	** 98

Component	% Species distribution			
	Species	Deionized H_2O	Moderately hard water	River water
Al^{3+}	Al^{3+}	6	7	7
	$AlOH^{2+}$	18	18	19
	$Al(OH)_2^+$	48	47	47
	$Al(OH)_3$	24	24	24
	$Al(OH)_4^-$	1	1	1
	$AlSO_4^+$	2	3	2
Cd^{2+}	Cd^{2+}	73	68	67
	$CdCl^+$	12	11	12
	$CdSO_4$	10	12	9
	$CdHCO_3^+$	4	9	11
$Cr(OH)_2^+$	Cr^{3+}	2	2	2
	$Cr(OH)^{2+}$	48	47	49
	$Cr(OH)_2+$	39	37	38
	$Cr(OH)_3$	1	1	1
	$CrOHSO_4$	11	13	11
Ni^{2+}	Ni^{2+}	76	66	65
	$NiSO_4$	7	8	6
	$Ni(CN)_2$	1	1	1
	$Ni(CN)_3^-$	1	1	**
	$NiH(CN)_4^-$	3	2	2
	$NiCO_3$	5	10	12
	$NiHCO_3^+$	5	10	12
CN^-	$CaFe(CN)_6^-$	4	5	6
	$Fe(CN)_6^{3-}$	38	39	38
	HCN	35	35	35
	$MgFe(CN)_6^-$	4	5	6
	$Ni(CN)_4^{2-}$	2	1	1
	$Ni(CN)_3^-$	3	3	3
	$NiH(CN)_4^-$	11	9	8
	NiH_2CN_4	1	**	**
	$Ni(CN)_2$	2	2	2
NH_4^+	NH_4^+	100	99	100
	$NH_4SO_4^-$	**	1	**
CO_3^{2-}	HCO_3^-	18	18	18
	H_2CO_3	81	81	81

Table 15 continued

Table 16: Comparison of dilution water effects on the speciation of the BG-11 - metal plating effluent system at 50% dilution

Component	% Species distribution			
	Species	Deionized H_2O	Moderately hard water	River water
Ca^{2+}	Ca^{2+}	92	90	92
	$CaSO_4$	8	10	8
	$CaHCO_3^+$	**	**	**
Cl^-	Cl^-	100	100	100
Na^+	Na^+	100	100	100
NO_3^-	NO_3^-	100	100	100
K^+	K^+	100	100	100
PO_4^{3-}	$MgH_2PO_4^+$	**	1	2
	HPO_4^{2-}	4	4	4
	$H_2PO_4^-$	93	92	91
	$CaH_2PO_4^+$	**	**	1
	$MgHPO_4$	**	**	**
Mg^{2+}	Mg^{2+}	92	91	92
	$MgSO_4$	7	9	7
	$MgHCO_3^+$	**	**	**
SO_4^{2-}	SO_4^{2-}	88	87	84
	$MgSO_4$	3	4	5
	$CaSO_4$	4	5	6
	$ZnSO_4$	2	2	2
H_3BO_3	H_3BO_3	100	100	100
Mn^{2+}	Mn^{2+}	92	90	92
	$MnSO_4$	8	9	8
	$MnHCO_3^+$	**	**	**
Cu^{2+}	Cu^{2+}	87	86	87
	$CuHCO_3$	3	3	4
	$CuSO_4$	8	10	8
	$CuCO_3$	**	**	**
Zn^{2+}	Zn^{2+}	89	87	89
	$ZnSO_4$	9	11	9
	$ZnHCO_3^+$	**	**	**
Fe^{3+}	$Fe(OH)_2^+$	84	84	84
	$Fe(CN)_6^{3-}$	5	5	5
	FeEDTA	**	**	**
	FeHCitrate	9	9	8

Component	% Species distribution			
	Species	Deionized H_2O	Moderately hard water	River water
$EDTA^{4-}$	NiEDTA	1	1	1
	FeEDTA	98	98	98
Citrate	FeCitrate	2	2	2
	FeHCitrate	96	96	96
Al^{3+}	Al^{3+}	7	7	7
	$AlOH^{2+}$	19	19	19
	$Al(OH)_2^+$	47	48	47
	$Al(OH)_3$	24	24	24
	$Al(OH)_4^-$	1	1	1
	$AlSO_4^+$	2	3	2
Cd^{2+}	Cd^{2+}	77	76	77
	$CdCl^+$	12	11	12
	$CdSO_4$	10	12	10
	$CdHCO_3^+$	**	**	**
$Cr(OH)_2^+$	Cr^{3+}	2	2	2
	$Cr(OH)^{2+}$	49	48	49
	$Cr(OH)_2+$	38	37	38
	$Cr(OH)_3$	1	1	1
	$CrOHSO_4$	10	12	10
Ni^{2+}	Ni^{2+}	84	83	84
	$NiSO_4$	7	9	7
	$Ni(CN)_2$	1	1	1
	$Ni(CN)_3^-$	1	1	1
	$NiH(CN)_4^-$	3	3	3
	$NiCO_3$	**	**	1
	$NiHCO_3^+$	**	**	1
CN^-	$CaFe(CN)_6^-$	4	5	6
	$Fe(CN)_6^{3-}$	38	38	37
	HCN	35	35	34
	$MgFe(CN)_6^-$	4	4	5
	$Ni(CN)_4^{2-}$	2	2	2
	$Ni(CN)_3^-$	4	3	3
	$NiH(CN)_4^-$	11	11	10
	NiH_2CN_4	1	1	1
	$Ni(CN)_2$	3	2	2
NH_4^+	NH_4^+	100	99	100
	$NH_4SO_4^-$	**	1	**
CO_3^{2-}	HCO_3^-	18	18	18
	H_2CO_3	81	81	81

Table 16 continued

Table 17: Comparison of dilution water effects on the speciation of the AAM - metal plating effluent system at 1% effluent

Component	% Species distribution			
	Species	Deionized H_2O	Moderately hard water	River water
Ca^{2+}	Ca^{2+}	98	91	94
	$CaSO_4$	2	7	3
	$CaHCO_3^+$	**	2	2
Cl	Cl	100	100	100
Na^+	Na^+	100	100	100
NO_3^-	NO_3^-	100	100	100
K^+	K^+	100	100	100
PO_4^{3-}	$MgH_2PO_4^+$	2	1	2
	HPO_4^{2-}	15	15	15
	$H_2PO_4^-$	83	78	73
	$CaHPO_4$	**	2	3
	$CaH_2PO_4^+$	**	**	1
	$MgHPO_4$	**	4	6
Mg^{2+}	Mg^{2+}	98	91	95
	$MgSO_4$	2	7	3
	$MgHCO_3^+$	**	2	3
SO_4^{2-}	SO_4^{2-}	96	91	85
	$MgSO_4$	3	5	8
	$CaSO_4$	**	3	7
H_3BO_3	H_3BO_3	100	100	100
Mn^{2+}	Mn^{2+}	97	90	93
	$MnSO_4$	2	7	3
	$MnHCO_3^+$	1	3	4
Cu^{2+}	Cu^{2+}	45	27	27
	$CuHCO_3$	12	24	29
	$CuSO_4$	7	2	**
	$CuCO_3$	15	29	35
	$Cu(OH)_2$	5	2	2
	CuEDTA	22	15	4
Zn^{2+}	Zn^{2+}	90	73	73
	$ZnSO_4$	2	7	3
	$ZnHCO_3^+$	6	17	20
	$ZnCO_3$	1	3	4
Fe^{3+}	$Fe(OH)_2^+$	61	57	81
	FeEDTA	36	39	16
	$Fe(OH)_3$	2	2	2
	FeOHEDTA	1	2	**

Component	% Species distribution			
	Species	Deionized H_2O	Moderately hard water	River water
$EDTA^{4-}$	NiEDTA	17	11	3
	FeEDTA	75	81	91
	FeOHEDTA	3	3	4
	CuEDTA	5	3	2
Al^{3+}	$AlOH^{2+}$	1	2	2
	$Al(OH)_2^+$	21	21	22
	$Al(OH)_3$	62	61	61
	$Al(OH)_4^-$	16	16	16
Cd^{2+}	Cd^{2+}	88	70	69
	$CdCl^+$	3	2	4
	$CdSO_4$	2	8	3
	$CdHCO_3^+$	6	16	19
	$CdCO_3$	1	4	4
$Cr(OH)_2^+$	$Cr(OH)^{2+}$	15	15	16
	$Cr(OH)_2+$	72	70	71
	$Cr(OH)_3$	13	12	12
	$CrOHSO_4$	**	3	1
Ni^{2+}	Ni^{2+}	53	31	31
	$NiSO_4$	**	3	**
	$NiCO_3$	25	47	55
	$NiHCO_3^+$	4	8	10
	NiEDTA	17	12	3
CN^-	HCN	100	100	100
NH_4^+	NH_4^+	100	100	100
CO_3^{2-}	HCO_3^-	53	54	54
	H_2CO_3	46	46	45

Table 17 continued

Chemical speciation of the metal plating effluent - bacterial growth medium mixture

In order to simulate the speciation of this system, the component concentrations of a 100% effluent and bacterial growth medium mixture were calculated. The speciation of this system is similar to that of the metal plating effluent on its own. The detailed speciation will thus not be discussed here. There is thus no chemical explanation for the lower sensitivity of this test when used to evaluate the metal plating effluent.

Chemical speciation of the metal plating - protozoan growth medium mixture

The speciation of this system is similar to that of the metal plating effluent on its own. Therefore, it will not be discussed here.

Chemical speciation of the paper mill effluent - algal growth media mixture

This effluent was in general not toxic. The sample collected on 93/02/15 exhibited slight toxicity. However, no chemical analysis was performed on this sample, which makes it impossible to calculate the speciation. It was observed that, in general, the paper mill effluent stimulated algal growth. Precipitation was also observed in the algal growth - effluent sample mixtures. These questions will be addressed here.

The observed stimulation of algal growth by the paper mill effluent may have two causes. First, addition of the paper mill effluent to the algal growth media may increase the total concentrations of essential elements in the mixture. Second, the paper mill effluent may change the speciation of essential elements with the net effect that the bioavailable fraction of the essential elements increase. Both of these possibilities were investigated.

Table 18 compares the component concentrations present in the AAM media with those in the AAM - paper mill effluent mixture. An undiluted effluent sample was assumed in the calculation of the component concentrations. Table 19 compares the relative abundance of species in the AAM medium with that of the AAM - paper mill effluent mixture.

It is immediately obvious from Table 18 that the growth medium - effluent mixture has higher component concentrations than the growth medium on its own. This follows from the fact that the effluent contributes to the component concentrations. Thus, the growth media -effluent sample contains higher total concentrations of essential elements than the AAM medium.

Apart from this, the results in Table 19 show that the effluent - growth medium mixture contains a higher percentage Zn^{2+} (35%), compared with the AAM medium where zinc is bound exclusively to EDTA. The mixture also has a lower percentage of CuEDTA present. The net effect of this is that the effluent - growth media mixtures have more bioavailable essential elements present than the AAM medium. This, together with the higher total component concentrations, explains the growth stimulation observed by the paper mill effluent.

Table 18: Component concentrations (mol/l) in the AAM medium and the AAM - paper mill effluent mixture

Component	AAM	AAM - Paper Mill mixture
Ca^{2+}	3.0E-05	2.1E-03
Cl^-	1.6E-04	2.3E-03
Na^+	4.8E-04	4.8E-04
NO_3^-	3.0E-04	3.2E-04
K^+	1.2E-05	1.2E-05
PO_4^{3-}	6.0E-06	1.8E-05
Mg^{2+}	1.1E-04	8.5E-04
SO_4^{2-}	5.9E-05	2.1E-03
CO_3^{2-}	1.8E-04	2.2E-03
H_3BO_3	3.4E-07	3.4E-07
Mn^{2+}	2.1E-06	8.7E-06
Cu^{2+}	8.9E-11	5.7E-07
Zn^{2+}	2.4E-09	1.2E-06
Fe^{3+}	5.7E-07	2.5E-06
$EDTA^{4-}$	8.1E-07	8.1E-07
Al^{3+}	**	1.5E-05
NH_4^+	**	1.8E-04

The precipitation observed in the effluent - growth media mixtures may be explained as follows. The paper mill effluent sample after secondary clarification taken on 93/05/10 are supersaturated with respect to a number of solids (cf Table 13, p 18). The algal growth media are also supersaturated with respect to HFO's (cf Table 9, p 12). Adding the two mixtures will result in a higher degree of supersaturation. Furthermore, the fact that the algal growth media is concentrated 20 times before being added to the test solution may also have an effect. It is feasible that in the 20 times concentrated growth media, a fine precipitate starts to form before addition to the test sample. This fine precipitate will then induce precipitation in the test system.

Table 19: Comparison of relative species abundance in the AAM algal growth medium with that in the paper mill - AAM medium mixture

Component	% Species distribution		
	Species	AAM	AAM - Paper Mill mixture
Ca^{2+}	Ca^{2+}	98	86
	$CaHCO_3$	**	1
	$CaSO_4$	**	12
Cl^-	Cl^-	100	100
Na^+	Na^+	100	100
NO_3^-	NO_3^-	100	100
K^+	K^+	100	100
PO_4^{3-}	$MgPO_4^-$	1	2
	$MgHPO_4$	5	13
	$CaHPO_4$	**	21
	$CaPO_4$	**	3
	HPO_4^{2-}	81	50
	$H_2PO_4^-$	11	12
Mg^{2+}	Mg^{+2}	99	87
	$MgHCO_3^+$	**	1
	$MgSO_4$	**	11
SO_4^{2-}	SO_4^{-2}	98	82
	$MgSO_4$	1	5
	$CaSO_4$	**	13
CO_3^{2-}	HCO_3^-	97	94
	$H_2CO_3^-$	2	4
	$CaHCO_3^+$	**	1
H_3BO_3	H_3BO_3	94	97
	$H_2BO_3^-$	6	3
Mn^{2+}	Mn^{2+}	99	86
	$MnSO_4$	**	11
	$MnHCO_3$	**	2
Cu^{2+}	CuEDTA	100	71
	$Cu(OH)_2$	**	19
	$CuCO_3$	**	9
Zn^{2+}	Zn^{2+}	**	35
	$Zn(OH)^+$	**	1
	$ZnSO_4$	**	6
	$ZnHCO_3$	**	6
	$ZnCO_3$	**	20
	$Zn(CO_3)_2^{2-}$	**	3
	ZnEDTA	100	28

Component	% Species distribution		
	Species	AAM	AAM - Paper Mill mixture
Fe^{3+}	FeEDTA	40	15
	FeOHEDTA	59	13
	$Fe(OH)_2^+$	**	40
	$Fe(OH)_3$	**	21
	$Fe(OH)_4^-$	**	12
$EDTA^{4-}$	CuEDTA	**	25
	FeEDTA	28	28
	FeOHEDTA	42	25
	ZnEDTA	**	22
	CaEDTA	27	**
	MgEDTA	2	**
Al^{3+}	$Al(OH)_4^-$	**	85
	$Al(OH)_3$	**	15
NH_4^+	NH_4^+	**	96
	NH_3	**	2
	$NH_4SO_4^-$	**	1

Table 19 continued

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GUIDELINES FOR WHOLE EFFLUENT TOXICITY TESTING

GUIDELINES FOR WHOLE EFFLUENT TOXICITY TESTING

CONTRACT DOCUMENT FOR THE WATER RESEARCH COMMISSION

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1. INTRODUCTION

The Department of Water Affairs and Forestry (DWA&F) has recently identified Whole Effluent Toxicity (WET) testing as an appropriate tool to evaluate the suitability of hazardous/toxic effluents for discharge into receiving waters (Slabbert *et al.*, 1996a,b). The WET approach involves the use of toxicity as control parameter. This is the alternative to the chemical-specific approach, where the focus is on individual chemicals. WET is the total effect of an effluent and is measured directly with tests employing living organisms/biological material (called toxicity tests/bioassays/biotests). Toxicity tests are important additions to chemical-specific measurements because they:

* Respond to compounds which are not readily identifiable or measurable by analytical techniques;
* Respond to unknown compounds;
* Detect effects due to chemical interaction, *e.g.* synergism, antagonism and addition;
* Provide information on the type of hazardous chemical activity in an effluent, *i.e.* toxicity, mutagenicity, potential carcinogenicity and teratogenicity; and
* Provide information on the impact on particular groups of target organisms.

In order to implement the WET testing approach in a structured manner, procedures for use in the South African context were needed. This document provides guidelines on WET testing. It is the product of a two year study for the Water Research Commission which has been conducted to develop and establish methodologies for WET testing (Slabbert *et al.*, 1996a). The document has been prepared to assist the DWA&F in its task to manage the discharge of hazardous effluents. The document contains general information on toxicity testing and provides guidance on specific applications of toxicity tests.

This document should be used in conjunction with another guideline document **Guidelines for toxicity bioassaying of waters and effluents in South Africa**, which provides guidance on toxicity tests, sampling and quality control (Slabbert, 1996). For additional information on WET testing refer to the report of Slabbert *et al.* (1996a) **Development of procedures to assess whole effluent toxicity.**

2. TERMINOLOGY

2.1 Concept of toxicity

Toxicity is a characteristic of a chemical (or a group of chemicals) that causes adverse effects in organisms. This may be influenced by physical-chemical conditions prevailing at the time. Adverse effects include lethality or those effects limiting an organism's ability to survive in nature. Such effects can be acute or chronic (US EPA, 1990; 1991a). Acute and chronic refer to the length of time organisms are exposed to toxicants before adverse effects are observed.

* **Acute** means a stimulus severe enough to rapidly induce an effect (short-term effects). In aquatic toxicity tests an effect observed within 96 h or less is usually considered acute. An acute effect is usually but not always measured in terms of lethality.

* **Chronic** means a stimulus that continues for a relatively long period of time (long-term effects of small doses and their accumulative effects over time). Chronic toxicity is measured in terms of sub-lethal effects such as reduced growth, reduced reproduction, etc. in addition to lethality. Most of the chronic tests in use today use a short exposure period of approximately 7 days, and are called short-term chronic tests.

Toxicity of a chemical substance is measured by observing the responses of organisms to increasing concentrations of the substance. One substance is more toxic than another when the same adverse effect is caused at a lower concentration. For any given substance, toxic effects are lowered when concentrations are reduced. Thus, when the toxicity of an effluent is reduced (concentrations of toxic constituents reduced) the toxic effect of that effluent on receiving waters is also reduced. Similarly, greater dilution of a toxic effluent will result in lower toxicity in receiving waters. The most effective way to control toxicity is to limit the measured toxicity in an effluent (US EPA, 1990).

Actual toxicity is the toxicity of an effluent without any dilution (100% of the effluent). The **inherent toxicity** of an effluent is established by using standard dilution water (*Slabbert, 1996*) for dilution. **Relative toxicity** is the toxicity of an effluent when it is diluted with the receiving water, to test for interaction after discharge (US EPA,1990; 1991a).

2.2 Toxicity test

A toxicity test is a technique that determines the effect of a chemical/water/effluent using living organisms or cellular/subcellular systems and defined test conditions. A toxicity test measures the degree of effect on the exposed test organisms/material.

Three general types of tests can be carried out:

* **Screening test** - In this type of test organisms/material are directly exposed to the effluent (100% effluent) or to one dilution of the effluent (50% effluent). Ambient water (instream water) is tested without dilution.

* **Range finding test** - This is a test conducted on a range of ten-fold dilutions of an effluent to determine the approximate level of toxicity, and the concentration range to be used for definitive testing.

* **Definitive test** - This test estimates the concentrations at which a certain percentage or number of organisms exhibit a certain response. Organisms are exposed to various proportions of 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 in each test concentration is observed and recorded, and the number of responses in relation to the test concentration analyzed.

A toxicity test is also described according to the way in which organisms are exposed to test solutions. In a **flow-through** test effluent and dilution water are mechanically renewed throughout the day. A **static test** is conducted in test chambers into which solutions are added manually at the start of the test. The **static renewal test** is a variation of the static test.

In this test the solutions are replaced on a predetermined basis (*i.e.* 24 h intervals). Fresh effluent samples are usually collected for renewal (US EPA, 1991a).

A flow-through and static renewal test is best applicable if an effluent is continuously discharged and is highly variable. The same systems will be applicable if the effluent is highly variable with intermittent discharge. If the effluent is not variable, *e.g.* a discharge from a 30 day retention dam, a static or renewal test would be the appropriate systems.

Toxicity tests may be conducted on-site (at the effluent discharge) or off-site at a central laboratory. In general, static and static renewal tests will be conducted off-site, while flow-through tests will be performed on-site. If an effluent contains volatile or biodegradable toxic compounds on-site testing may be the most appropriate.

2.3 Toxicity test results

The measured effects of definitive toxicity tests are usually brought into relationship with the corresponding pollutant concentrations or percentages of effluent using statistical calculations or graphic methods (DIN, 1982; ISO, 1989; US EPA, 1991a,b,c).

2.3.1 Toxicity endpoints

Acute toxicity endpoints commonly include lethal concentrations (LC's). The LC is the point estimate of the toxicant concentration (calculated value) at which a certain percentage of the test organisms die, *e.g.* the LC_{10} (10% lethality) or LC_{50} (50% lethality). The exposure duration is also included in the endpoint, namely 24, 48, 72 or 96 h (*e.g.* 96-h LC_{50}) (US EPA, 1991a).

Chronic toxicity endpoints normally include the no observed effect concentration (NOEC), the lowest observed effect concentration (LOEC) and the effect concentration (EC), based on observations of reduced reproduction, growth, and/or survival from life cycle, partial life cycle, and early life stage tests with aquatic organisms (US EPA, 1990; 1991a).

* The NOEC is the highest **tested** concentration where no adverse effects are observed on the test organisms at a specific time of observation and the LOEC is the lowest **tested** concentration where an adverse effect on test organisms is observed at a specific time of observation. These values are determined using hypothesis testing and are not point estimates (US EPA, 1991b), and are dependent on the concentrations initially selected for testing.

* The EC is the point estimate of the toxicant concentration at which a certain percentage of the test organisms would be affected (growth inhibition, respiration inhibition, immobility), *e.g.* the EC_{10} (10% effect) or EC_{50} (50% effect). For some species like *Daphnia* where the point of death is not certain, immobility is often used as a surrogate for death (ISO, 1989). Results for responses like immobility may be reported as an EC_{50}. However, usually no distinction is made between EC_{50}'s and LC_{50}'s when the response is a surrogate for death.

Point estimates such as LC's and EC's are statistically derived from dose-response curves, assuming a linear relationship (US EPA, 1991a,b,c).

2.3.2 Toxic units

Since toxicity involves an inverse relationship to effect concentrations (the lower the effect concentration, the higher the toxicity of an effluent), it is more understandable to translate concentration-based toxicity measurements into toxic units (TU's). The major advantage of using toxic units to express toxicity test results is that toxic units increase linearly as the toxicity of the effluent increases. Toxic units also make it easy to specify water quality criteria based on toxicity. One common method of deriving toxic units is to divide 100 (full strength effluent expressed as %) by the concentration (in %) that causes acute or chronic toxicity (OECD, 1987; US EPA, 1991a), *e.g.*:

(TU_a) **(acute toxicity unit)** = $100/LC_{50}$
(TU_c) **(chronic toxicity unit)** = **100/NOEC**

2.3.3 Acute-to-chronic ratio

The acute-to-chronic ratio (ACR) expresses the relationship between acute (LC_{50}) and chronic toxicity (NOEC) (obtained with the same organism). It is used as a factor for estimating chronic toxicity on the basis of acute toxicity data, or for estimating acute toxicity on the basis of chronic toxicity data.

3. TOXICITY TEST COMPONENTS

A toxicity test consist of five components, namely the test organisms/material, test system, effluent, dilution water and test results. In order to ensure successful implementation of toxicity tests it is important that each of the components meet certain general quality assurance requirements.

3.1 Test organisms

A large number and variety of test organisms/material can be used for toxicity testing (Slabbert *et al.*, 1996b). The most reliable data will, however, be obtained with those organisms/material which have been extensively evaluated and are used in standardized procedures. Test organisms used in toxicity tests should have a known origin and history, be disease free, and be acclimatized to test conditions. Care should be taken with their handling and treatment to limit variation in their responses. Organisms taken from natural waters are usually not appropriate for toxicity testing unless they are required for some specific reason and have been cultured in the laboratory for several generations. Such testing is usually more difficult to do and subject to more variability (disease, age) than standardized testing (US EPA, 1985a). Organisms collected directly from the receiving water should never be used because existing effects may mask toxicity.

The sensitivities of laboratory cultures should be periodically tested with reference toxicants to ensure that responses are within the required limits and that test precision is good.

Procedures which have been found suitable for local application, test conditions and test requirements are outlined in **Guidelines for toxicity bioassaying of waters and effluents in South Africa** (Slabbert, 1996).

3.2 Test system

Materials that come into contact with the effluent or dilution water should not release, absorb or adsorb toxicants, and should be cleaned thoroughly before use. Furthermore, test conditions should be appropriate for the test organisms (Slabbert, 1996). There are certain physical/chemical measurements which are carried out in conjunction with toxicity tests to ensure that conditions are within the required ranges for organism maintenance. These include temperature, dissolved oxygen and pH (US EPA, 1990):

Temperature -

Each toxicity test has a nominal temperature and a range of temperatures at which the test should be carried out. Because organisms may be more sensitive at the upper and lower limits of the range, it is important to keep within the temperature ranges to standardize responses.

Dissolved oxygen -

Test organisms used in tests at temperatures ≥20° C are stressed by oxygen levels <4,0 mg/ℓ. Aeration, should however, only be used as a last resort because toxicity may be affected by aeration (volatiles removed). If aeration is applied to individual test containers this should be applied very gently by means of a pasteur pipette and not by an air stone.

pH -

pH affects test organisms as well as the toxicity of chemicals in an effluent. It is, therefore, recommended to monitor pH on a daily basis.

3.3 Effluent

An effluent should be sampled and stored in such a way that the sample is representative of the effluent. The toxicity of an effluent can change because of volatilization of chemicals, precipitation, or biological degradation. If holding is necessary, samples should be stored at 4° C and for limited periods only (preferably used within 24 h) to ensure that the composition of the effluent does not change before testing (Slabbert *et al.*, 1996a). Containers should be clean to avoid contamination and should seal properly to ensure that volatiles are not lost. No preservatives are added to samples collected for toxicity testing.

Effluent variability will determine the sampling method and frequency (Slabbert *et al.*, 1996a). An effluent showing a large variability in quality may be sampled continuously or by means of a series of grab samples to be as representative as possible. A sample taken this way is called a **composite sample**. If an effluent does not exhibit much variation, single **grab samples** can be taken.

Grab samples collected during peaks of toxicity provide a measure of maximum toxicity if the toxicity of the effluent is highly variable. Grab samples may be necessary if there is little mixing of effluent with the receiving water. A grab sample will only reveal the toxicity peak in

an effluent if the sample has been collected at the time of the toxicity peak. A composite sample may catch the toxicity peaks, but the compositing process may tend to dilute the toxicity, resulting in misleading measures of the maximum toxicity of the effluent. A 24 h composite sample is usually recommended for a chronic toxicity test (where peak toxicity is of short duration), while grab samples taken at selected intervals are most appropriate for acute tests.

3.4 Dilution water

The dilution water used will depend on the objective of a test (see section 2.1). Either standard dilution water (Slabbert, 1996), receiving water or other natural waters are appropriate. Water of similar composition (standard test water, other surface water, or ground water) is usually used for dilution if receiving water is not available. Dilution water should not be toxic. Tests that show significant mortality or adverse responses in dilution water controls should be discarded. Dechlorinated tap water is generally not recommended for dilution and control testing. If it is the objective to establish the contribution of the effluent to receiving waters, receiving water should be used for dilution, whether or not it is toxic (US EPA, 1990; 1991a).

3.5 Test results

The expected result in all toxicity tests is an increasing response with increasing effluent concentrations. Often this increasing response is observed as one concentration eliciting no response and the next concentration showing a total response (100%). This pattern is particularly obvious with acute tests. If responses are inconsistent with the expected pattern (increased effect with increasing concentration) it is likely that dilutions were incorrectly labelled or that contamination occurred. Organisms/material in controls should not exhibit any significant responses. Controls are the basis to determine toxic effects and if responses occur, results based on the data will be inaccurate. Requirements for controls are specified in the **Guidelines for toxicity bioassaying of waters and effluents in South Africa** (Slabbert, 1996).

4. USE OF TOXICITY TESTS TO MANAGE HAZARDOUS EFFLUENTS

Toxicity testing could be used by the DWA&F for the following applications:

* To establish the suitability of an effluent for discharge into receiving water;
* To develop permit limits for effluent control;
* To monitor effluents to ensure compliance with the limits;
* To identify/categorize/prioritize toxic effluents;
* To assist industry to reduce effluent toxicity; and
* To monitor the receiving water body (ambient toxicity testing).

As the toxicity testing approach is different for each application, each application will be addressed separately. Guidelines and procedures given are based on the South African experience (Slabbert *et al.*, 1996a). Where necessary, recommendations from literature will be included.

4.1 Establishing the suitability of an effluent for discharge

When addressing the suitability of an effluent for discharge into receiving water, two questions are asked. Firstly: Is the effluent toxic/harmful? Secondly: Does the potential exist that the effluent could cause an impact on the receiving water?

Such an evaluation could be required for:

* Effluents already being discharged;
* Effluents originating from new facilities; and
* New effluents produced by existing facilities.

In case of new effluents the DWA&F requires industry to simulate the production process and to subject the effluent from the simulated process to toxicity evaluation.

4.1.1 Screening of existing information

The first step in establishing whether or not an effluent is suitable for discharge will be to present and evaluate ecological and historical data for the environment. Furthermore, it will be necessary to screen background information on the treatment facility/process and, if available, existing monitoring data. Information which should be considered include:

* **Type of industry** - Raw materials; products; presence of potentially toxic chemicals; management practices; treatment processes and efficiency; industrial load in case of sewage works;

* **Complexity and variability of effluent composition** - Compliance history; existing chemical-physical data from monitoring reports and applications; possible data on toxicity;

* **Receiving water** - Type and designated/existing uses; stream survey data; and

* **Dilution of the effluent with receiving water** - Dilution calculations.

If the screening process reveals that the effluent is potentially toxic/has the potential to cause adverse chemical activity upon discharge, it will be necessary to conduct toxicity tests to generate toxicity data for decision making. For example, if a combination of the following were found, there is a large possibility that the effluent could impact on the receiving water: low dilution, high quality receiving water, poor compliance record; many effluent dischargers in the area. Toxicity testing will also be required if the screening process does not provide clear-cut answers on potential toxicity.

4.1.2 Toxicity testing

There are two approaches to follow when evaluating effluents for toxicity, namely sequential (tiered testing) and simultaneous testing. In the first case simple, inexpensive and mainly lethality tests are initially carried out. This is followed by tests of increasing complexity (sublethal and chronic tests). After each stage a decision must be taken if further testing is necessary. This approach has the following shortcomings:

* The initial tests do not provide information on sublethal/chronic effects;
* Such testing can take too long and is very expensive.

Simultaneous testing involves the use of several tests from various levels of the biological organization at the same time (battery of tests). This approach provides the best possible assessment of adverse activity, is cost-effective and results are available in a short period of time. This approach has been adopted by most overseas laboratories (US EPA, 1991a; Blaise *et al.*, 1985) and has also been found the most favourable for local application.

It has been found (OECD, 1987; US EPA, 1991a) that the best assessment of toxic activity is made when using a fish, invertebrate and algal test for WET testing. Such tests should detect acute and chronic toxicity. In South Africa, acute toxicity tests are well established, but a great need for chronic toxicity tests has been identified (Slabbert *et al.*, 1996a). Chronic aquatic tests are particularly needed for those effluents showing low or no acute toxicity and which are discharged into receiving water with no or little dilution.

4.1.2.1 *Toxicity tests*

The following is an example of a battery of acute tests (representing organisms from different levels of the aquatic food chain) which could be used as the minimum requirement for local application to establish whether or not an effluent is toxic:

96 h Fish (*Poecilia reticulata* - guppy) lethality test;
48 h Water flea (*Daphnia pulex*) lethality test; and the
48/72 h Algal (*Selenastrum capricornutum*) growth inhibition test.

These tests have been found to be the most sensitive of the locally available toxicity tests, have a good reproducibility, are relatively simple to conduct, are not very labour-intensive, and above all, they are similar to the tests applied in other countries (Slabbert *et al.*, 1996a,b).

In addition to the battery of acute tests the following short-term chronic tests could be applied when effluent is to be discharged into water which serves as drinking water source:

Toad (*Xenopus laevis*) embryo teratogenicity test; and the
Ames *Salmonella* mutagenicity test.

New toxicity tests are continually being developed. As such tests become available they could be used to supplement the local battery of tests or to replace them.

The different tests and test requirements are described in the document **Guidelines for toxicity bioassaying of waters and effluents in South Africa** (Slabbert 1996). Summaries of the fish, water flea and algal test protocols are given in Table 1.

TABLE 1: Summaries of fish, water flea and algal test protocols

Parameter	Fish (*Poecilia reticulata*) lethality test	Water flea (*Daphnia pulex*) lethality test	Algal (*Selenastrum capricornutum*) growth test
Test type	Static	Static	Static
Temperature	23±2°C	20±1°C	25±1°C
Light quality	Daylight - no illumination	Daylight - no illumination	Cool white fluorescent light
Light intensity	Ambient laboratory levels	Ambient laboratory levels	85-95 $\mu E/m^2/s$
Photoperiod	9-14 h light per day	9-14 h light per day	Continuous illumination
Age of test organisms	2-3 weeks	<24 h	4-6 days
Size of test container	500 mℓ	50 mℓ	350 $\mu\ell$ (24 well plate)
Volume of test sample	350 mℓ	25 mℓ	1,8 mℓ plus 0,2 mℓ inoculum
Number of fish/container	5	5	200 000 cells/mℓ
Number of replicate containers	2	4	5 for screening, 3 for definitive testing
Total number of fish/test	10	20	-
Feeding	No feeding	No feeding	Algal culture medium
Aeration	No aeration[1]	No aeration[1]	No aeration
pH	As obtained[2]	As obtained[2]	As obtained[2]
Control water	Standard dilution water[3]/ receiving water	Standard dilution water[3]/ receiving water	Standard dilution water[4]/ receiving water
Test duration	96 h	48 h	48-72 h
Effect measured	Lethality	Lethality - no movement	Growth inhibition in terms of OD (microplate reader)
Detection limit	10%	10%	10%
Test acceptability	Control survival >90%	Control survival >90%	OD of control cell suspension in microwell: 0,13-0,18; Variation in controls <10%

1 If oxygen content is <40% saturation, a comparison test on aerated sample could be carried out

2 If pH is low, a comparison test on sample with adjusted pH could be carried out

3 Synthetic moderately hard water (US EPA, 1985b)

4 Deionized water

Fish and water flea tests are carried out according to international standard procedures. The algal test is a miniaturization of the standard flask test. If preferred, flask tests could be carried out

4.1.2.2 *Type of testing*

Not only is it necessary to establish whether or not an effluent is toxic, but it is also important to determine the extent of toxicity (how toxic?). For this reason tests should be applied as definitive tests (testing serial dilutions) (not necessary for Ames *Salmonella* mutagenicity test).

The effective concentration range should be established by means of a range finding test (testing 10-fold dilutions) carried out with water flea using a 24 h exposure period. Because effluents change in composition it is crucial that definitive tests start as soon as possible (preferably within 24 h of sampling (Slabbert *et al.*, 1996a). It is expected that a range finding test will only be necessary on the first date of testing, unless the effluent shows large variation. Effective concentration ranges for a few selected effluents are presented in Table 2.

Standard dilution water (deionized water: algae; moderately hard water: fish, toad embryos, water flea) is used for dilution because information on inherent toxicity is required (allowing comparison between effluents and effluent samples).

TABLE 2: Effective concentration range for a few selected effluents (%)[1]

Effluent	Fish lethality test	Water flea lethality test	Algal growth inhibition test	Toad embryo teratogenicity test	
				Lethality	Deformation
Secondary treated sewage effluent	10-100	10-100	10-100	10-100	10-100
Paper mill effluent before clarification	10-100	10-100	10-100	10-100	neg
Paper mill effluent after clarification	100	100	100	10-100	neg
Metal plating effluent	0,1-10	0,1-10	0,01-10	1-100	0,1-100

[1] Slabbert *et al.* (1996a)
Neg Negative

4.1.2.3 *Sampling frequency and sampling procedure*

Effluents vary in composition. It is, therefore, necessary to conduct repetitive tests to ensure the best assessment of the effluent quality.

> In order to effectively assess the variability in effluent toxicity (Slabbert *et al.*, 1996a) a possible approach is to conduct toxicity tests at quarterly intervals for one year (4-times).

Depending on the specific site, the sensitivity of the environment, and the type of effluent the frequency could be changed. If time is limited, the period could be reduced, but four sets of

tests at adequate intervals, would still be valuable. If short-term variation is expected, additional tests on samples taken at short time intervals (see section 4.2) could be carried out.

4.1.2.4 *Calculation of toxicity results*

Toxicity results are expressed in terms of effective concentrations (toxicity endpoints). Toxicity endpoints are derived from the data of definitive tests by means of statistical analysis (linear regression) using dose-response curves (% effect *versus* log concentration). Important endpoints to calculate include LC_{50}'s or EC_{50}'s and LC_{10}s or EC_{10}'s (minimum/lowest effect concentration). The minimum effect concentration (MEC) used for a particular test depend on the detection limit of the test (Slabbert, 1996b). Ideally, measured effects should range from 0% to 100% to ensure the best-fit-line and allow the derivation of the minimum effect level. It is thus important to select a sufficiently wide range of concentrations for definitive testing (see section 4.1.2.2). For each data set it is also necessary to establish how closely the calculated line fits the measured data. This information is provided by the correlation coefficient (R). R-values >0,9 is an indication of a good correlation. Figure 1 shows examples of measured data and regression lines.

In cases where toxicity is too low to use linear regression for the calculation of toxicity endpoints, endpoints could be presented as follows: EC_{50}: >100%; EC_{10}: 50-100% (Slabbert *et al.*, 1996a).

4.1.2.5 *Interpretation of toxicity results*

After toxicity endpoints have been calculated these values are used to establish the dilution required for an effluent to avoid toxic effects in receiving water. For example, for each effluent sample the MEC's (LC_{10}'s or EC_{10}'s) of the three acute toxicity tests mentioned in section 4.1.2.1 are compared. The result of the most sensitive test is considered to be the measured acute toxicity for the particular effluent sample. The lowest MEC of the four data sets will indicate the highest measured acute toxicity for the effluent. From this value the dilution could be calculated as 100%/MEC (full strength effluent divided by toxicity endpoint). An acute dilution factor (DF_a) >100%/MEC will be required to avoid acute toxic effects in the receiving water. An example is given in Table 3.

TABLE 3: MEC's obtained for a hypothetical effluent

Sample	96-h Fish LC10 (%)	48-h Water flea LC10 (%)	Algal EC10 (%)
1	3,2	**0,8**	0,9
2	1,0	0,4	**0,2**
3	1,8	**0,2**	**0,2**
4	4,1	0,9	**0,5**

Bold - lowest MEC for each sample
Lowest MEC for the four samples: 0,2% (100%/0,2% = 500-times dilution)

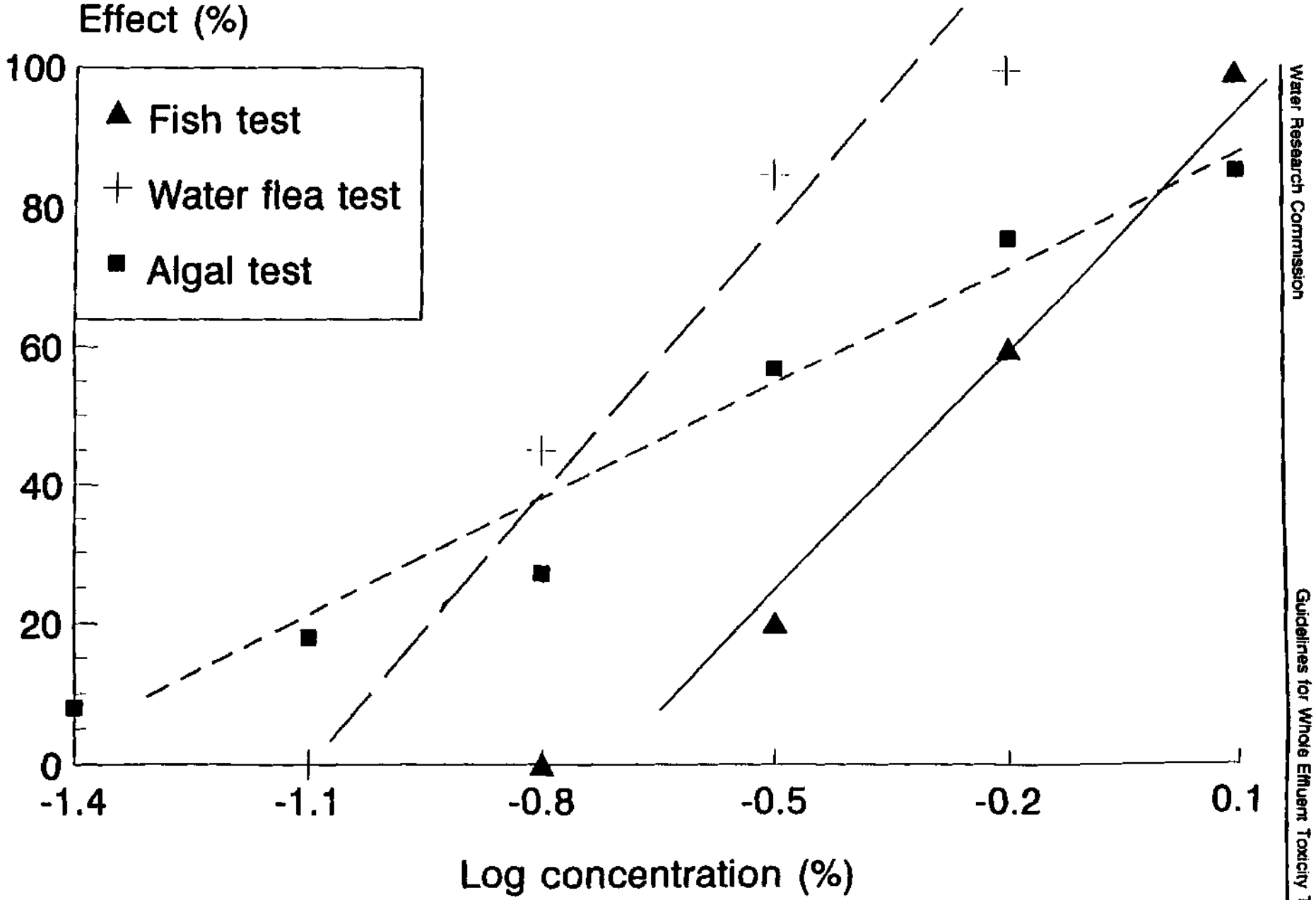

FIGURE 1: Examples of measured data and regression lines

If the toad embryo test was included in the battery of tests, and this test showed the highest sensitivity or the only response [as was sometimes observed with treated sewage effluent and paper mill effluent (Slabbert *et al.* (1996a)] these results could be used to determine the chronic dilution factor (DF_c).

Since chronic aquatic toxicity tests are not currently available, chronic data could be estimated on the basis of the acute data by using an application factor. It has been found that in most instances the ACR's (acute-to-chronic ratios) for data sets are approximately 10 (US EPA, 1991a). For instance, chronic data could be estimated by dividing the LC_{50} of the most sensitive of the three acute tests (section 4.1.2.1) by 10. From this data the dilution to avoid chronic toxicity (DF_c) could be established. For example, if an ACR of 10 is applied to the algal LC_{50} given for metal plating effluent in Table 4 (0,07%), the chronic test NOEC will be 0,007%. The DF_c will then be 14 286 (100%/0,007%).

Some countries express toxicity results in terms of toxic units (TU's) (OECD, 1987; US EPA, 1991a; Slabbert *et al.*, 1996a). Like DF's, TU's are derived by dividing the full strength effluent (100%) by the toxicity endpoint (*e.g.* LC_{50}). Table 4 shows the toxicity of selected effluents in terms of acute toxicity endpoints (LC_{50} or EC_{50}) and acute toxic units (TU_a).

TABLE 4: Toxicity expressed in terms of toxicity endpoints and toxicity units[1]

Effluent	LC_{50} or EC_{50} (%)	TU_a[2]
Secondary treated sewage effluent	~100 (96 h fish test)	~1
Paper mill effluent before clarification	~100 (48 h water flea test)	~1
Paper mill effluent after clarification	>100	>1
Metal plating effluent	0,07 (algal test)	1 429

1 Slabbert *et al.* (1996a)
2 100% divided by LC_{50} or EC_{50}

4.1.3 *Using toxicity data to establish potential toxic impact*

Having established the dilution factors from the toxicity data, these values are compared to the dilution possible in the receiving water. If there is a mixing zone, the dilution in the zone should be $\geq DF_a$ (no acute toxicity), and at the edge of the mixing zone it should be $\geq DF_c$ (no chronic toxicity). If there is no mixing zone, the dilution with stream water should be $\geq DF_c$ (no chronic toxicity at end of pipe). The dilution at the lowest flow should be considered.

The above mentioned comparison is very simple and is only based on dilution. Other countries make use of more sophisticated formulas including various application factors. If the dilution factors and the possible dilution with receiving water are in close proximity, it might

be more appropriate to use a formula which includes other factors. For example, the EPA recommends the use of a statistical analysis of effluent data which accounts for limited sample size and effluent variability. As alternative a stochastic dilution model which incorporates dilution and effluent variability could be used (US EPA, 1991a).

If it is not clear from data that an impact is possible, an element of judgement by the DWA&F might be required.

4.1.3.1 *Decisions on control*

- If the effluent is not toxic, no permit limit for WET would be required (conventional strategy followed for control).

- If the effluent is toxic but dilution with receiving water is very large (eliminating any possibility of impact), a permit limit for WET may not be necessary. It is, however, recommended that toxicity tests are carried out at each application/re issuance of a permit (*e.g.* once every three/five years).

- If the effluent is toxic, the potential for impact has been demonstrated, and discharge appears to be acceptable, a permit limit for WET should be developed.

- If the effluent is toxic and sufficient dilution is not possible a reduction in toxicity could be requested, retention dams could be required or an alternate disposal route could be followed*.

- Alternative disposal should be used if an effluent shows significant mutagenic activity (potential carcinogenicity) (Slabbert, 1996b).

* On occasion, treated sewage effluent and paper mill effluent have been found to exhibit low acute toxicity and deformation in toad embryos (mainly at the 100% effluent concentration) (Slabbert *et al.*, 1996a). Very little, if any, dilution is possible upon discharge into the receiving streams. It has also been found that the receiving water is toxic, and that discharge of effluent actually reduces (or eliminate) toxicity. In instances like these where the effluents constitute the main part of the stream, less strict criteria might be applicable (no acute toxicity at end of pipe, allowing some degree of chronic toxicity in the stream).

It should be kept in mind that dilution is not a solution to get rid of toxic chemicals. Because of limited resources, every attempt should be made to limit the discharge of highly toxic effluents.

4.2 Development of permit limits for WET

In order to set permit limits the data generated during the previous section (4.1) should serve as base-line information. However, additional testing should be carried out to establish effluent variability and to include dilution with the receiving water (relative toxicity - see section 2.1).

Usually the same tests used to establish whether or not an effluent is toxic (section 4.1.2) should be applied to develop permit limits for WET. If very low toxicity was found with the acute tests, chronic tests would be more applicable (as soon as they are available). Some

effluents (*e.g.* treated sewage effluent, paper mill effluent) stimulate algal growth because of high nutrient levels, or precipitation might occur (*e.g.* treated sewage effluent, paper mill effluent) (Slabbert *et al.*, 1996a). If such observations were made during the impact assessment phase (see section 4.1) the algal test could be omitted during permit limit development.

Effluent variability could, for example, be established by conducting the toxicity tests on four grab samples taken every 6 h during a 24 h period. This could be done once a month for any given period. The length of time will depend on the amount of variability and other factors given under section 3.2.1. The receiving water should be used for dilution to incorporate chemical interaction between effluent and receiving water. If large variation is expected, the sampling intervals during a 24 h period could be shortened to include more samples. Furthermore, if large variations is only expected during certain times of the day [*e.g.* paper mill effluent, metal plating effluent (Slabbert *et al.*, 1996a)], samples could be taken at 2 to 3 h intervals over a 8 to 12 h period. If short-term variation is unlikely, *e.g.* long retention periods [paper mill effluent after clarification (Slabbert *et al.*, 1996a)], tests on samples taken at short intervals would not be necessary. Testing on such samples could be carried out once a month for any given period. If mutagenicity tests are used, testing could be carried out once a month (tests are very expensive and labour intensive). In case of chronic tests (future inclusion) 24 h composite samples are usually tested.

The receiving water might be toxic [*e.g.* Small Blesbok Spruit (Slabbert *et al.*, 1996a)]. Before any dilutions are made with receiving water it is necessary to conduct a set of toxicity tests on the receiving water. If the receiving water is toxic, a water sample higher upstream/ standard dilution water/surface water of similar composition could be used for dilution. If it is the objective to establish the contribution of the effluent to receiving waters (*e.g.* in multiple-source discharge situations), receiving water should be used for dilution whether or not it is toxic. A standard control should always be included in all tests for quality assurance.

In case of new effluents, permit limits should be developed (if required) once the facility is in operation (not on simulated effluent). In order to obtain as much as possible information on the simulated effluent it is, however, recommended to also carry out dilutions with the receiving water when evaluating the potential for toxic impact (dilution with standard dilution water).

The results of the evaluation should be calculated and interpreted, and the potential for toxic impact established, in the same way as described in section 4.1. Advanced techniques might also be necessary (*e.g.* chemical fate modelling, physiological investigations and monitoring of instream organisms).

4.2.1 Derivation of permit limits

Permits should clearly state:

The specific endpoints (permit limit)-

This could be stated as a concentration or as a dilution factor (*e.g.* MEC: <10% or DF_a: >10; NOEC: 2% or DF_c: 50). Depending on the test detection levels/sensitivity either acute or chronic tests might apply to a given situation (*e.g.* when acute toxicity is low chronic tests for control are more applicable). However, while chronic tests are

not available, the limit should be stated in terms of acute tests. If acute tests are used and calculation of the true toxicity endpoints are not possible (*e.g.* LC_{50} >100%), an acute toxicity test could still be used for permit setting and compliance monitoring, but the endpoint could be changed to a greater sensitivity (*e.g.* it could be stated that no toxicity should be detected at the 100% effluent). Such tests would not accurately quantify any level of chronic toxicity present (if applying an ACR).

Which toxicity tests should be used (including test species and methodology) -

The best control mayl be obtained if fish, water flea and algae are used. However, in certain cases (*e.g.* effluent highly consistent; little variation in treatment) the two most sensitive, or even the one most sensitive test might be appropriate for control.

There are some rapid tests available (Slabbert, 1996) which could be calibrated against the standard tests (Hunt *et al.*, 1991), and which could be used in the place of a standard test/s for compliance monitoring. As such rapid tests are not nearly as sensitive as standard tests, the toxicity of the effluent should be fairly high to enable the use of such tests. In case of metal plating effluent, the urease enzyme test has been found to be very sensitive, and a possible candidate for calibration against the standard tests (Slabbert *et al.*, 1996a). In cases where there are highly sensitive species in the receiving water to be protected, it might be required to use a specific organism for compliance monitoring. Testing should then be conducted with this organism during permit development. See section 3.1 for information on test organisms and quality assurance.

Dilutions for testing -

A range of dilutions should be tested, as established during the permit development process. The use of a single concentration is not recommended as the error and variability will be too large. The range should be wide enough to calculate the required endpoints.

Monitoring frequencies -

There is no fixed guidance on the establishment of monitoring frequencies. These will depend on factors such as:

- Type of treatment process, including retention time;
- Environmental significance and nature of pollutant/s;
- Cost of monitoring relative to the discharger's capabilities and benefit obtained;
- Compliance history;
- Number of monthly samples used in developing the permit limit; and
- Effluent variability.

Germany collects and tests samples on a weekly basis (4 samples per month). The EPA recommends that samples are collected and tested on a monthly or bimonthly basis and that on each occasion several grab samples are collected *e.g.* four taken 6 h apart or six taken 4 h apart (Slabbert *et al.*, 1996a).

Statistical procedures for analyzing data -

Regression analysis, as discussed in section 4.1.2.4., could be applied.

Quality assurance -

See section 3 and **Guidelines for toxicity bioassaying of waters and effluents in South Africa** (Slabbert, 1996).

Other important information includes sampling points and sampling method (grab/composite).

4.3 Compliance monitoring

Once the permit containing limitations and conditions to control effluent has been issued, the permittee should be responsible for attaining, monitoring and maintaining compliance with the requirements of the permit. If required, the DWA&F could conduct its own inspection testing. All the tests specified in the permit limit could be applied, or if preferred the most sensitive test could be used.

4.4 Identifying/categorizing/prioritizing toxic effluent

The DWA&F might require information on effluent toxicity in general, without considering permit limits at the stage. Here, the tests specified in section 4.1 could be applied to a single effluent sample to identify toxic effluents, to place them in a category and to prioritize them for impact screening. If enough experience has been gained with toxicity testing, all the tests might not be necessary. In case of very toxic effluents, it might also be possible to use rapid tests.

4.5 Reducing effluent toxicity

Where monitoring data indicates unacceptable effluent toxicity, one mechanism to bring the discharger to compliance is a toxicity reduction evaluation (TRE). The purpose of such an evaluation is to investigate the causes of toxicity and to identify corrective actions for difficult toxicity problems. The tests recommended in section 4.1.2 are applicable.

4.6 Ambient toxicity testing

Ambient toxicity is measured by conducting a toxicity test on a sample taken from a water body. By means of such testing ambient interactions of effluent and receiving water, persistence and interferences can be established. Ambient toxicity testing is particulary important where impact is caused by multiple point sources. Ambient toxicity testing has mostly shown that multiple discharges result in additive (acute) toxicity.

Ideally sampling should be carried out under conditions of low flow in the receiving water so that worst case toxicity conditions become apparent. It is very important to closely replicate the worst case receiving water conditions in toxicity tests, because of the influence of environmental conditions. For instance, if tests are carried out at temperatures different from that in the receiving water, the toxicity in receiving water may be under or over predicted.

The basic ambient toxicity testing procedure consists of exposing organisms to water samples taken from selected sampling points above, at and below the point of discharge. With such testing the actual receiving water is directly tested. Since the concentrations after discharge is usually very low, chronic toxicity tests should be conducted. The location of sampling points should be determined on the basis of known mixing characteristics of the receiving water and the basis of knowledge of important sensitive areas within the receiving water. Such testing could be conducted by the DWA&F or the discharger might be requested to do so.

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