THE USE OF DAPHNIA SPP. AND INDIGENOUS RIVER INVERTEBRATES IN WHOLE EFFLUENT TOXICITY TESTING IN THE VAAL CATCHMENT

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FOREWORD

Aquatic toxicology is a well-established science and water quality management tool in the developed world, particularly in the United States and Europe. In South Africa, methods have been developed over the past two decades and we are now poised to integrate past experience and knowledge, with that from the developed world , to implement applied aquatic ecotoxicology in water quality management. This project makes a key contribution to this potential.

Important components of the present water quality management and policy environment include: a National Water Act (No. 36 of 1998) which requires resource protection in order to ensure sustainable resource use; a resource protection policy which will be implemented through 1) **resource directed measures** (*e.g.* ecological reserve assessment, environmental water quality guidelines, and the development of resource quality objectives), and 2) **source directed controls** (*e.g.* effluent standards and discharge licence criteria); and the recognition that risk assessment is a valuable resource protection tool.

Many pollutants enter the environment as complex mixtures - either directly or indirectly as part of sewage, or after irrigation. Chemical interactions over these indirect pathways are complex, and final chemical products are difficult to predict. These complex mixtures are often not acutely toxic, and many of the chemicals are present in below-measurable concentrations. They can, however, be seriously detrimental to the environment.

The most effective approach to complex effluent control is direct toxicity assessment, or whole effluent toxicity testing, exposing test organisms directly to the complex mixture. However, results depend on the type of diluent (*e.g.* laboratory culture medium, or receiving water) and test organism (*e.g.* standard toxicity test organisms, such *Daphnia* spp., or wild populations from the receiving environment). Exposures and end-points also differ: 1) short-term exposure and mortality (acute lethal tests) and 2) longer term exposure and growth or reproduction changes (chronic sub-lethal tests). Acute tests are cheaper, and the results less protective, compared with chronic tests.

The General Authorisation (No 1191, Government Gazette No 205526, 8/10/1999) prohibits the discharge of complex industrial effluents without a licence. It will therefore be possible to prescribe direct toxicity tests as part of licence conditions. It is important that requirements are cost effective. It is envisaged that a tiered approach to testing will achieve this. (For example, a screening procedure, using a suite of the simplest, cheapest tests, going on to site specific ecological risk assessment, including organisms and water from the receiving environment.)

This project provides support for this tiered approach. The most protective results came from the standard *Daphnia* tests in prepared laboratory water, and the most relaxed results from wild populations with receiving water diluent. This accords with the concept that more relaxed licence criteria could accompany site-specific risk-based investigations. This project is part of a suite of research efforts to formalise methods in applied aquatic ecotoxicology, to develop standard methods for the use of standard and wild populations, and for standard sub-lethal tests. Other associated research areas are environmental chemical fate and effect studies, and the use of toxicity results to link chemical and biological monitoring results by providing a causal relationship between a chemical concentration and a biological response.

EXECUTIVE SUMMARY

INTRODUCTION

Freshwater is vital to societal, and environmental, well-being and any changes in the distribution, abundance and quality of water resources and ecosystems are detrimental to this societal and environmental sustainability. Increasing socio-economic activities world-wide have been accompanied by increased pollution stress on the aquatic environment. The need for improved efficiency in water quality management is urgent and immediate and it is important that policy to manage freshwater systems is underpinned by sound science. Strategies to manage receiving water quality have been implemented world-wide and include chemical monitoring, biological monitoring and toxicological assessments, all of which are supported by on-going research.

Water quality management in South Africa has come a long way since 1919, when it was first promulgated (Union Health Act 36 of 1919; van der Merwe and Grobler, 1990) but only included sewage effluent. Later amendments broadened water quality management to include effluent discharge from industry, mining and storm-water runoff. However, despite these and uniform, and general, effluent standards (UES), as well as DWAFs recognition of the need for integrated water resource management, water quality in the resources continue to deteriorate (DWAF, 1995; Basson *et al.*1997).

The National Water Act (NWA) (No 36 of 1998) provides for *resource protection* by the implementation of *resource directed measures* - where the requirements of aquatic ecosystems are provided as quantitative and qualitative resource quality objectives; and through *source directed controls* - where the source of impact (in the case of water quality this means pollutants) is controlled using a licensing system. However, water quality management is particularly complex because it encompasses many different factors and variables - all of which interact.

Whole Effluent Toxicity testing in water quality management

Most pollutants enter water resources (aquatic ecosystems such as rivers, wetlands, dams, lakes, and aquifers) as mixtures. The water in the water resource is also a mixture of water molecules together with a variety of dissolved and suspended substances. These dissolved and suspended substances are termed "pollutants" when they adversely affect people and/or the natural environment.

When a waste-water enters a river or other freshwater system, the wastewater and river water mix and a new, unique chemical mixture forms. Effective water quality management requires that mixtures which threaten people and ecosystems be identified and controlled. Potentially polluting wastewater can enter water resources: directly from industrial outfalls and pipes; together with domestic sewage form the pipes or outfalls from sewage treatment works; or as run-off from the landscape, as non point-source pollution, when water flows over agricultural lands or over formal and informal urban areas.

To date in South Africa, as in many other places, pollution control has mainly been approached by controlling the concentrations of the individual chemical components of mixtures, but this

substance specific, chemical, approach is of limited use in controlling the effects of discharging the complex chemical mixtures.

This is because complex chemical mixtures may contain substances that cannot be individually identified; and might be too numerous, too expensive, and/or too difficult, to analyse. Some substances may be present in quantities that cannot be chemically detected, but still have a negative effect. As biological process occur, new mixtures can be formed that are either difficult or impossible to characterise. In addition, mixtures can have substantially different environmental effects than the sum of individual substance effects. Chemical, substance-specific limits, which are dependent on chemical analysis for enforcement, are therefore of limited use in authorising, and controlling the environmental consequences, of the discharge of complex chemical mixtures.

The question is, if chemical testing is inadequate to control the effects of complex mixtures how should they be managed and controlled? The United Kingdom, the United States, and the Netherlands have led the way in introducing direct toxicity assessment or whole effluent toxicity (WET) testing as the way to effectively manage these complex mixtures (Anon, 1989; UK Environment Agency 1996; Tonkes 1998; USEPA 1991;2000). This approach involves exposing a variety of test organisms to the complex mixture, and quantifying the effect on test organisms. (Grothe *et al.* 1996). Licence specifications are then in terms of a toxicity-test endpoint, rather than a chemical concentration. The test organisms respond to the integrated effect of exposure to the whole mixture.

AIMS

The project aims to investigate and compare the responses of *D. pulex* and site-specific indigenous invertebrates to a range of chemical mixtures in order to evaluate the use of WET testing in water quality management. The site-specific organisms, and some of the waste-waters tested were collected in the Vaal River barrage area, and the results would be particularly applicable in that area.

The specific aims of the project are listed below. During the project, it became obvious that the proposed approach of undertaking acute toxicity tests using selected industry effluents was not feasible as the none of effluents resulted in measurable acute toxic effects. As a result of these practical constraints, the Project Steering Committee agreed that a change in the approach was appropriate and it was decided to create an artificial effluent in order to evaluate *Daphnia pulex* and indigenous invertebrates for whole effluent toxicity testing in the Vaal catchment. Consequently, the proposed aim of relating real industry effluent toxicity test results to industry discharge permits and their actual discharges with a view to reviewing current discharge criteria and receiving water quality objectives set by Rand Water for the Vaal Barrage could not be undertaken. The Steering Committee agreed to the proposed change in application of the toxicity test results listed below (point 4).

i.) Identify and access test industries and effluents

- List and visit a range of industries which discharge effluents into the Vaal River (either directly or indirectly via a tributary).

- Select effluents from representative industries in collaboration with Rand Water and DWAF.
- Create an artificial complex effluent mixture.

Results are reported in Chapter 3.

ii.) Select indigenous invertebrates from a reference site in the Vaal River as test organisms in whole effluent toxicity tests

- Visit a range of possible reference sites identified by previous SASS biomonitoring, and identify one or two abundant indigenous riffle-dwelling (benthic) invertebrate species for use in the artificial streams.
- Set up artificial stream systems at Rand Water Scientific Services.
- Decide on the test medium for the toxicity tests, *i.e.* river water from the reference site or dechlorinated tap water.
- Undertake preliminary tests to assess the suitability of using the selected test organisms in the artificial streams.

Results are reported in Chapter 3 and Appendix 1.

iii.) Use Daphnia pulex and selected indigenous organisms as test organisms in whole effluent toxicity tests

- Undertake acute toxicity tests exposing the standard organism *Daphnia pulex*, with culture medium and river water as dilution waters, to selected industry effluents and an artificial effluent.
- Undertake acute toxicity tests exposing selected indigenous invertebrates, with river water medium as the dilution water, to selected industry effluents and an artificial effluent.

Results of the toxicity experiments are reported in Chapter 4.

iv.) Relate toxicity test results to the application of WET testing in water quality management

- Evaluate the use of WET testing to the source directed control of complex effluents in the Vaal barrage area.
- Evaluate the use of *D. pulex* and indigenous invertebrates in WET testing.

The discussion is presented in Chapters 5 and 6.

An M.Sc student, VJ Everitt, was funded in part by the project and a detailed summary of the major findings of her project can be found in Appendix 2. The M.Sc was awarded to Ms Everitt at the Rhodes University Ceremony in Grahamstown in 2000. A brief summary of the project is included in the Executive Summary. Ms Everitt's research contributed to project K5/815 by examining sources of variability in toxicity testing associated with *D. pulex*. The research contributed to the on-going discussion on advantages and disadvantages of using laboratory-reared toxicity test organisms and wild-caught indigenous invertebrates in both reference toxicity testing and whole effluent toxicity testing.

BACKGROUND

Whole effluent toxicity (WET) tests are considered useful tools in protecting the environment from the potentially harmful effects of effluents (Chapman, 2000). An underlying assumption of WET tests is that laboratory toxicity tests are predictive of effects in the receiving water resource (Dorn, 1996). Toxicity assessments of effluents is becoming increasingly more common, as it is not possible to test all combinations of the thousands of chemicals in use, for monitoring and regulatory purposes, to try and establish cause-and-effect links (Roux 1994).

There is sufficient international experience to recommend WET testing as an efficient, and cost-effective approach to the management of complex industrial waste- waters. However, *within* the WET assessment approach, a wide range of tests and test organisms can be used, and it is important that we select the most appropriate combination for South African conditions.

Complex industrial waste-waters can range widely in the threat they pose to human and water resource health. It is therefore important to effectively screen those which pose a low or minimal risk to health, using the shortest and cheapest tests, and then require the lowest level of compliance monitoring. Generally a tiered approach is favoured, where the level of risk posed by the effluent is related to the extent of the testing and monitoring required.

The chemistry of the natural resource into which the effluent flows may mitigate the toxicity of the effluent, and in addition, wild, natural populations may be more tolerant than laboratory-bred test organisms. It may therefore be possible to allow the disposal of effluents that are identified as posing a hazard after testing with standard test organisms. However, as the risk to the environment is increased, the level of knowledge about the effluent; the receiving water; and the response of organisms; that is required increases. Therefore a more relaxed licence condition will carry more onerous testing and monitoring requirements. This is a good example of the "polluter pays" principle.

Within any application of WET testing, the discharger should evaluate the comparative benefits of toxicity identification and reduction compared with the more comprehensive testing required to more accurately assess the risk posed by the effluent. More detailed testing may result in a more relaxed licence criterion, but would also identify unacceptable risks and effluents which should simply not be discharged to the environment without toxicity identification and reduction.

In this study, *D. pulex* were exposed to an artificial effluent first using laboratory medium as the test diluent; then using river water as the diluent - thus taking into account the possible mitigating affect of receiving water chemistry. Then followed tests using indigenous, site specific riverine organisms as test taxa, which took into account any differences in tolerance between the wild populations from the receiving water resource as compared with a standard test organism.

Whole effluent toxicity (WET) testing and aquatic toxicology

Since a purely chemical analytical approach is unable to protect the aquatic environment, and chemical monitoring does not take into account either environmental factors which affect toxicity (*e.g.* hardness, pH, temp, suspended inorganics, dissolved organics); or the potential transformations such as speciation which chemicals can undergo and may result in altered toxicity; WET methods are considered suitable for predicting instream effects.

Aquatic organisms integrate the effects of their environment during their lifetime, and can therefore be used in experiments (bioassays) and also to reflect conditions to which they may have been exposed to earlier (biomonitoring). Bioassays, both toxicity tests and bioaccumulation studies, have been shown to be highly effective in water quality monitoring programmes in developed nations, where improvements in environmental water quality have been achieved (Chapman, 1995a).

Bioassays, complemented by chemical and biomonitoring data, are essential tools for the assessment of effluents: both biological data and chemical data together are important for the management of the quality of waste-water and the receiving water bodies.

There are numerous ways to assess the toxicity of inputs into the aquatic environment and these are mainly standard acute and chronic tests (bioassays) in order to show either "end-of-pipe" toxicity or receiving water toxicity (Roux, 1994). Acute toxicity tests are generally less than four days, and usually mortality is measured, while chronic toxicity tests assess the effects of long-term exposure to a toxicant, typically expose the test organisms over at least part of their life-cycle and measure responses such as growth and reproduction (Rand, 1995). Acute toxicity tests tend to be cheaper to perform and are performed most commonly.

The specifics (*i.e.* choice of test organism, exposure system, dilution water) of undertaking WET tests are determined by the fundamental question being addressed; and need to take variabiltiy in to account.

MATERIALS AND METHODS

Laboratory toxicity tests are considered the first step in a tiered approach in establishing acceptable levels of effluents and standard experimental protocols for toxicity tests are accepted as suitable methods in establishing the toxicity of effluents. The standard methods for undertaking acute toxicity tests were for a static test system and a recirculating test system. The static test uses the standard laboratory-reared test organism *D. pulex*, while the recirculating test method uses field-collected, site-specific, indigenous invertebrates. The field-collected organisms used were two species of mayfly, namely *Afronurus peringueyi* (Heptageniidae) and *Euthraulus elegans* (Leptophlebiidae). The site-specific organisms were collected from the Vaal River at Engelbrechtdrift Weir, which is downstream of the Vaal Dam and upstream of the Vaal Barrage. The Vaal Barrage receives input from several tributaries draining the industrial complexes of the Vaal Triangle and Johannesburg; data presented in Appendix 1 show that Engelbrechtdrift Weir is significantly less impacted than a site downstream of the Vaal Barrage.

In collaboration with Rand Water, a number of industry effluents from the Vaal Triangle were selected for toxicity testing. However, these effluents proved to have sublethal rather than acute effects, and an artificial effluent was created based on results by Slabbert *et al.* (1998). The artificial effluent was based on a metal plating industry effluent, and was known to have an acutely toxic effect on both *D. pulex* and fish. A stock solution was made at the start of the experimental period, and the same stock was used to make effluent concentration ranges for both *D. pulex* and site-specific indigenous invertebrate experiments.

The experimental design followed was a regression design and, in order to improve the prediction of point estimates (*e.g.* LC_{50}), the minimum number of effluent concentrations used in the experiments was 9 for the site-specific indigenous invertebrate experiments and 8 for the *D. pulex* experiments. In order to test the proposed tiered approach for WET testing, and increase environmental realism, both receiving water and culture water were used to make effluent concentration ranges. Receiving water was used as dilution water to make effluent concentrations for the recirculating experiments, while both receiving water and culture water were used to make effluent concentrations for the *D. pulex* experiments in order to establish whether dilution water affected toxicity of the artificial effluent.

In order to standardise between the two experimental methods as far as possible, the experimental end-point measurement for all test species was mortality as this is widely accepted to be an unambiguous measure of response. The standard method for the static test was the 48-hour acute toxicity test method as specified by USEPA (1991) and the standard method for the recirculating test was the 96-hour acute toxicity test method for artificial streams as specified by DWAF (2000). Four experiments were undertaken using the site-specific mayflies and 3 experiments were undertaken using *D. pulex*.

Data were analysed using probit and trimmed Spearman-Karber; lethal point estimates and confidence intervals were obtained. Various methods (*e.g.* the APHA formula and coefficient of variation) were used to assess variability both within species (*i.e.* between experiments) and between species, and whether differences between LC values were significant.

RESULTS AND DISCUSSION

Acclimation mortality for the site-specific indigenous invertebrates was low (<3% across all four experiments) and there were no control mortalities. Similarly, there were no control mortalities for the *D. pulex* experiments. Therefore, the toxicity test results for all species were considered acceptable.

Species differ considerably in their sensitivity to toxicants and using site-specific indigenous invertebrates in toxicity tests allows for a more accurate prediction of the effects of pollutants in receiving water. *D. pulex* in culture water had the lowest LC_{50} value (0.2-0.4% effluent), which was significantly lower than the LC_{50} value for *D. pulex* tested in receiving water (0.4-1.3% effluent) and the LC_{50}s for *D. pulex* was significantly lower than for both the site-specific indigenous invertebrate species tested. There was a significant difference in sensitivity between the two site-specific invertebrates tested, with the leptophlebid (*E. elegans*) being significantly more sensitive than the heptagenid (*A. peringueyi*) (LC_{50}s were 8.9-12% effluent and 40-214% effluent, respectively).

The coefficient of variation (%CV) between experiments was lowest for *E. elegans* (18%) suggesting that between experiment variability for this species was acceptable (there was also no statistically significant difference in point estimates between experiments). The %CV for *A. peringueyi* was considered too high (76%) (although no statistically significant difference could be established between the experiments), but this may have been a result of the organisms tolerance to the artificial effluent: 100% cumulative mortality was not reached in any of the experiments and the confidence intervals for the predicted point estimates were wide. Similarly,

although due to their sensitivity rather than their tolerance, it was difficult to find an appropriate concentration-range for *D. pulex* and %CVs are high. The confidence intervals for *D. pulex* tested in receiving water are wider (53%) than those for *D. pulex* tested in culture water (49%), which concurs with the general view that increasing environmental realism (*i.e.* use of receiving water) results in increased variability in experimental results but this increased variability is no greater than variability experienced in effluent toxicity tests. There was no more variability in the toxicity test results (as assessed by %CV) as a result of the selected test system, *i.e.* static or recirculating test system. This suggests that the recirculating test system using artificial streams is suitable for assessing the toxicity of effluents in the Vaal River catchment.

The intention of effluent testing programmes world-wide has been to reduce the toxic effects of discharges of receiving water resources and WET testing has been shown to be effective in achieving the aim of reducing the discharge of toxic discharges from point sources although there are a number of sources of variability which may affect the interpretation of toxicity test results. Variability is an inherent part of effluent toxicity tests, and it has been suggested that rather than attempting to reduce the sources of variability, by *e.g.* developing further new acute toxicity tests, the sources of variability should be acknowledged, and incorporated, in the interpretation of the test results.

The results presented in this pilot study are for acute toxicity tests using an artificial metal plating industry effluent. They provide good evidence that the tiered approach for the assessment and effective management of complex waste discharge, in this case metal plating industry effluent, to the Vaal River catchment may be useful and appropriate. The first level of testing would require a standard laboratory-reared organism, such *D. pulex*, to be tested in culture water; this is the cheapest test method, but would also be the most protective for the receiving environment (*i.e.* most stringent criteria). The next tier of testing would increase environmental realism, by introducing use of receiving water in toxicity tests. This increases variability of the toxicity test result, which may make the test result more complex to interpret, but may provide more relaxed criteria for discharge by industry. The third level, which is more expensive, but increases environmental realism further through use of site-specific indigenous invertebrates may show that less stringent criteria may be applied and still be protective of the receiving water.

However, it is important to note that acute toxicity tests are short-term tests (48-hours for the *D. pulex* and 96-hours for the site-specific indigenous invertebrates), represent an extreme response (mortality) and the effluent used in the toxicity tests was an artificial effluent. The data provided therefore only provide evidence that the selected management approach is appropriate for acutely toxic effluent, where the end-point measurement is mortality. Numerous experiments undertaken over a period of two years, using a range of industry effluents from the Vaal Triangle, indicated more subtle and sublethal effects on the selected test organisms (these data will be archived in the toxicology database currently housed at CAT-IWR). This has implications for the effective management of water quality of the receiving water resource, the Vaal River.

The fact that no acutely toxic effluent was found during the experimental periods does not mean that no acutely toxic effluent is being discharged to the Vaal River. It rather indicates that there are numerous discharges to the receiving water which have sublethal and chronic effects which will not be adequately assessed during short-term (acute) toxicity tests. Short-term toxicity tests do not provide accurate predictions of longer-term environmental effects and it is imperative that

more ecologically relevant toxicological data be generated in order to more accurately predict environmental effects of effluents.

Therefore, in order to effectively manage the water quality of the Vaal River catchment, it is important that sublethal and chronic toxicity testing be undertaken so that all the effluents can be adequately assessed.

Data and experience obtained through the course of this research project has highlighted gaps and shortcomings in the current toxicological knowledge-base in South Africa. A number of recommendations for immediate future aquatic toxicology research in order to address these and obtain useful toxicological data and understand how best to apply the data for effective management of South Africa's water resources are listed:

i.) Continue to undertake acute toxicity tests using (site-specific) indigenous test organisms, and using both single-substances and whole effluents, in order to better understand variability in toxicity data associated with using different organisms, different chemicals and different site-specific receiving waters.

ii.) There is an urgent need for the development of chronic and sublethal test methods, and appropriate test end-point measurements, using indigenous organisms.

iii.) Develop methods which can link laboratory toxicity test results, both chronic and acute, to instream biological assessments.

iv.) Development of new toxicity test methods to assess effects of pulsed exposure to chemicals, undertake *in situ* toxicity assessments and assess sediment toxicity.

THE USE OF INDIGENOUS MACROINVERTEBRATES AND *DAPHNIA PULEX* IN ACUTE TOXICITY TESTING (APPENDIX 2)

Aquatic toxicology has been identified as a valuable tool in the identification and management of chemical pollution in aquatic ecosystems. Standardised methodologies for acute aquatic bioassays in South Africa have been adopted from international agencies. As a result of these standard methods, the use of laboratory cultured organisms (*e.g. Daphnia pulex*) for toxicity testing has been more popular than that of indigenous field-caught organisms. The suitability of these cultured organisms for representing the tolerance limits of indigenous aquatic organisms is however questioned. The popular *D.pulex* is a lentic aquatic species (Pennack 1978), whereas the predominant aquatic environment in South Africa is riverine (DWAF 1997). This study investigated and compared the use of *D.pulex* and indigenous macroinvertebrates as aquatic toxicity test organisms in South Africa.

The specific aims and objectives of the study were as follows:

1. To investigate the use of the standardised laboratory organism, *D.pulex,* as a test organism for setting and revising water quality guidelines to protect aquatic ecosystems from the adverse effects of pollution in South Africa.
2. To investigate the tolerances of different *D.pulex* cultures, set up from different populations, to a single-substance toxicant (zinc sulphate).
3. To assess the use of indigenous site-specific macroinvertebrates in toxicity testing by comparing the sensitivities of selected indigenous macroinvertebrates to those of *D. pulex*, using both a single-substance toxicant (zinc sulphate) and selected whole effluents.
4. To assess the practicality of whole effluent toxicity testing in South Africa in general.

D.pulex has been extensively used in aquatic bioassays in the past because they are easily cultured, individuals of a known age and background are available year round, daphnid biology is well documented, their sensitivity to numerous chemicals has been determined, and they were found to provide the most toxicological information per unit effort (Eagleson *et al.* 1986). As a result, culturing techniques and optimal test methodologies have been thoroughly investigated and standardised. An advantage of standardised methodologies is that experimental variability should be reduced (Rand *et al.* 1995). In this study however, variability between separate acute 48 h daphnid experiments, was high (CV above 130%), even though the same cultures were used and standard methods were followed. Variability between experiments has important implications when setting a numerical guideline determined to represent the tolerance limit for a species, and in determining compliance to whole effluent and water quality guidelines aimed to protect aquatic ecosystems (Parkhurst *et al.* 1992). The greater the variability, the less confidence is placed in the results (Thursby *et al.* 1997). Thus the importance of controlling and monitoring factors which may influence toxicity results is stressed by this study.

Laboratory *D.pulex* cultures tend to be kept for many years and no studies were found to indicate if the time spent under laboratory conditions influences the tolerance of individuals. It is assumed that the parthenogenetic *D.pulex* cultures remain genetically homogenous (Weider 1993), *i.e.* no mutation or genetic recombination is expected to occur. However, in adverse conditions males are produced and therefore may contribute to the genetic makeup of neonates, thus altering the genetic structure of the culture (Cooney 1995). Two *D.pulex* cultures were established in 1998, with the different generations being kept separate from one another, to try to determine if culture age (time spent under laboratory conditions) influenced the tolerance to a single-substance toxicant. No conclusion could be made however to whether or not the susceptibility to zinc changed with culture age or generation. It is however recommended that the assumption of genetic structure homogeneity over time (and hence sensitivity to chemicals) is further investigated, as this may be a source of experimental variability over time.

To investigate if population source influenced the susceptibility to a single-substance toxicant, two mature cultures (established for at least 10 years from independent populations) were compared with each other in terms of their 48-hour LC_{50}s. Unfortunately, due to high experimental variability, it could not be stated with any confidence that the differences in tolerance between the cultures were due to genetic differences. Inter-populational differences to toxic stress have been shown to occur in other species and it may therefore be important that *D. pulex* cultures in different laboratories originate from one female. This is particularly important if a species, such as *D.pulex*, is to be used in the setting of numerical water quality guidelines, such as discharge licences. By ensuring the same genotype between laboratories, some potential for experimental variability may be reduced.

Acute 96-hour toxicity tests using suitable site-specific indigenous invertebrates were compared to the standard acute 48-hour *D.pulex* toxicity test, using both a single-substance toxicant (zinc sulphate) and selected whole effluents. No concentration-response was recorded in the selected whole effluents for either *D.pulex* or the selected indigenous invertebrates, thus sensitivities to the effluents could not be compared. However, *D.pulex* appeared to be more sensitive to zinc than the three selected mayflies (*Afronurus peringueyi, Euthraulus elegans* and the Baetidae) and the shrimp, *Caradina nilotica*. The Baetidae and *E.elegans* showed 96-hour LC_{50}s that were similar to the 48-hour LC_{50}s calculated for *D.pulex*, and could potentially be used in the future as indigenous test organisms.

Numerous advantages and disadvantages for using *D.pulex* and indigenous invertebrates as aquatic toxicity test organisms were noted during this study. The advantages of using *D.pulex* include the convenience of laboratory cultures (animals of known age and background are available year round) and standardised toxicity test methodology. However in this study, the reliability of *D.pulex* cultures varied. Neonates were not always available for testing and tolerance to a reference chemical (zinc sulphate) appeared to fluctuate, reducing the confidence in results. The choice of reference sites, species abundance and availability, and their suitability to experimental systems were noted as important considerations in this study with regards to the use of indigenous invertebrates as toxicity test species. The above factors may make the use of indigenous invertebrates impractical, however their use is vital in the setting of water quality guidelines, as they are the organisms the guidelines are designed to protect (Buikema *et al.* 1982).

When setting a guideline aimed at protecting the aquatic ecosystem, it is recognised that a suite of indigenous test organisms from different trophic levels should be used, as different species are known to exhibit different susceptibilities to the same chemical (Roseth *et al.* 1996). By establishing a single-substance guideline or whole effluent discharge licence based on the tolerance of a suite of indigenous species, the guideline aimed at protecting the aquatic ecosystem will be more realistic (Buikema *et al.* 1982). The use of indigenous invertebrates in the monitoring for compliance may however prove to be impractical, whereas the use of *D.pulex* in compliance monitoring is more practical.

CAPACITY BUILDING

Capacity building is an integral part of the Centre for Aquatic Toxicology (IWR, Rhodes University) and as a result of WRC funding, training students in aquatic toxicology, and technology transfer of methods and research findings, has been possible through:

- undergraduate courses offered at Rhodes University: a semester in aquatic ecology, at third year level, which includes a 4 week module on water quality and aquatic toxicology;
- supervision of postgraduate aquatic toxicology projects (honours and MSc projects);
- training courses which either incorporate aquatic toxicology (*e.g.* National short course on the "Role and use of biological monitoring in aquatic resource management") or are wholly centred on aquatic toxicology (*e.g.* Lever Ponds "Introduction to Applied Aquatic Toxicology"). These courses attract delegates from a range of backgrounds, including regulatory agencies (water boards, DWAF and DEAT), industry, consultancies and other tertiary organisations.

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LIST OF ABBREVIATIONS

AEV	Acute effect value
ANOVA	Analysis of variance
CEV	Chronic effect value
CV	Coefficient of variation
DWAF	Department of Water Affairs and Forestry
EC	Effect concentration
LC	Lethal concentration
LOEC	Lowest observed effect concentration
NOEC	No observed effect concentration
QA/QC	Quality assurance/Quality control
RWQO	Resource water quality objective
SASS	South African Scoring System
TU	Toxic units
USEPA	United States of America Environmental Protection Agency
WET	Whole effluent toxicity

GLOSSARY OF TERMS

The following terms are used in the report and are defined here to ensure clarity and consistency of use. Reference sources are indicated.

Absolute toxicity — The toxicity of an effluent without considering dilution (Slabbert *et al.*, 1998).

acclimation — The time period before the onset of a toxicity test during which the test organisms are kept in untreated and toxicant-free dilution water with physical and chemical characteristics similar to the dilution water to be used in the toxicity test (Rand, 1995).

acute — "Having a sudden onset, lasting a short time. Of a stimulus, severe enough to induce a response rapidly. Can be used to define either the exposure or the response to an exposure (effect). For clarity, the length of the exposure (short, medium, or long) and the nature of the effect end point (lethal or nonlethal) should be specifice. The duration of an acute aquatic toxicity test is generally 4 d or less and mortality is the response measured" (Rand 1995, p939).

adverse effect — An effect which limits an organisms ability to survive; these effects can be acute or chronic (Slabbert *et al.*, 1998).

bioassay — An assay using a biological system (Chapman, 1995a).

chronic — "Involving a stimulus that is lingering or continues for a long time; often signifies periods from several weeks to years, depending on the reproductive life cycle of the aquatic species. Can be used to define either the exposure or the response to an exposure (effect). For clarity the length of the exposure and the nature of the effect end point should be specified. Chronic exposure typically induces a biological response of relatively slow progress and long continuance. The chronic aquatic toxicity test is used to study the effects of continuous, long-term exposure to a chemical or other potentially toxic material on aquatic organisms" (Rand 1995, p940).

concentration-response curve — The line (often a curve) describing the relationship between different exposure concentrations of a test substance and the response (percentage) of the exposed test population (Rand, 1995).

culture medium — Reconstituted water used to culture test organisms under known laboratory conditions.

definitive test — Organisms are exposed to a range of concentrations of the test substance and the response of the organisms at each concentration is used to establish a concentration- (or dose-) response curve (Slabbert *et al.*, 1998).

dilution water	Water which is used to dilute the test substance to prepare a range of concentrations for an aquatic toxicity test (Rand, 1995).
EC_{50}	The effective concentration of the test substance at which 50% of the test population responds (Rand, 1995).
ecotoxicology	"The science of how chemicals, at toxic concentrations, influence basic ecological relationships and processes" (Brown, 1986, cited in Chapman, 1995, p20).
effluent	A complex liquid industrial discharge which is discharged to the environment (Rand and Petrocelli, 1985). Effluent can also be considered to be the toxin or stressor in toxicity experiments.
end point	The biological, usually adverse, response which is measured in toxicity tests. These can range from acute (lethality) to chronic (growth, reproduction *etc.*) (Rand, 1995).
environmental impact	Any change to the environment, whether adverse or beneficial (ISO 14000 definition).
flow-through test system	An toxicological experimental exposure system in which the test solutions flow into and out of the test chambers on a once-through basis (Rand, 1995).
inherent toxicity	The toxicity of an effluent established by using a standard dilution water (such as reconstituted water or the organism's culture medium) for making the dilutions for the toxicity test (Slabbert *et al.*, 1998).
LC_{50}	The lethal concentration of the test substance at which 50% of the test population dies (Rand, 1995).
lethal	Causing death (Rand, 1995).
Lowest Observed Effect Level (LOEC)	The lowest concentration of a toxicity test solution that has a statistically significant adverse effect, compared to a control group, on the exposed population of test organisms (Rand, 1995).
No Observed Effect Level (NOEC)	The highest concentration of a toxicity test solution that has a statistically significant adverse effect, compared to a control group, on the exposed population of test organisms (Rand, 1995).
pollutant	A general term used to describe chemical, or non-chemical, agents present in the environment which result in an adverse effect (Rand, 1995).

pollution The introduction, either directly or indirectly, as a result of anthropogenic activity of substances which result in deleterious effects such as harm to living resources, hazards to human health, impairment of water quality with respect to water use in agricultural, industrial and other economic uses (Chapman, 1992). Pollution may result from point sources or diffuse (non-point) sources. It is the presence of contaminants that result in an adverse biological effect (Chapman, 1995a).

prevention of pollution Use of processes, practices, materials or products that avoid, reduce or control pollution, which may include recycling, treatment, process changes, control mechanisms, efficient use of resources and material substitution. The potential benefits of prevention of pollution include the reduction of adverse environmental impacts, improved efficiency and reduced costs (ISO 14000 definition).

range-finding test A toxicity test used to estimate the concentration range to be used in a definitive test (Rand, 1995).

Receiving Water Quality Objectives Water quality objectives in receiving waters are specified and point and non-point sources of pollution are controlled so that these water quality requirements are met.

relative toxicity The toxicity of an effluent when it is diluted with the receiving water, to test for interactions after effluent discharge (Slabbert *et al.*, 1998).

Reserve The quantity and quality of water required -

a) to satisfy basic human needs by securing a basic water supply, as prescribed under the Water Services Act, 1997 (Act No. 108 of 1997), for people who are now or who will, in the reasonably near future, be -
   i) relying upon;
   ii) taking water from; or
   iii) being supplied from,

the relevant water resource; and

b) to protect aquatic ecosystems in order to secure ecologically sustainable development and use of the relevant water resource (National Water Act, No. 36 of 1998).

screening test A short test usually used early in a testing programme to establish the potential of an effluent or chemical to elicit an adverse effect (Rand, 1995).

static test system An exposure system for toxicity tests in which the test solution in the chambers is still (Rand, 1995).

stressor Any physical, chemical or biological entity or process that can induce an adverse response (Murray and Claassen, 1999).

sublethal Below the concentration which causes death but may result in effects on behaviour, biochemical or physiological functioning and histology (Rand, 1995).

toxicant A chemical, agent or material which is able to produce an adverse response in a biological system and results in injury to structure and/or function and may result in death (Rand, 1995).

toxicity "The inherent potential or capacity of a material to cause adverse effects in a living organism" (Rand and Petrocelli, 1985).

toxicity test The experimental means by which the toxicity of a chemical/pollutant (the toxin) is used to measure the response of organisms to specific levels of the toxin (Rand and Petrocelli, 1985).

toxicology Toxicology is the study of chemical/agent effects on any physiological functions on living systems or animals (i.e. ultimately chronic effects; reproduction, accummulation of toxins, histology), to be able to predict hazards to humans and the environment (Rand, 1995).

water quality assessment An evaluation of the physical, chemical and biological nature of the water (or effluent) in relation to natural water quality and human effects and intended uses, with particular emphasis on aquatic ecosystem health and human health (Chapman, 1992).

water quality criteria Scientific judgements of concentrations of chemicals (or effluents) which, provided they are not exceeded, will not have an adverse effect on aquatic life in receiving water resources (Denton and Norberg-King, 1996). Water quality criteria are intended to protect most of the species most of the time.

water quality guideline A set of information provided for a particular water quality constituent, and consists of water quality criteria, CEV, AEV, the Target Water Quality Range, *etc.* (DWAF, 1996).

water quality monitoring Collection of information (e.g. chemical and biological data) at set locations and regular intervals in order to establish trends and define current conditions (Chapman, 1992).

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CHAPTER 1:
INTRODUCTION AND AIMS

1.1 INTRODUCTION

Freshwater is vital to societal, and environmental, well-being and any changes in the distribution, abundance and quality of water resources and ecosystems are detrimental to this societal and environmental sustainability (Naiman *et al.*, 1998). Increasing socio-economic activities world-wide have been accompanied by increased pollution stress on the aquatic environment. Generally, remedies to halt the trend for decreasing water quality are only instituted once there is a public perception of decreasing water quality and possible negative effects (Chapman, 1992) and frequently this is linked with progress in socio-economic development. Major point sources of pollution to aquatic ecosystems are considered to be a result of increasing pollutant concentrations as a result of water abstraction from receiving water resources, and the discharge of domestic wastewater, industrial wastes together with some agricultural activities such as animal husbandry to receiving water resources. Diffuse pollution is a result of atmospheric fall-out, urban run-off and agricultural activities such as pesticide and fertilizer application (Chapman, 1992). The effects of point source and non-point source pollution on receiving water are affected by both temporal and spatial variations in flow and natural water chemistry (Pegram *et al.*, 1998)

The need for improved efficiency in water quality management is urgent and immediate and it is important that policy to manage freshwater systems is underpinned by sound science (Naiman *et al.*, 1998). However, it is important that sound and accurate science underpins policy in order to achieve sound environmental decision-making (Policansky, 1998). Strategies to manage receiving water quality have been implemented world-wide and include chemical monitoring, biological monitoring and toxicological assessments, and are supported by on-going research.

1.1.1 Water quality

Water quality is defined on the basis of its physical, chemical, biological and aesthetic characteristic, and determines the health and integrity of aquatic ecosystems (DWAF, 1996). Physico-chemical water quality variables are measures of (amoungst others) dissolved oxygen, chemical oxygen demand, pH, temperature, alkalinity, conductivity, water hardness, turbidity and other dissolved chemicals. Water quality guidelines are commonly used by managers to protect aquatic resources (Warne, 1998), but internationally most water quality guidelines are in terms of individual chemical constituents.

1.1.2 The National Water Act and water quality management in South Africa

Water quality management in South Africa has come a long way since 1919, when it was first promulgated (Union Health Act 36 of 1919; van der Merwe and Grobler, 1990) but only included sewage effluent. Later amendments broadened water quality management to include effluent discharge from industry, mining and storm-water runoff. However, despite these and uniform, and general, effluent standards (UES), as well as DWAFs recognition of water resource management, water quality in the resources continue to deteriorate (DWAF, 1995; Basson *et al.*,

1997). The Receiving Water Quality Objective (RWQO) approach allowed for specification of water quality in receiving water, and in this way, point and non-point sources of pollution were to be controlled (van der Merwe and Grobler, 1990). The approach has taken in to account the assimilative capacity of the receiving water body and is site specific. However, the RWQO approach demands a thorough investigation of the fate of pollutants in the water body, and the impacts that the pollutants could have on water uses and users, and is considered inappropriate for dangerous pollutants and for which the pollution prevention approach is considered a more appropriate approach.

The RWQO shifted the emphasis of water quality management away from national standards toward a decision-based management system where the state and use of the water resource is defined and strategies implemented to achieve these. However, water quality has continued to deteriorate. This is partly due to lack of implementation of receiving water quality objectives - general and special standards have remained the basis for discharge permits, and have now been replaced by General and Special Waste Limits (Government Gazette No. 20526).

However, the National Water Act (NWA) (No 36 of 1998) gives natural aquatic ecosystem the *right* to the water quality that is required for a state of ecosystem health that will ensure sustainable use of the resource. (The ecological Reserve is "the quality and quantity required to protect aquatic ecosystems in order to secure ecologically sustainable development and use of the relevant water resource"). This means that water quality has to allow for use of the resource as well as for long term healthy ecosystem functioning. This is a difficult balance to achieve, and in a developing, and water-scarce country, there is an urgent need for knowledge of the effects of pollutants (toxins) in aquatic ecosystems.

The NWA provides for *resource protection* by the implementation of *resource directed measures* - where the requirements of aquatic ecosystems are provided as quantitative and qualitative resource quality objectives; and through *source directed controls* - where the source of impact (in the case of water quality this means pollutants) is controlled using a licensing system. However, water quality management is particularly complex because it encompasses many different factors and variables - all of which interact.

1.1.3 Whole Effluent Toxicity testing in water quality management

Most pollutants enter water resources (aquatic ecosystems such as rivers, wetlands, dams, lakes, and aquifers) as mixtures. The water in the water resource is also a mixture of water molecules together with a variety of dissolved and suspended substances. These dissolved and suspended substances are termed "pollutants" when they adversely affect people and/or the natural environment.

When a waste-water enters a river or other freshwater system, the wastewater and river water mix and a new, unique chemical mixture forms. Effective water quality management requires that mixtures which threaten people and ecosystems be identified and controlled. Potentially polluting wastewater can enter water resources: directly from industrial outfalls and pipes; together with domestic sewage form the pipes or outfalls from sewage treatment works; or as run-off from the landscape, as non point-source pollution, when water flows over agricultural lands or over formal and informal urban areas.

To date in South Africa, as in many other places, pollution control has mainly been approached by controlling the concentrations of the individual chemical components of mixtures, but this substance specific, chemical, approach is of limited use in controlling the effects of discharging the complex chemical mixtures.

This is because complex chemical mixtures may contain substances that cannot be individually identified; and might be too numerous, too expensive, and/or too difficult, to analyse. Some substances may be present in quantities that cannot be chemically detected, but still have a negative effect. As biological process occur, new mixtures can be formed that are either difficult or impossible to characterise. In addition, mixtures can have substantially different environmental effects than the sum of individual substance effects. Chemical, substance-specific limits, which are dependent on chemical analysis for enforcement, are therefore of limited use in authorising, and controlling the environmental consequences, of the discharge of complex chemical mixtures.

The question is, if chemical testing is inadequate to control the effects of complex mixtures how should they be managed and controlled? The United Kingdom, the United States, and the Netherlands have led the way in introducing direct toxicity assessment or whole effluent toxicity (WET) testing as the way to effectively manage these complex mixtures (Anon, 1989; UK Environment Agency, 1996; Tonkes, 1998; USEPA 1991b, 2000). This approach involves exposing a variety of test organisms to the complex mixture, and quantifying the effect on test organisms. (Grothe *et al.* 1996). Licence specifications are then in terms of a toxicity-test endpoint, rather than a chemical concentration. The test organisms respond to the integrated effect of exposure to the whole mixture. Fisher *et al.* (1989) showed that 40 to 50% of a range of discharges (municipal and industrial) assessed were toxic to either fish or daphnids, based on single acute WET toxicity trials. In order to protect aquatic ecosystems, it is important to be able to quantify the effects of pollutants (*e.g.* effluents) on these resources (Sarakinos and Rasmussen, 1998).

Toxicity tests, and in particular WET tests, are considered suitable for predicting instream effects, although there may be many factors which can confound and detract from accurate predictions (Ausley, 2000; Chapman, 2000). One of the underlying assumptions of WET tests is that laboratory toxicity tests are predictive of effects in the receiving water resource (Dorn, 1996). Effluent toxicity testing is carried out to protect the quality of receiving waters and, in the USA, is primarily used as a regulatory tool (Stewart, 1996).

The Department of Water Affairs and Forestry has identified whole effluent toxicity testing (WET) as a tool for assessing effluents for discharge into receiving waters (Slabbert, 1994; Slabbert *et al.*, 1998). WET tests can be used i.) to establish the suitability of a discharge; ii.) to develop permit limits to control effluent; iii.) to monitor effluent; iv.) to identify and prioritize effluents and v.) to reduce effluent toxicity (Slabbert, 1994; Slabbert *et al.*, 1998). However, toxicity test organisms are only useful in bioassays if they show little test-to-test variation and including toxicity-based limits in effluent discharge permits relies on the use of standardised toxicity tests to assess the effects of complex effluents. Robinson (1989) recognised the need for reliable, precise and accurate standard methods for toxicity tests and emphasized the need to select appropriate biological indicators (*i.e.* test organisms) for use in bioassays and acknowledged that the acceptability of a toxicological technique is important in establishing the usefulness of a method.

Aquatic toxicity tests in South Africa have traditionally used standard laboratory test organisms, such as *Daphnia pulex*, although it is not yet certain whether guidelines set using standard organisms will adequately protect South Africa's water resources, and/or provided accurate information about the site specific impacts of pollutants. Despite the good experimental protocols which exist for *D. pulex* there is still an issue of repeatability (variability) of tests, which may be linked to genetics, environmental conditions and any interactions between these two (Baird *et al.*, 1989). One of the applications of the artificial stream research (Palmer and Scherman, 2000) is the contribution of data for the inclusion of toxicity tests using indigenous invertebrates in effluent permits. A basic requirement of routine WET laboratory testing is that the techniques and organisms used in the toxicity tests have been reviewed and are accepted by the scientific community, but also that the test species are representative of local and regional species (Roux, 1994).

In this project, comparative experiments using site-specific invertebrate stream organisms in artificial streams and the standard test organism *Daphnia pulex* were carried out to contribute to data to assess inclusion of toxicity tests in waste-water (effluent) licences in order to prevent further deterioration of the receiving water resources.

1.2 AIMS

The project aims to investigate and compare the responses of *D. pulex* and site-specific indigenous invertebrates to a range of chemical mixtures in order to evaluate the use of WET testing in water quality management. The site-specific organisms, and some of the waste-waters tested were collected in the Vaal River barrage area, and the results would be particularly applicable in that area.

The specific aims of the project are listed below. During the project, it became obvious that the proposed approach of undertaking acute toxicity tests using selected industry effluents was not feasible as the none of effluents resulted in measurable acute toxic effects. As a result of these practical constraints, the Project Steering Committee agreed that a change in the approach was appropriate and it was decided to create an artificial effluent in order to evaluate *Daphnia pulex* and indigenous invertebrates for whole effluent toxicity testing in the Vaal catchment. Consequently, the proposed aim of relating real industry effluent toxicity test results to industry discharge permits and their actual discharges with a view to reviewing current discharge criteria and receiving water quality objectives set by Rand Water for the Vaal Barrage could not be undertaken. The Steering Committee agreed to the proposed change in application of the toxicity test results listed in below (point 4).

i.) Identify and access test industries and effluents

- List and visit a range of industries which discharge effluents into the Vaal River (either directly or indirectly via a tributary).
- Select effluents from representative industries in collaboration with Rand Water and DWAF.
- Create an artificial complex effluent mixture.

Results are reported in Chapter 3.

ii.) Select indigenous invertebrates from a reference site in the Vaal River as test organisms in whole effluent toxicity tests

- Visit a range of possible reference sites identified by previous SASS biomonitoring, and identify one or two abundant indigenous riffle-dwelling (benthic) invertebrate species for use in the artificial streams.
- Set up artificial stream systems at Rand Water Scientific Services.
- Decide on the test medium for the toxicity tests, *i.e.* river water from the reference site or dechlorinated tap water.
- Undertake preliminary tests to assess the suitability of using the selected test organisms in the artificial streams.

Results are reported in Chapter 3 and Appendix 1.

iii.) Use *Daphnia pulex* and selected indigenous organisms as test organisms in whole effluent toxicity tests

- Undertake acute toxicity tests exposing the standard organism *Daphnia pulex*, with culture medium and river water as dilution waters, to selected industry effluents and an artificial effluent.
- Undertake acute toxicity tests exposing selected indigenous invertebrates, with river water medium as the dilution water, to selected industry effluents and an artificial effluent.

Results of the toxicity experiments are reported in Chapter 4.

iv.) Relate toxicity test results to the application of WET testing in water quality management.

- Evaluate the use of WET testing to the source directed control of complex effluents in the Vaal barrage area.
- Evaluate the use of *D. pulex* and indigenous invertebrates in WET testing.

The discussion is presented in Chapters 5 and 6.

CHAPTER 2:
BACKGROUND

Whole effluent toxicity (WET) tests are considered useful tools in protecting the environment from the potentially harmful effects of effluents (Chapman, 2000). An underlying assumption of WET tests is that laboratory toxicity tests are predictive of effects in the receiving water resource (Dorn, 1996).

In the USA, the focus of WET testing policy has been to protect receiving water resources from the toxic effects of industry (point sources) and non-point source discharges, and has formed an important component in the integrated approach to water resource management (Heber *et al.*, 1996). The logical reasoning for using a toxicity testing approach was that chemical-specific criteria are designed to protect 95% of aquatic organisms 95% of the time. However, not all chemicals are, or can be, routinely monitored. Toxicity assessments of effluents is becoming increasingly more common, as it is not possible to test all combinations of the thousands of chemicals in use, for monitoring and regulatory purposes, to try and establish cause-and-effect links (Roux 1994).

There is sufficient international experience to recommend WET testing as an efficient, and cost-effective approach to the management of complex industrial waste- waters. However, *within* the WET assessment approach, a wide range of tests and test organisms can be used, and it is important that we select the most appropriate combination for South African conditions.

Complex industrial waste-waters can range widely in the threat they pose to human and water resource health. It is therefore important to effectively screen those which pose a low or minimal risk to health, using the shortest and cheapest tests, and then require the lowest level of compliance monitoring. Generally a tiered approach is favoured, where the level of risk posed by the effluent is related to the extent of the testing and monitoring required.

The chemistry of the natural resource into which the effluent flows may mitigate the toxicity of the effluent, and in addition, wild, natural populations may be more tolerant than laboratory-bred test organisms. It may therefore be possible to allow the disposal of effluents that are identified as posing a hazard after testing with standard test organisms. However, as the risk to the environment is increased, the level of knowledge about the effluent; the receiving water; and the response of organisms; that is required increases. Therefore a more relaxed licence condition will carry more onerous testing and monitoring requirements. This is a good example of the "polluter pays" principle.

Within any application of WET testing, the discharger should evaluate the comparative benefits of toxicity identification and reduction compared with the more comprehensive testing required to more accurately assess the risk posed by the effluent. More detailed testing may result in a more relaxed licence criterion, but would also identify unacceptable risks and effluents which should simply not be discharged to the environment without toxicity identification and reduction.

In this study, *D. pulex* were exposed to an artificial effluent first using laboratory medium as the test diluent; then using river water as the diluent - thus taking into account the possible mitigating

affect of receiving water chemistry. Then followed tests using indigenous, site specific riverine organisms as test taxa, which took into account any differences in tolerance between the wild populations from the receiving water resource as compared with a standard test organism.

2.1 WHOLE EFFLUENT TOXICITY (WET) TESTING

A purely chemical analytical approach is unable to protect the aquatic environment, especially as some chemicals may be present at levels too low for detection, but never-the-less have an adverse effect in the environment (Braginsky, 1995). Chemical monitoring does not take into account environmental factors which affect toxicity (*e.g.* hardness, pH, temp, suspended inorganics, dissolved organics) nor does it consider potential transformations and speciation which chemicals can undergo and may result in altered toxicity. Complex effluents may contain organic chemicals which are not readily identifiable and may enter a water resource undetected. Frequently stormwater discharges from industries are not considered in effluent permits and chemicals present in these may escape detection altogether. Effluent discharge permits are usually based on a small number of pollutants present in the effluent that are potentially harmful to the environment (Garric *et al.*, 1993) but it is difficult to apply permits for single substances to complex effluents.

Persistent pollutants can accumulate in slow-flowing waters and reservoirs to unacceptably high levels (Braginsky, 1995) and result in deterioration of the water quality of a resource. Acute toxicity tests are rapid and reliable methods for screening chemicals with unknown toxicity and frequently form the basis for subsequent sublethal and chronic toxicity tests (Roseth *et al.*, 1996). Toxicity tests can be proactive (will there be an effect?) or reactive (has there been an effect?) (Chapman, 1995a). WET methods are considered suitable for predicting instream effects, although there are many factors which confound the prediction, such as the extrapolation of effects from one species to others (Ausley, 2000). Bioassays, complemented by chemical data, are essential tools for the assessment of effluents: both biological data and chemical data together are important for the management of the quality of waste-water and the receiving water bodies.

2.1.1 Aquatic toxicology

Aquatic organisms integrate the effects of their environment during their lifetime, and can therefore be used in experiments (bioassays) and also to reflect conditions to which they may have been exposed to earlier (biomonitoring). Bioassays, both toxicity tests and bioaccumulation studies, have been shown to be highly effective in water quality monitoring programmes in developed nations, where improvements in environmental water quality have been achieved (Chapman, 1995a).

Bioassays are used to determine the potential of substances to cause environmental harm (Cairns and Pratt, 1989; Chapman, 1995a) and are sometimes able to elicit responses at concentrations below chemical detection limit. Advantages and disadvantages are summarised in Table 2.1. Bioassays were first introduced to assess the effects of pollution on fish in 1945 (Rand, 1995). Initially, single species were used, and results were extrapolated from species to species and then from species to ecosystems (Cairns and Pratt, 1989). These early tests established the effects of known toxicants on understood species, with little understanding of the impact on the environment.

TABLE 2.1 Advantages and disadvantages of toxicity tests (adapted from Chapman, 1995a)

Advantage	Disadvantage
A holistic approach, integrating effects of all stressors (especially useful for investigating whole effluent).	Tests do not indicate which stressors are causing the observed effect
Tests can be simple and cost-effective	Tests can be too simple and result in environmentally unsound answers
The effect on the selected organism(s) is (are) observed at the exposure(s) tested	Not all organisms, exposures and end-points can be tested
Tests are normally carried out under controlled laboratory conditions	Field conditions are different from laboratory conditions and extrapolating results from laboratory to field is associated with its own set of complications

The objective of an ecotoxicological assessment for effluent discharge control is to maintain or improve the quality of the receiving water (Garric *et al.*, 1993) and the first step in this process is the establishment of an acute toxicity data-base: single-species bioassays are useful in determining limitations for discharge levels (Garric *et al.*, 1993). However, these tests are not able to predict sublethal effects and should therefore be followed by chronic testing. These laboratory lethal and chronic (sub-lethal) tests can be undertaken to define concentrations that should not be accepted in the environment (which can be tested by routine chemical and biological monitoring of the receiving water).

2.1.2 Standard toxicity tests

An objective of toxicity tests is to assess whether the measured end-point in one treatment (usually contains the test substance, *e.g.* an effluent) is significantly different from the measured endpoint in another treatment, usually the control (Denton and Norberg-King, 1996). The formulated null hypothesis is that there is no difference between these two treatments, while the alternative hypothesis states that there is a measurable statistical difference between the treatments. Obviously then an important component of aquatic toxicity, and WET, testing is the ability to detect either (and/or) acute or chronic toxicity (Chapman *et al.*, 1996).

There are numerous ways to assess the toxicity of inputs into the aquatic environment and these are mainly standard acute and chronic tests (bioassays) in order to show either "end-of-pipe" toxicity or receiving water toxicity (Roux, 1994). Acute toxicity tests are generally less than four days, and usually mortality is measured, while chronic toxicity tests assess the effects of longer-term exposure to a toxicant, typically expose the test organisms over at least part of their life-cycle and measure responses such as growth and reproduction (Rand, 1995). Acute toxicity tests tend to be cheaper to perform and are performed most commonly.

An important factor in assessing whether an acute test method is acceptable as a standard method is whether the results can be replicated and statistical measures of certainty can be ascertained (Kimball and Levin, 1985). Well-established and accepted (by the scientific community) test protocols must be available for the test in order for it to be accepted as a standard method (Rand, 1995).

There are several designs of experimental, or exposure, systems and selection of the system to be used is determined by the question being addressed (Williams *et al.*, 1984) and each of these is associated with advantages and disadvantages. In static tests the test organisms are exposed to the toxicant in still water while in recirculating tests the test organisms are exposed to the toxicant in flowing water (Rand, 1995). In these systems, the toxicant is not replaced for the duration of the test. The choice of experimental system may be influenced by the choice of test organism, *i.e.* it may be more appropriate to use a flow-through experimental system when establishing water quality guidelines for lotic systems (Williams, *et al.*, 1984).

Stewart (1996) suggests that, despite short-comings of laboratory tests (*e.g.* the ability to extrapolate test results from laboratory experiments to field conditions) and the need for more cost-effective, new and better testing methods, there is a place for laboratory tests in order to generate sorely needed accurate and environmentally relevant data which can be used in ecological risk assessments, as well as for regulatory purposes.

2.1.3 Whole effluent toxicity tests

WET tests can be used to i.) set whole effluent toxicity standards; ii.) verify permit compliance; and iii.) can be used to revise permit standards. But these can only work if routine monitoring of the effluent is carried out to assess the effluent comprehensively. Several overseas countries have implemented strategies to reduce the toxicity of waste-water by including whole effluent toxicity tests in effluent permits to ensure that the effluents do not adversely affect aquatic life (Garric *et al.*, 1993; Grothe, 1996). Permits are generally site-specific and include specification on the frequency of testing, the organism/s to be used and the LC_{50} for the effluent. When the effluents do not conform to standards, further toxicity testing has to be undertaken so that the cause of toxicity can be identified and corrective action taken by the industry (toxicity identification evaluations and toxicity reduction evaluations; Rand, 1995).

The objective of WET tests is to ensure that effluents which are discharged do not adversely affect aquatic life (Grothe *et al.*, 1996). WET test methods currently used by the USEPA have repeatedly demonstrated their performance (Chapman *et al.*, 1996) but this does not preclude further refinement of these tests or even the development of new effluent toxicity test methods. The major assumption of WET testing is that the test results are accurate predictors of environmental effects and that, allowing for some test variability, WET tests are reproducible (Ruffier, 1996) but there are a number of conditions which can affect WET test variability.

2.1.4 Selecting test conditions

The approach to evaluating the toxicity of an effluent depends on the test method and conditions, the test organism and the chemicals present in the effluent (Warren-Hicks and Parkhurst, 1996). The specifics (*i.e.* choice of test organism, exposure system, dilution water) of undertaking WET tests are determined by the fundamental question being addressed.

2.1.4.1 Test species

Regulatory monitoring is usually carried out on limited test species (for practical and economic reasons), although these species may not necessarily be the most sensitive species since organism sensitivity to toxicants is species specific and effluent specific. In order to assess compliance of effluents to specified toxicity end-points, Roux (1994) recommended that at least three test species be assessed and these should include a fish, an invertebrate and an algal species. In a summary of international legislation of WET testing by Slabbert *et al.* (1998) the most frequently recommended test organisms are fish, *Daphnia* species, algae and bacteria, usually in a battery of tests. These tests are usually a combination of acute and chronic tests.

The choice of test organism for WET tests is guided by several considerations and these are well established (Rand, 1995). Test organisms should be sensitive to a range of chemicals and show rapid responses, and the responses should be easy to measure. The organisms should be abundant, available all year round and in good physical condition. And importantly, the organisms should be easy to handle and withstand laboratory conditions. There are two approaches to the choice of test organism. One is the approach used by the USEPA, which uses standard laboratory organisms which have been reared in the laboratory under stringent conditions, while the other uses organisms representative of the study area in question and may therefore be more directly relevant to answering the question and avoid uncertainty between extrapolation between species (Chapman, 1995a).

Standard toxicity tests use indicator (or surrogate) species which may not necessarily be found in the water resource in questions; most often, these indicator species are laboratory reared so that their age and background is known. *D. pulex* is a standard laboratory toxicity test organism for a range of reasons which include its short life cycle, ease of laboratory culture (requires little space, is easy to handle and count), its maintenance in a strictly defined culture medium and small water requirements both for culture and undertaking toxicity tests (Maki and Bishop, 1979).

The predominant use of pelagic taxa (e.g. cladocera species, fish, algae) in toxicity tests may result in inaccurate predictions from toxicity tests as the route of exposure to the chemicals may not be the same for benthic species (Sarakinos and Rasmussen, 1998) and Chapman (2000) cautions against protecting only the "white rat" test species instead of protecting the environment.

Gruber (2000) described various methods, as recommended by US EPA guidelines, to modify water quality standards in order to be applicable to site-specific conditions. One of these methods is the use of indigenous organisms in a battery of tests. Using indigenous species for toxicity testing may eventually replace standard organisms, although it is not anticipated that the standard organisms will be abandoned entirely, although the significance of their use will decrease (Cairns, 1993).

In developing site-specific water quality criteria and using site-specific organisms instead of the recommended standard indicator, or surrogate, test species, there are a number of criteria which should be considered when selecting the site-specific organism (Chapman *et al.*, 1996). These are that the site-specific test organism be ecologically, recreationally or commercially important; that the test species be at least as sensitive as those recommended by the USEPA; that an early life-stage is used and that this stage be available all year round; the organism should be easy to handle and give constant and reproducible results; that test endpoints be quantifiable and the

results must be able to be subjected to statistical analysis; and that inter- and intra-laboratory toxicity testing should be feasible. In order to achieve a more environmentally realistic answer the selection of appropriate test species and test end-points is important: it is best to test what you are trying to protect, or at least a good approximation thereof, and to carry out definitive testing with a range of organisms (Chapman, 1995a).

Populations are made up of individuals who respond in varying ways to changes in their environmental conditions. It has been hypothesized that genetic variation in test populations may affect test variability (Ausley, 1996) and several studies have reported that certain genetic strains of a test species are more sensitive to certain chemicals than other strains of the same species. This will affect the reproductive success of the individuals but will ultimately also affect the survival of the population or species at a higher level of organisation (Depledge, 1990). Measures of individual variability are usually measured as a deviation from the mean, and it is often viewed that responses which are far removed from the mean are atypical and do not reflect population responses. However, it is the variability in responses which allow the continued existence of a population, and one of the criticisms of using laboratory-based toxicity tests using standard test organisms and an emphasis on reducing variability is that these tests fail to provide useful information for predicting long-term ecological effects precisely because of the reduced variability (Depledge, 1990).

Australia has developed laboratory test methods using indigenous and introduced species for use in both acute and chronic toxicity tests (Chapman, 1995a), and there are ongoing attempts to adapt tests from the USA for Australian species. Macroinvertebrates have been shown in some instances to be more sensitive to pollutants than a range of other organisms (Williams *et al.*, 1984) and are frequently used in toxicity tests as alternatives to indicator species. Cairns (1993) recommends that standard single-species tests be accompanied by tests using indigenous organisms in order to establish relative sensitivity and tolerance. The development of test methods using indigenous species is optimised through simultaneous testing with a "benchmark" species *i.e.* those species which comprise a substantial database (such as standard laboratory organisms) (Chapman, 1995a) and hence the need for simultaneous testing of indigenous organisms and *D. pulex*.

2.1.4.2 Exposure Test systems and dilution water

In order to be able to improve the quality of the receiving water, both laboratory and field data need to be considered when defining wastewater discharge conditions for receiving waters (Garric *et al.*, 1993). It is necessary to make laboratory tests as environmentally realistic as possible. While it is possible to simulate field conditions under certain laboratory conditions (Kimball and Levin, 1985), obtaining environmentally relevant results remains a challenge and part of the challenge is selecting exposure (test) systems which are appropriate. The choice of test system will largely be determined by the choice of test organism: for example, it is considered inappropriate to expose lotic organisms to the test toxicant in a static system.

The lack of toxicity data for lotic organisms may be reflected by the lack of experimental systems to generate the necessary data (Williams *et al.*, 1984). The development of artificial stream systems world-wide has allowed for the generation of data for riffle-dwelling invertebrates (Guckert, 1993; Palmer *et al.*, 1996). In the manner that laboratory standard tests using static systems and lentic organisms attempt to predict the effects of chemicals and effluent to lentic

systems, artificial streams attempt to predict effects to lotic systems (Guckert, 1993). A range of designs exist ranging from once flow-through systems to recirculating systems, and with varying degrees of hydraulic control to suit requirements of specific organisms.

The choice of dilution water will affect the measure of toxicity and is determined by the nature of the question being asked. When receiving water is used to dilute the toxicant, a measure of relative toxicity is obtained, while a measure of inherent toxicity is obtained when the toxicant is diluted in standard water or the laboratory test organisms' culture medium (Slabbert *et al.*, 1998). In the interests of environmental realism, using site-specific organisms in receiving water in a suitable test system may be more appropriate than using a standard indicator laboratory-reared organism tested in standard water.

2.2 VARIABILITY IN WET TEST DATA

WET tests can be used effectively to establish and set permissible limits for effluent discharges, monitor for compliance of effluent discharges, reduce toxicity of effluent, identify toxic effluents and identify potentially threatened water resources and monitor receiving water resources and establish the effectiveness of a water quality control programme (Slabbert *et al.*, 1998). There are a number of intrinsic and extrinsic factors which affect effluent test variability (Ausley, 1996). Intrinsic factors are inherent to the toxicity test method, such as the dilution water used, the organism used, feeding regime, temperature and duration of the test *etc.* Extrinsic factors are those which are introduced by the analyst, such as changing the test protocol, using test organisms of the wrong age, using unacceptable dilution water *etc.*

From an industry perspective, site-specific water quality standards and effluent discharge criteria can be developed based on WET test. The license specifications can then be either a narrative standard or numeric limit (Dorn, 1996). In order to generate water quality criteria for single constituents there is a minimum data requirement and part of this is that generally eight or more species are tested for each constituent (Denton and Norberg-King, 1996). However, for practical reasons, this data intensive approach is not feasible for effluent monitoring or setting water quality criteria for complex effluents. WET tests are therefore used to estimate the effluent concentration which will still provide aquatic ecosystem protection.

There are a number of issues which remain of concern to industries undertaking WET toxicity tests (Dorn, 1996): extrapolating laboratory results to field situations (where organisms often are able to display avoidance behaviour, there are a greater range of effluent dilutions, and communities and populations of organisms are complex), false positive test results due to an insufficient number of effluent dilutions, variability in the reporting of results and the use of surrogate or standard species in toxicity tests.

Test precision is a measure of how reproducible the toxicity test is, both within and between laboratories, when the same test method, test species and toxicant are used. A measure of test precision is a measure of the coefficient of variation (%CV) of point estimates of a number of tests (Denton and Norberg-King, 1996). One of the factors which increases the precision of a test is the method of the test: if a method is clearly defined, the precision increases. Generally, standard toxicity test methods have a good precision. However, toxicity tests should not only be

judged on their precision, but also on their sensitivity, accuracy and ecological relevance (Denton and Norberg-King, 1996).

Dorn (1996) cautions against developing new species for toxicity tests in an attempt to gain (possibly) better toxicological data than that which can be obtained from using the surrogate species. Attempts to find "the most sensitive species" may be misleading, and the surrogate species may well be the more sensitive species. In his opinion, Dorn (1996) suggests that unless differences between the surrogate species and the new test species (*e.g.* a site-specific indigenous invertebrate) are significant, it may well be better to use the standard surrogate species with its associated well-researched and developed test methods. However, some species are more sensitive to some chemicals than others, and it may then be feasible to select a test species for a particular generic industry effluent (*e.g.* petro-chemical industry, sewage treatment works *etc.*).

CHAPTER 3:
MATERIALS AND METHODS

Laboratory toxicity tests are considered to be the first step in a tiered approach to establishing guidelines for minimum acceptable concentrations of pollutants (Chapman, 1995) and acute toxicity tests are relatively rapid, simple and, in most cases, a cost effective method for assessing the potential effects of chemicals and effluents (Kimball and Levin, 1985). Although longer-term toxicity tests are considered more ecologically relevant, Williams *et al.* (1984) concede that there are occassions where it is more appropriate to use acute toxicity tests. In this study, acute toxicity tests were undertaken to assess the toxicity of effluents.

Standard experimental protocols are accepted as methods to assess the toxicity of pollutants to a range of organisms and in this project, two standard methods, for site-specific indigenous invertebrates and *Daphnia pulex*, were compared to assess their use in Whole Effluent Toxicity testing in the Vaal Catchment.

3.1 EXPERIMENTAL CONDITIONS

As the project was a comparison of the responses of site-specific invertebrates and a standard invertebrate test organism, the experimental conditions for both were the same. Detailed specifications for the two test methods are outlined in Sections 3.2 and 3.3.

3.1.1 Experimental design

Careful consideration of the experimental design allows for proper data analysis and is largely determined by the purpose of the experiment.

Hypothesis tesing (or an experimental design which allows for Analysis of Variance (ANOVA) statistical analysis) allows for the use of many replicates and may result in a good test statistic (Chapman *et al.*, 1996). However, confidence intervals cannot be obtained and the results (NOEC and LOEC) are dependent on the concentrations selected (*i.e.* were the test concentrations sufficiently different from the control in order to be able to measure an effect).

When point estimates, such as LCs and ECs, are used to predict an effluent concentration at which impacts are predicted, then confidence limits must be included (Chapman *et al.*, 1996). Regression models, such as those employed by the probit and Spearman-Karber models, provide point estimates and confidence estimates. However, the point estimates and confidence intervals are dependent on the concentrations selected for the toxicity test and the model selected for data analysis. An advantage of using a point-estimate technique is that all the information from the dose-response relationship is used to derive the point estimate and confidence intervals are provided (Denton and Norberg-King, 1996). A disadvantage of using the technique is that the point estimate is model dependent, and the model used may depend on a range of factors such as the effluent, test species, concentration range, toxicity of the test substance or even the dilution water.

The number of concentrations most frequently selected for WET tests is five effluent concentrations and one control, with typically one of the concentrations being 100% effluent and a dilution range of 0.5 or 0.3 (Burton *et al.*, 1996). Increasing the number of concentrations will result in increased test precision. The experimental design selected for the purpose of the study was an unreplicated regression design, using a minimum of 8 effluent concentrations and 1 control, in order to increase the number of points on the regression line and thus improve the accuracy of the point estimates. Guckert (1993) supported the use of increased concentrations with no replicates to assess the responses of single species to a test compound.

3.1.2 Experimental end-points

It is important to select a laboratory test end-point which bears some ecological relevance and this is particularly important when the test is used for regulatory and impact assessment purposes (Chapman, 1995). However, the measure should also be made easily as the more complex the method is, the more likely that errors will occur (Dorn, 1996). When selecting test organisms for toxicity tests, and appropriate test end-point measurements, it is important to consider the effort required to obtain results and whether the additional information yields significantly more information. For example, Roseth *et al.* (1996) found that growth rate studies of fish were time-consuming and required some skill to undertake. The information obtained from the growth-rate study did not yield significantly more information, nor was it significantly more sensitive, than the lethality test.

End-points for toxicity tests are often selected for replicability rather than sensitivity (Cairns, 1986) but should also reflect the test exposure (Chapman *et al.*, 1996). There are a range of toxicity test endpoints which can be used, but not all are ecological relevant. The most frequently measured test endpoints in WET tests are survival, growth and reproduction (Chapman *et al.*, 1996) and while mortality is not necessarily the most sensitive, or even environmentally relevant, end-point, it is an unambiguous measure of response. Lethality is the most commonly measured and used test endpoint (Chapman *et al.*, 1996) and can provide point estimates (*e.g.* 48-hour LC50) or No Observed Effect Concentrations (NOEC) and Lowest Observed Effect Concentrations (LOEC). Studies in which the responses of mayflies were compared to the responses of other standard organisms concluded that the experimental end-point mortality was comparable between different species (Diamond *et al.*, 1992).

For the purpose of this study, mortality was used as the end-point in all experiments.

3.1.3 Test effluent

A number of potential industry effluents were identified by Steynburg and Heath (Rand Water; pers. comm.) and final selection of effluents for testing was founded primarily on accessibility to the effluent and distance from laboratory. In a series of experiments which were undertaken during four fieldtrips in 1997 and 1998, none of the effluents (from a range of industries) yielded concentration-responses which could be adequately analysed using the regression models (*i.e.* probit or Trimmed Spearman-Karber) due to the data not fulfilling the assumptions of either of the models.

As a result, the project Steering Committe recommended using an artificial effluent. The artificial effluent was designed (Wade, pers. comm), based on the toxicity test results reported by Slabbert

et al. (1998), similar to a metal-plating industry effluent. Sufficient stock solution of the artificial effluent was made at the start of the experimental period, and aerated for 36-hours before its first use and remained aerated for the entire experimental period (4 weeks). Predetermined amounts of the stock solution were used to prepare the concentration-dilution series for all the toxicity tests. With this approach, the repeatability of the whole effluent toxicity test results for the site-specific indigenous invertebrates over a short experimental time period could be assessed.

The artifical metal-plating effluent contained aluminium, cadmium, chromium, copper, manganese, nickel and zinc, and these chemicals were specifically monitored for in chemical analyses.

3.1.4 Laboratory conditions

Laboratory toxicity tests need to be carried out under clearly defined, controlled and reproducible conditions (Brown, 1986, cited in Chapman, 1995). The experiments were undertaken at Rand Water Analytical Services, Vereeniging, in the Hydrology section. A laboratory was made available for the duration of the experimental period and all experiments, including *Daphnia pulex* tests, were carried out in this laboratory.

The air temperature in the laboratory was maintained between 16-18 °C and the light regime was maintained at 14L:10D.

3.1.5 Chemical analysis

Chemical analysis was undertaken by Rand Water Analytical Services (Organic Chemisty and Inorganic Chemistry Laboratories). Results were not received for all samples submitted.

3.1.6 Waste disposal and cleaning equipment

Waste generated in experiments was disposed of by Rand Water. All equipment used in the experiments was cleaned according to DWAF (2000).

3.2 ACUTE TOXICITY TESTS USING SITE-SPECIFIC INDIGENOUS INVERTEBRATES

3.2.1 Reference site

The Vaal River catchment is a highly impacted area, and it proved challenging to find a reference site with riffles, which was also close to the Rand Water laboratory in Vereeniging. Tributaries of the Vaal River were not suitable, either due to surrounding land-use impacts or due to river seasonality. A suitable reference site was found immediately downstream of Engelbrechtdrift Weir (Figure 3.1), which is between the Vaal Dam and Vaal Barrage.

Standard procedures for WET testing recommend that field-collected organisms are not used in toxicity tests (Slabbert *et al.*, 1998) due to the unknown effects of stress that the organisms have been exposed to in the field (Burton *et al.*, 1996). However, laboratory-cultured indigenous invertebrates are not yet available for use in toxicity tests, and the artificial streams rely on field-

collected organisms for use in tests. Great care is taken to minimize handling and transport stress, and emphasis is placed on reduced holding time in the transport vessels (DWAF, 2000). For this reason, it is desirable to have a reference site close to the laboratory where the experiments are undertaken. The selected reference site at Engelbrechtdrift Weir was approximately 30-45 minutes drive from the laboratory.

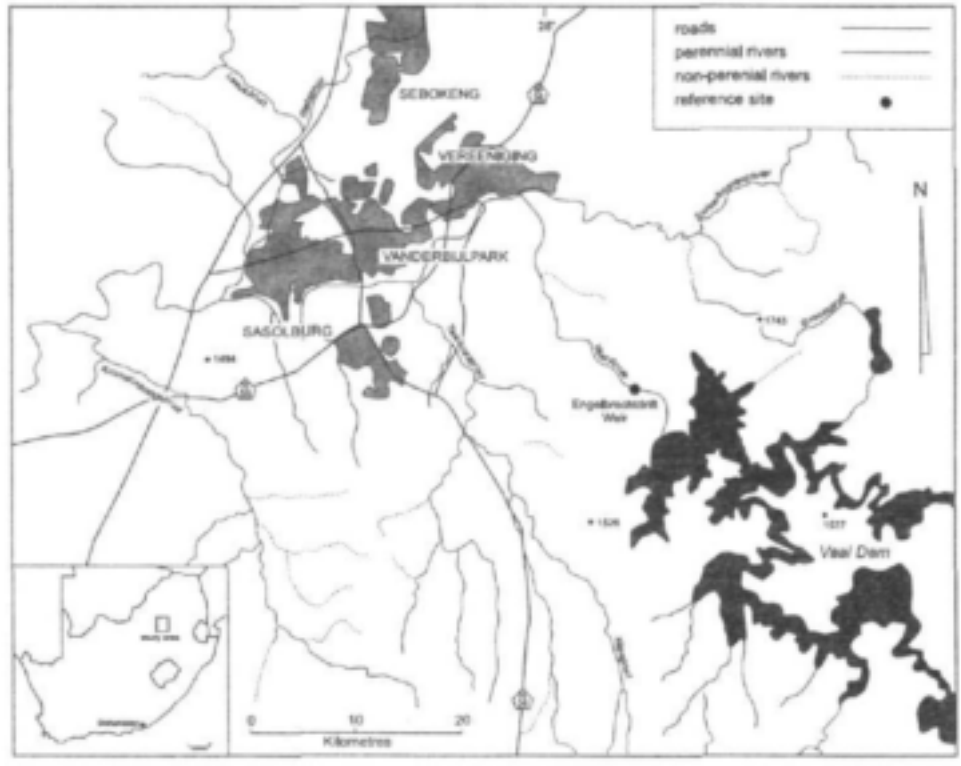

FIGURE 3.1: Map showing the study area and relative position of the reference site to the laboratory in Vereeniging

3.2.2 Site-specific indigenous invertebrates

Toxicity tests typically employ fish, invertebrates or algae, for acute and chronic tests, and these organisms have also been the focus for the development of new methods for carrying out toxicity tests. Development of new methods has also included development of new test species for inclusion in the repertoire of available toxicity test methods. However, the importance of selecting ecologically relevant organisms for testing is stressed often in the literature.

Not only is it important to develop regional and site-specific pollutant standards, it is equally important to select ecologically appropriate species for bioassay testing (Kimball and Levin, 1985). These ecologically appropriate species may not necessarily be those species selected by the USEPA for use in their standard methods, but may include those species which are economically or recreationally important, or those species which can serve as early warning systems, ecological dominants, keystone predators or those critical to ecosystem structure and function.

There are several assumptions made when selecting "the most sensitive species" for toxicity tests (Cairns, 1986), the most significant of which are that the response of the selected organism corresponds to the response of a larger group of organisms in a wide range of ecosystems and that the selected end-points chosen for the species in the toxicity test will adequately protect the ecosystems (Cairns, 1986). The question then arises, what if the selected species is not indigenous to the natural system being tested? Imposing safety factors to protect a species which does not occur in the system being tested may incur expensive management decisions (Cairns, 1986). Chapman (1995b) lists a number of criteria for selecting appropriate test organisms and these are often based on properties which are not related to their importance in natural systems. These properties are related to their availability in numbers, ease of collection, handling, transport, rearing, ability to tolerate laboratory conditions. It is recommended that new test species be benchmarked against established species to determine their relative tolerance (Chapman, 1995b).

Age significantly affects the toxicity of chemicals and effluents, and it is commonly accepted that younger organisms are more sensitive than older organisms and variability in toxicity test results can occur when organisms of different ages are used in toxicity tests (Burton *et al.*, 1996). As the age of the field-collected test organisms is unknown, care was taken to select mayflies which were similar in size in order to minimize the source of variability. However, it is known that in mayflies within age groups there is great variability in size, and so it is not certain how successfully this source of variability was controlled.

The ability of the test organism to withstand handling is vitally important, as this will influence the test precision and variability (Burton *et al.*, 1996). Field-collected test organisms are acclimated to laboratory test conditions for varying lengths of time before their use in experiments (Burton *et al.*, 1996). In this series of experiments, organisms were acclimated for a minimum of 36 to 48 hours. Once the mayflies were in the laboratory, they were randomly assigned to artificial streams, making sure they had all six legs and three cerci (*i.e.* no obvious signs of damage or stress).

The natural distribution patterns of indigenous invertebrates may be a result of undetermined water quality parameters (such as pH or hardness) and this may affect their sensitivity to pollution (Williams *et al.*, 1984). For this reason, it may be useful to expose site-specific organisms from unimpacted sites in WET tests, especially to establish site-specific water quality guidelines.

Preliminary testing using the two identified mayfly species indicated their suitability for use in the toxicity tests. The species used were *Afronurus peringeyi* (Ephemeroptera: Heptageniidae) and *Euthraulus elegans* (Ephemeroptera: Leptophlebiidae). The species occurred together at the reference site and were both available in abundance for the duration of the study period.

3.2.3 Dilution water and experiment concentration-range

Dilution water can affect the toxicity of effluents by modifying the bioavailability of toxic chemicals and the diluent used in a WET test should therefore have characteristics similar to the receiving water (Burton, *et al.*, 1996). Organism sensitivity to the effluent can be influenced by the dilution water and, in order to reduce variability, it is recommended that organisms are exposed in dilution water which is most similar to receiving water (Burton *et al.*, 1996). The dilution water used for the experiments with site-specific indigenous invertebrates was Vaal Dam

water. The water was taken directly from the pipeline which feeds water from the Vaal Dam to the Rand Water Vereeniging Treatment Works. Water from the Vaal Dam is released downstream and as there are no major land-use impacts or point-sources of polllution, it is assumed that the water from Vaal Dam is the same quality as that of the reference site at Engelbrechtdrift Weir.

Concentration ranges selected for WET and ecotoxicology studies tend to be selected in order to assess the potential for a detrimental effect in the environment, and may frequently be higher than may be encountered in the field (Guckert, 1993). The experiments undertaken for this project were acute toxicity tests, and as a result, concentrations selected may be higher than would normally be encountered under field-conditions.

Experiment 1 was a range-finding experiment and since little was known of the sensitivities of the selected mayflies, it was decided to use a dilution range which would cover both high and low concentrations of the effluent. This resulted in 19 effluent concentrations and 1 control. In subsequent Experiments 2 to 4, the number of concentrations was reduced to 9 effluent concentrations and 1 control. A total of 4 acute Whole Effluent Toxicity experiments was undertaken to test the artificial metal-plating effluent.

3.2.4 Experiment protocol

Experiments using site-specific indigenous invertebrates were carried out using 1m artificial streams. The design of the artificial stream was based on the original design by Williams (1996, in Palmer and Scherman, 2000) but modified to reduce evaporation (Figure 3.2). The 1m PVC gutter was fitted with a U-bend and down-pipe at the outfall end of the stream. The down-pipe, which fits through a hole in the bucket lid, minimised evaporation from the bucket and return-flow to the bucket. The 20ℓ bucket contains the submersible pump which pumps water through the tubing to the top of the stream. A perspex cover over the inlet pipe at the top of the stream reduced loss due to splash where the water enters the stream. Test organisms are confined to the artificial stream by a screen in front of the down-pipe, and stones and mesh are provided in the stream as substrate.

A 96-hour test period was initially developed so that a toxicity test could be completed within a week, but this period has since been supported by research (Chapman, 1995a). This test period is now specified for acute toxicity tests for specific test organisms. The acute toxicity test used in this study is 96-hours and is the same as that outlined in DWAF (2000).

Selected test organisms were collected from Engelbrechtdrift Weir and allowed to acclimate prior to exposing them to the test toxicant. Mayflies were hand-picked off rocks with fine paintbrushes to prevent damaging them and placed in reference site river water in cooler boxes containing an ice-pack to prevent increases in water temperature. The water in the cooler box was aerated using a battery operated air pump. Care was taken not to extend the collecting period beyond 2½ hours in order to minimise stress to the organisms. Mayflies were sorted into the artificial streams, filled with Vaal Dam water, as quickly as possible, taking care to select only those mayflies that were undamaged, did not have wingbuds and were within 50% size-range. A minimum of 25, sometimes 30, mayflies per species, was introduced into each streams. They were allowed to acclimate to the laboratory conditions for 36 to 48-hours before the artificial effluent was introduced.

Vaal Dam water was collected prior to the start of the toxicity test and the test effluent concentrations were made. Acclimation water was drained from the streams, taking care not to leave any of the mayflies stranded, and the test solution introduced immediately; changing water did not take more than 3 to 5 minutes per stream. Monitoring for mortality commenced within 6 to 12 hours (depending on the time the experiment was started), and thereafter was recorded twice daily (am and pm) approximately 12 hours apart. Dead mayflies and moults were recorded, removed from the streams and preserved in 80% alcohol for species identification.

After 96 hours, the final mortality and moult count was undertaken, and the pumps stopped. Any live mayflies were counted and preserved for later identification. The final number of exposed mayflies was recorded as the total live mayflies at the end of the experimental period plus the total number of dead mayflies; these numbers were used for data analysis. Mayflies which emerged were not included in the count.

Laboratory and stream water temperature were monitored daily.

Vaal Dam water at the start of the experiment was sent for chemical analysis, as were samples of all the test concentrations at the end of the test period.

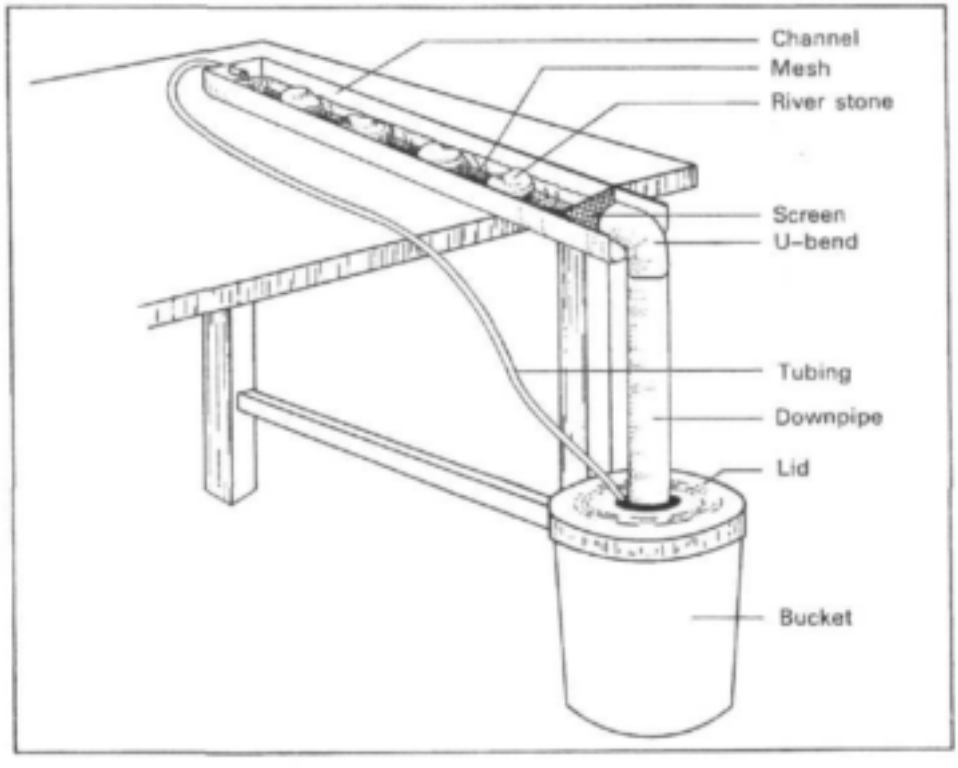

FIGURE 3.2: Diagram of the artificial stream, showing the modifications to reduce evaporation (down-pipe and bucket lid)

3.3 ACUTE TOXICITY TESTS USING A STANDARD TEST ORGANISM

The test organism used was *Daphnia pulex* (Cladocera: Daphniidae), an organism cultured successfully for routine monitoring by Rand Water Analytical Laboratory (Hydrology).

3.3.1 Dilution water and experiment concentration-range

For the standard organism experiments, both culture medium and Vaal Dam water were used in separate concentration-response experiments in order to assess whether either the culture water or Vaal Dam water affected the sensitivity of the test organism to the artificial effluent.

In the first experiment, the concentration-range selected was the same as the concentration-range for the site-specific indigenous invertebrate Experiment 2 to 4. However, the *D. pulex* were significantly more sensitive than the site-specific organisms and the second experiment was a range-finding experiment to assess toxicity of the effluent to the organisms. A third experiment was undertaken with a different concentration-range in order to refine the LC_{50}.

3.3.2 Experiment protocol

The experimental protocol for testing *D. pulex* was the standard method as outlined by USEPA (1991a) as this is the method followed by Rand Water. *D. pulex* are cultured under rigorous laboratory conditions and a strict feeding regime following the EPA guidelines and routinely tested for sensitivity using a reference chemical (du Preez and Mulder, pers. comm; Rand Water participate in the Aquatox forum Proficiency Scheme and have recently achieved accreditation for the *D. pulex* acute toxicity test).

The day before the effluent test, *D. pulex* adults with embryos were placed in 100mℓ beakers containing culture medium and placed aside overnight. The following day, the hatched neonates (< 24 hours) were used for the artificial effluent acute toxicity tests. The solutions for the concentration-range for the experiment were prepared and 25 mℓ of each concentration decanted into each of 4 beakers. Five *D. pulex* were placed in each of the beakers, and standard mortality monitoring procedures commenced; mortalities were checked at 30 min., 1, 2, 4, 8, 24 and a final count at 48 hours, and the mortalities recorded. The mortality data were used for the data analysis.

Samples of the "100% artificial effluent" (used to make the concentration-range) were submitted for chemical analysis.

3.4 DATA ANALYSIS

Acute toxicity test results are usually reported as lethal, or effect, concentrations, while chronic test endpoints are commonly reported as NOECs and LOECs (Grothe *et al.*, 1996). For this project, data were analysed using probit and Trimmed Spearman-Karber software, available from the USEPA, in order to get lethal point estimates (LC_1, LC_5, LC_{10} and LC_{50}) and confidence intervals, so that comparisons between the species could be undertaken. The most frequently accepted level of toxicity is indicated by 10% response, provided that the control response

remains less than 10% (Slabbert *et al.*, 1998). In the acute toxicity tests undertaken there were no control mortalities, and therefore the same level was used to indicate toxicity.

The probit model assumes a normal distribution; the concentration-response relationship, which is sigmoidal, is linearized through a probit transformation and log transformation of the concentrations to produce a regression line (Baird *et al.*, 1996). The model is sensitive to outliers and failure to meet the requirements of the model can be detected by the χ^2 test for heterogeneity. An important requirement of the probit model is that partial effects must be present in at least two of the concentrations. This requirement is frequently overlooked and a study by the USEPA found that 82% of toxicity tests had fewer than two partial effects thereby invalidating them for probit analysis. Non-parametric models, such as the Trimmed Spearman-Karber, do not assume a concentration-response relationship, and in data sets where a monotonic concentration-response does not exist, a smoothing procedure is used to obtain the relationship (Baird *et al.*, 1996). The Spearman-Karber model also has a partial effect requirement.

Traditionally, a significance of 50% (LC/EC_{50}) is selected for making comparisons between species (or chemicals) because at that point the confidence interval is at its narrowest. The variability indicated by the confidence interval (*i.e.* the 95% confidence interval) around the point estimate should not be confused with the variability associated with WET test variability (coefficient of variation) (Fulk, 1996).

While it is important to remember that statistical significance does not necessarily equate to biological (ecological) significance, it is a useful tool to analyse and assess available data (Thursby *et al.*, 1997). For the purposes of this project, it was important to assess whether the different test species responded differently to the same test effluent, and whether the responses within a test species could be repeated over a number of experiments. A number of analytical tools are available to do this.

Assessing variability has been an important component of this project and the most commonly used measure of variability has been to use the % Coefficient of Variation (%CV) to assess endpoint precision (Burton *et al.*, 1996). The %CV of LC_{50}s are considered appropriate measures of the precision of WET tests (Fulk, 1996). Norberg-King (pers. comm. with Everitt, project student) indicated that a %CV of 20-30% around the LC_{50} appears to be acceptable for conditions where the same experimental test protocol, same water, same organisms, same conditions, same person and same stock solutions are used. Chapman (1995a) suggests that, especially for effluents whose toxicity can be highly variable, results within up to a factor of two are not different.

Toxicity test results can be usefully be presented in Toxic Units (Slabbert *et al.*, 1998):

$$TU_a = \frac{100}{LC_{50}}$$

where TU_a is the Toxic Units for an acute toxicity test, and LC_{50} is the point estimate for that particular toxicity test. The higher the value of TU, the more toxic the effluent is to the organism, and this provides a quick measure of the relative toxicity of toxicant to different test species. However, this measure does not take variability into account and relies on the accuracy of the LC_{50} obtained.

In order to establish whether LC_{50} values were statistically significantly different, the following formula, from APHA (1992) was used:

$$f_{1.2} = anti\log\sqrt{(\log f_1)^2 + \log(f_2)^2}$$

where f = factor for 95% confidence limits, *i.e.* $\frac{upper95\%}{LC_{50}}$.

If the ratio of the greater LC_{50}:lower LC_{50} is greater than $f_{1.2}$ then the LC_{50}s are significantly different.

A further assessment tool used was to compare the slopes of the probit regression lines using the Generalized Linear Model (Statistica). However, this relies on data meeting the assumptions and requirements for probit analysis, and was not possible for all comparisons.

CHAPTER 4:
RESULTS

4.1 SITE-SPECIFIC INVERTEBRATE 96-HOUR ACUTE TOXICITY TESTS

The first experiment using site-specific invertebrates exposed to artificial metal plating industry effluent, based on data from Slabbert *et al.* (1998), comprised a range-finding test, using 19 concentrations of the effluent and a single control. This was done to assess which concentrations to focus on for subsequent toxicity experiments.

Data from Slabbert *et al.* (1998) using metal-plating industry effluent indicated that the effluent was highly toxic, it was decided to obtain a wide range of effluent concentrations, ranging from 100% effluent to 0.01% effluent concentration. Results from Experiment 1 indicated that the two species of mayfly displayed significantly different responses to the toxicant. In this chapter, the responses of each of the different organisms will first be discussed separately.

As the two experimental protocols required different exposure times (*i.e.* 96-hours for the site-specific invertebrates *vs* 48-hours for the *Daphnia pulex*), only the final cumulative mortalities are considered in order to compare the two standard methods.

4.1.1 *Afronurus peringueyi* (Ephemeroptera: Heptageniidae)

There were no control mortalities for *A. peringueyi* during any of the experiments over the experimental period (96 hours).

Cumulative mortality (expressed as %) for Experiments 1 to 4 are presented graphically in Figure 4.1. The LC_{50} values (in effluent concentration) and 95% confidence intervals for each of the experiments are presented in Figure 4.2. The artificial effluent resulted in spurious toxicity at effluent concentrations below 12.5%; above this, cumulative mortality increased with increasing effluent concentrations (except for an apparent spurious result in Experiment 4) although never exceeded 70% (Figure 4.1). The spurious mortalities at low concentrations and the fact that 100% cumulative mortality was not reached meant that the assumptions of the probit and TSK models were not met and may account for the wide 95% confidence intervals (Figure 4.2).

Analysis of the entire Experiment 1 data set indicated that the probit model was inappropriate for the data and therefore TSK analysis was undertaken (Table 4.1). However, when the data set was trimmed to include only those effluent concentrations which corresponded with concentrations used in subsequent experiments (in order to undertake comparisons), the probit model fit the data. Results for both the untrimmed and trimmed analyses are presented in Table 4.1, and show a similar LC_{50} value (49% and 40% for the probit and TSK respectively) using the two different models, with similar 95% confidence intervals. Using the formula provided in APHA (1992), no statistically significant difference could be established between the two results and subsequent comparisons were undertaken using the trimmed data set, as the % trim for the TSK was considered too great (30%).

The LC_{50} values for *A. peringueyi* ranged from 49% to 214%, and, despite the apparent large differences in LC_{50} values between the experiments, they were not statistically significantly different (APHA, 1992). The % Coefficient of Variation (%CV) at the LC_{50} estimate was higher than considered acceptable (Norberg-King, pers. comm.)(Table 4.1) but was significantly lower at the LC_1, LC_5 and LC_{10} point estimates. The LC_{10}, frequently used to indicate toxicity, ranged from 7 to 27% effluent concentration.

A comparison of the probit regression lines (using the Generalized Linear Model, Statistica) indicated that there was no significant difference between the slopes of the regression lines.

4.1.2 *Euthraulus elegans* (Ephemeroptera: Leptophlebiidae)

There were no control mortalities for *E. elegans* during any of the experiments over the experimental period (96 hours).

Figure 4.3 depicts the % cumulative mortality for *E. elegans* and the LC_{50} values (and 95% confidence intervals) are shown in Figure 4.4. Point estimates are listed in Table 4.1, and indicate that for Experiments 1, 3 and 4 the data were not appropriate for probit analysis. The data for Experiment 1 were not appropriate for probit analysis, indicated by high χ^2-heterogeneity, even after the data set was trimmed to only include those concentrations which were similar to those used in Experiments 2 to 4. It is suspected that the spurious mortality, in Experiments 1,3 and 4, at effluent concentrations below 6.25% effluent may have affected suitability of the data for probit analysis (Figure 4.3).

The trim undertaken by TSK was considered within acceptable limits (<12%) and the LC_{50} values were included in the variability analysis. The %CV for LC_{50} was 18% and within the limit considered acceptable by Norberg-King; this suggests low variability in the point estimate between the experiments. The LC_{50}s of the four experiments were not statistically significantly different (APHA, 1992) and ranged from 8.9 to 11% effluent concentration. Figure 4.4 indicates that the LC_{10} for *E. elegans* is between the 3.13 and 6.25% effluent concentration.

Only the data for Experiment 2 were appropriate for probit analysis and as a result was the only experiment to provide point estimates for LC_1, LC_5 and LC_{10} (Table 4.1). Similarly, a comparison of the probit regression lines between experiments was not possible as only data for Experiment 2 fit the probit model.

4.1.3 Comparing *A. peringueyi* and *E. elegans* responses to the metal-plating industry artificial effluent

A comparison of the LC_{50} point estimate indicated that they were significantly different with *E. elegans* being significantly more sensitive that *A. peringueyi* (Table 4.1) (APHA, 1992). Although *E. elegans* were more sensitive to the artificial effluent, 100% cumulative mortality was only achieved in one experiment (Experiment 2), although cumulative mortality was close to 100% in the remainder of the experiments

A comparison of probit regression lines, which was only possible for Experiment 2 where data for both species were appropriate for the probit model, revealed that the slopes were significantly different (p=0.03) and suggested that the response rate of the species to the artificial effluent

differed. This is supported by the onset of toxicity at lower effluent concentrations for *E. elegans* than *A. peringueyi* (Figures 4.1 and 4.3). The onset of toxicity (*i.e.* greater than 10% cumulative mortality) for *E. elegans* is between 3.13 and 6.25%, while that for *A. peringueyi* is between 6.25 and 25% effluent concentration.

The changes in LC_{50} between the two species between experiments showed similarities: for both species, the highest LC_{50} values were obtained in Experiments 4 and 2 (increasing LC_{50} respectively). There was no pattern for Experiments 1 and 3. When considering the chemical composition of the artificial effluent, there are no obvious patterns in the reported metal concentrations which can be associated with the patterns in mortality in Experiments 1 to 4 (Table 5.2). A reason for the reduced LC_{50} for both *A. peringueyi* and *E. elegans* in Experiment 4 is not obvious when considering the chemical analyses since the concentration of the listed constituents are within the range experienced across the 4 experiments.

The onset of toxicity for *E. elegans* between 3.13 and 6.25% effluent concentration may be associated with sharp increases in zinc and nickel concentrations (the other metals remained below chemical detection limit), while the onset of toxicity for *A. peringueyi* between 12.5 and 25% effluent concentration may be associated with a general increase in TDS and conductivity to above 30mS/m. However, further speculation of relationships may prove misleading and chemical speciation studies may explain more precisely the cause of toxicity for the species.

4.2 STANDARD *D. PULEX* 48-HOUR ACUTE TOXICITY TESTS

The concentration-range for the first set of *D. pulex* experiments was the same as that used for the site-specific indigenous invertebrate Experiments 2 to 4. However, these concentrations resulted in rapid 100% cumulative mortality, for both dilution waters. This may account for a significant difference in LC_{50} between Experiment 1 and subsequent experiments where artificial effluent was diluted in culture medium and also for a lack of confidence intervals for Experiment 1 where the artificial effluent was diluted in Vaal Dam water.

4.2.1 *D. pulex* in culture medium dilution water

There were no control mortalities for *D. pulex* in the culture medium during the experimental periods for Experiments 1 to 3.

Although there was a significant difference in LC_{50} between Experiment 1 and Experiments 2 and 3, this is considered an experimental artifact as a result of the chosen effluent concentration range; there was no significant difference in LC_{50} between Experiments 2 and 3 (APHA, 1992) (Figures 4.5 and 4.7). The %CV for all the point estimates were high (Table 4.1), although this may be an artefact of the small sample size (only 2 samples for the LC_1, LC_5 and LC_{10}), or the significantly higher LC_{50}.

Despite the data for Experiments 1 and 2 fitting the probit assumptions, a comparison of the probit regression slopes could not be undertaken.

4.2.2 *D. pulex* in Vaal Dam dilution water

There were no control mortalities for *D. pulex* in Vaal Dam water during the experimental periods for Experiments 1 to 3.

Cumulative mortalities (%) for *D. pulex* exposed to the artificial effluent in Vaal Dam water are shown in Figure 4.6. None of the data for the experiments where *D. pulex* were exposed to the artificial effluent with Vaal Dam water fit the assumptions for the probit model, and TSK analysis was undertaken. This may be due to increased variability as a result of using Vaal Dam water as dilution water, and is reflected by the wide 95% confidence intervals (Figure 4.7). As a result, only LC_{50} point estimates are provided (Table 4.1). The % trim in Experiment 1 was high, and confidence intervals could not be determined. As a result, it could not be established whether the LC_{50} was similar to those obtained for Experiments 2 and 3, as the APHA (1992) formula relies on use of 95% confidence intervals to establish whether point estimates are statistically significantly different. However, it is thought that the reduced LC_{50}, (compared to subsequent experiments) is an artefact of the effluent-concentration range selected for the experiments. This may be reflected by the %CV, which is high (Table 4.1).

There was no significant difference in the LC_{50} between Experiments 2 and 3 which ranged from 0.9 to 1.3% effluent concentration.

4.2.3 Comparison between *D. pulex* in culture medium and Vaal Dam water

Using the APHA method (1992), it was established that the difference in LC_{50} between *D. pulex* exposed to the artificial effluent in culture medium and Vaal Dam water as dilution waters were significantly different, with the LC_{50} for *D. pulex* in Vaal Dam water being significantly higher (Figure 4.7 and Table 4.1).

Since the data did not meet the assumptions for probit analysis (for *D. pulex* in Vaal Dam water), a comparison of the regression slopes could not be undertaken.

As it was difficult to establish a reasonable concentration-response line for the *D. pulex* in either culture medium or Vaal Dam water, it was considered an unnecessary expense to submit effluent concentration samples for chemical analysis. However, samples of the "100% artificial effluent" samples, in Vaal Dam dilution water and culture medium were submitted for analysis for Experiments 2 and 3. These results are presented in Table 4.3. There were no obvious differences in concentrations of the selected water quality constituents which could have resulted in *D. pulex* in culture medium being significantly more sensitive than those in Vaal Dam water. However, TDS in the culture medium effluent concentration-range was significantly lower than that in the Vaal Dam effluent concentration-range and may account for the differences in tolerances between the experiments.

4.3 COMPARISON BETWEEN SPECIES

D. pulex were significantly more sensitive to the metal plating industry artificial effluent than either *A. peringueyi* or *E. elegans* (Table 4.1), and *D. pulex* exposed to the effluent in culture

water as dilution medium were the most sensitive. This is also reflected in the expression of their toxicity as toxic units (Table 4.1).

The trend for differences in end-point estimates and TUs between experiments is the same for both site-specific indigenous invertebrate species (Table 4.1) but does not appear to be associated with any noticeable trends in the chemical analyses (Table 4.2). Simlarly, the difference in sensitivity of *D. pulex* between experiments does not appear to be associated with any obvious trends in chemical analyses.

Chemical analysis of samples shows significant differences in concentrations of zinc, nickel, cadmium and copper at 100% effluent concentration, with levels of these constituents being significantly higher in the *D. pulex* experiments (Tables 4.2 and 4.3). It is not clear why this should be as the same stock solution was used to make up concentrations for both the site-specific invertebrates and *D. pulex* experiments. Comparison of sample analysis between the site-specific invertebrate experiments showed that there was a marginal difference between experiments: there was a trend for increasing concentrations for some chemicals (*e.g.* zinc) from Experiment 1 to Experiment 4, with no such apparent trend for others. However, the *D. pulex* experiments were undertaken during the site-specific experiments, and there is no obvious explanation to account for the large differences in zinc, nickel, copper and cadmium concentrations. Samples for analysis were taken at different times in the experimental process (at the end of the experiment for the site-specific experiments and at the start of the experiment for the *D. pulex* experiments) and may account for at least some of the differences.

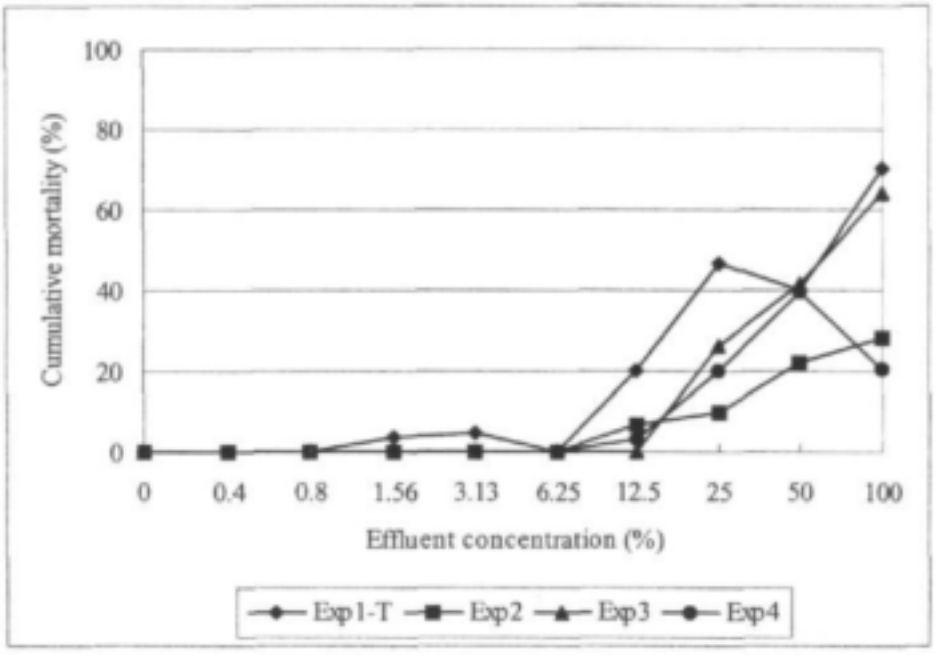

FIGURE 4.1: 96-Hour cumulative mortality (%) for *A. peringueyi* (Heptageniidae) in artificial effluent Experiments 1 to 4. (Results reported for Experiment 1 are from the trimmed data set)

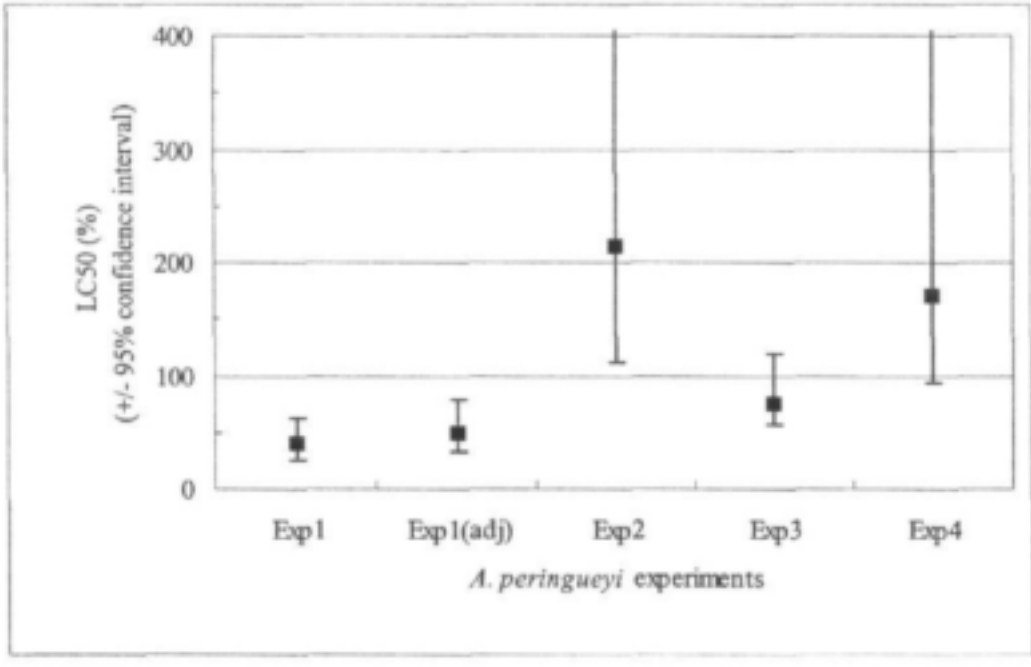

FIGURE 4.2: 96-Hour LC_{50} values (expressed as % effluent concentration) and 95% confidence intervals for *A. peringueyi* (Heptageniidae) exposed to artificial effluents in 4 experiments. Results plotted for Experiment 1 are from the Trimmed Spearman-Karber, and Exp1(adj) is the probit prediction from the trimmed data set for Experiment 1; all other values are probit predictions.

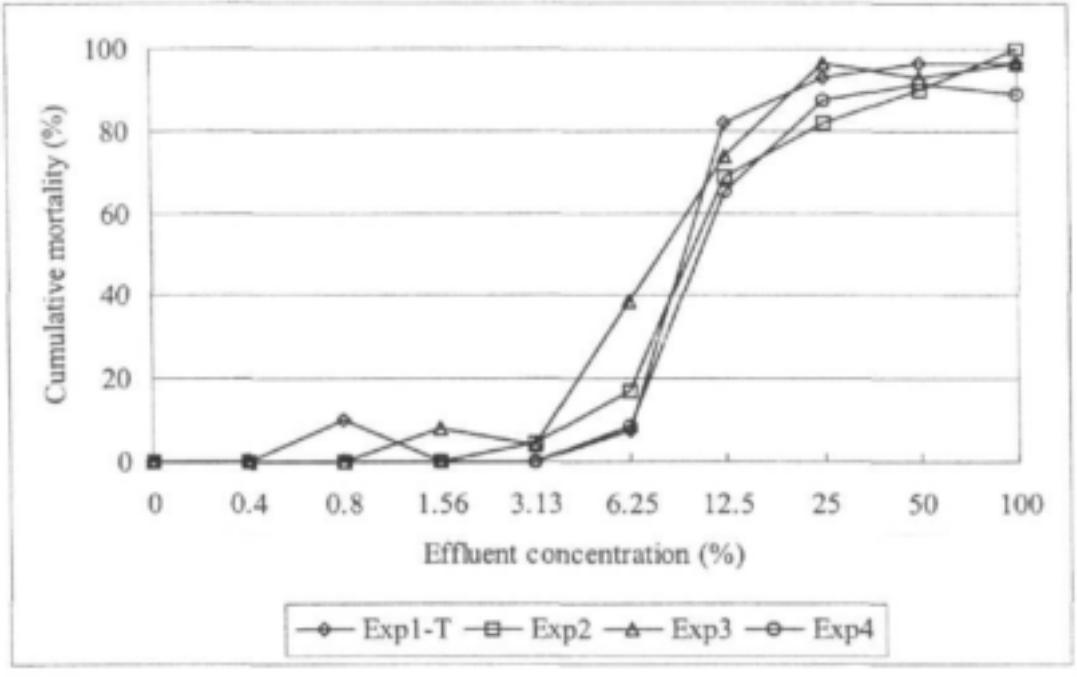

FIGURE 4.3: 96-Hour cumulative mortality (%) for *E. elegans* (Leptophlebiidae) in artificial effluent Experiments 1 to 4. (Results reported for Experiment 1 are from the trimmed data set)

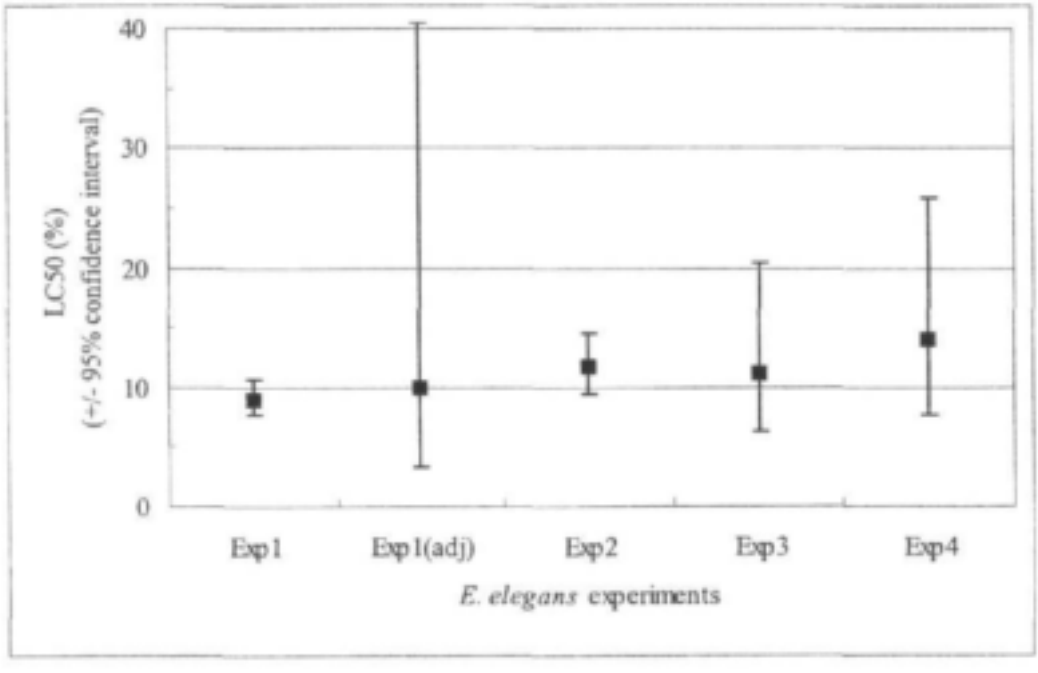

FIGURE 4.4: 96-Hour LC_{50} values (expressed as % effluent concentration) and 95% confidence intervals obtained for *E. elegans* (Leptophlebiidae) in 4 experiments. Results plotted for Experiment 1 are from the Trimmed Spearman-Karber, and Exp1(adj) is the probit prediction from the reduced data set for Experiment 1; all other values are probit predictions.

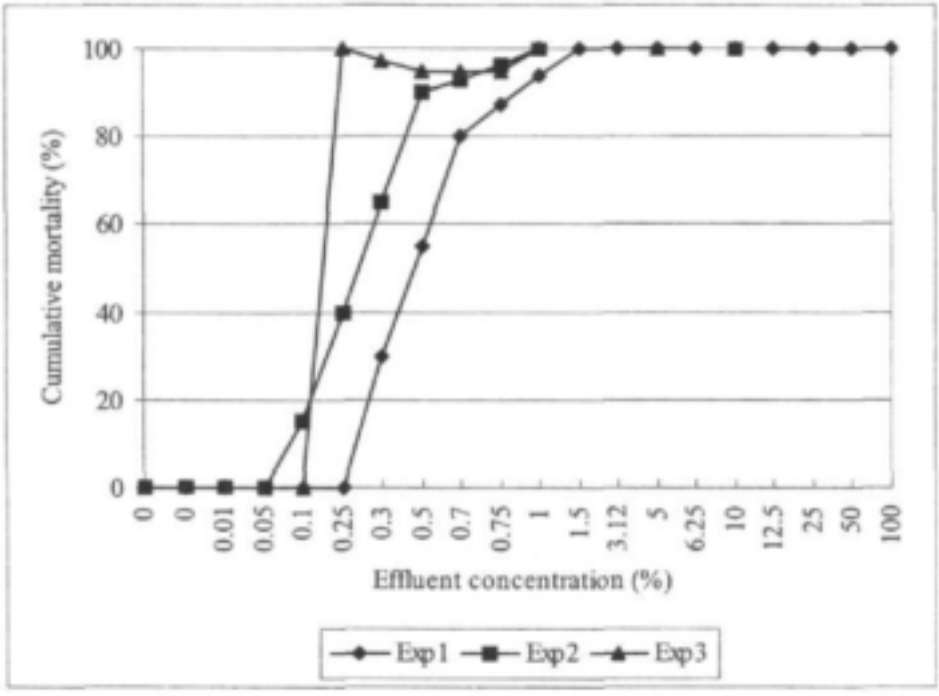

FIGURE 4.5: 48-Hour cumulative mortality (%) for *D. pulex* (Daphniidae) exposed to artificial effluent, Experiments 1 to 3, using culture medium as dilution water

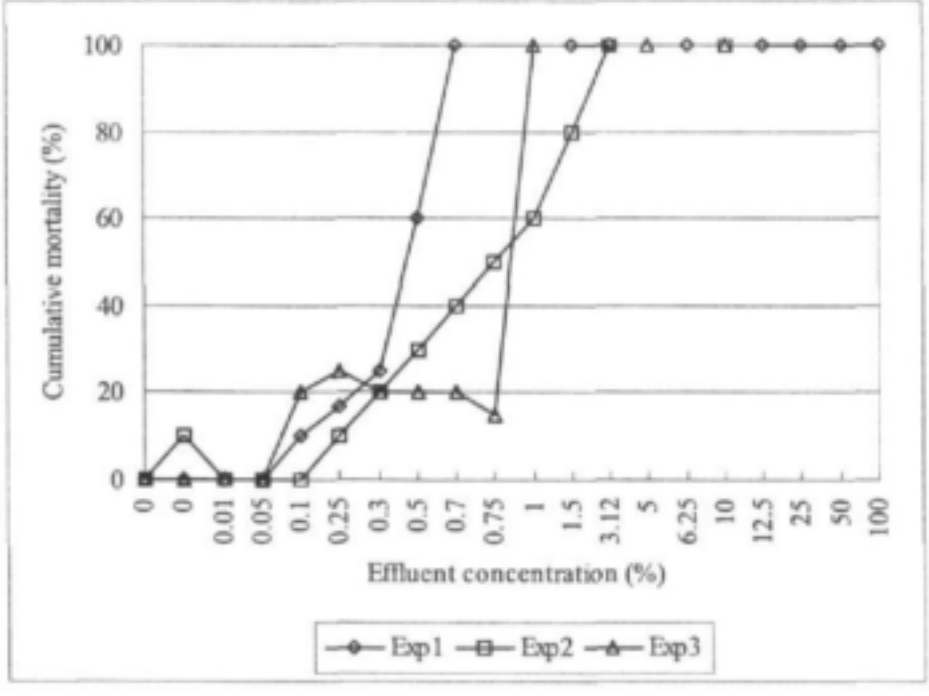

FIGURE 4.6: 48-Hour cumulative mortality (%) for *D. pulex* (Daphniidae) exposed to artificial effluent, Experiments 1 to 3, using Vaal Dam water as dilution water

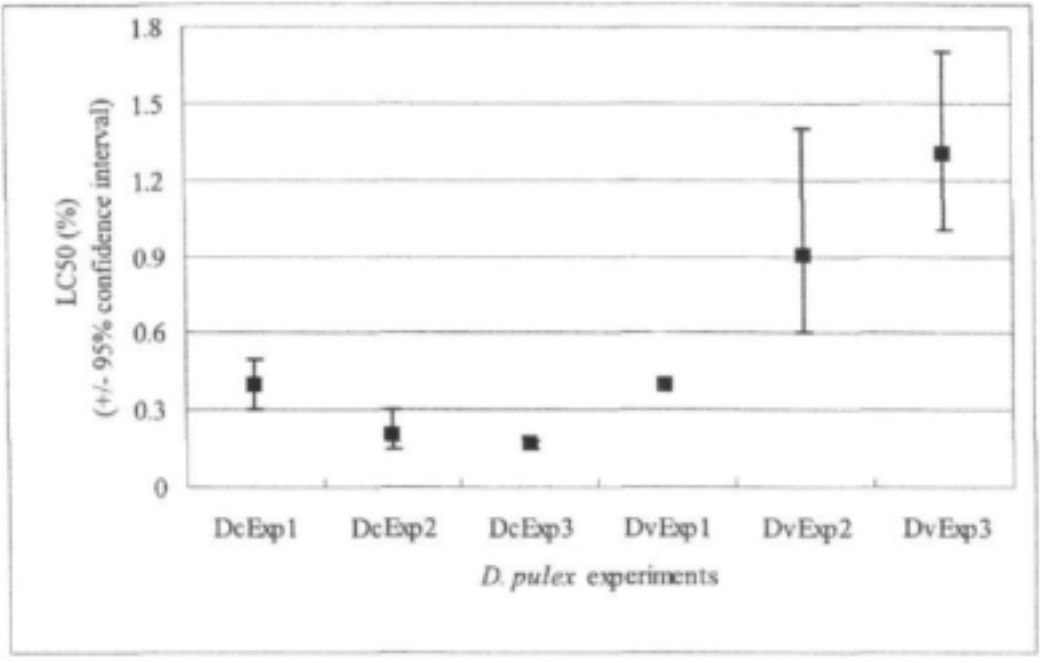

FIGURE 4.7: 48-Hour LC_{50}s, and 95% confidence intervals, for *D. pulex* exposed to artificial effluent in culture medium (DcExp1, DcExp2 and DcExp3) and Vaal Dam water (DvExp1, DvExp2 and DvExp3). Confidence intervals for DvExp1 could not be estimated due to the high % trim by TSK.

TABLE 4.1: Lethal end-point predictions (LC_1, LC_5, LC_{10} and LC_{50}) (95% confidence intervals) and Toxic Units for the artificial metal-plating industry effluent (% effluent concentration) for *A. peringueyi* (Heptageniidae), *E. elegans* (Leptophlebiidae) and *D. pulex* (Daphniidae). Where the probit model did not fit the data, Trimmed Spearman-Karber LC_{50} results are presented and no further point-estimates are listed.

Experiment	LC_1	LC_5	LC_{10}	LC_{50}	Model	TU
A. peringueyi						
1	-	-	-	40 (26-61)	TSK (30%)[1]	2.5
1-trimmed	1.3 (0.4-2.6)	3.8 (1.8-6.2)	6.7 (3.7-10)	49 (34-79)	Probit	2.04
2	4.9 (0.8-10)	14.7 (5.6-24)	27 (14-41)	214 (112-959)	Probit	0.47
3	7.9 (2.8-13)	15 (7.6-22)	22 (13-30)	76 (56-120)	Probit	1.31
4	3.7 (0.7-7.9)	11 (4.3-19)	21 (11-32)	171 (94-604)	Probit	0.59
%CV	26	3	12	76		
E. elegans						
1	-	-	-	8.9 (7.6-11)	TSK (3.6)	11.2
1-trimmed	-	-	-	9.1 (7.6-11)	TSK (3.6)	10.9
2	1.7 (0.9-2.5)	2.9 (1.8-4.1)	3.9 (2.6-5.3)	12 (9.4-15)	Probit	8.3
3	-	-	-	8.9 (7.1-11)	TSK (12%)	11.2
4	-	-	-	11 (9.1-14)	TSK (10%)	9.1
%CV	-	-	-	18		

Experiment	LC_1	LC_5	LC_{10}	LC_{50}	Model	TU
***D. pulex* in culture medium dilution water**						
1	0.11 (0.03-0.2)	0.17 (0.07-0.2)	0.2 (0.09-0.3)	0.4 (0.3-0.5)	Probit	250
2	0.05 (0.02-0.07)	0.07 (0.04-0.1)	0.09 (0.06-0.1)	0.2 (0.15-0.3)	Probit	500
3	-	-	-	0.17 (0.15-0.18)	TSK (0%)	588
%CV	57	54	52	49		
***D. pulex* in Vaal Dam dilution water**						
1	-	-	-	0.4 (could not be estimated)	TSK (25%)	250
2	-	-	-	0.9 (0.6-1.4)	TSK (2.5%)	111
3	-	-	-	1.3 (1-1.7)	TSK (0%)	77
%CV				53		

[1] Denotes the % Trim for the Trimmed Spearman-Karber analysis.

TABLE 4.2: Chemical analyses of selected chemicals from Experiments 1 to 4 for the site-specific indigenous invertebrate experiments at each of the effluent concentrations as well as the diluent (Vaal Dam water) at the start of the experiment (# indicates inconsistent results; - indicates that results were not received from analytical laboratory). ([1] Cond = Conductivity, measured as mS/m; [2] measured as mg/ℓ; [3] Hard = Hardness, measured as $CaCO_3$ mg/ℓ; [4] Alk = Alkalinity, measured as $CaCO_3$ mg/ℓ)

Effluent concentration	Exp.	pH	Cond[1]	TDS[2]	Al[2]	Cd[2]	Cr[2]	Cu[2]	Mn[2]	Ni[2]	Zn[2]	Hard[3]	Alk[4]
0-start	1	-	-	-	0.13	<0.05	<0.05	<0.1	<0.1	<0.1	<0.1	90	96
	2	-	-	175	0.26	<0.05	<0.05	<0.1	<0.1	<0.1	<0.1	78	90
	3	-	-	160	0.14	<0.05	<0.05	<0.1	<0.1	<0.1	<0.1	90	-
	4	-	-	-	-	-	-	-	-	-	-	-	-
0-end	1	-	-	-	0.13	<0.05	<0.05	<0.1	<0.1	<0.1	<0.1	90	96
	2	8.1	28	180	0.26	<0.05	<0.05	<0.1	<0.1	<0.1	<0.1	90	95
	3	8	28	-	0.14	<0.05	<0.05	<0.1	<0.1	<0.1	<0.1	90	100
	4	8	28	165	0.14	<0.05	<0.05	<0.1	<0.1	<0.1	<0.1	-	-
0.4%	1	8.1	29.2	-	0.29	<0.05	<0.05	<0.1	<0.1	<0.1	0.12	97	100
	2	8.1	28	175	-	<0.05	<0.05	<0.1	<0.1	<0.1	<0.1	90	94
	3	7.9	28	-	0.12	<0.05	<0.05	<0.1	<0.1	<0.1	0.17	90	100
	4	7.9	28	170	0.14	<0.05	<0.05	<0.1	<0.1	<0.1	0.36	95	96
0.8%	1	8.1	28.8	-	<0.1	<0.05	<0.05	<0.1	<0.1	<0.1	0.12	93	98
	2	8	28	165	<0.1	<0.05	<0.05	<0.1	<0.1	<0.1	0.14	90	95
	3	7.9	28	-	0.11	<0.05	<0.05	<0.1	<0.1	<0.1	0.14	90	98
	4	7.7	28	170	0.1	<0.05	<0.05	<0.1	<0.1	<0.1	0.22	95	95
1.56%	1	8.1	29.2	-	0.12	<0.05	<0.05	<0.1	<0.1	<0.1	0.26	93	98
	2	8	28	175	-	<0.05	<0.05	<0.1	<0.1	<0.1	0.22	90	94
	3	7.8	28	-	<0.1	<0.05	<0.05	<0.1	<0.1	<0.1	0.18	90	98
	4	7.8	28	165	0.2	<0.05	<0.05	<0.1	<0.1	<0.1	0.31	95	95
3.13%	1	8.1	29.3	-	<0.1	<0.05	<0.05	<0.1	<0.1	<0.1	0.24	93	97
	2	8	28	170	<0.1	<0.05	<0.05	<0.1	<0.1	<0.1	0.4	90	94
	3	7.9	28	-	<0.1	<0.05	<0.05	<0.1	<0.1	<0.1	0.37	90	97
	4	7.7	28	175	0.12	<0.05	<0.05	<0.1	<0.1	<0.1	0.54	95	94

Effluent concentration	Exp.	pH	Cond[1]	TDS[2]	Al[2]	Cd[2]	Cr[2]	Cu[2]	Mn[2]	Ni[2]	Zn[2]	Hard[3]	Alk[4]
6.25%	1	8.1	29.5	-	<0.1	<0.05	<0.05	<0.1	<0.1	0.12	0.67	93	93
	2	7.9	28	175	<0.1	<0.05	<0.05	<0.1	<0.1	0.11	1	90	93
	3	7.7	29	-	<0.1	<0.05	<0.05	<0.1	<0.1	0.11	0.77	90	96
	4	7.4	28	165	<0.1	<0.05	<0.05	<0.1	<0.1	0.15	1.3	95	92
12.5%	1	8	30.2	-	<0.1	<0.05	<0.05	<0.1	<0.1	0.26	1.5	93	91
	2	#	#	180	<0.1	<0.05	<0.05	<0.1	<0.1	0.29	#	93	#
	3	7.6	29	-	<0.1	<0.05	<0.05	<0.1	<0.1	0.31	1.4	93	90
	4	7.6	29	180	<0.1	<0.05	<0.05	<0.1	<0.1	0.35	#	95	91
25%	1	7.9	30.4	-	<0.1	<0.05	<0.05	<0.1	<0.1	0.51	2.1	93	83
	2	7.7	31	200	<0.1	<0.05	<0.05	<0.1	<0.1	0.72	#	93	86
	3	7.5	30	-	<0.1	<0.05	<0.05	<0.1	<0.1	0.68	2	93	88
	4	7.9	31	185	<0.1	<0.05	<0.05	<0.1	<0.1	0.73	2	98	88
50%	1	6.9	33	-	<0.1	0.14	<0.05	<0.1	0.11	0.9	3	95	#
	2	7.7	34	220	<0.1	0.16	<0.05	<0.1	0.1	1.6	3.7	93	75
	3	7.3	33	-	<0.1	<0.05	<0.05	<0.1	<0.1	1.7	3.9	93	83
	4	7.6	34	230	<0.1	0.12	<0.05	<0.1	0.19	1.7	3.6	98	83
100%	1	6.5	39.4	-	<0.1	0.35	<0.05	<0.1	0.54	2.4	9.2	#	#
	2	6.8	39	320	<0.1	0.43	<0.05	<0.1	0.39	2.8	10	95	78
	3	6.7	40	-	<0.1	<0.05	<0.05	<0.1	#	2.8	10	95	68
	4	6.8	40	340	<0.1	0.38	<0.05	<0.1	0.45	3	11	98	56

TABLE 4.3: Chemical analyses of selected chemicals from Experiments 2 and 3 for the stock solution ("100% artificial effluent") used to make up the concentration ranges for the respective *D. pulex* experiments (- indicates that results were not received from analytical laboratory). ([1] Cond = Conductivity, measured as mS/m; [2] measured as mg/ℓ; [3] Hard = Hardness, measured as $CaCO_3$ mg/ℓ; [4]Alk = Alkalinity, measured as $CaCO_3$ mg/ℓ)

Dilution water	Exp.	pH	Cond[1]	TDS[2]	Al[2]	Cd[2]	Cr[2]	Cu[2]	Mn[2]	Ni[2]	Zn[2]	Hard[3]	Alk[4]
Culture medium	2	6.8	46	325	<0.1	0.57	<0.05	0.12	0.55	5	50	70	54
	3	6.8	46	-	<0.1	0.51	<0.05	0.56	0.56	5	37	59	56
Vaal Dam	2	7	47	345	<0.1	0.54	<0.05	0.22	0.53	4.9	50	95	83
	3	7	47	-	0.26	0.49	<0.05	0.33	0.55	4.9	54	83	85

CHAPTER 5:
DISCUSSION AND CONCLUSION

The intention of the whole effluent toxicity (WET) testing programme in the USA, and in the direct toxicity assessment approach in the UK and the Netherlands was to identify, characterize and minimize the toxic effects of discharges on receiving water resources. For this purpose, WET testing has been a useful, although not perfect tool (Chapman, 2000) and has been shown to be effective in reducing the discharge of toxic substances in toxic amounts of point-sources (Ausley, 2000).

There are three, interacting, factors which contribute to the toxicity of a toxicant. These are the dose of the chemical, what the chemical does to the organism (and over what time frame) and what the organism does to the chemical (and over what time frame) (Miller *et al.*, 2000). Within these, there are a number of sources of variability which may affect interpretation of toxicity test results and when WET testing is being considered for inclusion in a water quality monitoring programme, these sources of variability should not be ignored as they contribute significantly to the outcome of the acceptability of the test result (Ruffier, 1996).

Sources of variability in WET tests include aspects relating to the test, *e.g.* the number of concentrations and replicates, the number of organisms exposed and the control, as well as aspects relating to test organism health, seasonal differences as well as performance of the analyst (Burton *et al.*, 1996). Quality of the test organism, analyst experience, deviation from temperature and test duration, dilution water and effluent sample representivity are considered to have the most influence on WET test variability. WET test variability affects the interpretation of test results, and unfortunately the variability of effluent test methods have not been adequately or accurately determined (Burton *et al.*, 1996). While there are sources of variability that can be controlled, such as analyst experience, there remain a number of sources of variability that remain elusive.

This chapter discusses sources of variability in standard toxicity tests, using both site-specific indigenous invertebrates and *D. pulex*, for whole effluent toxicity testing in light of the proposed tiered approach for the assessment of complex waste discharges to water resources (DWAF, in prep.). The tiered approach to whole effluent toxicity testing assumes a conservative approach where *D. pulex*, in culture medium as dilution water, are used to assess the toxicity of an effluent in the first instance. This is the cheapest option, but may also be the most protective, with associated costs for the discharging industry (polluter pays principal). With further testing, and increasing the environmental realism of the toxicity tests (*i.e.* using *D. pulex* in receiving water or site-specific indigenous invertebrates in artificial streams), dischargers may be "rewarded" with more relaxed criteria which are acceptable for the receiving resource.

5.1 WET TESTING VARIABILITY

Laboratory toxicity tests are usually carried out under rigorous conditions which bear little resemblance to natural conditions. However, they are valuable in that they are able to assess the potential impact of effluents independent of external (field) influences, such as physical and

chemical conditions of the receiving water, which play an important role in transforming the effluent. The US EPA concluded that toxicity test data from whole effluent toxicity tests provide useful and valuable information for water quality control programmes (Fisher *et al.*, 1989). Variability is an inherent component of WET tests, but this does not preclude them from being used in effluent discharge permits and in a regulatory environment (Ausley, 1996). Interestingly, Parkhurst *et al.* (1992) report that the %CVs for acute toxicity tests using single chemicals and standard laboratory organisms (*e.g. Daphnia* spp.) were greater than those for effluent toxicity tests, and that the %CVs for chronic toxicity tests were lower than those for acute toxicity tests.

Variability in toxicity tests results may be a result of the effluent itself: if an effluent is moderately toxic to an organism, the test variability is likely to be higher than if the effluent is highly toxic or has low toxicity (Fulk, 1996). Effluents are not constant (Slabbert *et al.*, 1998) and can vary considerably over time, either short-term or long-term. This may contribute significantly to the outcome and recommendations of a WET testing programme, as the sample may have been collected during a period when the effluent was either at peak toxicity or low toxicity; regular monitoring should establish the pattern of effluent toxicity. In the experiments undertaken for this study, an attempt was made to keep the effluent constant (samples were made from a stock solution), although chemical analysis suggests that this was not entirely successful (Tables 4.2 and 4.3).

5.1.1 Experimental variability

5.1.1.1 Exposure systems

As different organisms were used in the two test systems (static and recirculating artificial streams) a direct comparison between the methods is not possible. However, comment can be made on the practical aspects of use of the test systems.

The static test, using *D. pulex*, requires less space, less test solution (with implications for less waste to be dealt with) and less time in setting up (if one does not consider the time taken to culture *D. pulex*). The organisms are easy to collect (from a laboratory culture) and it is relatively easy to ensure that sufficient organisms are available to be able to undertake a toxicity test. The results are available in 48-hours, which may be desirable. The recirculating test, using site-specific riffle-dwelling invertebrates in artificial streams, leaves more room for sources of error to occur, such as organism availability (when using field collected organisms), more complex equipment (*e.g.* submersible pump) which can fail and a more complex experimental protocol. The test is over 96-hours (excluding the acclimation period), which increases the period for potential test failure.

However, despite these factors, the %CVs for the site-specific invertebrates in artificial streams was lower than that for the *D. pulex* tests (Table 4.1). Although it may have partly been an artefact of the unknown effects of the artificial effluent, and selecting a suitable concentration-range for testing, it may indicate there may be no more variability introduced as a result of the selected test system and that the artificial streams are suitable for whole effluent toxicity testing. This is supported by Parkhurst *et al.* (1992) who suggest that the %CVs for static and flow-through toxicity tests, even using effluent, should be similar. In a comparison of intralaboratory effluent toxicity testing using *D. pulex*, the %CVs ranged from 0 to 49% (Parkhurst *et al.*, 1992), which were similar to the range obtained in this study for *D. pulex* experiments.

5.1.1.2 Dilution water

Slabbert *et al.* (1998) found that the choice of dilution water significantly affected the toxicity of a metal plating industry effluent to *Daphnia*. In some cases, there was a 10-fold difference, with *Daphnia* exposed to the effluent in river water being more tolerant than those in standard (culture medium) water. Similarly, Ivorra *et al.* (1995) found differences in responses of algae when exposed to copper in synthetic and natural water, although in this case, the algae were more tolerant when exposed in synthetic water. *Ceriodaphnia* were found to have increased tolerance in receiving water (Stewart, 1996) and this was attributed to the presence of food particles in the water. In contrast, Pontasch *et al.* (1989) found negligible differences in the response of *Daphnia magna* when exposed to effluent in different types of water.

Studies by Slabbert *et al.* (1998) and Ivorra *et al.* (1995) serve to stress the importance of making up synthetic water (or culture medium) exactly as specified, as slight variations may result in significantly different results in toxicity of the test species. These differences in tolerances between organisms exposed to toxicants in standard water (or culture water) and receiving water may affect the choice of dilution water used by an industry for compliance monitoring and may need to be considered, or even specified, for effluent discharge permit requirements.

D. pulex were exposed to artificial effluent in both culture water and Vaal Dam water, and there was a significant difference in the resultant LC_{50}s between the two dilution types. *D. pulex* exposed in Vaal Dam water were significantly more tolerant than those in the culture water (Table 4.1). This has implications for setting site-specific water quality criteria and should be considered carefully in permit requirements. Further studies would be required to determine how the receiving water is resulting in increased tolerance of *D. pulex*.

In this study, the site-specific invertebrates were only exposed to the artificial effluent in Vaal Dam water. Previous experiments using the same species in dechlorinated tapwater resulted in high mortality of the mayflies during acclimation during two separate experiments (> 5%, the maximum allowable by APHA (1992)), indicating that the two species were not suitable for testing in dechlorinated water (Everitt, 1999). The acclimation mortality in this study was less than 3% (over all four experiments) and there was 0% cumulative mortality in the controls for both *A. peringueyi* and *E. elegans* (over all four experiments). It is unsure whether, and to what extent, the receiving water increases the tolerances of the site-specific invertebrates.

Increasing environmental realism by using receiving water in the toxicity tests appears not to introduce more variability than generally experienced (Parkhurst *et al.*, 1992), and should be considered when setting permit requirements.

5.1.1.3 Concentration-range

The amount of effluent relative to the receiving water into which it is being discharged can vary and will result in exposure variability (Slabbert *et al.*, 1998). This may affect selection of the dilution-range to be used in a definitive test in order to increase environmental realism of the toxicity test.

The importance of selecting an appropriate concentration-range for WET tests is often overlooked, but introduces a measure of test result variability. In those experiments which

resulted in high mortality in most of the concentrations, there was poor fit of the data to either of the statistical models (Table 4.1). Increasing the number of partial effects between no-effect and 100% effect improves the fit of the data, reduces the 95% confidence interval (especially at the lower concentrations) and may result in reduced variability.

Selecting an appropriate concentration-range for *D. pulex* experiments proved difficult, possibly due to their sensitivity. One of the requirements for undertaking the statistical analyses is that there are at least two partial responses, but this was difficult to achieve (Figures 4.5 and 4.6) and may account for the high %CV (Table 4.1). This is an important consideration when selecting appropriate concentration-ranges for permit requirements.

5.1.2 Organism variability

It has been shown repeatedly that species vary quite considerably in their sensitivity to toxicants and that using site-specific invertebrates in toxicity tests allows for a more direct prediction of effects in the receiving water (Pontasch *et al.*, 1989). A study by Gruber *et al.* (1994) found that *D. pulex* was more sensitive than resident fish species, but less sensitive than the resident mayfly species (*Stenonoma* sp.) in acute toxicity tests using a fabricated plate effluent; similarly, Pontasch *et al.* (1989) found that mayflies were significantly more sensitive than *Daphnia*.

This is in contrast to the findings of this study where *D. pulex*, in both culture water and receiving water, were found to be significantly more sensitive than site-specific mayflies (Chapter 4). This result is supported by Everitt (1999) who used a single toxicant (zinc) to test the responses of the same species and showed that the organisms showed similar responses: *D. pulex* were significantly more sensitive than *E. elegans* and both species were significantly more sensitive than *A. peringueyi* (Appendix 2).

These results confirm the convention that WET tests should be undertaken using a range of taxa in order to obtain a range of sensitivities of the biological community and therefore increasing predictability of environmental effects (Cairns, 1983; Sarakinos and Rasmussen, 1998). It would be dangerous to use only simple and short-term toxicity tests using single species to protect the environment (Cairns, 1986), especially as the selected species may not be representative of the ecosystem being studied or be highly adaptable to natural abiotic stressors (Koivisto, 1995); genetic differences within single test species but between laboratories may confound inter-laboratory test results (Everitt, 1999; Duan *et al.*, 1997). Different species may be responding to different components of a complex effluent, and this may be an important consideration in effluent toxicity testing and choice of test organisms. Toxicity tests, especially single-species acute toxicity tests, should be viewed as part of the management decision-making process and not as the management answer.

It is highly unlikely that the most sensitive species will ever be tested, mostly because these organisms are not suited to laboratory conditions (Cairns, 1983). Rather than trying to find the elusive "mythical most sensitive species", it is more appropriate to focus on incorporating variability in toxicity tests (Dorn, 1996). Even when using standard, or surrogate species, variability is encountered (Chapter 4). The question remains "how much variability is normal" and this can only be answered by undertaking more toxicity tests and increasing the South African toxicological database. Dorn (1996) proposed that research needs for WET testing should focus on assessing variability in responses to effluent (or classes of effluent) within species and

the development of test methods to assess the toxicity of effluents which have low effects, *i.e.* where the NOEC is greater than 50% effluent concentration.

Slabbert *et al.* (1998), recommend that field collected organisms are not used in WET tests unless there is compelling reason for doing so. However, genetic variability in a test population, such as when collected directly from the field, is not necessarily a drawback especially when doing site-specific testing (Diamond *et al.*, 1992). Although there will be increased variability in the repeatability of the test, due to the fact that most species are not genetic monoclones, field collected organisms serves to increase environmental realism. There are, however, a number of limitations of field collected test organisms which should be considered, such as the range in size (Kiffney and Clements, 1996), no information on organism history (*e.g.* acclimation of test population to local conditions, which may make them more sensitive/tolerant to certain effluents) or organisms may not be available all year round (Diamond *et al.*, 1992).

In Australia, there has been an emphasis on developing toxicity test methods using indigenous invertebrates with the view that these be included in water quality management programmes (Chapman, 1995a). These tests are being developed with the following criteria:

- realistic end-points, which can be interpreted by managers and not only by scientists;
- appropriately sensitive organisms;
- simple methods which can be used by all laboratories and not only highly specialised research laboratories;
- cost-effectiveness.

A similar approach in South Africa would contribute significantly to management of water quality in aquatic resources. Although toxicity tests using lotic indigenous invertebrates in recirculating or continuous flow test systems are relatively more expensive (than static tests), often have complicated structures and produce large amounts of toxic waste, they can give better estimates of environmental toxicity (Williams *et al.*, 1984).

The results presented here indicate that the %CV for the site-specific *E. elegans* was significantly less than that obtained for *D. pulex* experiments (Table 4.1). Differences in end-point estimates for *E. elegans* did not differ significantly between experiments and the species may therefore be appropriate for use in site-specific toxicity tests in the Vaal River catchment. Confidence in *A. peringueyi* as a test organism for setting water quality criteria for the Vaal River catchment is much reduced, as the end-point estimates were more variable (although not significantly different, possibly a result of the large confidence intervals).

5.1.3 Measurable end point variability and sensitivity

The selection of the test end-point should be considered carefully and appropriate statistical methods selected accordingly (Ruffier, 1996). The end-point may well affect the test design (*i.e.* ANOVA *vs* regression experimental design). The more complex the test end-point is to measure, the potential for increased variability increases. This may not be as important for acute tests, as most often the measured effect is mortality and this is a fairly unambiguous measurement. However, sublethal and chronic toxicity tests are more complex.

LC_{50} (or other point estimates) values are insufficient to protect the environment, and measures of LOEL and NOEL should be used as guidelines instead (Chapman, 1995a; Diamond *et al.*,

1992). However, the use of NOECs for regulatory purposes is controversial and it has been suggested that they are inappropriate due to the high variability between results (Chapman *et al.*, 1996). NOECs are highly dependent on the selected concentration-range (they rely on statistically significant differences between the control and the LOEC) while point estimates, which are interpolated, are more reliable and less variable.

In this study, NOECs could not be obtained due to the experimental design (regression approach, with increased concentrations, rather than a replicated design). The %CV of the end-point estimates was variable (Table 4.1), but improved at the lower point estimates (LC_{10} and LC_5). It is suggested that these may be appropriate for regulatory purposes, should only acute toxicity tests be required. However, environmental monitoring would have to be undertaken to confirm this.

5.2 CONCLUSION

The variable nature of flows in South Africa results in uncertainty when attempting to assess assimilative capacity and setting target limits and water quality guidelines (Ashton *et al.* 1995). Added complications are the unknown effects of temperature (and other climatic factors) and chemical interactions (*e.g.* synergism) on the toxic effects on biota, both indigenous and standard laboratory organisms. DWAFs move towards a risk-based approach for resource water quality management is to be commended (Jooste *et al.*, 2000). However, the method requires a large database on which to base the outcomes and assessment of a risk assessment. An important component of the database includes toxicological data and it now becomes imperative that DWAF supports the collection of toxicological data and relevant experimental end-points on which to base the risk-based approach.

> "*Theory without sufficient data is sterile and fragile; data without theoretical basis to explain and relate them can lead only to a confounding morass of unusable information*" (Kimball and Levin, 1985, p168).

Toxicity tests are only part of the answer in achieving sound water quality management as they provide information on pollutants and allow prioritization of efforts to control the pollution (Chapman, 1995a). The need for the inclusion of toxicity testing in effluent regulation was recognised in South Africa in 1993 (Roux, 1994) and the data presented here can usefully contribute to a toxicity database and used to make informed decisions for the management of water resources.

5.2.1 Using WET test data

Despite the negative assertions of the "myth of the most sensitive species", carrying out single-species tests can serve a function in protecting ecosystem function provided a sensitive parameter at sublethal effects can be found (Cairns, 1986). To suggest that sound management decisions can be made on the provision of a single-species sublethal test with a sensitive end-point, would be foolish. However, acute WET tests provide an important first step in assessing the toxicological effects of chemicals and effluent but it is important to have in place biomonitoring systems which will allow biologists and water resource managers to assess predictions and provide a database for further management decisions (Kimball and Levin, 1985). Williams *et al.*

(1984) suggest that the systematic generation of toxicological data is a good approach in determining maximum permissible limits of pollutants, but methods should be optimised in order to make results from different laboratories more comparable. And, although often forgotten, WET tests should not be undertaken without consideration of site-specific applications (Chapman, 2000). Not only is it important to assess the toxicity of effluents, it is also important to assess whether the receiving water may be toxic prior to the addition of effluents (Stewart, 1996). In South Africa, inclusion of receiving water toxicity testing may prove useful in testing the accuracy of Reserve water quality guidelines.

Variability is a reality of using organisms in experiments. Rather than focussing on trying to find the ultimate toxicity test system and experimental organism which results in no test variability, it is more useful to incorporate the variability in the analysis of WET tests and situation analysis, especially for water resource management (Chapman, 2000). Kussatz (1994) suggests that it may be more useful to increase the range of laboratory tests by increasing the number of species tested and finding more sensitive methods (*e.g.* chronic tests) as indicators for higher levels of organization. This should be coupled with an increase in field bioassessments (biological monitoring) to assess whether the laboratory results and predictions are appropriately sensitive and adequate.

When toxicity tests are included in effluent discharge licenses, the violation criteria should also be considered. For example, in the USA a single WET test violation is not subject to lawsuit (Heber *et al.*, 1996), but Toxicity Reduction Evaluations are encouraged so that the offense is not repeated. The selection of appropriate effect levels to be incorporated in license discharge permits is influenced by a number of factors relating to both the test organisms used in the toxicity tests (their sensitivity and representivity of the ecosystem as well as the relevance of the test endpoint used in the toxicity test), the sensitivity of the statistical analysis method, the precision of the test methods employed and the temporal and spatial pattern of effluent discharge (Chapman *et al.*, 1996).

In specifying permit requirements, the more conservative approach would be not to allow toxicity in the effluent prior to its discharge to a receiving water resource (Slabbert *et al.*, 1998). However, this may not be a reasonable or feasible expectation, and it may be more practical to allow a mixing zone within which some detrimental effects will occur. The duration, size and extent of toxicity within this mixing zone can be specified in the permit requirement (Slabbert *et al.*, 1998).

Effluent grab samples are frequently used to assess toxicity of effluents (Slabbert *et al.*, 1998). While a sample taken during peak-toxicity period may lead to a worst-case scenario assessment, and potentially result in over-protection, it may be wiser to err on the side of caution (the precautionary principal approach) as South Africa's water resources are poorly researched and biological diversity remains improperly documented or catalogued.

The data presented here support a tiered approach for WET testing in the Vaal River catchment where using a standard laboratory organism, such as *D. pulex* (in culture water), will result in the most protective criteria, while increasing environmental realism, through use of receiving water and/or site-specific indigenous invertebrates, may well result in more relaxed criteria but still be suitable for the receiving water body in question.

However, the shortcomings identified most frequently in WET tests have been identified as inadequately trained personnel who undertake effluent toxicity tests and insufficient quality assurance and quality control (QA/QC) procedures (Chapman *et al.*, 1996, Burton *et al.*, 1996). It is essential that these be addressed. Development of standard toxicity tests, with easy-to-follow test protocols, has contributed to reducing between-test variability but it is now equally important that personnel be trained to undertake whole effluent toxicity tests. The importance QA/QC programmes in laboratories undertaking WET tests should not be underestimated (Warren-Hicks and Parkhurst, 1996) and QA/QC requirements are not sufficiently prescribed in WET protocols (Ruffier, 1996). There is no purpose in developing new tests, or even refining current tests, unless personnel appointed to carry out the toxicity tests are competent to do so.

5.2.2 Implications of the results for management of the Vaal River catchment

The results presented in this pilot study are for acute toxicity tests using an artificial metal plating industry effluent. They provide good evidence that the tiered approach for the assessment and effective management of complex waste discharge, in this case metal plating industry effluent, to the Vaal River catchment may be useful and appropriate. The first level of testing would require a standard laboratory-reared organism, such *D. pulex*, to be tested in culture water; this is the cheapest test method, but would also be the most protective for the receiving environment (*i.e.* most stringent criteria). The next tier of testing would increase environmental realism, by introducing use of receiving water in toxicity tests. This increases variability of the toxicity test result, which may make the test result more complex to interpret, but may provide more relaxed criteria for discharge by industry. The third level, which is more expensive, but increases environmental realism further through use of site-specific indigenous invertebrates may show that less stringent criteria may be applied and still be protective of the receiving water.

However, it is important to note that acute toxicity tests are short-term tests (48-hours for the *D. pulex* and 96-hours for the site-specific indigenous invertebrates), represent an extreme response (mortality) and the effluent used in the toxicity tests was an artificial effluent. The data provided therefore only provide evidence that the selected management approach is appropriate for acutely toxic effluent, where the end-point measurement is mortality. Numerous experiments undertaken over a period of two years, using a range of industry effluents from the Vaal Triangle, indicated more subtle and sublethal effects on the selected test organisms (these data will be archived in the toxicology database currently housed at CAT-IWR). This has implications for the effective management of water quality of the receiving water resource, the Vaal River.

The fact that no acutely toxic effluent was found during the experimental periods does not mean that no acutely toxic effluent is being discharged to the Vaal River. It rather indicates that there are numerous discharges to the receiving water which have sublethal and chronic effects which will not be adequately assessed during short-term (acute) toxicity tests. Short-term toxicity tests do not provide accurate predictions of longer-term environmental effects and it is imperative that more ecologically relevant toxicological data be generated in order to more accurately predict environmental effects of effluents.

Therefore, in order to effectively manage the water quality of the Vaal River catchment, it is important that sublethal and chronic toxicity testing be undertaken so that all the effluents can be adequately assessed.

CHAPTER 6

6.1 RECOMMENDATIONS FOR FUTURE RESEARCH

Data and experience obtained through the course of this research project has highlighted gaps and shortcomings in the current toxicological knowledge-base in South Africa. This section lists a number of recommendations for immediate future aquatic toxicology research in order to address these and obtain useful toxicological data and understand how best to apply the data for effective management of South Africa's water resources.

i. Continue to undertake acute toxicity tests using (site-specific) indigenous test organisms, and using both single-substances and whole effluents, in order to better understand variability in toxicity data associated with using different organisms, different chemicals and different site-specific receiving waters

It is recommended that acute tests using a wide range of indigenous organisms, exposed to a range of reference toxicants (and effluents) and a standard response such as death, continue to be undertaken. Although acute toxicity tests are not suitable indicators of safe or harmful concentrations in aquatic ecosystems as affects on growth, reproduction and other physiological and histological effects occur at sublethal and chronic levels (usually at levels far below the acute levels), these data will provide valuable insight into the nature of variability of toxicity data and how best to incorporate this variability in the application of toxicity data in water resource management.

After this, appropriate organisms can be selected for sublethal and chronic testing; these test taxa should not necessarily be standard organisms, but rather sensitive site-specific indigenous species (Diamond *et al.*, 1992).

ii. There is an urgent need for the development of chronic and sublethal test methods, and appropriate test end-point measurements, using indigenous organisms

Acute toxicity results in short-term disruption of the ecosystem before recovery can begin while long-term effects, at chronic levels, are more disruptive of the ecosystem (affecting growth, reproductive output and mortality) (Cairns and Pratt 1989). The effect of single-substances on single-species in acute toxicity tests often fail to accurately predict the environmental effects because they fail to take into account sublethal effects (*e.g.* effects on growth and reproduction of organisms, and subsequent recruitment into the affected area), fate and effects of pollutants (in the natural environment) and effects of the pollutants up the foodchain (Kimball and Levin, 1985). There exists a need for more ecologically relevant toxicological data, and particularly important are the effects of exposure of organisms, including site-specific indigenous organisms, to chemicals at sublethal and chronic levels, as well as pulsed exposures (Williams *et al.*, 1984). These data are not only important for single-substances (*e.g.* reference toxicants) but vital for the monitoring and management of effluent discharged to receiving water.

A study by Sarakinos and Rasmussen (1998) showed that instream invertebrate taxonomic richness declined most significantly above 16% instream effluent concentration, while

invertebrate density decreased most significantly above 2% instream effluent concentration. Although there was a decrease in taxonomic richness, it was evident that tolerant species became more abundant at these low effluent dilutions. Chronic effluent toxicity tests which were carried out simultaneously indicated MATCs which were higher than the thresholds indicated by the field trials (Sarakinos and Rasmussen, 1998). This may be due to one, or more, of several factors: test end-point sensitivity is inadequate; variables and factors which confound toxicity; not all exposure routes are considered; and use of non-indigenous test taxa.

Where the effluent forms the greater part of the stream, decisions to manage the water resource based on laboratory WET test results should suffice (La Point and Waller, 2000). However, in the vast majority of cases the effluent forms a fraction of the stream and current laboratory toxicity test methods (acute and chronic) may then be inadequate to predict the environmental impact. This indicates an urgent need for the development of more sensitive chronic toxicity tests than those currently available in order to adequately predict instream effects from laboratory-based toxicity tests (Chapman *et al.*, 1996). It is particularly important that laboratory test end-points are considered and developed carefully so that they will yield environmentally relevant and useful information, and at the same time avoid false positive or negative interpretation of results (Chapman, 1995b). It is preferable that the selected laboratory test taxa also be found in the field, *i.e.* site-specific indigenous organisms (Chapman, 1995b).

iii. Develop methods which can link laboratory toxicity test results, both chronic and acute, to instream biological assessments

The importance of combining laboratory and field studies has been emphasized by a number of studies, especially as the issue of extrapolating from laboratory experiments to field conditions remains uncertain (Kimball and Levin, 1985). Results obtained by Sarakinos and Rasmussen (1998) suggest that laboratory obtained results should be verified by instream assessments, as toxicity tests may be under- (or over-) protective of field conditions. A comprehensive review by La Point and Waller (2000) indicated the need for making direct links between laboratory toxicity test results (acute and chronic whole effluent tests) and field predictions (toxicological field studies and bioassessments). This is particularly due to the indirect effect of complex effluents which may occur at significantly lower concentrations than those which elicit direct toxic effects.

By their nature, chemical and WET testing are predictive, while instream biomonitoring assesses whether criteria and guidelines developed and set using chemical and toxicity testing are sufficiently protective (Heber *et al.*, 1996). Since permit requirements are frequently overly conservative (Ruffier, 1996) it may be appropriate to combine WET tests with instream biomonitoring.

Instream surveys of organisms should be included routinely in assessment programmes in addition to chemical monitoring and toxicity testing, as the biological communities act as continual monitors of environmental monitors and are good indicators of the health of ecosystems. Measuring water chemistry and undertaking toxicity tests are insufficient to protect ecological integrity and should be accompanied by direct ecological assessments (Karr, 1993). Chemical monitoring, laboratory bioassays and *in situ* biological monitoring are all required to adequately assess the effects of pollution (Roux, 1994; Chapman, 1995a).

iv. Development of new toxicity test methods to assess effects of pulsed exposure to chemicals, undertake *in situ* toxicity assessments and assess sediment toxicity

A valid criticism of the research undertaken by this project is that the focus of the research has been on the dose effect of the effluent, while time has not been considered. Even the best designed toxicology experiments have uncontrolled variables, such as the kinetics of the chemicals used in the experiments, and in a recent paper, the frequency or duration of exposure was shown to be an important, and often neglected, consideration in estimating toxicity of substances (Rozman, 2000). Toxicity test methods which are able to incorporate a time factor, *e.g.* pulsed effluent dosing, would be particularly useful to increase the predictability of likely environmental impacts from laboratory toxicity tests.

In situ toxicity testing may present a solution to the problems associated with laboratory-based toxicity tests, especially where the toxic effects of effluents are chronic rather than acute. Results from a study by Crane *et al.* (1995) suggest that instream biomonitoring with *in situ* toxicity testing provide complimentary information, especially in polluted streams, and the development of methods to measure *in situ* toxicity should be considered (Gruber *et al.*, 1994; Sarakinos and Rasmussen, 1998).

There are a number of issues that laboratory toxicity tests are not able to address when attempting to accurately predict environmental impacts based on laboratory tests (La Point and Waller, 2000). For example, the effects of bioaccumulation and bioconcentration are not considered in laboratory tests, the receiving water conditions are not always considered (especially the effects of eutrophication), genotoxicity effects (low-grade toxicity) are not often considered, it is difficult to simulate pulse effects under laboratory conditions, and sensitive or endangered species are infrequently used as test species (for obvious reasons). Sediment toxicity testing is frequently omitted from toxicity test programmes yet sediment can contribute significantly to the toxicity of a water body. Future research programmes should consider this.

6.2 CAPACITY BUILDING

Capacity building activities for Dr Muller are listed; Prof. CG Palmer's activities are included as part of Project K5/955.

As a result of WRC funding for Project K5/815, training students in aquatic toxicology has been possible through undergraduate courses offered at Rhodes University, supervision of postgraduate aquatic toxicology projects and offering outside training courses which either incorporate, or are wholly centred on, aquatic toxicology.

Teaching and training

- Coordinator of a third year semester titled "Applied Freshwater Ecology" (ECL301) which is offered annually to students in the Departments of Zoology and Entomology, Environmental Science and Geography since 1998; lectured on the course in 2000. The course teaches students how to apply ecological concepts and principles to the management of water resources and includes a 4 week module on water quality and aquatic toxicology.

- *Ad hoc* lecture on aquatic toxicology and biomonitoring to 2nd and 3rd year entomology students at University of Fort Hare (1997).

- Co-coordinator of, with Dr P Scherman, and lecturer on the DWAF/CSIR National short course on the "Role and use of biological monitoring in aquatic resource management" in 1999 and 2000. The course provides a basic understanding of the concepts, advantages, uses and limitations associated with different biomonitoring techniques, and includes lectures and practicals on toxicity bioassays. The composition of course delegates was as follows:

	Females		Males	
	non-white	white	non-white	white
1999	3	7	11	12
2000	6	8	13	11

- Co-coordinator of, with Prof CG Palmer, and lecturer on the Lever Ponds "Introduction to Applied Aquatic Toxicology" in 2000. The course offers an overview of aquatic toxicology and how methods and data can be applied by water resource managers and industry. The first course was held in 2000, and the composition of course delegates was as follows:

	Females		Males	
	non-white	white	non-white	white
2000	1	4	4	2

Student supervision

Undergraduate and Honours research projects, for students from various departments (Zoology and Entomology, Environmental Science and Geography) are offered annually. Masters and Doctoral supervision is also undertaken. *Ad hoc* input to research projects (at all levels) is also undertaken through informal discussions. Only projects related to aquatic toxicology have been listed.

Honours students

1999:

- Co-supervised Mr T Mlilo (Department of Chemistry) with Dr P-A Scherman and Prof. T Nyokong (Department of Chemistry).

2000:

- Co-supervised Ms N. Mkize (Department of Zoology and Entomology) with Prof. CG Palmer.
- Co-supervised Ms S. Mbande (Department of Zoology and Entomology) with Prof. CG Palmer.

MSc student

- Co-supervised Ms V. Everitt with Dr P-A Scherman. Ms Everitt submitted her thesis in May 1999 (Rhodes University, Grahamstown) and the degree was awarded in April 2000.

6.3 ARCHIVING DATA

The data (both for site-specific indigenous invertebrates and *D. pulex* toxicity tests) will be incorporated in the toxicology database developed, and currently housed, at CAT-IWR.

6.4 PROJECT ACTIVITIES AND PRODUCTS

Project activities and products for Dr Muller are listed; Prof. CG Palmer's activities are included as part of Project K5/955.

Contracts:

A number of contracts have been undertaken but only those relating to aquatic toxicology, and the application of aquatic toxicology, are listed.

1998:

- Investigation of the feasibility of using a risk-based approach to set integrated environmental objectives for the protection of water resources (1998/1999)

1999:

- Undertook a site-specific toxicology and biomonitoring contract for a petroleum company in Gauteng (1999/2000)

2000:

- Report on the workshop to determine the future direction of research and implementation of ecotoxicology and bioassaying

Posters and papers:

Everitt V, Muller WJ, Villet MH, Mendelow J & Steele G (1997) Water quality in the Jukskei River: An invertebrate study. Proceedings of the 34th Annual Symposium of the Southern African Society of Aquatic Scientists, University of Zululand

Palmer CG, Scherman P-A and Muller WJ (1998) The role of aquatic toxicology, ecotoxicology and biomonitoring in the risk-based management of rivers, and a consideration of the future of aquatic toxicological research, in South Africa. National Rivers Initiative, Southern African Society of Aquatic Scientists Symposium, Pietermaritzburg

Muller WJ, Heath RGM and Villet MH (1998) Finding the optimum: Fluoridation of potable water in South Africa. Water SA 24(1): 21-27

Muller WJ (1999) A comparison of indigenous macroinvertebrates and *Daphnia pulex* in acute whole effluent toxicity testing. Proceedings of the 35th Annual Symposium of the Southern African Society of Aquatic Scientists, Swakopmund, Namibia

Muller WJ (1999) Comparing indigenous macroinvertebrates and *Daphnia pulex* in acute whole effluent toxicity tests. Proceedings of the 20th Annual Meeting of the Society of Environmental Toxicology and Chemistry, Philadelphia

Jooste S, MacKay HM, Scherman P-A and Muller WJ (2000) Feasibility of using a risk-based approach to set integrated environmental objectives for the protection of water resources. WRC Report No 914/1/00

Water Research Commission steering committees:

- The Orange River blackfly *Simulium chutteri*: investigations into the physiology of the aquatic and non-aquatic stages so as to adjust the existing control programme to overcome summer outbreaks
- The gender dimension of the water policy and its impact on water and sanitation provision and management (attended meetings in place of Prof. CG Palmer)

Other Activities:

In order to promote awareness of science in general, and aquatic toxicology in particular, a number of activities have been undertaken:

- Judged School Science Expo projects (annually, from 1996)
- Served on the Eastern Cape River Health Programme Core Group, tasked with initiating the RHP in the Eastern Cape (1997 onwards)
- Served as a member of the Regional Water Supply and Management Task Team (1997/1998)
- Participated in the South African Women in Science and Education exhibition at SciFest (1998)
- Attended a 2-day US EPA course on Environmental Risk Assessment (1998)
- Attended a 3-day Royal Society of South Africa conference titled "Implications of the new water policy - problems and solutions" (1998)
- Attended a 5-day course titled "Introduction to Environmental Impact Assessment" (2000)

CHAPTER 7:
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APPENDIX 1

The study required the use of riffle-dwelling indigenous invertebrates which were site-specific to the Vaal River. As there were limited suitable sites between the Vaal Dam and Vaal Barrage which had riffles for the collection of indigenous invertebrates, there was a need to assess whether the selected reference site on the Vaal River, at Engelbrechtdrift Weir (downstream of Vaal Dam), was a suitable reference site for collecting indigenous invertebrates for whole effluent toxicity testing. Using a standard invertebrate biomonitoring method, SASS4, a comparison of the selected site at Engelbrechtdrift Weir, upstream of Vaal Barrage, and a site at Parys, downstream of the Vaal Barrage, was undertaken. The Vaal Barrage receives a range of industrial effluents via several small tributaries. Downstream of the Vaal Barrage there are industrial discharges directly into the Vaal River.

Results of the SASS4 biomonitoring are presented in Tables A.1.1 and A.1.2. Table A.1.1 is a record of families present during the sampling episode at the 2 sampling sites, and the SASS4 scores (sample score, number of families and Average Score Per Taxon), per habitat and a total for each of the sites, is recorded in Table A.1.2.

Table A.1.2 clearly shows that the diversity of families is higher at Engelbrechtdrift Weir than at Parys and indicates that Engelbrechtdrift Weir is a suitable reference site. The number of sensitive families and the sensitivity of the families was greater at the reference site. This is reflected not only in the total site scores, despite there being more habitats present at the Engelbrechtdrift Weir site, but also for individual habitats. Where the same habitats were sampled at both of the sites, the scores for Engelbrechtdrift Weir were consistently higher. While the difference between the two sites was only slight for stones-in-current, it was significant for stones-out-of-current and marginal vegetation.

Furthermore, it should be noted that families which were found routinely during collecting events at Engelbrechtdrift Weir, *e.g.* Plectoptera, were not collected during the SASS4 biomonitoring survey.

There was evidence of nutrient enrichment at the Parys site (extensive algal growth) and the water had a foul odour. Water samples were collected and submitted to the Rand Water Analytical Services for analysis. Results of the analysis are listed in Table A.1.3, and clearly indicate the poorer water quality at the Parys site. However, it should be cautioned that this was a single sampling event. Several water quality constituents were measured at levels significantly higher at Parys than Engelbrechtdrift Weir.

Table A.1.1: Families recorded in the SASS4 biomonitoring undertaken at the reference site at Engelbrechtdrift Weir, upstream of the Vaal Barrage, and at Parys, downstream of the Vaal Barrage (SIC = stones-in-current; SOOC = stones-out-of-current; MV = marginal vegetation; Bottom = sandy/muddy/gravel river bottom)(the letters a to c indicate abundance, where a = 1-10, b = 10-100 and c = 100-1000).

	ENGELBRECHTDRIFT WEIR				PARYS		
TAXON	**SIC**	**SOOC**	**MV**	**BOTTOM**	**SIC**	**SOOC**	**MV**
Turbellaria							
Planarians		a	a		b	a	a
Annelida							
Oligochaeta		b				a	
Hirudinea							
Leeches		a			a		
Crustacea							
Shrimps			a				a
Hydracarina							
Hydrachnellae			b	c			
Ephemeroptera							
Baetidae: 1 species					b	b	
2 species				a			b
> 2 species	c	c	c				
Heptgeniidae	b	b					
Leptophlebiidae	a	b			b	b	
Prosopistomatidae		a					
Caenidae	a	b	c	a	a	b	a
Odonata							
Protoneuridae							a
Coenagriidae			a		a	a	
Gomphidae			a				
Hemiptera							
Corixidae			a	a		a	
Vellidae							a
Trichoptera							
Hydropsychidae: 1 sp.	a	a	a		b		
Movable case larvae		a	a				
Coleoptera							
Gyrinidae		a	a		a	a	a
Diptera							
Psychodidae						a	
Simuliidae	b	a			c	a	a
Chironomidae	b	c	b	b	a	a	a
Ceratopogonidae	b				b	a	
Gastropoda							
Planorbidae			a				
Physidae							a
Ancylidae	a	a					

Table A.1.2: Sample scores, number of families and Average Score Per Taxon (ASPT) recorded at Engelbrechtdrift Weir and Parys, per habitat and per site

	ENGELBRECHTDRIFT WEIR				PARYS		
SASS4 SCORES PER HABITAT							
	SIC	**SOOC**	**MV**	**BOTTOM**	**SIC**	**SOOC**	**MV**
Sample Score	63	95	66	25	58	54	53
No. of families	9	11	12	5	11	12	10
Score/ASPT	7	8.6	5.5	5	5.3	4.5	5.3
SASS4 SCORES PER SITE							
Sample Score	132				91		
No. of families	21				19		
Score/ASPT	6.3				4.8		

Table A.1.3: Water quality of samples from biomonitoring event at Engelbrechtdrift Weir and Parys

Water Quality Constituent	Engelbrechtdrift Weir	Parys
Hardness (as $CaCO_3$ in mg/ℓ)	83	290
Alkalinity (as $CaCO_3$ in mg/ℓ)	87	120
Ca (mg/ℓ)	17	71
Mg (mg/ℓ)	9.8	27
Na (mg/ℓ)	17	71
K (mg/ℓ)	4.1	12
Cd (mg/ℓ)	<0.05	<0.05
Cr (mg/ℓ)	<0.05	<0.05
Co (mg/ℓ)	<0.1	<0.1
Cu (mg/ℓ)	<0.1	<0.1
Mn (mg/ℓ)	<0.1	<0.1
Pb (mg/ℓ)	<0.1	<0.1
Zn (mg/ℓ)	<0.1	0.11
Ni (mg/ℓ)	<0.1	<0.1

Water Quality Constituent	Engelbrechtdrift Weir	Parys
Al (mg/ℓ)	0.88	<0.1
Fe (mg/ℓ)	0.48	<0.05
B (mg/ℓ)	<0.1	<0.1
V (mg/ℓ)	<0.1	<0.1
Mo (mg/ℓ)	<0.1	<0.1
SiO_2 (mg/ℓ)	1.5	<1
Ammonia as N (mg/ℓ)	0.1	0.08
Nitrite as N (mg/ℓ)	<0.03	<0.03
Nitrate as N (mg/ℓ)	0.16	1.3
Ortho phosphate as P (mg/ℓ)	<0.03	0.22
Total phosphate as P (mg/ℓ)	<0.3	0.36
SO_4 (mg/ℓ)	22	180
S (mg/ℓ)	8	81
Cl (mg/ℓ)	<10	62
F (mg/ℓ)	0.19	0.36
CN (mg/ℓ)	<0.03	<0.03
Br (mg/ℓ)	0.27	1.5
COD (mg/ℓ)	19	47
DOC (mgC/ℓ)	10	14

APPENDIX 2

THE USE OF INDIGENOUS MACROINVERTEBRATES AND *DAPHNIA PULEX* IN ACUTE TOXICITY TESTING

by

VJ Everitt

Appendix 2 is a summary of the M.Sc research funded by project K5/815 and a copy of the thesis has been submitted to the WRC. The M.Sc. was awarded to VJ Everitt at the Rhodes University Graduation Ceremony in Grahamstown in 2000.

The contribution of the M.Sc research to the WRC Project K5/815 "The use of *Daphnia* spp and indigenous river invertebrates in whole effluent toxicity testing in the Vaal catchment" was to examine sources of variability in toxicity testing associated with *D. pulex*. The research contributed to the on-going discussion on the advantages and disadvantages of using laboratory-reared toxicity test organisms and wild-caught indigenous invertebrates in both reference toxicity testing and whole effluent toxicity testing, with an emphasis on implications for whole effluent toxicity testing in South Africa.

TABLE OF CONTENTS

1. INTRODUCTION

South Africans rely on the country's water resources for both their basic human needs and as a means of domestic and industrial waste disposal (DWAF 1997). One of the most scarce resources in South Africa is water (Schulze 1986), thus water resources must be managed carefully, in terms of both quantity and quality (DWAF 1997). Aquatic toxicology has played an important role in water quality management programmes internationally (Richardson and Martin 1994) and can provide the knowledge, through toxicity tests, about the potential adverse effects of toxicants on the aquatic community. It can therefore play an important role in ensuring the sustainable use of aquatic environments through the setting of water quality guidelines and by providing information on the tolerances of aquatic organisms.

1.1 Motivation

Aquatic toxicology, although established in international water quality programs, is still a relatively new field in South Africa, and standardised methodologies for aquatic bioassays were adopted from international agencies (such as the United States Environmental Protection Agency (USEPA) and the American Public Health Association (APHA)). There is however a danger in adopting international standardised methodologies without testing whether the methods and organisms are appropriate for testing under South African conditions. These methods are however being investigated in numerous toxicity studies around South Africa (e.g. DWAF 1999).

An example of a species used widely in international bioassays is the freshwater crustacean *Daphnia pulex*. *D.pulex* lives in the water column and is found largely in standing water (lentic) systems, such as dams and lakes (Pennak 1978). However in South Africa rivers have been identified as the most important aquatic ecosystem in the country (DWAF 1997). Thus the relevance of *D.pulex* as a test organism for setting water quality guidelines to protect the aquatic environment in South Africa is questionable, as it can be argued that a lentic species can not represent those organisms adapted to flowing (lotic) water. *D.pulex* is also a very common species, and has been described as cosmopolitan (Weber 1991). If they are cosmopolitan, is it not because they are tolerant to a wide range of ambient conditions, and may therefore not be ideal as a toxicity test species setting guidelines designed to protect other aquatic organisms? It is also argued that indigenous species and toxicological methodologies adapted for local conditions should be used instead of methodologies adopted from countries with very different aquatic environments.

Ten years ago, the lack of data on the water quality tolerances of South Africa's indigenous riverine organisms was identified (Palmer *et al.* 1996) and the need for environmental research on the water quality requirements of indigenous riverine organisms was highlighted at a Kruger Park Rivers Research Programme (KNPRRP) workshop (Moore 1990). The Institute for Water Research (IWR) in Grahamstown became extensively involved in investigating the use of indigenous macroinvertebrates in toxicity testing. Three different artificial stream systems (including the Channel systems) were designed with the aim of using the artificial streams in toxicity testing (Palmer *et al.* 1996, Scherman and Palmer in press). Their work contributed both to the increase of toxicological knowledge of South Africa's indigenous macroinvertebrates and toward developing a method protocol for the use of indigenous macroinvertebrates in aquatic toxicity testing (DWAF 1999). The need for site-specific whole effluent toxicity testing however

became apparent, along with the need to compare the use of indigenous macroinvertebrates to that of standardised laboratory test species (e.g. *D.pulex*).

1.2 Aims of this study

The aims of this study are summarized below, then discussed in more detail:

- To investigate the use of a standardised laboratory organism, *D.pulex*, as a test organism for setting water quality guidelines to protect aquatic ecosystems from the adverse effects of pollution in South Africa.
- To investigate the tolerances of different *D.pulex* cultures, set up from different populations, to a single-substance toxicant.
- To assess the use of indigenous site-specific macroinvertebrates in toxicity testing by comparing the sensitivities of selected indigenous macroinvertebrates to those of *D. pulex*, using both a single-substance toxicant and selected whole effluents.
- To assess the practicality of whole effluent toxicity testing in South Africa in general.

The primary aim of this thesis was to investigate and discuss the use of *D.pulex* and indigenous macroinvertebrates as toxicity test organisms in South Africa. *D.pulex* are used extensively in other countries as standard aquatic toxicology test species, because they were found to be among the more sensitive of aquatic species tested (Wall and Hanmer 1987, Roux 1994), and were found to produce the most useful toxicological information per unit effort (Eagleson *et al.* 1986). As a result, culturing techniques and optimal test methodologies have been thoroughly investigated and standardised. *D.pulex* is however adapted to still waters, and as most of South Africa's aquatic environments are flowing, the suitability of this species to represent South African aquatic organisms is questioned. Therefore the susceptibility to a single-substance (reference) toxicant (zinc sulphate) and selected whole effluents is compared between *D.pulex* and selected indigenous site-specific macroinvertebrates. The use of indigenous macroinvertebrates in aquatic toxicology is still relatively new in South Africa, with methodologies still being developed and information limited. The practicality of using field-caught site-specific macroinvertebrates in aquatic toxicology tests, versus the use of a standardised, laboratory cultured organism such as *D.pulex*, is therefore discussed.

Laboratory cultured organisms have long been favoured in aquatic toxicity testing (APHA 1992, Rand *et al.* 1995), as they are convenient, with individuals of known age and background being available year round (Adema 1978, Leeuwangh 1978). Many *D.pulex* cultures have been established in laboratories in South Africa and are used in the routine monitoring of potable water quality (e.g. Rand Water Hydrobiology laboratories). However, these cultures have been set up independently between laboratories from females caught from different populations. If *D.pulex* are to be used as a standardised test species in aquatic toxicology, the effect of cultures originating from different populations needs to be quantified. This study compares tolerances between cultures of *D.pulex* set up from different populations, to determine if there are any significant implications when using different populations of a species in toxicity testing.

2. MATERIALS AND METHODS

This study was divided into three main sections, with experiments in each section being designed to address the question being asked. To summarise, the sections are outlined in Table A.2.1.

Table A.2.1: A summary of each major section of the study and its aim, including a brief outline of the methodology, toxicant, test organisms and place of conduct. (IWQS: Institute for Water Quality Studies, Pretoria; CSIR: Center for Scientific and Industrial Research, Pretoria).

Section 1: Differences in zinc tolerance between *Daphnia pulex* cultures started from separate populations.	
Aim	To determine whether source population or culture age influence the tolerance to zinc.
Test organism	2 mature cultures: (used in Experiments 1-6) IWQS (originating from Roodeplaat Dam, Pretoria in 1990). CSIR (Originating from Daspoort Reclamation Works, Pretoria in 1985). 2 immature cultures: (used in Experiments 4-6) RD (collected from Roodeplaat Dam, Pretoria in 1998). SW (Collected from Sewage Works, Grahamstown in 1998).
Methodology	Standard 48 h acute *D.pulex* toxicity tests (with cultures run simultaneously in each experiment).
Toxicant	Zinc sulphate.
Dilution medium	Culture medium.
Place of conduct	Experiments 1-3 at IWQS Experiments 4-6 at IWR
Section 2: Single-substance acute toxicity testing: A comparison between selected indigenous macro-invertebrates and *D.pulex*.	
Aim	To compare the tolerance of *D.pulex* and selected indigenous invertebrates to zinc.
Test organism	*D.pulex* (CSIR and IWQS cultures). Available site-specific indigenous macroinvertebrates (see Table A.2.2)
Methodology	Standard 48 h acute *D.pulex* toxicity tests. 96 h acute toxicity tests with available site-specific indigenous macro-invertebrates. (Unfortunately, because *D.pulex* cultures were not in a suitable condition for reliable experiments when site-specific experiments were conducted, Experiments 5&6 from Section 1 used in the comparison.)
Toxicant	Zinc sulphate.

Dilution medium	*D.pulex*: culture medium. Indigenous invertebrates: Vaal Dam water.
Place of conduct	*D.pulex*: IWR. Indigenous invertebrates: Rand Water.
Section 3: A case study: whole effluent toxicity testing	
Aim	To compare the tolerance of *D.pulex* and selected indigenous invertebrates to selected whole effluents, and to investigate the practicality of using field-caught indigenous invertebrates in aquatic toxicity tests.
Test organism	*D.pulex* (Rand Water cultures, which originated from CSIR). Available site-specific indigenous invertebrates (see Table A.2.2).
Methodology	Standard 48 h acute *D.pulex* toxicity tests. 96 h acute toxicity tests with available site-specific indigenous macro-invertebrates. (Both *D.pulex* and indigenous invertebrate experiments were run simultaneously to ensure effluent composition was the same for both test methodologies.)
Toxicant	Whole effluents A and B.
Dilution medium	*D.pulex*: culture medium. Indigenous invertebrates: Vaal Dam water.
Place of conduct	Rand Water.

2.1 *Daphnia pulex* culturing and test methodology

Standard daphnid culturing and test methodologies are set out by the USEPA (Weber 1991), and these methodologies were followed. Cultures were kept in 3 *l* glass beakers filled with 2 *l* of culture medium. The culture medium, was made up from stock solutions of $CaSO_4.2H_2O$, KCl, $MgSO_4.7H_2O$ and $NaHCO_3$ dissolved in deionised water (IWQS 1997). Cultures were cleaned regularly to prevent the build up of metabolic waste. Temperature in the culture room was kept between 18° and 22°C, with a day-night cycle set at 17 h light and 7 h dark to simulate natural conditions (APHA 1992). The cultures were fed daily on 4 m*l* of artificial food, made by blending trout pellets, dried yeast and dried alfalfa in deionised water (Weber 1991).

Females with embryos were caught the day before the toxicity test using a pipette and allowed to hatch overnight in fresh medium in 150 m*l* beakers. Twenty neonates were then divided between 4x30 m*l* beakers filled with 25 m*l* of test solution (i.e. 5 neonates per beaker), with mortalities recorded after 0.5, 1, 2, 4, 8, 24 and 48 h.

The concentration range of zinc sulphate (prepared in culture medium) was selected to cover 0 and 100% mortalities, with the number of concentrations being run determined by neonate availability. Effluent dilution ranges (prepared in culture medium) were chosen to include 0 and 100% effluent, with the number of dilutions being run primarily determined by the number of

Channel systems (used for the indigenous invertebrate experiments) available in the laboratory. The concentration range for both zinc sulphate and whole effluent experiments were calculated to follow a geometric progression (APHA 1992).

One of the essential properties of a standardised laboratory method, such as the *D.pulex* bioassay, is that test results should be repeatable (Canton and Adema 1978, Baird *et al.* 1989), i.e. consistent results should be obtained when the same test is repeated through time by the same or different analysts. Despite standardised methodology, inter-and intra-laboratory variation exists around the measured end-points. As acceptable levels of variation tend to be set independently for each toxicant, end-point, and test species, it was decided in this study that a level of variability above 15% was high, based on the study by Rue *et al.* (1988) where it was shown that the average CV in effluent studies was 15.8%.

2.2 Indigenous site-specific macroinvertebrate test methodology

2.2.1 Study area

All site-specific experiments were conducted at Rand Water, Vereeniging. Vereeniging is part of the Vaal Triangle, which is made up of Vereeniging, Vanderbjilpark and Sasolburg. This area is largely industrial and borders one of South Africa's largest rivers, the Vaal River. The Vaal River catchment provides water to an extensive area of northern South Africa (i.e. the Free State and Gauteng) and is used for domestic, agricultural and recreational uses, as well as a source of waste disposal for local industries.

The indigenous invertebrates used in this study were collected from two reference sites in this area. Namely the Taaibosspruit, a tributary of the Vaal River, and Engelbrechtdrift weir, a site on the Vaal River. These sites were chosen because they are above the local Sasol/Vereeniging industrial areas and were the best available sites in the area at the time of indigenous invertebrate collection.

The industrial whole effluents selected for use were collected from two industries in this area. Industry A was located in Vanderbjilpark, and Industry B in Sasolburg. Industry A and B both discharge effluent, via effluent canals, into the Rietspruit and Taaibosspruit, respectively. Industry A discharges an effluent composed primarily of heavy metals into the Rietspruit via an effluent canal. This effluent was used in three separate experiments, with effluent samples being referred to as A1, A2 and A3. Industry B's effluent is known to be highly variable and known to contain polyvinyl chloride (PVC), cyanide and fertilizer. One experiment was conducted using this effluent.

2.2.2 Experimental systems and test methodology

The Channel systems have been widely used by the IWR in other toxicity studies and were made from 1 m lengths of polyvinyl chloride (PVC) guttering, with a screen of fine netting at one end (to prevent animals from being flushed through the system), through which the water flowed into a 20 *l* bucket, and was recirculated via a submersible aquarium pump (IDRA IP68 or Shott Centrifugal Pump 12.10, both of which have adjustable flow rates from 400-1300 *l*/h and 200-1400 *l*/h respectively). Evaporation from the system was minimised by adding a "U-bend" and "down-pipe" to the front of the Channels and placing a lid on each bucket. A perspex screen was

placed over the back of the Channel to prevent splash from the inlet pipe. A strip of mosquito netting was provided as a substrate for the animals. All systems were cleaned thoroughly before and after use with extran, then acid-washed using 4% HC*l* and rinsed with deionised water.

Experiments were conducted in a laboratory where the temperature and light regime could be controlled. Air temperature was set in order to maintain a Channel water temperature of 20°C. Temperature was recorded in each channel daily. The light regime was chosen to reflect that experienced naturally in the field (APHA 1992), and was set at a 17 h day, 7 h night cycle.

Up to three test species were placed in a Channel at one time, with at least twenty healthy individuals for each being added at random (APHA 1992). To avoid overcrowding no more than 25 of each species were added. The animals were acclimated for at least 48 h in the dilution water (Vaal Dam water) before the start of the experiment. If more than 5% of the test organisms died during acclimation, the test was terminated (APHA 1992). Where possible the same test species were used for all experiments, but this was dependent on availability of the organism. Acclimation water was completely replaced with 20 *l* test solution at the beginning of the experiment. Concentrations were positioned at random to avoid any positional effects which may occur in the laboratory (APHA 1992). Mortality was recorded twice daily and the dead specimens preserved in 70% alcohol for later identification at the Albany Museum, Grahamstown (which houses the national freshwater invertebrate collection).

At the end of each indigenous invertebrate experiment, a full chemical profile was not undertaken due to cost constraints, however pH, nutrients (ammonia, nitrite, nitrate and total phosphate), water hardness (as calcium carbonate), sulphate and zinc concentrations for each treatment were determined by Rand Water. Standard methods require that nutrients, temperature, electrical conductivity and pH be measured in each experimental system daily, as these factors are known to influence the toxicity of metals (APHA 1992) and were therefore measured during the zinc experiments. Conductivity and pH were not recorded daily during effluent experiments as they are a characteristic of the effluent.

A sample of each effluent was used for chemical analysis to determine the effluent composition. It was accepted that the chemical analysis could not be definitive, as all constituents (e.g. organics) could not be tested for due to cost constraints, and some constituents may have been below current method detection limits (Roux *et al.* 1993, Heber *et al.* 1996). The chemical analysis does however provide an indication of effluent composition and variability.

The range of nutrient concentrations determined for each experiment was compared to chemical data obtained from Rand Water for Engelbrechtdrift weir. Water samples were analysed by Rand Water monthly and data from 1992 to 1998 was summarised as the maximum, minimum and median values experienced over this period. Nutrient concentrations recorded in the experiment were also compared to the TWQR in the Guidelines for the Protection of Aquatic Ecosystems. These comparisons were made to give an indication of previous exposure and whether concentrations recorded during the experiment were to be considered high.

Waste was discarded into Wastetech containers for disposal by Rand Water.

By using the receiving water as the dilution medium in both zinc sulphate and whole effluent experiments, the interactions and resultant toxicity between the chemical components of the

water and the toxicant are determined during the toxicity tests. The environmental realism of the experiment is therefore increased, because these interactions are incorporated into the end-point and the relative toxicity of the toxicant is determined (Buikema *et al.* 1982, Guckert 1993). This is opposed to the inherent toxicity of the toxicant which is established by using a standard dilution water, prepared from distilled or deionised water and known chemicals (Slabbert *et al.* 1998), such as in the *D.pulex* method where the culture medium is used as dilution media.

2.2.3 Test organisms

The indigenous invertebrates chosen for this study were selected on the basis of: their abundance and their suitability to the conditions of the test system. Thus those species which lived in a flowing water environment and proved easy to collect, quick to identify and abundant were chosen as test organisms. A freshwater shrimp, *Caradina nilotica* (Atyidae), and the mayfly family Baetidae, were first selected as potential test organisms as they had been used in previous toxicity studies (Scherman and Palmer in press, and Binder 1999 respectively). Due to the eventual abstraction of water at the Taaibosspruit reference site (at the beginning of 1998, the second year of this study), these species were no longer available and potential test species were collected from Engelbrechtdrift weir reference site instead. *C.nilotica* and Baetidae were collected along with the mayfly species, *Afronurus peringueyi* (Heptageniidae), *Euthraulus elegans* (Leptophlebiidae), and a waterbug species (in the family Corixidae).

Most test organisms were easily identified to species level in the field, except for the Corixidae and Baetidae. These were however easily recognisable at family level. The Corixidae appeared to consist of one species, which remains unidentified, but the Baetidae were known to be a complex of species, and were identified to species level by the Albany Museum, Grahamstown.

Table A.2.2 lists where test organisms were collected for each experiment. Test organisms were collected by either picking individuals off the rocks with soft paintbrushes (mayfly species) or sweeping with a net (shrimps and Corixidae). Organisms were then placed in river water in a cooler box with an ice brick and aerated, in order to prevent an increase in temperature and ensure high oxygen levels on the drive back to the laboratory, where they were sorted. Organisms of different ages (within the same species) have been reported to exhibit different tolerance to the same chemical, so to ensure that test organisms are of similar age only those within a size range of 50% of one another were placed into the channels (APHA 1992). Only uninjured individuals were placed in Channels as the cause of death of injured organisms may not necessarily be due to the toxicant.

Table A.2.2: A summary detailing from where indigenous macroinvertebrates were collected for single-substance and whole effluent experiments.

	Experiment	Test Organism	Reference Site
Single substance reference toxicant (Zinc) experiments	1	Baetidae *A.peringueyi* *C.nilotica*	Engelbrechtdrift weir
	2	Baetidae *A.peringueyi* *E.elegans*	Engelbrechtdrift weir
	3	Baetidae *C.nilotica* Corixidae	Engelbrechtdrift weir
	4	Baetidae *C.nilotica* Corixidae	Engelbrechtdrift weir
Whole effluent experiments	Effluent A1	*C.nilotica*	Emfuleni Park
	Effluent A2	Baetidae	Taaibosspruit
	Effluent A3	Baetidae	Taaibosspruit
	Effluent B	*C.nilotica* Corixidae	Engelbrechtdrift weir

2.3 Statistics

A toxicity test is acceptable only if control mortality is 10% or below (APHA 1992), thus only data sets where control mortality was below 10% were included in the analyses.

Experiments in this study were designed to take advantage of the linear regression approach (Stephan and Rogers 1985), as opposed to the more traditional ANOVA approach (APHA 1992). The linear regression was advantageous in this study for the following reasons:

- *D.pulex* neonates were often only available in limited numbers.
- Laboratory space in which to run the Channel systems was limited.
- Precise $ZnSO_4$ replicates were difficult to maintain throughout the experiments.

It was decided that being able to run a wide range of dilutions had greater value than few replicated dilutions as the strength of the statistical estimate of the LC_{50} increases with an increased number of concentrations (i.e. the linear regression becomes more rigorous with increased dilutions and both Probit and Trimmed Spearman-Karber statistical models (used in this study) estimate LC_{50}s based on a linear regression).

LC_{50} values (concentration at which 50% of the test population dies) (Hoekstra 1993) were calculated for each test organism using both Probit and Trimmed Spearman-Karber methods

(Gelber *et al.* 1985). In this study, if the data did not fit a model's assumptions, data were analysed using the alternate model. Most data fitted the Trimmed Spearman-Karber model. The percentage trim which was considered to be unacceptable could not be found in the literature, thus in this study a percentage trim greater than 20% was considered to be high, as 20% on both sides of the concentration-response curve had to be trimmed off to calculate the LC_{50} (Hamilton *et al.* 1977) (i.e. only 60% of the data was used to calculated the LC_{50}).

To determine whether there were any significant differences between two LC_{50}s the following formula was used (APHA 1992):

$$f_{1.2} = antilog\sqrt{(\log f_1)^2 + (\log f_2)^2}$$

where f = the factor for 95% confidence limits of the LC_{50}, i.e. calculated by dividing the upper confidence limit by the LC_{50}. If the ratio (greater LC_{50})/(lower LC_{50}) exceeds the value for $f_{1.2}$ the LC_{50}s are considered to be significantly different.

If LC_{50}s between experiments were shown not to be significantly different for a particular test organism, the data were pooled and one LC_{50} calculated; if shown to be significantly different, a LC_{50} range is discussed.

3. RESULTS

3.1 Section 1: Differences in zinc tolerance between *Daphnia pulex* cultures started from separate populations

Within all four *D.pulex* cultures (i.e. 2 mature cultures: IWQS and CSIR; 2 immature cultures: RD and SW), experimental variability was high (i.e. greater than 15%), with each culture showing a CV above 130%. Due to the significant variability occurring between the LC_{50}s for each experiment within one culture, comparisons made between the cultures was limited to each separate experiment. Therefore a comparison was made between IWQS and CSIR Experiment 1, and treated independently from a comparison made between IWQS and CSIR Experiment 2, etc.

3.1.1 The influence of different genetic populations on the tolerance of *D.pulex* neonates to zinc

When the 48 h LC50 values were compared between the IWQS and CSIR cultures in each experiment, the two populations were statistically different using the APHA formula. For example, the CSIR cultures in Experiment 1 showed a 48 h LC_{50} value of 12.10 $\mu g/l$ Zn compared to 18.51 $\mu g/l$ Zn calculated for the IWQS culture (Table A.2.3).

Table A.2.3: The 48 h LC_{50}s (Trimmed Spearman-Karber), with confidence limits and percentage trim for each CSIR and IWQS *D.pulex* experiment. The high percentage trim in Experiments 3 and 4 is due to a non-monotonic increase in mortality with an increase in zinc concentration.

Experiment	CSIR culture: 48 h LC_{50} ($\mu g/l$ Zn) (% trim)	IWQS culture: 48 h LC_{50} ($\mu g/l$ Zn) (% trim)
1	12.10 (10.64-13.76) (0%)	18.51 (16.79-20.42) (0%)
2	7.15 (6.19-8.25) (12.5%)	10.13 (8.31-12.34) (12%)
3	37.39 (29.27-47.78) (30%)	27.78 (24.28-31.78) (20%)
4	1280 (1210-1350) (20%)	1070 (940-1230) (20%)
5	320 (290-350) (0%)	220 (200-250) (0%)
6	470 (440-510) (0%)	600 (570-640) (0%)

The fact that the LC_{50} values for CSIR and IWQS populations were significantly different in each experiment suggests that these two populations are different with respect to their susceptibilities to zinc. When comparing the response of both cultures after 48 h, only in Experiments 1, 2 and 6 did the CSIR show a greater sensitivity to the zinc than the IWQS culture after 48 h. In Experiments 3-5, the IWQS cultures showed a greater sensitivity to the zinc than the CSIR culture. Neither culture was therefore consistently more tolerant than the other, and the results may have been confounded by the factors contributing to experimental variability.

3.1.2 The influence of culture age/generation on zinc tolerance

A new *D.pulex* culture was set up at the beginning of 1998 using a female from Roodeplaat Dam (RD), i.e. the same population from which the IWQS culture originated. Thus comparisons could be made between a mature culture (IWQS) which has been kept in the laboratory for over eight years and an immature culture (RD), which has been kept in the laboratory for less than a year.

In each experiment, the 48 h LC_{50}s calculated for the IWQS culture were significantly different from those calculated for the RD culture. For example, the 48 h LC_{50} calculated for the IWQS culture in Experiment 5 was 37% less than that of the RD culture (220 $\mu g/l$ Zn compared to 350 $\mu g/l$ Zn respectively) (Table A.2.4).

Table A.2.4: The 48 h LC_{50} values (Trimmed Spearman-Karber), with confidence limits and percentage trim for IWQS and RD *D.pulex* experiments 4-6. The high percentage trim in RD Experiment 4 is due to a non-monotonic increase in mortality. IWQS culture LC_{50} values for each experiment are significantly different from those calculated for the RD culture.

Experiment	IWQS culture: 48 h LC_{50} ($\mu g/l$ Zn) (% trim)	RD culture: 48 h LC_{50} ($\mu g/l$ Zn) (% trim)
4	1070 (940-1230) (20%)	1370 (1160-1610) (40%)
5	220 (200-250) (0%)	350 (320-390) (0%)
6	600 (570-640) (0%)	460 (430-490) (0%)

Although all three experiments show that the two cultures were significantly different in terms of their response to zinc, no one culture was consistently more tolerant than the other. In Experiments 4 and 5, the IWQS culture showed a greater sensitivity to zinc than the RD culture, but this was reversed in Experiment 6, where RD showed the greater sensitivity to zinc. The trend for increased sensitivity by one culture was therefore not consistent, and more experiments need to be carried out before a conclusion can be made as to whether or not age of a culture influences the response to a chemical.

In the two immature cultures (RD and SW), each generation of *D.pulex* was kept separate, allowing for a comparison to be made between the generations of RD and SW populations. In the SW culture, the 48 h LC_{50} calculated for the thirteenth generation was significantly greater than that calculated for the sixteenth and seventeenth generations. The sixteenth and seventeenth generation 48 h LC_{50}s showed no significant differences from one another (Table A.2.5). This trend was not repeated in the RD culture, where all three generations were significantly different in their response to zinc.

The LC_{50} calculated for the thirteenth generation (Experiment 4) in both SW and RD cultures was significantly greater than that calculated for the subsequent generations, however the seventeenth generation (Experiment 6) in both cultures show a greater tolerance than the sixteenth (Experiment 5) (Table A.2.5). Thus there is no consistency in earlier (or later) generations being more tolerant to zinc in either SW or RD cultures (over the generations tested). Due to the confounding factors which resulted in high experimental variability and low experiment number, no conclusions can be made as to whether or not the amount of time spent cultured in the laboratory influences neonate tolerance to zinc.

Table A.2.5: The 48 h LC_{50} values (Trimmed Spearman-Karber), with confidence limits and percentage trim for each SW and RD generation tested. The high percentage trim was recorded in Experiment 4 due to the non-monotonic increase in mortality with increasing zinc concentration.

Experiment	Generation	SW culture: 48 h LC_{50} ($\mu g/l$ Zn) (% trim)	RD culture: 48 h LC_{50} ($\mu g/l$ Zn) (% trim)
4	F13	1500 (1340-1670) (40%)	1370 (1160-1610) (40%)
5	F16	450 (410-490) (5%)	350 (320-390) (0%)
6	F17	470 (440-510) (0%)	460 (430-490) (0%)

3.2 Section 2: The comparison between site-specific invertebrate and *Daphnia pulex* tolerance to acute zinc concentrations

It is important to note that a comparison between different species and two different methodologies was made. The *D.pulex* methodology followed was the standard 48 h acute toxicity test using a synthetic water (culture medium) as dilution medium. Experiments therefore determined the inherent toxicity of zinc to *D.pulex*. The indigenous invertebrate methodology determined the relative toxicity of zinc, using the receiving water as dilution medium, over 96 h. These factors should therefore be remembered whilst comparing the *D.pulex* and indigenous invertebrate tolerance data.

Only LC_{50}s from *D.pulex* experiments 5 and 6 were used in the comparison between daphnid and indigenous invertebrate tolerance, because chemical analysis to determine exact zinc concentration was carried out on stock solutions for Experiments 4-6 and not for Experiments 1-3. As Experiment 4 showed a percentage trim above 20%, it was not included in the analysis.

The Baetidae LC_{50}s calculated for Experiments 1 and 2 were found to be similar (according to the APHA formula) and similarly *A.peringueyi* Experiments 1 and 2. The results were therefore pooled and one LC_{50} for each test organism used in the comparison with *D.pulex*.

The *D.pulex* were significantly more sensitive to zinc than the indigenous site-specific macroinvertebrate species (Figure A.2.1, Table A.2.6), with the 48 h daphnid LC_{50}s being significantly less than the 96 h LC_{50}s calculated for the indigenous invertebrates. The fact that the *D.pulex* were consistently more sensitive to zinc after 48 h than the indigenous invertebrates after 96 h suggests that an environmental water quality guideline set according to *D.pulex* sensitivity would protect the selected indigenous invertebrates from the Vaal River system against zinc pollution.

A.peringueyi was the most tolerant to zinc of all the test species tested. The results from Experiments 1 and 2 were pooled as LC_{50}s were not significantly different. The resultant mortality curve was not monotonic, thus resulting in large confidence intervals for this test species (ranging from 10.67-28.43 mg/*l* Zn) (Table A.2.6). The results however suggest that *A.peringueyi* is not an ideal test species to use in the setting of guidelines aimed to protect aquatic ecosystems, as they were significantly more tolerant than the other three indigenous invertebrates tested. *E.elegans* may, however, be the more suitable indigenous macroinvertebrate to use for setting environmental guidelines to protect indigenous aquatic invertebrates against zinc pollution (Figure A.2.1, Table A.2.6).

Table A.2.6: Calculated Spearman-Karber LC_{50}s (mg/*l* Zn) and the confidence limits for each test organism from Experiments 1 and 2 are presented. The LC_{50}s calculated for the Baetidae (Experiments 1 and 2) and *E.elegans* were not significantly different from one another (denoted by an *). All other LC_{50}s calculated were significantly different from one another.

Organism	LC_{50}s (mg/ℓ Zn)	Experiment
Baetidae	0.94 (0.86-1.02)*	Pooled (Experiment 1 & 2)
A.peringueyi	17.42 (10.67-28.43)	Pooled (Experiment 1 & 2)
E.elegans	0.98 (0.84 - 1.14)*	Experiment 2
C.nilotica	3.17 (2.39 - 4.20)	Experiment 1
D.pulex (CSIR)	0.32 (0.29-0.35)	Experiment 5
D.pulex (CSIR)	0.47 (0.44-0.51)	Experiment 6
D.pulex (IWQS)	0.22 (0.2-0.25)	Experiment 5
D.pulex (IWQS)	0.6 (0.57-0.64)	Experiment 6

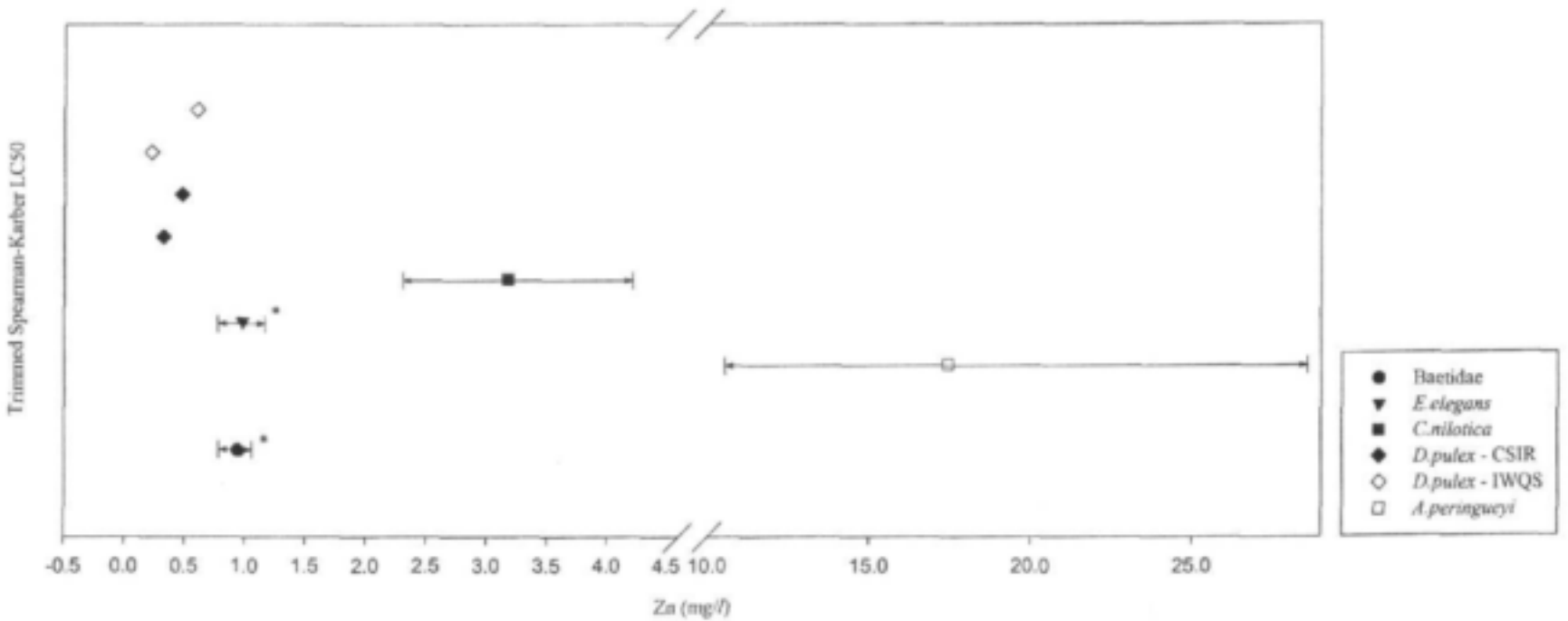

Figure A.2.1: The graph summarises all the LC_{50} values (with 95% confidence limits) calculated for the indigenous site-specific organisms (96 h LC_{50}s) and *D.pulex* (48 h LC_{50}s) (both CSIR and IWQS). All the LC_{50}s were significantly different from one another, except for those calculated for the Baetidae and *E.elegans* (denoted with an *) (see Table A.2.6).

3.3 Section 3: A case study: whole effluent toxicity testing

3.3.1 Industry A

A1 effluent appeared to be the most toxic of the three tested, and no concentration-response curve was recorded for either *D.pulex* or selected macroinvertebrates in Effluents A2 and A3. The only monotonic concentration-response curve recorded for Industry A effluent was recorded in Effluent A1 for *D.pulex*, after 48 h. The *D.pulex* and indigenous invertebrate mortality in Experiments A2 and A3 appeared to be unrelated to increasing effluent dilution, and test organisms did not show a concentration response to either effluent samples.

D.pulex was more sensitive to Effluent A1 than *C.nilotica* (Figure A.2.2, Table A.2.7). A 48 h *D.pulex* LC_{50} was calculated at 39.1 (32.5-47.0)% A1 Effluent, whereas no 96 h *C.nilotica* LC_{50} could be calculated, as only 22.5% mortality was reached at the end of the experiment. In the 100% effluent treatments, 93.3% 24 h *D.pulex* mortality was recorded, compared to only 10.2% by the *C.nilotica* after 24 h.

Although only 22.5% *C.nilotica* mortality was recorded in the 100% A1 effluent dilution after 96 h, *C.nilotica* mortality increased over the experimental period (96 h) in the two higher dilutions (75% and 100% effluent treatments) (Table A.2.7). *C.nilotica* mortality increased from 5.9% after 24 h to 11.8% at 96 h in the 75% effluent treatment, similarly 24 to 96 h mortality increased from 10.2% to 22.5% respectively in the 100% effluent treatment. *C.nilotica* was therefore affected by the A1 effluent and it can be speculated that mortality may have increased with increased exposure time.

Table A.2.7: The cumulative percentage mortalities recorded for *C.nilotica* (96 h mortality) and *D.pulex* (48 h mortality) in response to Industry A1 effluent. *D.pulex* 48 h LC_{50} was calculated at 39.1% (32.5% - 47.0%). Due to *D.pulex* neonate shortage, treatments 3.12 and 6.25% could not be run. The rate of mortality within each treatment can be read by reading down a column, and a comparison between treatments recorded at one time can be made by reading along a row.

		A1 Effluent							
	Hours	100%	75%	50%	25%	12.5%	6.25%	3.12%	Control
C.nilotica	24	10.2	5.9	0	0	0	0	0	0
	48	14.3	9.8	2	0	0	2	0	0
	72	18.4	11.8	2	0	0	2	0	0
	96	22.5	11.8	2	0	0	2	0	0
D.pulex	24	93.3	46.7	33.3	6.7	0	-	-	0
	48	100	86.7	73.3	13.3	0	-	-	0

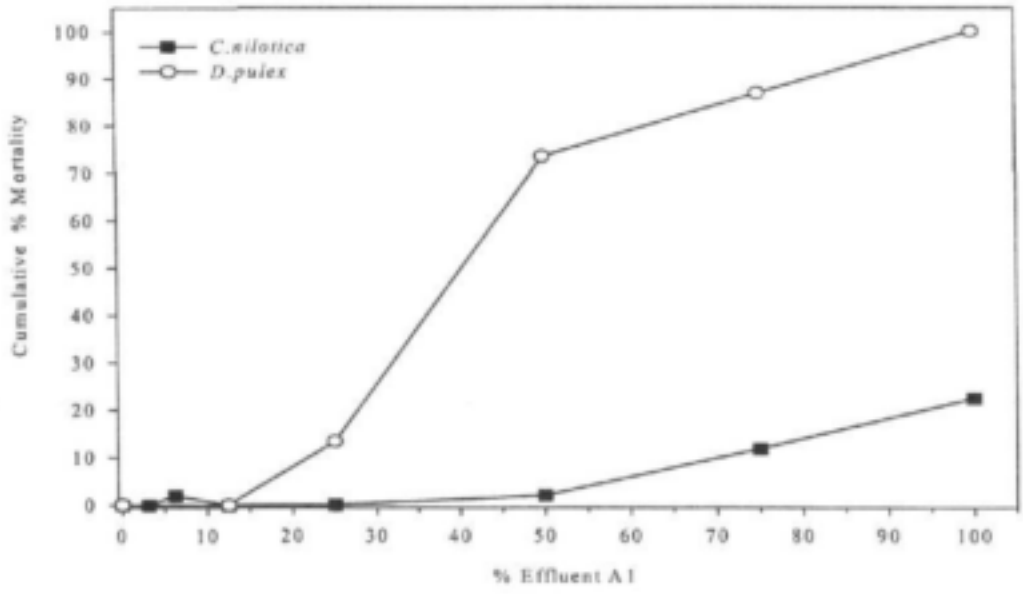

Figure A.2.2: The 96 h *C.nilotica* and 48 h *D.pulex* percentage mortalities recorded in response to Effluent A1. The Spearman-Karber LC_{50} was at 39.1% (32.5%-47.0%) A1 Effluent. No LC_{50} was calculated for *C.nilotica*.

Most chemical variables in Effluent A1 were greater than those measured in Effluents A2 and A3. For example, electrical conductivity (EC) in A1 was almost double that of A2 and A3, and ammonia was recorded as being approximately 3 times greater in A1 than that measured in A2 and A3. When comparing the concentrations measured in the three effluent samples to those measured in the field, most variables exceeded their respective median values as recorded at Engelbrechtdrift weir. Of the variables, only pH and aluminium did not exceed the maximum recorded value at the reference site in all three effluent samples. Ammonia, manganese, zinc and fluoride concentrations exceeded either the Acute Effect Value (AEV), Guideline for the Protection of Aquatic Life (GPAL) values or both in all three effluent samples. These constituents may therefore have contributed to the toxic effects of the effluents. This is however pure speculation, as the synergistic and antagonistic effects between chemicals within the effluent are unknown.

3.3.2 Industry B

This effluent did not result in any significant *C.nilotica*, Corixidae or *D.pulex* mortality (Figure A.2.3, Table A.2.8). The Corixidae appeared to respond at random, suggesting that mortality displayed natural variability and was unrelated to the effluent. For example, 50% Corixidae mortality was recorded in the 6% effluent dilution, whereas only 10% corixid mortality was recorded in the 100% effluent treatment at 96 h. Ten percent mortality occurred in the *D.pulex* control (culture medium) after 24h and mortality in the effluent dilutions appeared again to be

random (Figure A.2.3). Fifteen percent *D.pulex* mortality occurred in the 24% effluent dilution, whereas no daphnid mortality was recorded in the 34.5% and 49% effluent dilutions, with only 5% *D.pulex* mortality occurring in the 70% and 100% effluent dilutions. Effluent B therefore was not acutely toxic to *D.pulex* or to the two selected indigenous invertebrates.

Table A.2.8: The cumulative percentage mortalities recorded at the end of each experiment for *C.nilotica* (96 h), Corixidae (96 h) and *D.pulex* (48 h) in response to the increasing percentage of Industry B effluent. The rate of mortality within each treatment can be read by reading down a column, and a comparison between treatments recorded at one time can be made by reading along a row.

		Effluent B									
	Hours	100%	70%	49%	34.5%	24%	17%	12%	8.5%	6%	Control
C.nilotica	24	0	0	0	0	0	0	0	0	0	0
	48	0	0	0	0	0	0	0	0	0	0
	72	0	0	0	0	0	0	0	0	0	0
	96	0	0	2	0	0	0	0	0	0	0
Corixidae	24	0	20	10	0	0	0	20	0	0	0
	48	0	20	20	0	0	0	20	0	10	11.1
	72	0	30	20	20	0	10	20	0	10	11.1
	96	10	40	40	40	20	40	40	30	50	11.1
D.pulex	24	5	0	0	0	0	0	0	0	0	10
	48	5	5	0	0	15	5	0	0	0	10

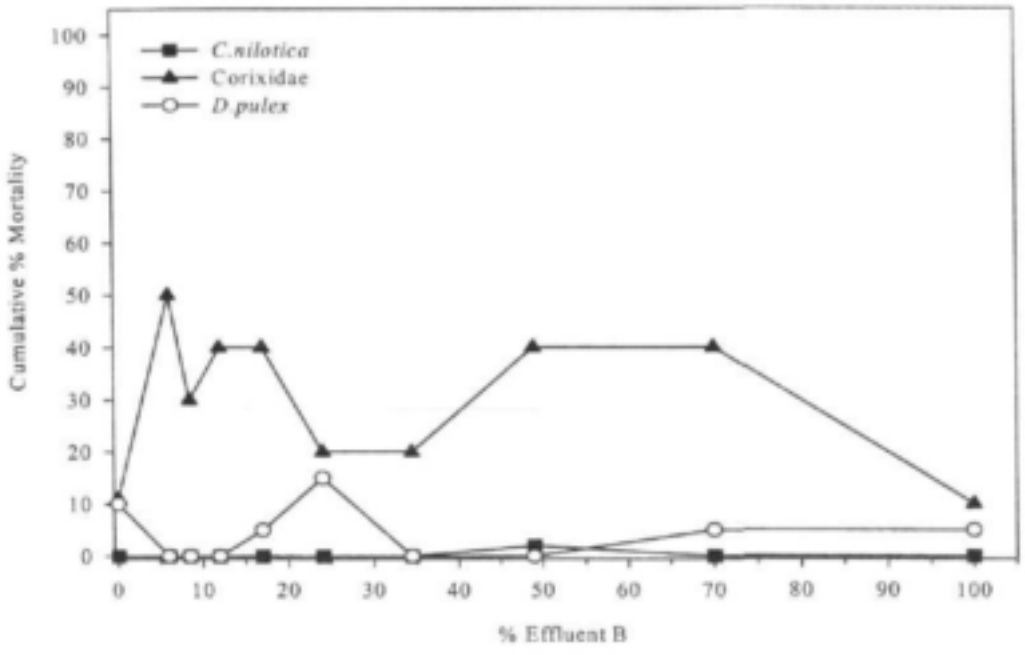

Figure A.2.3: The 96 h *C.nilotica*, Corixidae and 48 h *D.pulex* percentage mortalities in response to Effluent B. LC_{50}s could not be calculated.

4. DISCUSSION

4.1 Section 1: Differences in zinc tolerance between *Daphnia pulex* cultures started from separate populations

4.1.1 End-point variability between experiments and implications for aquatic toxicology

The variability around the measured end-point (48 h LC_{50}) in experiments in this study was high (over 130%) and the same pattern of variability was noted between different cultures. For example, Experiment 4 consistently showed greater LC_{50} values than the other experiments in all four cultures. This would suggest a consistent error in the experiments and which may have be due to an error in the make up of Zn solutions or an extreme variability around *D.pulex* acute Zn tolerance. Despite any uncertainty surrounding the Zn concentration in each experiment the cultures were found to be significantly different from one another in all experiments (based on the APHA formula). Thus it can still be argued that variability existed around the measured end-point in the *D.pulex* cultures and should be confirmed with further toxicity testing.

These findings stress the importance of adhering to standardised methods, and those variables known to influence tolerance (such as temperature and diet) should be monitored continuously. Variability between experiments has important implications when setting a numerical guideline representing the tolerance limit for a species, and in determining compliance to whole effluent and water quality guidelines aimed to protect aquatic ecosystems (Parkhurst *et al.* 1992). The greater the variability, the less confidence is placed in the results (Thursby *et al.* 1997).

4.1.2 The implication of genetic-related tolerance differences between *D.pulex* cultures

Populations are continually adapting in order to survive and different genetic populations result when local populations become isolated in different localities, due to the heterogenous nature of river systems (Duan *et al.* 1997). Differences in genotypes are known to reflect differences in toxicological response, thus cultures established from individuals caught at different locations may well show different sensitivities to the same chemical (e.g. Baird *et al.* 1989). Examples of studies which have shown that populations separated geographically shown differences in tolerance to the same chemicals are Naylor *et al.* (1990) and Crane (1995).

The culturing of *D.pulex* involves asexual reproduction (Pennack 1978) and because a *D.pulex* culture is started from one female and there is no recombination of genes due to sexual reproduction, all individuals are presumed to be genetically homogenous (i.e. identical to the original female). Therefore asexual genotypes are transmitted intact (barring no significant mutational input) from generation to generation (Weider 1993). If no mutation occurs, and tolerance to a chemical is genetically based, the expressed sensitivity to the chemical will not be disrupted by culturing (Baird *et al.* 1989). Therefore, if there were differences between populations in toxicological response to chemicals, this adaptation, if a genetic response, would be expressed in the respective cultures despite standard laboratory rearing.

In this study, because problems with experimental variability were experienced, it can not be stated with any confidence that the differences in tolerance between the cultures was due to genetic differences. Inter-populational differences to toxic stress have however been shown to occur in other species (Lauglin and French 1989, Munzinger and Moncelli 1991), and are thought to be either due to genetic factors (Baird *et al.* 1989) or due to differences in diet, disease, temperature and other factors (Clark and LaZerte 1987). Therefore, if cultures are to be comparable with one another, they should originate from the same population. This is particularly important if a species, such as *D.pulex*, is to be used in the setting of numerical water quality guidelines, such as discharge licences. Standard methods do not currently include specifications of culture origin, but by ensuring the same genotype between laboratories, the potential for experimental variability is reduced.

4.1.3 The influence of culture age/generation on tolerance to zinc

Laboratory *D.pulex* cultures tend to be kept for many years and no studies were found to indicate if the time spent under laboratory conditions influences the tolerance of individuals. It is assumed that the *D.pulex* cultures remain genetically homogenous (Weider 1993), i.e. no mutation or genetic recombination is expected to occur. However in adverse conditions, such as low temperatures or high densities, where there is a subsequent accumulation of excretory products and/or a decrease in available food, males tend to be produced (Cooney 1995). The males may contribute to the genetic makeup of neonates, thus altering the genetic structure of the culture (Cooney 1995). Therefore with every clutch there is a potential for a genetic mutation to occur, and over time, the genetic structure of a culture may change (Baird *et al.* 1989). In this study no conclusion could be made to whether or not the susceptibility to zinc changed with culture age (time spent under laboratory conditions) or generation. Too few experiments were run and it can be argued that results between the IWQS and RD cultures (mature and immature cultures collected from the same locality) were confounded by populational differences. Although both cultures stem from the same dam, the genetic structure of the population may have changed in the eight years that separate their collection. It is recommended that the assumption of genetic structure homogeneity over time (and hence sensitivity to chemicals) is further investigated.

4.2 Section 2: The comparison between site-specific invertebrate and *Daphnia pulex* tolerance to acute zinc concentrations

The tolerance to zinc was not significantly different between the baetids and *E.elegans*. *A.peringueyi* was the most tolerant of the indigenous invertebrates tested to zinc, in terms of 96 h LC_{50}s, and therefore should not be used to set a protective guideline for zinc pollution. Similarly, *C.nilotica*, because of its apparent tolerance to zinc, would not be a good species with which to set a protective ecosystem guideline.

Test repeatability and reliability are important considerations in the choice of test species (Rand *et al.* 1995) and although the baetids appeared consistent in their response to zinc, they are not an ideal family for future toxicity testing. Baetid populations may be made up of a complex of species which are difficult to separate in the field. In toxicity testing, confounding factors such as the mixing of an unknown portion of different species within test concentrations, should be avoided. The baetids also tended to emerge during experiments in this study, which resulted in

low test numbers. Although baetids have been extensively used as test species in the past (Williams *et al.* 1985, Kiffney and Clements 1994), this study recommends that unless a single species population can be identified with confidence and emergence rate controlled, baetids should be avoided as a toxicity test organism.

The *D.pulex* were found to be more sensitive to zinc than the indigenous invertebrates in this study and had lower LC_{50} concentrations. Although limited, this study shows that *D.pulex* could be used in the setting of a guideline designed to protect the aquatic ecosystem against zinc pollution, and the selected indigenous invertebrates used in this study would be protected. However it is important to note that the inherent toxicity of zinc to *D.pulex* was measured, whereas the relative toxicity of zinc to the selected indigenous invertebrates was determined. Also *D.pulex* may not be representative of the Vaal River system. Therefore, although the indigenous invertebrates tested in this study would be protected if a guideline was set using the standard *D.pulex* test methodology, the guideline may be too stringent and not truly reflective of indigenous invertebrate tolerance. The baetids and *E.elegans* were the most similar of the indigenous invertebrates to *D.pulex* in terms of acute sensitivity to zinc, suggesting that these two indigenous invertebrates could also potentially be used to set protective guidelines for the aquatic ecosystem against zinc pollution.

4.3 Section 3: A case study: whole effluent toxicity testing

None of the effluents investigated in this Chapter (excepting Effluent A1) resulted in an acute toxic response over the measured period (i.e. 96 h and 48 h mortality in the selected indigenous invertebrates and *D.pulex* experiments, respectively). During the test period, random mortality was recorded in both the selected indigenous invertebrates and *D.pulex* test populations, and did not appear related to effluent dilution. The effects of the exposure varied from severe in some individuals to none in others, within the same test population. These differences in response to a toxicant within a population may be due to natural biological variation and reflect the genetic make-up of the population and condition of the individuals within the population (Rand *et al.* 1995). Natural variability in test population response will be dampened if the effluent produces a measurable effect on the test population (Grothe *et al.* 1996). In other words, if the effluent is not toxic over the test period, natural variability becomes more marked in the test situation. For example, Effluent A1 was toxic to the *D.pulex* test population, and consequently less variability was evident in test population response compared to the other experiments, where the effluents tested were not acutely toxic.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Choice of test species

A number of criteria for the choice of test species have been set by the USEPA (1979) and APHA (1992), however the choice of test species is largely dependent on the question being addressed (Sprague and Fogels 1977). The primary advantage of using field caught indigenous organisms in aquatic toxicology is that they are representative of local conditions. Consideration of their tolerance is therefore important in the setting of site-specific water quality guidelines. The advantage of laboratory cultured organisms, such as *D.pulex*, is their convenience (Leeuwangh 1978). Recommendations concerning the use of *D.pulex* and indigenous site-specific invertebrates in aquatic toxicity testing in South Africa are discussed below.

5.2 Factors to consider in the choice of indigenous invertebrate test organisms

A primary consideration in the choice of test species for any aquatic toxicity test is the suitability of the species to the test system. The experimental system must meet the species' hydraulic requirements (Statzner *et al.* 1988, Lowel *et al.* 1995). As flowing water environments have been characterised as one of the more dominant aquatic environments in South Africa (DWAF 1997) the Channels, designed for using riffle-dwelling organisms (Palmer *et al.* 1994) in toxicity testing, were seen as an advantage.

When selecting appropriate test species their known sensitivity to chemicals should be considered (Cairns *et al.* 1993, Rand *et al.* 1995). With the assumption being that if sensitive species are chosen, the more tolerant species will be protected (Cairns 1956). As very little is known of the tolerance limits and life histories of most of South Africa's aquatic invertebrates, indigenous invertebrates used in this study were selected for their availability, abundance and suitability to the test system. This is not ideal, as the species in abundance may be those which are more tolerant. A population with high numbers is however required as standard methods require more than 20 individuals at each concentration (APHA 1992). The constant collection of a rare species or a population slow at recovery will quickly deplete the supply of test species. This is an important consideration in areas such as the Vaal Triangle, where industry is numerous and reference sites are sparse.

The stability of a chosen reference site for collection of selected test organisms may be a limiting factor (or disadvantage) in the regular use of indigenous invertebrates in aquatic toxicology. A reference site should be representative of the desired state for the receiving water in question. In an industrial area such as the Vaal Triangle, it can be difficult to find an unimpacted site which is still representative of the receiving water. It may therefore be necessary to settle for the best available site, which may not necessarily reflect the "most natural" conditions. Reliability or stability of the reference site is an important consideration, as changes in flow for example, can result in changes to community composition, making selected test species unavailable for testing. It may become necessary to have a number of reference sites from which the same or different test species are available. However, it is not recommended that a test species is collected from more than one site for an experiment, because differences in tolerance are known to exist between different populations of the same species (Naylor *et al.* 1990, Cairns *et al.* 1993, Crane 1995).

The use of numerous populations may also increase the potential for experimental variability. Thus the choice of the reference site is important and its stability may be a factor to consider in the choice of test species.

5.3 Factors to consider when using cultured *D.pulex* as a test organism

The advantages of laboratory cultured organisms include their availability for testing year round, their documented background biology, known previous exposure to chemicals, and standardised test methodologies (Leeuwangh 1978, Koivisto 1995). An advantage is also that, because methodologies are standardised, experimental variability should be reduced (Rand *et al.* 1995). In this study however, variability between experiments was high, and the importance of controlling and monitoring abiotic factors, such as temperature, which may influence toxicity results, was stressed. Also, it is recommended that the assumption that *D.pulex* cultures remain genetically homogenous over time (Weider 1993) be investigated, as experiments in this study could not conclude that culture age did not play a role in experimental variability. Based on other studies (Buikema 1983, Woods *et al.* 1989), it can be concluded that genetic differences between cultures will result in differences in tolerance to the same chemical. It is therefore recommended that cultures originate from one female.

5.4 The choice of test species for the setting of water quality guidelines

It is recognised that cost and man power are important considerations in aquatic toxicology and not all aquatic species can be tested for their tolerance to all toxicants. It is therefore stressed that the test species and experimental design be chosen according to the aims and questions being asked. When setting a guideline aimed at protecting the aquatic ecosystem, it is recognised that a suite of indigenous test organisms from different trophic levels should be used (Cairns *et al.* 1993, Chapman 1996), as different species are known to exhibit different susceptibilities to the same chemical (McKinney and Wade 1996, Roseth *et al.* 1996). Therefore, the setting of a guideline using one species may not protect all other species, and could be either over or under protective. By establishing a single-substance guideline or whole effluent discharge licence based on the tolerance of a suite of indigenous species, the guideline aimed at protecting the aquatic ecosystem will be more realistic (Buikema *et al.* 1982). The use of indigenous invertebrates in the monitoring of compliance may however prove to be impractical due to the limitations mentioned above, whereas the use of *D.pulex* in compliance monitoring is more practical.

5.5 Recommendations

It is recommended by this study that the setting of discharge licences and environmental water quality guidelines should be based on a suite of indigenous invertebrate and standard laboratory culture experiments. The use of indigenous invertebrates as test species in the setting of a guideline or discharge licence is important as they are the organisms these guidelines are designed to protect. These species are integral components of a complex system, and because the predictive value of cultured populations is still debatable, an end-point determined using site-specific invertebrates will be more reflective of the actual receiving water impacts. *D.pulex* are however more suitable for regular toxicity testing than indigenous invertebrates and it is therefore recommended that they be used in compliance monitoring. If these laboratory populations are to

be used in monitoring however, their tolerance must be compared to that of indigenous populations to determine if they are more or less sensitive than the indigenous invertebrates to the toxicant studied.

Acceptable levels of biological variability around a measured end-point need to be defined, and further research into the differences between biological and statistical differences needs to be initiated. However, factors known to result in experimental variability must be strictly controlled if experimental variability is to be kept to a minimum. These factors include temperature and population differences. Chemical analysis of test solutions at the end of the toxicity test is also recommended in order to determine exact exposure concentrations (for both *D.pulex* and indigenous invertebrate methodologies). The potential for genetic changes, due to mutation, within a *D.pulex* culture also warrants further investigation. Regular testing of laboratory cultures with a reference toxicant may help determine changes in tolerance within a culture over time.

Toxicity tests to determine guidelines and discharge licences should incorporate indigenous population variability (Slabbert *et al.* 1996) and thus be conducted on a wide range of test species (Chapman 1996) to increase the protective value of the guideline. It is therefore recommended that a suite of organisms be used, which are representative of a number of trophic levels and aquatic habitats (Cairns *et al.* 1993).

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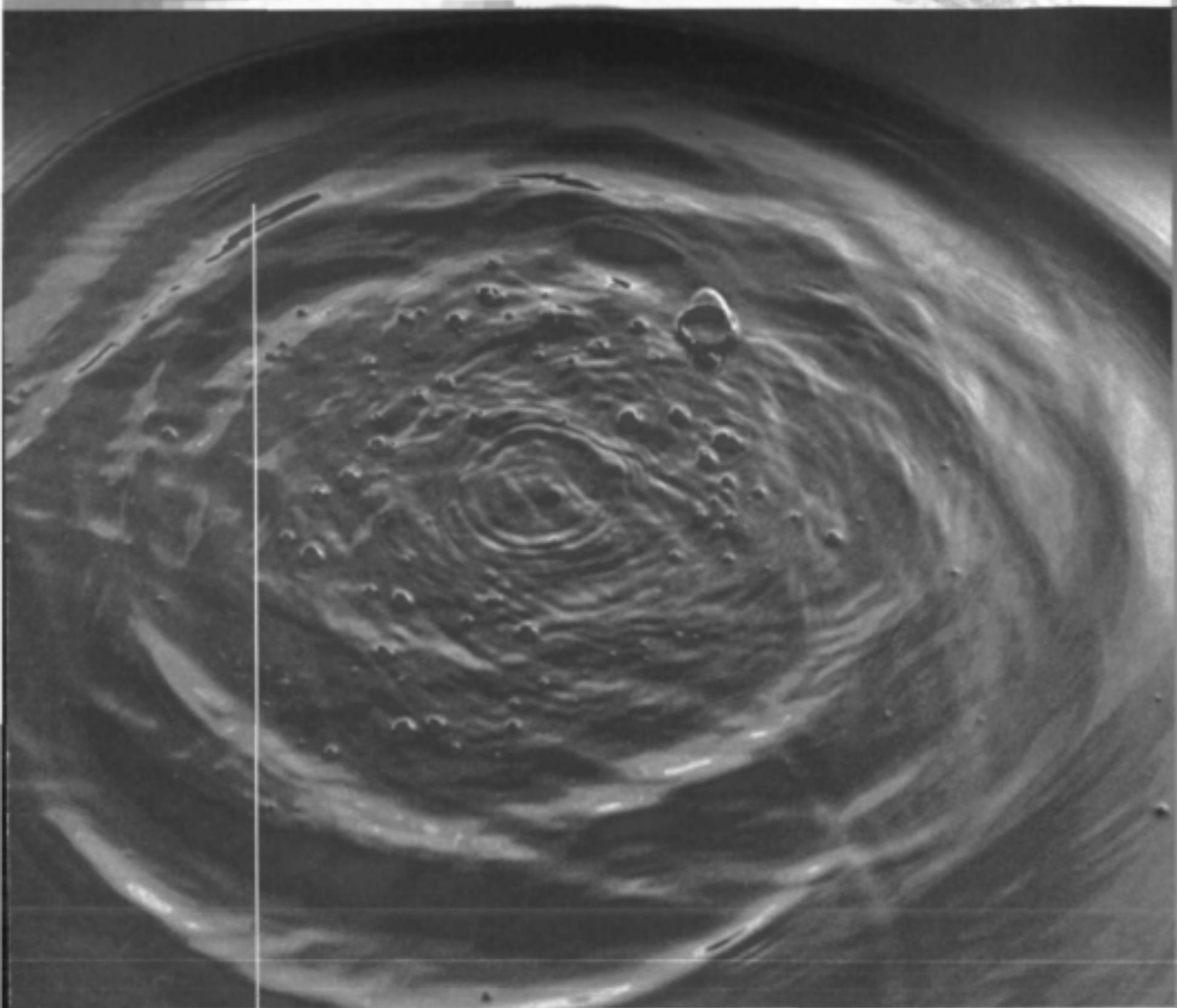

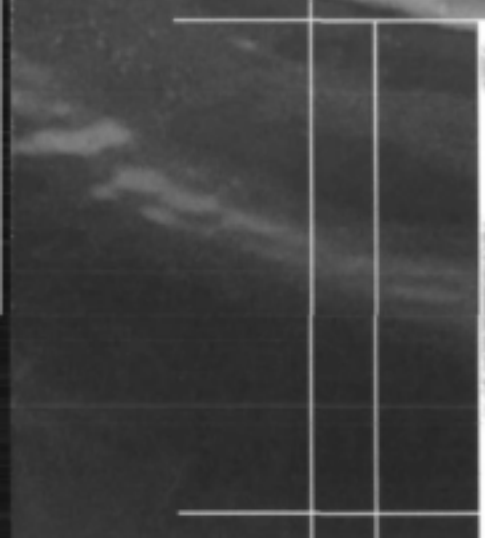

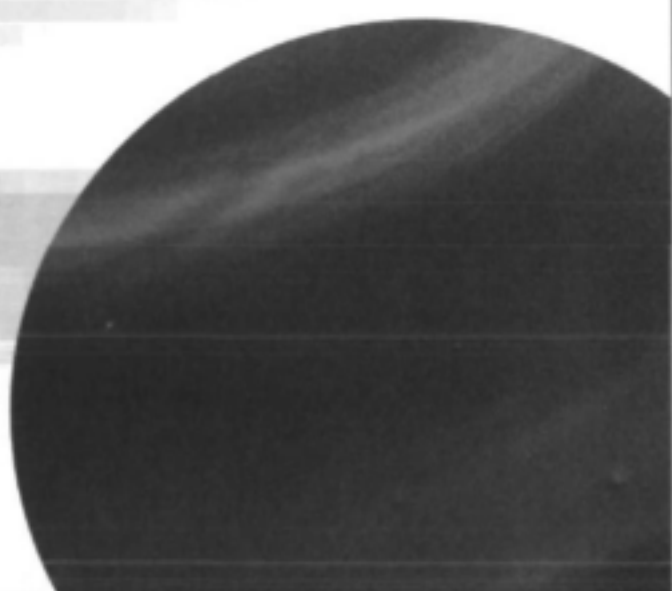

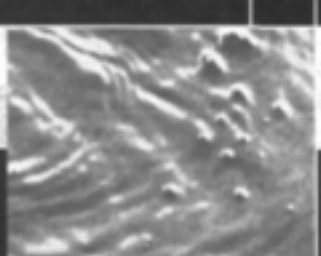

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