The use of indigenous macroinvertebrates and *Daphnia pulex* in acute toxicity testing

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Victoria Jane Everitt

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ABSTRACT

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 have been adopted from international agencies. As a result of these standard methods, the use of laboratory cultured organisms for toxicity testing has been more popular than that of indigenous field-caught organisms. Included in these adopted methods are those for the cultured crustacean *Daphnia pulex*. *D.pulex* is adapted to living in standing water and the suitability of this species to determine toxic effects for South African riverine environments, which are largely flowing, has been questioned. Thus this thesis is a case-study of the use of *D.pulex* and indigenous site-specific macroinvertebrates as toxicity test organisms for setting acute water quality guidelines to protect aquatic ecosystems. The study highlights site-specific problems such as reference sites and organism identification. The acute tolerance of selected indigenous invertebrates was compared to that of *D. pulex*, using both a single-substance reference toxicant (zinc) and selected whole effluents. The significance of source population and culture age as a potential source of biological variability between *D.pulex* cultures was also investigated.

D.pulex cultures have been initiated in South Africa from females collected from a number of different local populations; also it is assumed that no genetic change (due to mutation) occurs within a *D.pulex* culture over time. In order to establish if source population and culture age are a source of biological variability between *D.pulex* experiments, the acute tolerance to zinc of two different *D.pulex* populations and three different generations within a population were compared. Due to experimental variability results were inconclusive, and differences in tolerance as a result of population difference or culture age could not be determined with confidence.

The acute tolerance of *D.pulex* to a single reference chemical (zinc) and selected whole effluents was compared to that of selected indigenous invertebrates. Acute 48 h *D.pulex* zinc tolerance (LC50 range: 0.22 - 0.60 mg/*l* Zn) was found to be more sensitive than acute 96 h tolerances shown by mayfly species *Afronurus peringueyi* (Heptageniidae) (LC50: 17.42 mg/*l* Zn), *Euthraulus elegans* (Leptophlebiidae) (LC50: 0.98 mg/*l* Zn), Baetidae (LC50: 0.94 mg/*l* Zn) and shrimp, *Caradina nilotica* (Atyidae) (LC50: 3.17 mg/*l* Zn). This result suggests that guidelines

for zinc set using *D.pulex* will protect the selected indigenous invertebrates.

Selected whole effluents were not acutely toxic to either *D.pulex* or selected indigenous invertebrates. These experiments were used as a case study for method development regarding the comparative use of *D.pulex* and indigenous invertebrates in acute whole effluent toxicity testing.

Finally, it is recommended that a suite of indigenous organisms (e.g. macroinvertebrates, fish and algae), as well as laboratory cultured *D.pulex*, be used in the initial setting of guidelines and that *D.pulex* be used for routine compliance monitoring. It is futher recommended that a suite of available monitoring methods, such as chemical and biomonitoring methodologies, be used in conjuction with toxicity testing in water quality management.

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GLOSSARY OF TERMS

(Taken from Rand *et al.* 1995 unless otherwise stated)

Acclimation: the time period prior to the initiation of a toxicity test in which aquatic organisms are maintained in untreated, toxicant-free dilution water.

Acute: a stimulus severe enough to induce a response rapidly. The length of exposure and nature of the effect are specified, for example a 48h lethal response.

Acute effect value: the concentration at or above which statistically significant acute adverse effects are expected to occur (DWAF 1996g).

Aquatic toxicology: the study of the effects of manufactured chemicals and other anthropogenic and natural materials and activities (collectively termed toxic agents or substances) on aquatic organisms at various levels of organisation, from subcellular through individual organisms to communities and ecosystems.

Assimilative capacity: the capacity of the aquatic environment to assimilate pollutants without damage to the ecological communities or processes.

Bioaccumulation: a process by which chemicals are taken up by aquatic organisms directly from water as well as through exposure through other routes, such as consumption of food and sediment containing the chemicals.

Bioassays: a biological experiment for estimating the nature, constitution, or potency of a chemical, by means of a reaction that follows its application to living matter.

Bioavailablity: the portion of the total quantity or concentration of a chemical in the environment or a portion of it that is potentially available for biological action, such as uptake by an aquatic organism.

Biological integrity: the ability of an ecosystem to support and maintain a "balanced, integrated, adaptive community of organisms having a species composition, diversity and functional organisation comparable to that of the natural habitat of the region" (Karr 1993).

Chronic: a stimulus that continues over a long period of time. The length of exposure and nature of the effect are specified, for example a 10d growth inhibition.

Concentration-response curve: a curve describing the relationship between different exposure dosages of the test material and percentage response of the exposed test population.

Dilution water (diluent): the water used to dilute the test material in an aquatic toxicity test in order to prepare either different concentrations of a test chemical or different percentages of an effluent for the various test treatments.

Ecological Reserve: the quality, quantity and reliability of water needed to protect both basic human needs, and the structure and function of ecosystems so as to secure ecologically sustainable development and utilisation (DWAF 1997).

Effluent: a complex waste material that may be discharged into the environment.

End-point: the biological response measured. Used in toxicity tests as criteria for effects, for example lethality or growth inhibition.

Exposure: the contact reaction between a chemical or physical agent and a biological system.

Hazard Assessment/Evaluation: identification and assessment of the potential adverse effects that could result from manufacture, use and disposal of a material in a specified quantity and manner.

LC50: the concentration of a chemical that is lethal to 50% of the test organisms as a result of exposures for the periods specified, for example a 96h LC50.

Lethal: causing death by direct action of the toxicant.

Lentic: standing water environment.

Lotic: flowing water environment.

Mixing zone: an area where an effluent discharge undergoes initial dilution and mixing in the ambient water body. An allocated area where water quality criteria can be exceeded as long as acute toxic conditions are prevented.

Probit: a parametric statistical model used to calculate LC50s and the concentration-response relationship between the test material and test organism (Hoekstra 1993b).

Pollutant: a general term for a chemical or non-chemical (e.g. increased suspended solids) agent that is present in the environment and causes adverse effects.

Reference site: a relatively unimpacted site used to define the best available physical habitat, water quality and biological parameters for a river (Eeckhout *et al.* 1996).

Reference toxicant: a chemical used in an aquatic toxicity test as a positive control (i.e. deliberately causes known adverse effects) in contrast to the negative control provided by exposure water without the test chemical. Information collected is used to determine the general health and viability of the test organisms and assess consistency in testing protocol.

Recirculating test system: an artificial stream system where the test solution and dilution water are recirculated through the system via a pump (Williams *et al.* 1984).

Ring test (or round-robin test): a conjoint test conducted under strictly standardised and uniformly applied conditions to assess the precision and accuracy with which different laboratories can determine the toxicity of a chemical or effluent.

SASS: South African Scoring System, an invertebrate biotic assessment method used to give an indication of water quality (Chutter 1995).

Screening test: a short-term test used early in a testing program to evaluate the potential of a chemical to produce some selected adverse effect (e.g. mortality).

Static test: an exposure system for aquatic toxicity tests in which the test chambers contain concentrations of the test material or control water, which is remains static throughout the test period.

Statistically significant effects: effects (responses) in the exposed population that are different from those in the controls at a given statistical probability level, typically $P \leq 0.05$. The statistical approach differs with the type of toxicity test conducted.

Spearman-Karber: a non-parametric statistical method used to calculate LC50 concentrations (Hamilton *et al.* 1977).

Test species: the organism exposed to the test solution in an aquatic toxicity test.

Test material: the chemical or effluent under investigation in an aquatic toxicity test.

Test solution (or test treatment): medium containing the material to which organisms are exposed in a toxicity test. Different test solutions contain different concentrations of the test material.

Toxicity Identification Evaluation (TIE): a set of procedures to identify the specific chemicals responsible for the toxicity of effluents.

Toxicity Reduction Evaluation (TRE): a site-specific study conducted in a stepwise process designed to identify the causative agents in a toxic effluent, isolate the sources of toxicity, evaluate the effectiveness of toxicity control options, and then confirm the reduction in

effluent toxicity.

Toxicant: an agent or material capable of producing an adverse response (effect) in a biological system, seriously injuring structure and/or function or producing death.

Toxicity: the inherent potential or capacity of an agent or material to cause adverse effects in a living organism when the organism is exposed to it.

Toxicity test: the means by which the toxicity of a chemical or other test material is determined. Used to measure the degree of response produced by exposure to a specific levels of stimulus (or concentration of a chemical).

Water quality criterion: an estimate, based on scientific judgements, of the concentration of a chemical or other constituent in water which, if not exceeded, will protect an organism, organism community, or a predetermined water use or quality with an adequate degree of safety. Criteria also refer to the scientific principles and data that are used to formulate guidelines or limits.

Water quality guideline (or limit): a scientifically based numerical concentration limit or narrative statement recommended to support and maintain a designated water use.

Water quality objective: a scientifically based numerical concentration limit or narrative statement that has been established to support and protect designated uses of water, often at a specified site or sites. Objectives reflect desired conditions in receiving waters, usually do not carry the force of the law (i.e. are not water quality standards), and often consider various receiving waters separately.

Water quality standard: a standard, numerical criterion or narrative that is based on the limiting concentration of a chemical (or degree of intensity of some adverse condition) which is permitted in an effluent or receiving water. A water quality standard is typically dependent on the use of the water to be protected. Standards are established for regulatory purposes.

Whole effluent toxicity (WET): the total toxic effect of an effluent measured directly with aquatic organisms in a toxicity test.

LIST OF ABBREVIATIONS

AEV: Acute Effect Ratio.

APHA: American Public Health Association.

CSIR: Center for Scientific and Industrial Research.

DWAF: Department of Water Affairs and Forestry.

IWQS: Institute for Water Quality Studies.

IWR: Institute for Water Research, Rhodes University, Grahamstown.

GPAL: Guideline for the Protection of Aquatic Life.

PP: Pollution Prevention approach proposed by DWAF to prevent the input of hazardous effluents into the aquatic environment.

RWQO: Receiving Water Quality Objectives approach.

TWQR: Target Water Quality Range.

UES: Uniform Effluent Standard approach.

USEPA: the United States Environmental Protection Agency.

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CHAPTER 1

AN INTRODUCTION TO AQUATIC TOXICOLOGY AND SOUTH AFRICAN WATER QUALITY MANAGEMENT

1.1. AQUATIC TOXICOLOGY

Aquatic toxicology is a multidisciplinary field concerned with the effects of chemical substances on the aquatic environment (Rand *et al.* 1995). It involves the understanding of chemical, physical and biological factors which affect environmental concentrations of chemicals found in aquatic systems. Aquatic toxicology is therefore the qualitative and quantitative study of adverse or toxic effects of chemicals on aquatic organisms (Rand and Petrocelli 1985). It tries to explore the deterministic relationship between the exposure of an organism to a substance and the effect of the substance to an organism under defined, controlled and reproducible conditions (Brown 1986).

1.1.1. A brief history of aquatic toxicology

Basic toxicological research can be traced back to the 1800s (Buikema *et al.* 1982). In 1815 the first book devoted to the harmful effects of chemicals on organisms was published by M.J.B Orfila, and discussed many aspects of toxicology recognised as valid today (Rand *et al.* 1995). Research was initiated on rodents, fish and insects, with major developments in pharmacology, toxicology and applied entomology occurring from that time onward (Buikema *et al.* 1982). The systematic study of toxic effects in laboratory animals (mostly mammals) began in the 1920s, in response to concerns about the unwanted side effects of food addictives, drugs and pesticides (Rodricks 1992). In the 1940s and 1950s concerns about the effects of wastes and chemicals on nonhuman biota became a public issue and the years after World War 2 saw efforts to standardise earlier method descriptions and suggestions for test organisms (e.g. cladocerans and fish) (Hunn 1989). Hart *et al.* (1945) introduced the single-species fish bioassay and since then fish have been used to study the effects of various environmental factors on toxic responses as a way of predicting field impacts (Rand *et al.* 1995). The concept of selecting representative organisms from different trophic levels which would display the range of response to toxicants evolved in

the 1950s, thus introducing the concept of using a range of organisms in aquatic toxicology (Cairns 1956). The 1960s saw the advancement in experimental system design (in both static and flow-through systems) and in the measurement of sublethal end-points (growth, development and behaviour) on a range of species from micro-organisms to fish. The American Society for Testing Materials (ASTM) expanded its coverage of bioassay protocols to include organisms other than fish, and in the 1970s journals such as Water Research and Environmental Pollution were started (Rand *et al.* 1995). In response to increased public, scientific and government awareness over water pollution, the number of laboratories employing toxicity testing increased in the 1970s (Johnstone 1977). Acute fish toxicity was accepted as a valid parameter for both government regulations and guidelines for water pollution control (Rand *et al.* 1995). In the 1980s aquatic toxicology was integrated into the hazard assessment process (Cairns and Pratt 1989). It was recognised that the science of aquatic toxicology needed to move away from a reliance of single-species bioassays, even though they have remained attractive to regulators, and toward the use of both multispecies approaches and full field ecosystem end-points to verify laboratory predictions (Cairns and Pratt 1989).

The 1990s have seen continued growth in the field of aquatic toxicology and particularly in the United States of America and Europe, environmental protection agencies have restated the value of applying aquatic hazard assessment principles and procedures to effluents and their component chemicals and properties (Rand *et al.* 1995). Testing of effluents via "end-of-pipe" and "receiving water" approaches has served a number of countries well in their efforts to curb aquatic pollution. There is also a better understanding of the applications, advantages and limitations of the various laboratory and field approaches. It is commonly accepted now that biology or ecotoxicology (effects identification) and chemical sampling (cause identification) are inseparable components of the various hazard assessment approaches being developed and employed by environmental protection agencies (Blaise *et al.* 1988). Biological procedures of many kinds, including toxicity tests, are being built into current policies, frameworks, strategies, guidelines, regulations and codes of good practice in many countries, and on most continents (Rand *et al.* 1995), because biological tests and toxicological end-points offer rapid, reliable and unequivocal, integrated measures of environmental effects or their potential effects (Rand *et al.* 1995). The Organisation for Economic Cooperation and Development (OECD) Water

Management Policy Group and the Expert Group on Biological Testing of Effluents concluded that toxicity testing can be successfully used for measuring toxic effects of effluents on biota of receiving waters, and should be used to generate quantitative data in order to provide the regulative basis for the control of toxic substances (OECD 1987).

1.1.2. Concepts and principles in aquatic toxicology

A toxicant is an agent that can produce an adverse response in a biological system, e.g. by damaging its structure or function, even resulting in death (Rand *et al.* 1995). Toxicants can be introduced deliberately or accidentally into the aquatic ecosystem, impairing water quality and making it unfavourable for aquatic life. Toxicity is a relative property reflecting a chemical's potential to have a harmful effect on a living organism. It is a function of the concentration and composition (or properties) of the chemical to which the organism is exposed (i.e. externally and internally) and the duration of exposure (Rand *et al.* 1995).

Toxicity can be divided into direct and indirect toxicity. Direct toxicity results from the toxic agent acting more or less directly at sites of action in and/or on organisms; indirect toxicity occurs as a result of the influences of changes in the chemical, physical and/or biological environment (e.g. changes in food quality and quantity or habitat changes). Direct toxicity is largely the result of internal biochemical changes, whereas indirect toxicity is more a function of changes in general organism viability produced by factors external to the organism. Laboratory toxicity studies tend to focus on direct toxic effects, whereas field studies include indirect effects and community changes. The direct toxicological information gained from toxicity tests is used to estimate indirect effects or interpret site-specific situations, although experimental examination of indirect toxicity is increasing (Rand *et al.* 1995).

The fundamental assumptions of toxicology are as integral and applicable for aquatic toxicology as they are for human or mammalian toxicology, where they were initially developed (Filov *et al.* 1979). The most basic toxicological principle is the cause-effect relationship, in which it is assumed that the effect or response in question is a direct or indirect result of exposure of the organism to the toxic agent being examined. This principle is followed by the assumption that

a concentration-response or concentration-response relationship exists, where the effect or response in question is a result of the toxic agent reaching and interacting with the site of toxic action in or on the organism. Thus it is assumed that the amount of toxic agent reaching the site of toxic action is some function of the exposure of the organism to the toxic action, and that above an effect threshold, the magnitude of effect is proportional to the amount of toxicant reaching the site of action. The third fundamental principle in toxicology is that the toxic effect can be quantified. That is that the observed effects or responses of toxic action can be measured and quantified in a reproducible way that is relevant to the toxic process under examination (Rand *et al.* 1995).

Causality in toxicology is critical and for quantitative purposes it must be reasonably certain that there is a causal relationship between the observed effect or response and the presence of the toxic agent. There may be some doubt about the identification of the chemical (which may change during the exposure), the actual exposure concentration in the water, and the specificity in response, because aquatic organisms may respond similarly to a variety of stresses. Thus, until proved, it is only a reasonable working presumption that the effect or response being observed is a result of exposure to the known concentration of chemical (Rand *et al.* 1995).

No chemical is completely safe and no chemical completely harmful, and one factor that determines whether a chemical agent is potentially harmful or safe is the relationship between the concentration of the chemical to which the organism is exposed and the duration of exposure. This concentration-response relationship implies that all chemicals are capable of producing an adverse effect if in high enough concentrations (Rand *et al.* 1995).

1.1.3. Factors influencing toxicity

The toxicity of a particular substance to an aquatic population is determined by a variety of factors. For example, the time during which the organism is exposed to the chemical (i.e. the duration of exposure), the concentration of the chemical, frequency of exposure and kind of exposure, have a direct influence on the toxicity of the chemical (Rand *et al.* 1995). Rates and patterns of metabolism and excretion can substantially affect the susceptibility of an organism

to a chemical (Rainbow and Dallinger 1991, Pritchard 1993), and this may account for different species showing different tolerance limits to the same chemical. Differences in tolerance to a chemical have also been attributed to genetic factors (Baird *et al.* 1989), and populations previously exposed to a chemical may become selectively more tolerant than unexposed populations (Naylor *et al.* 1990, Crane 1995). Dietary factors and nutritional status of the organism may also influence toxicity (Jooste 1998) by producing changes in body composition, physiological and biochemical functions. Age of the organism also appears to play a role in the tolerance to a chemical (Jarvis 1989, Hoekstra *et al.* 1993), with immature organisms tending to be more susceptible than adult organisms. This may be due to differences in rates of excretion and body size (Jarvis 1989, Jooste 1998).

External environmental factors may also influence the toxicity of a chemical by altering the form of the chemical or the nature of exposure (Pontasch *et al.* 1989a). Factors associated with the bioavailability of the chemical in water include dissolved oxygen, temperature and dissolved solids (Buikema *et al.* 1982, Abel 1989). Other chemicals in the water may have a synergistic or antagonistic effect on the toxicity of the chemical (Marking 1985). The toxicity of a chemical can be influenced by its composition, and impurities or contaminants may be more toxic than the chemical itself. Physical properties, such as solubility, vapour pressure and pH, may affect the persistence, transformation, bioavailability and ultimate fate of the chemical in water (Rand *et al.* 1995). The presence of other toxicants in the water may also influence the toxicity of a chemical (Jooste 1998).

1.2. TOXICITY TESTING

Aquatic toxicity tests are procedures in which the responses of aquatic organisms are used to detect or measure the presence or effect of one or more substances, wastes, or environmental factors, alone or in combination (APHA 1992). During a toxicity test aquatic organisms are exposed to a chemical or effluent and the effects quantified in terms of behavioural and reproductive changes, physiological effects or mortality. An objective of a toxicity test therefore is to determine the concentration of a test material (e.g. chemical or effluent) that produces a harmful effect on a group of test organisms under controlled conditions (Rand and Petrocelli

1985).

1.2.1. Toxicity test criteria

The criteria established to determine the suitability of a standard test procedure includes the acceptance of the method by the scientific community. Toxicity test protocols should be clearly defined, standardised and repeatable; data should ideally be useful for risk assessment and have some field predictive capability for similar organisms; the test should be economical and easy to conduct; and the test protocol design should be as sensitive and realistic as possible to detect and measure effects (Rand *et al.* 1995). Current test methods have been designed to examine the responses of a few individuals within a species (i.e. single-species toxicity test) and less attention has been given to the impact of chemicals on species interactions (multi-species toxicity tests) and ecosystems.

There are three general approaches which have been used to conduct toxicity tests. Tests can be conducted in a controlled laboratory with a limited number of variables, in an experimental model ecosystem (i.e. simulated indoors or outdoors), or toxicity effects can be studied in a natural environment (i.e. *in situ*). Laboratory toxicity tests are the most straightforward to conduct, are easier to standardise and replicate, and thus are the more popular approach (Leeuwangh 1978).

1.2.2. Toxicity test classification

Aquatic toxicity tests can be classified according to different factors, including duration or period of exposure (acute, sub-chronic or chronic) and purpose (lethality, behavioural changes, etc.) (APHA 1992). They can also be classified according to the method used (static or flowing), and organisms tested (Rand *et al.* 1995).

Acute exposure to a toxicant applies to a single episode (Timbrell 1989), such as an accidental spill of a chemical, and acute toxicity tests are designed to determine the short-term toxicity of the toxicant (Rand *et al.* 1995). Acute end-points are commonly (but not exclusively) mortality,

with toxicity expressed as a lethal concentration (LC). The point at which a certain percentage of the test organisms are affected and the duration of exposure are included in the end-point (e.g. a 96 h LC50 refers to the lethal concentration at which 50% of the population died after 96 h). Acute or short-term toxicity tests are often used for the routine monitoring of effluent discharge licenses and for exploratory tests, where the effects of a chemical are unknown. These tests are typically 96 h or less, depending on the organism's life cycle (Janssen and Persoone 1993). For example, acute toxicity tests using mayfly nymphs as the test organism are typically 96 h, whereas acute toxicity tests using *Daphnia pulex* (a cladoceran) are only 48 h (APHA 1992), because of their relatively shorter life span. Acute toxicity tests are useful because they are quick and can be used to determine compliance to a set licence and whether a particular treatment has resulted in the decreased toxicity of a substance (Rand and Petrocelli 1985). A disadvantage of short-term tests is that long term effects of the toxicant are not investigated. A short-term exposure may be tolerated without adverse effects, but if the organism is exposed for long periods, even at low concentrations, a harmful or chronic effect may be experienced (Jarvis 1989).

The repeated exposure to a substance can lead to the accumulation of the substance by the exposed organism, resulting in a chronic toxic effect. Chronic toxicity applies to an event which can occur many weeks, months or years after exposure to repeated doses of a chemical (Timbrell 1989). Chronic toxicity tests therefore permit the evaluation of possible long-term effects at sublethal concentrations. In some chronic tests, the test population is exposed for an entire reproductive life cycle (Rand *et al.* 1995), i.e. life cycle tests. Partial life cycle tests involve only the sensitive life stages, often the juveniles or only the aquatic stages in the life-cycle (e.g. mayfly nymphs). Chronic toxicity end-points are normally expressed as no observed effect concentrations (NOECs), the lowest observed effect concentration (LOEC), the effect concentration (EC) and the inhibition concentration (IC) based on observations of reduced reproduction, growth, and/or survival (Slabbert *et al.* 1998).

Other tests include short-term sub-chronic, early life stage and bioaccumulation tests. Short-term sub-chronic tests, i.e. 7-9 d, focus on the most sensitive life stages of the test organism. Early life stage tests involve the continuous exposure of the early life stages to a chemical for 1-2 months,

depending on the species. Bioaccumulation tests include measurements on the rate of chemical uptake by the organism through various physiological pathways such as feeding, the gills etc., as well as the amount of the chemical stored in the organism's tissues and the rate of excretion of the chemical (Rand and Petrocelli 1985).

1.2.3. Toxicity test organisms

Test species are often chosen for a wide variety of reasons, including: wide distribution and abundance; known sensitivity to the chemical under investigation (Cairns *et al.* 1993); indigenous or representative of the ecosystem that may receive the toxic impact; ecologically or commercially important; amenable to routine maintenance in the laboratory with available culture and experimental techniques so as to facilitate both acute and chronic toxicity testing; and whether the aquatic species is well known with background information available on its physiology, genetics and behaviour, allowing the data from a toxicity test can be more easily interpreted (USEPA 1979, APHA 1992, Rand *et al.* 1995). The species selected for testing may differ from site to site and the selection will often be based on site-specific considerations. The objective of the testing will also influence the choice of test species (Sprague and Fogels 1977).

There is no standard aquatic test species for all questions and all ecosystems, and the kinds and numbers of test species chosen will depend on the complexity of the ecosystem influenced by chemical pollution (Rand *et al.* 1995). Freshwater toxicity tests have traditionally been performed on species representative of algae, fish and invertebrates (APHA 1992). Test species can be collected from wild populations in relatively unpolluted areas, purchased from commercial suppliers or cultured in the laboratory (Rand *et al.* 1995).

1.2.4. Experimental systems

Aquatic toxicity tests are also grouped according to the experimental method and addition of test solution. In a static toxicity test, the test population is exposed to the toxicant (or effluent) in standing water (Rand *et al.* 1995). These tests are therefore essentially useful only for those organisms living in still waters, for example the crustacean *Daphnia* (Williams *et al.* 1984). The

test system is often a beaker, or similar test chamber, and the test solution is added at the beginning of the test. In a static non-renewal toxicity test there is no replacement of the test solution during the test period. These toxicity tests are often acute, as the build-up of metabolic wastes can result in adverse effects (APHA 1992). If the test solution is replaced periodically through-out the test or the organisms moved to another test chamber, the test is referred to as a renewal toxicity test (Rand *et al.* 1995).

A recirculation test is similar to most static tests in that the test solution is not replaced during the test period, except that the test solution is recirculated in the test chamber. This helps maintain high oxygen levels, and the test solution may be pumped through a filter to help maintain water quality (Rand *et al.* 1995). Recirculating toxicity tests are more expensive to conduct than a simple static test, but the advantage is that they are suitable for organisms adapted to flowing water environments (Williams *et al.* 1984). In a flow-through test, the dilution medium (e.g. water) flows through the test chamber once, with the test solution being added intermittently or continuously (Rand *et al.* 1995). Flow-through toxicity tests are generally very expensive and generate large volumes of waste (APHA 1992). They are, however, ideal for long-term tests, as metabolic waste products are continuously removed from the system and therefore unlikely to reach toxic levels (Frutiger 1984, Guckert 1993).

The test system and experimental design chosen in a toxicity study is often dependent on the selected test organism, its specific hydraulic requirements and the objective of the experiment (Lowel *et al.* 1995). A toxicity test initiated to assess an industry's compliance to its discharge licence, for example, will be different to that required to assess the effect of a toxicant on reproductive output of a test population. The former example is most often an acute toxicity test and the latter a chronic toxicity test. In general, three types of tests can be carried out to assess the toxicity of a single-substance or whole effluent. A screening test, where the test organisms are exposed to a range of concentrations of a single chemical or directly exposed to the effluent (no dilution) or to one dilution of the effluent. An unreplicated range-finding test (usually a 24 h test, depending on the test organism being used) is conducted on a wide range of dilutions to determine the approximate level of toxicity and the concentration range to be used for definitive testing. Replicated definitive tests estimate the concentration at which a certain percentage

(usually 50%) of test organisms exhibit a predetermined response (Slabbert *et al.* 1998).

The test system used in a toxicity test is largely dependent on the selected test organism (Williams *et al.* 1984) and its hydraulic requirements (Statzner *et al.* 1988). Those species adapted to still water can be tested in beakers, for example standard *D.pulex* experiments are conducted in a static environment, whereas artificial streams are designed to suit species adapted to living in flowing water. An artificial stream system can be defined as any constructed channel that has a controlled flow of water and is used to study a physical, chemical or biological property of natural streams (Lamberti and Steinman 1993). If specific hydraulic preferences and requirements of the test species are not met, the resultant stress will confound the cause-effect test result (Lowell *et al.* 1995).

Many artificial stream systems have been designed in the past to suit specific test organisms and to meet specific needs (Traaen 1978, Ryer *et al.* 1979, Shriner and Gregory 1984, Gillett 1989, Belanger *et al.* 1990, Crossland and Mitchell 1992, Guckert 1993, Brooks *et al.* 1996, Culp *et al.* 1996). The Institute for Water Research (IWR), Rhodes University, in collaboration with the Water Research Commission and the Department of Water Affairs and Forestry (DWAF), developed three different types of artificial streams, which differ in terms of scale (Palmer *et al.* 1994) and are designed to suit the hydraulic requirements of indigenous stream invertebrates. The use, advantages and limitations of each test system have been outlined in a Masters thesis by Binder (1999) and a full explanation of each system can be found in Palmer *et al.* (1996) and Scherman and Palmer (in press). The system used in this study is described in Chapter 2.

The aim of the three test systems designed at IWR was to enable the use of indigenous macroinvertebrates as toxicity test species in South Africa. Historically, the most commonly used aquatic toxicity test species were fish (Williams *et al.* 1984). Fish were seen as economically important and due to the bioconcentration of toxins up the trophic levels, it was assumed that fish were exposed to greater concentrations of toxins than those organisms at lower trophic levels. However, the tolerance to toxins by fish are often greater than the tolerances of other aquatic organisms and aquatic invertebrates are now also widely used in toxicity studies, with test species ranging from planktonic crustaceans such as *Daphnia*, to benthic macroinvertebrates such as

mayflies (Weber 1991, APHA 1992).

1.2.5. Standardised toxicity test methods

Internationally, toxicity studies are used extensively in water quality management (e.g. the Environmental Protection Agency in the United States (USEPA)) (Warren-Hicks and Parkhurst 1992). Daidoroff *et al.* (1951) first recognised the need to develop uniform, standardised test procedures for fish in order to maximise the comparability of data from toxicity experiments. The advantages of standardised test procedures include the easy initiation of new test protocols, increased data accuracy and the allowance for test replication (Davies 1977). Standardised methods also facilitate data comparison, thus increasing the usefulness of published data. There are also legal advantages if the test protocol is accepted by the judicial system, particularly during environmental disputes, as more confidence is placed in a standard recognised and accredited method (Davies 1977). Numerous standardised toxicity test procedures have been established, particularly for fish and invertebrates. For example, the US EPA and American Public Health Association (APHA) method manuals include standard methodologies for *Pimephales promelas* (fathead minnow), *Oncorhynchus mykiss* (rainbow trout), *Ceriodaphnia dubia, Daphnia magna* and *D.pulex* (Weber 1991, APHA 1992). To provide a constant supply of test organisms, many of the standard test species are cultured in the laboratory. Laboratory cultures provide organisms for toxicity tests of a known age and background, organisms are conveniently available at the time of testing, and information about the organism's general biology and sensitivity to chemicals is often readily available. Unfortunately, with the convenience of culturing, standard test species may be used to assess the effects of a toxin on a system where that species is not even found, thus neglecting the tolerances of indigenous species.

1.2.6. Toxicity data and environmental regulation

Rivers have the ability to assimilate a certain amount of waste without damage to the natural functioning of the river and its biotic community (Ashton *et al.* 1995). Thus the ability of aquatic systems to assimilate waste should be preserved and managed sustainably, if rivers are to continue to provide water for domestic and agricultural use and for the disposal of domestic and

industrial waste. The assimilative capacity of a river is influenced by its state of health (Cairns and McCormick 1992). A river can be considered healthy when its condition is stable, its capacity for self-repair when disturbed is preserved and a minimal external support for management is needed to maintain acceptable water quality conditions for the aquatic community (Karr *et al.* 1986, Karr 1993). If managed correctly, a river therefore has the potential to dispose of industrial (and other) waste without damage to the natural functioning of the river and its aquatic communities (Cairns and McCormick 1992).

In order to prevent deterioration in water quality, it is important to assess the potential hazard and risk resulting from the discharge of municipal and industrial wastes into water bodies. Aquatic toxicity tests can be used to evaluate these potential environmental hazards and risks, including the effects of long-term chemical releases under controlled conditions and accidental spills. Toxicity testing can be used in the prosecution and defence of chemical-related activities in environmental legislation, and can be used to confirm or refute a relationship between observed effects and the concentration of a chemical shown to exist or have existed in the aquatic environment (Rand *et al.* 1995). Toxicity tests are also used in the derivation of numerical water and sediment quality guidelines (criteria) for protection of aquatic organisms.

1.3. WATER QUALITY MANAGEMENT IN SOUTH AFRICA

Water is one of South Africa's most scarce resources (Schultze 1986) and in order to meet both the country's growing human population and industrial needs, available water resources have to be managed carefully, in terms of both quantity and quality (DWAF 1997). South Africa faces two problems. Firstly, South Africa is a semi-arid country, with uneven rainfall and high annual variability (Schultze 1986), and secondly, South Africa's cities were often built in historically important areas due to mining potential, and not near available water resources (Ashton *et al.* 1995). As a result large inter-basin transfer schemes have been developed and dams built to supply water to urban areas, for example the Lesotho Highlands Project.

South Africa is not only dependent on water for basic human needs, but also as a means of domestic and industrial waste disposal (DWAF 1997). Water quality is closely linked with water

quantity, as water acts as a diluent and helps to reduce or minimise the effects of industrial (and other) effluents on the aquatic environment (Hutcheson 1992). If dilution effects are limited by low volumes of water in a river, then water quality will deteriorate quicker than if the river was full. Many South Africans do not have access to potable water (i.e. that supplied through the municipality or an official water board) or formal water supplies and rely on rivers for their basic human and domestic needs (DWAF 1997). Water is also extracted from rivers for use in the irrigation of crops and the watering of livestock. Therefore water quality must be of a standard to meet the requirements of these basic needs.

Water quality management was initiated in South Africa in 1919 with the Union Health Act (Act No. 36 of 1919). The Chief Health Officer of the Public Health Department was conferred the responsibility of controlling pollution in aquatic environments, by ensuring that 'the best known or only the most practical methods' for sewage disposal were used (Van der Merwe and Grobler 1990). The Water Act of 1956 (Act No. 54) broadened water quality management to recognise increased industrial inputs (effluents) into South Africa's rivers and the limited availability of water in the country. The Act stated that industrial and domestic effluent had to be treated and then returned to natural water bodies in order to augment freshwater supplies in the country (Roux 1994). Although this policy resulted in an increase in water quantity, the quality of returned water and its effects on the rivers had to be considered further as water quality was deteriorating as a result of effluent disposal. General and Special Effluent Standards were introduced and applied through the Uniform Effluent Standards (UES) approach (Van Vliet 1996). The UES approach aimed to control the input of effluents to the aquatic environment by requiring effluent to comply with set uniform standards. These standards were determined primarily at technologically and economically feasible levels for industry and could be negotiated and relaxed, while technological, economic and socio-political issues were considered (Van der Merwe and Grobler 1990).

The UES approach helped limit the rate of deterioration in water quality and focused attention on pollution issues, resulting in improvements to waste-water treatment techniques and water quality management. However, although simple, comprehensive and easy to apply, the approach did not adequately address the quality of the receiving water environment (i.e. the aquatic system

involved in meeting the general effluent standard, there were no incentives for industry to locate themselves in the most advantageous environmental location, and no framework existed for the control of non-point source pollutants. As a result the UES approach was heavily criticised and an approach which focused on the receiving waters (the Receiving Water Quality Objectives (RWQO) approach) was offered as an alternative management strategy (Van Vliet 1996).

DWAF recognised that a protective approach to water quality management was required to prevent the continued deterioration in water quality. In 1991, the RWQO approach was proposed as an alternative to the UES approach to water quality management (DWAF 1991, Van Vliet 1996). The RWQO approach involved the establishment of a limit on levels of pollution according to water quality requirements of the users of a particular water system (i.e. river or dam) (Roux *et al.* 1993). This was the first approach to water quality management that was protective toward the aquatic environment, with the recognition that receiving waters have a capacity to assimilate pollution (Van der Merwe and Grobler 1990), and that this capacity should be protected in order to continue the sustainable use of aquatic systems. The aquatic environment is known to have a certain resilience to the pressures and demands of utilisation, and in order to preserve this resilience a certain level of ecological integrity and function must be maintained (MacKay 1998). This level is referred to as the Resource Base and guidelines were quantified with the aim of protecting and maintaining the Resource Base. Thus the aquatic environment's needs were recognised as important (second only to human needs) and which should be maintained above the needs of other users of aquatic systems, including industrial, agricultural and recreational users.

Based on defined users and their specific needs, the limits for water quality variables for South Africa were specified in a series of water quality guideline documents. These documents included guidelines for the following: Domestic Water Use (DWAF 1996a); Recreational Water Use (DWAF 1996b); Industrial Water Use (DWAF 1996c); Agricultural Water Use: Irrigation (DWAF 1996d); Agricultural Water Use: Livestock Watering (DWAF 1996e); Agricultural Water Use: Aquaculture (DWAF 1996f); Aquatic Ecosystems (DWAF 1996g). Water quality criteria can be defined as the limits which must not be exceeded in order to maintain the chemical, physical and biological characteristics which render a certain portion of water fit for its various defined uses (Roux 1992). The water quality guidelines were designed to serve as a primary

criteria can be defined as the limits which must not be exceeded in order to maintain the chemical, physical and biological characteristics which render a certain portion of water fit for its various defined uses (Roux 1992). The water quality guidelines were designed to serve as a primary source of information for determining the water quality requirements for the different users and for the maintenance of aquatic ecosystem health. Due to the lack of local toxicological data on South African aquatic organism tolerances to chemical substances, international standards were used in the setting of guidelines for the protection of aquatic ecosystems. These included the Canadian (CCREM 1987), American (APHA 1992) and Australian (ANZECC 1992) water quality criteria. When information becomes available on the tolerance of indigenous organism to chemicals, DWAF aims to modify the guidelines, thus making them more representative of South African conditions (DWAF 1996g).

The RWQO approach addresses the issue of setting objectives for water quality within a particular aquatic system and managing the aquatic resource, as well as the sources of impact and pollution (Van der Merwe and Grobler 1990). The approach is now used together with the General and Special Effluent Standards in an attempt to prevent the deterioration in water quality in South Africa's rivers. This was formalised in 1998 in the National Water Act (Act No. 36).

The Policy document (DWAF 1997), which preceded the Water Act, grants all South Africans two rights. The right to water to meet basic human needs (25*l* per person per day), and the right to an environment that is not harmful to their health or well being and that is protected for the benefit of present and future generations. The water required to fulfill these needs was termed the Reserve, and defined as the quality, quantity and reliability of water needed to protect both basic human needs, and the structure and function of ecosystems so as to secure ecologically sustainable development and utilisation (specifically named the Ecological Reserve). The Water Law states that the Reserve shall enjoy priority of use by right, and the use of water for all other purposes shall be subject to authorisation (Principle 10) (DWAF 1997). Thus the sustainable use of water is in effect law in South Africa.

Sustainable utilisation of aquatic resources requires the balance between the acceptable level of long term protection of water resources and water users, and society's present requirements for

economic growth and development (MacKay 1998). Industry provides many with employment and contributes significantly to South Africa's economic growth and development, and it would therefore not be in the interest of the nation's prosperity to unnecessarily constrain industrial usage of water (DWAF 1997). Thus to ensure the protection of water resources so that utilization can be sustained in the long term, policy, including the following regulatory activities, has been implemented:

- resource-directed measures, i.e. defining a desired level of protection for a water resource (Resource Quality Objectives), and on that basis, setting clear numerical or descriptive goals (Receiving Water Quality Guidelines),
- source-directed controls, i.e. controlling impacts on the water resource through the use of regulatory measures such as registration, licences, directives and prosecution, and economic incentives such as levies and fees, in order to ensure that the Resource Quality Objectives are met (e.g. effluent discharge permits) (Van Vliet 1996, MacKay 1998).

1.4. AQUATIC TOXICOLOGY IN SOUTH AFRICA

The new approach to water quality management in South Africa is based on several principles, which include that:

- the desired quality (objective) of a water resource be determined by its present and/or intended users and this quality should then be listed as a set of water quality objectives
- it is accepted that the aquatic environment has a capacity to assimilate pollutants without detriment to predetermined quality objectives and that this capacity is part of the resource and, as such, should be managed judiciously and shared in an equitable manner amongst all users for the disposal of their waste
- for those pollutants which pose the greatest threat to the environment because of their toxicity, extent of bioaccumulation or persistence, a precautionary approach aimed at minimising or preventing their inputs to the aquatic environment should be adopted (i.e. DWAF's Pollution Prevention Approach) (Van der Merwe and Grobler 1990).

It is assumed in the above principles that water quality objectives (based on the maintenance of ecosystem health and biological integrity) and assimilative capacity can be determined and

quantified for a particular water body. These principles also assume a knowledge of which toxicants pose a threat to the aquatic environment and the manner in which they pose the threat. Aquatic toxicology 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. In the past South Africa had to rely on international toxicological data bases to set water quality guidelines to protect the aquatic environment. DWAF, however, aim to modify guidelines as toxicological data becomes available for indigenous South African riverine organisms (DWAF 1996g). The toxicity data required in the setting of a guideline in South Africa includes results of acute tests with a least one species of freshwater organism in at least eight families, including a cold and warm water fish, planktonic crustacean (e.g. *Daphnia* species), benthic crustaceans, insects (e.g. mayflies) and a family other than Arthropoda or Chordata (e.g. Rotifera) (Roux *et al.* 1996).

Aquatic toxicology has played an important role in water quality management programmes internationally (Richardson and Martin 1994), with a number of standardised methodologies being developed. In South Africa toxicological bioassays are not yet a routine component of water quality monitoring (DWAF 1998), but have been recognised as a valuable tool in water quality management. As aquatic toxicology is still a relatively new field in South Africa, standardised methodologies for aquatic bioassays were adopted from international agencies, such as the USEPA and the 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).

A species which is widely used internationally, with standardised culture and test methodologies, 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 most of the aquatic systems are rivers, with rivers having 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?

Water quality criteria set to protect the aquatic environment are aimed at safeguarding freshwater ecosystems in South Africa from the adverse effects of pollution. This is best achieved by using South African species in the determination of environmental water quality criteria. It can be argued therefore 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 IWR in Grahamstown became extensively involved in investigating of the use of indigenous macroinvertebrates in toxicity testing, with the aim of assisting in the development of water quality guidelines set to protect the aquatic environment from the adverse effects of pollution. Three different artificial streams, designed to suit the hydraulic requirements of riffle dwelling organisms, were designed with the aim of using the artificial streams in toxicity testing (Palmer *et al.* 1996, Scherman and Palmer in press). Riffle-dwelling macroinvertebrates were chosen as potential test organisms, as little is known of South African aquatic macroinvertebrates, and riffles are the main habitat sampled in bioassessment studies (Chutter 1995).

The use of indigenous macroinvertebrates as test species and the use of the IWR artificial streams in toxicity testing was initiated using single-substance toxicants (e.g. salts and metals) (Scherman and Palmer in press). This 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 became apparent, along with the need to compare the use of indigenous macroinvertebrates to that of standardised laboratory test species (e.g. *D.pulex*). IWR is involved in a number of projects which are investigating the use of setting whole effluent licences (discharge permits) and in the comparison of test species. This study forms part of an investigation entitled "The use of *Daphnia* and indigenous invertebrates in whole effluent toxicity testing in the Vaal River catchment". This project is jointly funded by the Water Research Commission and Rand Water and is a collaboration with IWR and Rand Water.

1.5. AIMS OF THIS STUDY

The aims of this study are summarized below and also later discussed in more detail:

- To investigate the use of a standardised laboratory organism, *Daphnia 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 is 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, as they have proved to be among the more sensitive species and quality guidelines set according to their tolerances appear to adequately protect other aquatic species (Wall and Hanmer 1987, Roux 1994).They were also found to produce the most useful toxicological information per unit effort (Eagleson *et al.* 1986). *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. Zinc sulphate was chosen as the single substance toxicant as research on an indigenous mayfly, *Adenophlebia auriculata*, and zinc sulphate had recently been carried out at IWR (Everitt *et al.* in press). A fairly comprehensive international toxicity database is also available for zinc (e.g. the USEPA ECOTOX database). 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.

CHAPTER 2

MATERIALS AND METHODS

This study aims to compare the use of *Daphnia pulex* and site-specific macroinvertebrates in aquatic toxicology. To achieve this aim, the methodologies used were suited to each test organism and therefore vary in a number of aspects. As *D.pulex* are short lived and live in the water column, they are best suited to a static environment (Williams *et al.* 1984) and standard 48 h acute toxicity tests were conducted in glass beakers. The site-specific indigenous invertebrates were collected from flowing water environments and to accommodate their specific hydraulic requirements, a flowing test environment had to be provided (Frutiger 1984, Horne and Bennison 1986, Lowell *et al.* 1995). Acute toxicity tests were therefore conducted in recirculating artificial streams (see Section 2.2.1.). The selected invertebrates also have a longer life cycle than *D.pulex* and therefore require a longer exposure period for acute toxic effects. Acute 48 h static test methodology was therefore followed for *D.pulex* and acute 96 h flowing water test systems required for the testing of selected site-specific indigenous macroinvertebrates. Zinc sulphate (Chapter 4) and selected whole effluents (Chapter 5) were used as toxicants in these experiments.

2.1. *Daphnia pulex* CULTURING AND TEST METHODOLOGY

Standard daphnid culturing and test methodologies, described in the United States Environmental Protection Agency (Weber 1991), were followed and are described briefly below.

2.1.1. Culturing

Two *D.pulex* cultures were sub-cultured from existing mature IWQS (Institute for Water Quality Studies) and CSIR (Center for Scientific and Industrial Research) cultures, and two new cultures were initiated from a single female from Roodeplaat Dam, Pretoria, and the Sewage Works, Grahamstown for experiments in Chapters 3 and 4. These cultures were kept at the Institute for Water Research (IWR) in Grahamstown. Rand Water *D.pulex* cultures (sub-cultured from the CSIR culture) were used for experiments in Chapter 5. Standardised culturing methodologies are

essential for ensuring a healthy reliable source of neonates and for the standardisation of test organism quality (Persoone and Janssen 1993). A number of variables are known to influence *D.pulex* growth and reproduction, and are therefore of particular importance when trying to culture healthy neonates for toxicological tests. These variables, which include culture medium, diet and temperature, are discussed with regard to culturing and testing techniques.

Culture medium

A synthetic water, which meets the specific needs of *D.pulex*, is used for daphnid culturing and as the diluent in toxicity experiments (Adema 1978, Elendt 1990). The use of a standardised culture medium in toxicity tests enhances the reproducibility and comparability of the tests (Persoone and Janssen 1993). River water is not used for either culturing or as the diluent in toxicity tests due to practical difficulties, such as the accessibility of water of the same composition all year round, availability of the selected water, and suitability of the water in terms of quality, e.g. chlorine levels (Elendt 1990). It is important to ensure that the culture medium meets the specific requirements of the daphnids to be cultured. For example, Elendt and Bias (1990) found when comparing mediums, one medium caused low reproductive rates, reduced growth, production of high numbers of undeveloped parthenogenetic eggs and high mortality of neonate and adult *Daphnia*. This was later found to be due to trace element deficiencies, such as selenium.

A medium, made up from stock solutions of $CaSO_4.2H_2O$, KCl, $MgSO_4.7H_2O$ and $NaHCO_3$ dissolved in deionised water, was found to be suitable for the successful culturing of *D.pulex* (IWQS 1997). The culture medium can be prepared as follows:

i. Add 18.4 *l* deionised water to a clean aspirator bottle

ii. Add 1.2 g CaSO4.2H2O to 1 *l* deionised water and dissolve

iii. Add 2.4 g MgSO4.7H2O, 1.8 g NaHCO3 and 0.08 g KCl to 500 ml deionised water and dissolve

iv. Add above two stock solutions to aspirator bottle and aerate for at least 24 h before use.

Cultures must be maintained so as to prevent the build up of waste products and the depletion of oxygen, and were therefore cleaned by transferring the daphnids into 2 *l* of fresh culture medium weekly.

Diet

Maternal nutrition plays an important role in both the resistance of the neonates to toxic stress and their ability to withstand starvation (Enserink *et al.* 1993, Persoone and Janssen 1993). Both the quantity (Baird *et al.* 1989) and the quality (Belanger *et al.* 1989) of food are important, and female *D.magna* have been shown to produce many small neonates under favourable conditions, while only a few heavy, stress-resistant neonates are born when food is sparse (Cowgill *et al.* 1985, Enserink *et al.* 1990). High food concentrations may not always result in increased productivity, for example Cowgill *et al.* (1985) obtained the largest mean brood size from a mixed algal diet, whilst the smallest mean brood size was produced by a synthetic diet.

D. pulex have been successfully cultured on a diet of artificial food, made by blending trout pellets, dried yeast and dried alfalfa in deionised water, and cultured algae (Weber 1991). The artificial food used in this study was prepared as follows:

i. Add 6.3 g crushed trout pellets, 2.6 g dried yeast and 0.6 g dried alfalfa into 500 ml deionised water
ii. Blend together for approximately 10 minutes
iii. Leave mixture in fridge overnight allowing it to settle
iv. Pour off top 300 ml into containers and freeze, discard bottom 200 ml
v. Thaw food when required.

Approximately 4 m*l* of artificial food was given to each 2 *l* culture daily.

Temperature

Temperature affects the length of time taken to reach maturity, the time between broods, the number of eggs, life span and the proportion of males produced in the culture (Lewis and Horning 1991). *D.pulex* are sensitive to sudden and large temperature changes and it is therefore important

that the temperature in the culture room remains constant (Weber 1991). Ringelberg (1973) reported that a temperature of 20°C was an acceptable temperature for survival and reproduction of *Daphnia* cultures, whereas Goss and Bunting (1983) concluded that a regime between 15 - 20° C was optimal. Most authors agree that temperatures should not exceed 20°C (Adema 1978, Lewis and Horning 1991) as reproductive output and survival decrease as temperatures increase from 20 to 35°C (Craven *et al.* 1983).

Temperature has also been shown to influence the sensitivity of daphnids to several chemicals such as heavy metals (Scherban 1977, Braginskiy and Scherban 1979) and herbicides (Cairns *et al.* 1978, Stephenson and Watts 1984). Lewis and Horning (1991) recommended a test temperature of 20°C, as test results were found to be more reliable and control mortalities lower at this temperature. Temperature in the culture room at IWR is kept between 18° and 22°C, with a day-night cycle set at 17 h light and 7 h dark (APHA 1992) to simulate natural conditions.

2.1.2. Test methodology

Standard acute 48 h *D.pulex* test methodology (Weber 1991) was followed for all *D.pulex* experiments in this study. Females with embryos were caught the day before the test using a pipette and allowed to hatch overnight in fresh medium in 150 ml beakers. Twenty neonates were then divided between 4x30 ml beakers filled with 25 ml of test solution (i.e. 5 neonates per beaker), with mortalities recorded after 0.5, 1, 2, 4, 8, 24 and 48 h.

Zinc sulphate experiments (Chapter 3) were used to determine whether source population or culture age influence *D.pulex* tolerance. Two established (mature) cultures (collected from the CSIR and IWQS) and two new (immature) cultures (each initiated from a female from Roodeplaat Dam and Grahamstown Sewage Works) were used. The CSIR culture was started with a female caught from Daspoort Reclamation Works, Pretoria in 1985 and the IWQS cultures started with a female from Roodeplaat Dam, Pretoria, 1990. These cultures were of mixed generations. The immature cultures were started with a gravid female from Roodeplaat Dam (Pretoria) and Sewage Works (Grahamstown) populations, and successive generations were kept separate from one another in order to investigate generation-tolerance relationships. The experimental protocol

described above was followed, with all four cultures being run simultaneously when possible, using the same zinc sulphate stock solution to reduce experimental error. The concentration range of zinc sulphate (prepared in culture medium) was selected to cover 0 and 100% mortalities, with as many concentrations being run as possible. The range was calculated to follow a geometric progression (APHA 1992). Experiments were conducted at IWQS, Pretoria and at IWR, Grahamstown under controlled conditions.

Chapter 3 test results were also used in the comparison between site-specific invertebrates and *D.pulex* tolerance to zinc (Chapter 4). Ideally site-specific invertebrate and *D.pulex* experiments should have been run simultaneously for comparative purposes. Unfortunately *D.pulex* cultures were not in a suitable condition for reliable experiments when site-specific experiments were conducted at Rand Water.

In Chapter 5 (i.e. the whole effluent testing), the site-specific invertebrate and *D.pulex* experiments were run simultaneously in order to ensure that effluent composition was the same for both test methodologies. If neonates were unavailable for testing on the elected day, the site-specific invertebrate test was begun, effluent samples were kept at 4°C until the following day, and the *D.pulex* experiment was initiated. By keeping the effluent samples at 4°C, any chemical reactions within the effluent and between effluent and container were minimised, and it was unlikely that the composition of the effluent changed significantly (Creed *et al.* 1995). 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. Experiments were conducted at Rand Water using their established cultures, which were originally obtained from the CSIR, Pretoria.

2.2. INDIGENOUS SITE-SPECIFIC MACROINVERTEBRATE TEST METHODOLOGY

All site-specific experiments were conducted at Rand Water, Vereeniging (Figure 2.1). Vereeniging is part of the Vaal Triangle, made up 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.

All 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 (refer to Section 2.2.2.) (Figure 2.1). The industrial whole effluents selected for use in Chapter 5 were collected from two industries in this area. Industry A is located in Vanderbjilpark, and Industry B in Sasolburg. Industry A and B both discharge effluent, via effluent canals, into the Rietspruit and Taaibosspruit, respectively (refer to Section 2.3.2.) (Figure 2.1).

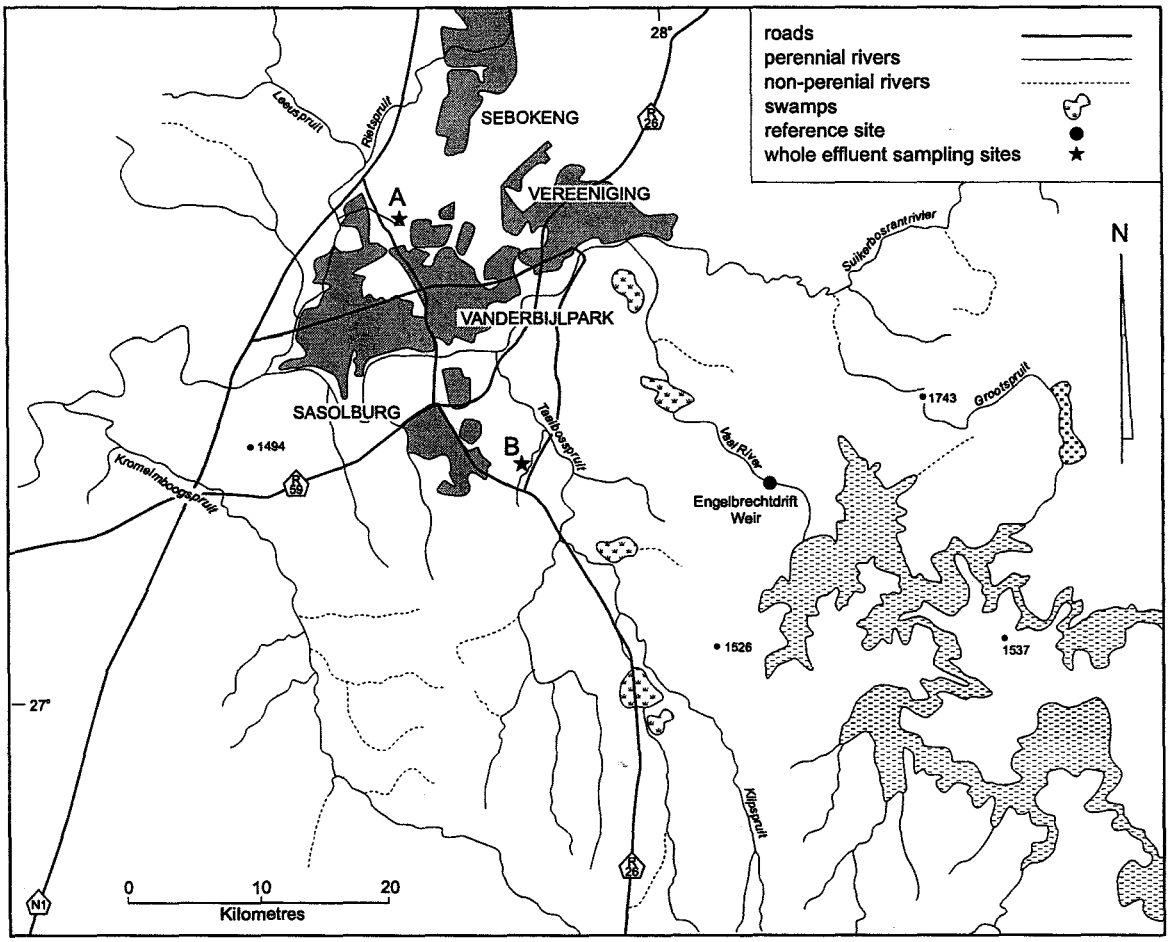

Figure 2.1: Map of the Vaal Triangle, showing the reference and whole effluent sampling sites

2.2.1. Experimental systems

The Channel systems have been widely used by the IWR in other toxicity studies, e.g. to determine the effects of chlorinated sewage effluent on macro invertebrate communities (Williams 1996), and the effects of salinity (Scherman and Palmer in press) and heavy metals on selected Ephemeroptera (Everitt *et al.* in press, Scherman and Palmer in press). The Channel systems were found to be the more useful test system when compared with other stream designs (Binder 1999), such as the large Artificial Streams and the Raceways (Palmer *et al.* 1996).

The Channel systems (Figure 2.2) were made from 1m 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). PVC is a relatively inert plastic and is considered a suitable material as it does not release toxins into the system (Palmer *et al.* 1996). 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.

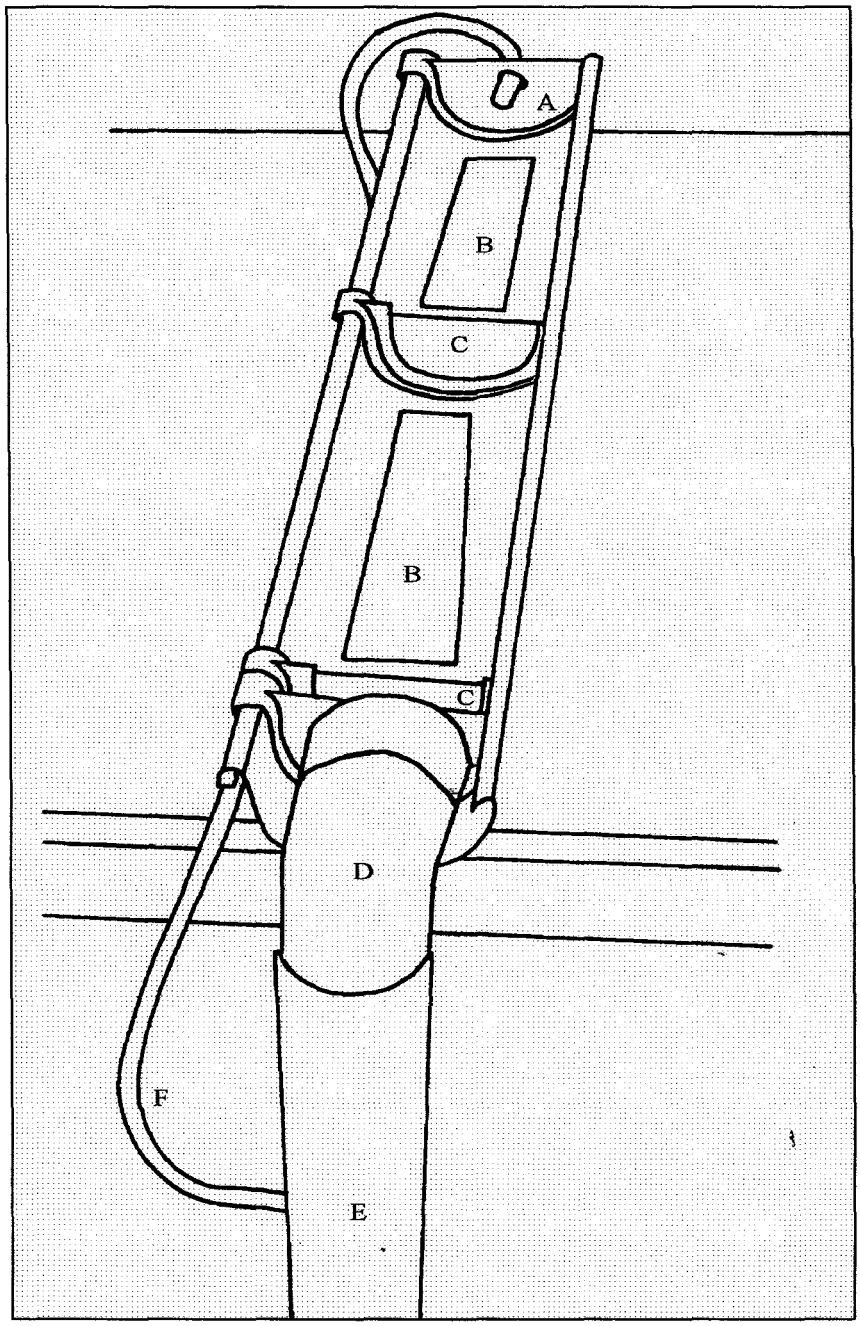

Figure 2.2: The artificial stream system, the Channel, designed to meet the hydraulic needs of riffle-dwelling invertebrates. A = the inlet, B = mosquito gauze, which acts as a substrate, C = channel divider with netting, to prevent test organism escape, D = U-bend, E = down-pipe leading to 20 *l* bucket with lid, designed to prevent splashing and minimise evaporative water loss, F = plastic tubing, taking water from the pump back to the inlet.

2.2.2. Reference sites

A reference site, from which indigenous test organisms are collected, should be unimpacted by pollutants (Eekhout *et al.* 1996). Reference sites are usually above industrial discharge and non-point source pollutant sources; organisms are therefore previously unexposed to pollutants.

A number of reference sites were chosen during this study as natural and man-induced flow changes resulted in some sites being unreliable in providing suitable test species. All sites were chosen because they are above the local Sasol/Vereeniging industrial areas and were the best available site in the area at the time of collection. The sites include the Taaibosspruit, Emfuleni Park and Engelbrechtdrift weir (Figure 2.1).

The Taaibosspruit flows into the Vaal Barrage and was chosen as a reference site based on a previous Rand Water study (Muller pers comm.) and biological water quality assessment using SASS4 (South African Scoring System). This site later became unsuitable as water was extracted by upstream users.

The Vaal Barrage is downstream of industrial inputs. Although Emfuleni Park falls on the banks of the Vaal Barrage, the site was only used once when flooding for several days was experienced, and an alternative reference site was needed as the other sites were unsuitable due to flooding.

Engelbrechtdrift weir was chosen as a reference site based on a SASS4 evaluation of water quality. SASS4 requires the sampling of invertebrates in a number of in-stream riverine habitats according to a set protocol using standardised equipment. The invertebrates are identified to family level and scored (1-15) according to their tolerance to water quality conditions, e.g. a family known to be tolerant would score 1, whereas a family known to be sensitive would score 15 (Table 2.1). These scores are summed to give a *sample score.* The sample score is divided by the number of taxa to give the *average score per taxon (ASPT).* The SASS4 results are compared to a reference site and because SASS results are influenced by habitat availability, the same habitats at each site should be compared when estimating water quality.

SASS4 scores at Engelbrechtdrift weir indicated that this site was in a better condition than a downstream, effluent-impacted site in Parys and was therefore suitable as a reference site. ASPT and SASS4 scores suggested that water quality was natural (Table 2.2) according to the categories as in Table 2.3 (Chutter 1995), with both tolerant and sensitive species being found, although more sensitive species occurred than at the site in Parys. For example, at Engelbrechtdrift weir, 17% of the taxa found were within the most sensitive of the tolerance classes (score of between 12-15), compared to only 6% found at the Parys site. One family known to be very sensitive to pollution, the Prosopistomatidae (Ephemeroptera), which scores 15 was found at Engelbrechtdrift weir but not at Parys; also more than two species of Baetidae were found at Engelbrechtdrift weir, whereas only two species were found at Parys, resulting in a lower score at Parys. The Parys site showed a greater percentage of taxa scoring within the lower tolerance classes (below a tolerance score of 8) when compared with Engelbrechtdrift weir, 94% compared with 77% respectively. Habitat influences the types of species found in an area and it is therefore important to take the condition of the habitat into consideration when using a biotic index to determine water quality (Uys *et al.* 1996). At each site a general habitat assessment was carried out, using the Habitat Assessment Matrix (HAM) (Uys *et al.* 1996). A high HAM score at both sites suggests that the habitat condition is excellent (Table 2.2), further suggesting that the low SASS4 and ASPT scores at the Parys site were due to the pollution experienced at that site.

SASS4

River..... Date..... Time.....
Sampling point.....
Temp °C..... pH..... Cond.(mSm^{-1}).....

Biotopes sampled:
SIC.....(Type/time.....)
Marg veg... Dom Sp.....
Aq veg... Spp.....
SOOC..... Sand..... Mud..... Gravel.....
Other.....

PROCEDURE PROTOCOLS:

1. If stones in current (SIC) all kickable, sample for 2 min. otherwise for maximum of 5 min.
2. Gravel - 1/2 min.
3. Mar/Aq veg, back & forward sweep 2m.
4. Stones out of current (SOOC) kick +/- 1m^2.
5. Sand/Mud stir with feet & sweep net over disturbed area for 1/2 min.
6. Any other biotopes - 1/2 min.
7. Complete top of form.
8. Tip net contents into tray. Remove leaves, twigs and trash.
9. Check taxa present on above list FOR THE LESSER of 15 min. or 5 min. since the last taxon was found.
10. Estimate abundances on scale:
    A- 1 to 10 B- 10 to 100
    C- 100 to 1000 D> 1000
11. BEFORE LEAVING CHECK THAT THIS FORM HAS BEEN FULLY COMPLETED!

A* -score allocated to taxon

Taxon	A*					Taxon	A*					Taxon	A*				
Porifera	5					Corduliidae	8					Diptera					
Coelenterata						Libellulidae	4					Blepharoceridae	15				
Hydra sp.	1					Hemiptera						Tipulidae	5				
Turbellaria						Notonectidae*	3					Psychodidae	1				
Planarians	5					Pleidae*	4					Culididae*	1				
Annelida						Naucoridae*	7					Dixidae*	13				
Oligochaeta	1					Nepidae*	3					Simuliidae	5				
Hirudinea						Belostomatidae*	3					Chironomidae	2				
Leeches	3					Corixidae*	3					Ceratopogonidae	5				
Crustacea						Gerridae*	5					Tabanidae	5				
Amphipoda	15					Veliidae*	5					Syrphidae*	1				
Crabs	3					Megaloptera						Athericidae	13				
Shrimps	8					Corydalidae	8					Emphididae	6				
Hydracarina						Trichoptera						Ephydridae	3				
Hydrachnellae	8					Hydropsychidae 1 sp.	4					Muscidae	1				
Plecoptera						2 spp or	6					Gastropoda					
Notonemouridae	12					>2 spp.	12					Lymnaeidae*	3				
Perlidae	12					Philopotamidae	10					Melaniidae*	3				
Ephemeroptera						Polycentropodidae	12					Planorbidae*	3				
Polymitarcyidae	10					Psychomyiidae	8					Physidae*	3				
Ephemeridae	15					Ecnomidae	8					Ancylidae	6				
Baetidae 1sp.	4					Hydroptilidae	6					Hydrobiidae*	3				
2 spp. or	6					Other movable case						Pelecypoda					
>2 spp.	12					larvae:						Sphaeridae	3				
Oligoneuriidae	15											Unionidae	6				
Heptageniidae	10					Case type, family											
Leptophlebiidae	13					1 1	8					Sample score					
Ephemerellidae	15					2 1	15										
Tricorythidae	9					3 1	20					No. of families					
Prosopistomatidae	15					4 2	30										
Caenidae	6					5 2	40					Score/taxon ASPT					
						>5 3	50										
Odonata												Air breathers fam.					
Chlorolestidae	8					Lepidoptera											
Lestidae	8					Nymphulidae	15					Air breathers score					
Protoneuridae	8					Coleoptera											
Platycnemidae	10					Dytiscidae (adults*)	5					Other families present:					
Coenagriidae	4					Elmidae/Dryopidae	8										
Calopterygidae	10					Gyrinidae (adults*)	5										
Chlorocyphidae	10					Haliplidae (adults*)	5										
Zygoptera juvs.	6					Helodidae	12										
Gomphidae	6					Hydraenidae (adults*)	8										
Aeshnidae	8					Hydrophilidae (adults*)	5										
						Limnichidae	8										
						Psephenidae	10										

Table 2.2: SASS4 and HAM scores for both Engelbrechtdrift weir (reference site) and Parys (a downstream, effluent impacted site). The families found were grouped into four tolerance classes to provide an indication of the relative percentage of tolerant organisms at the two sites. (E.weir = Engelbrechtdrift weir; MV = marginal vegetation; SOC = stones-out-of current; SIC = stones-in-current; TOT = the total sample score at the site, calculated by adding the families found at each sampled habitat to give the SASS4 score for the site in total).

	Habitat sampled (SASS4 sample score ASPT)				Tolerance classes (% of tot. families found)					Habitat	
Site	**MV**	**SOC**	**SIC**	**TOT**	**1-4**	**5-8**	**9-11**	**12-15**	**Water quality**	**HAM**	**Condition**
E.weir	60 6	95 8.6	63 7	117 5.85	27	50	6	17	Water quality approaching natural	112	Excellent
Parys	53 5.3	54 4.5	58 5.27	91 4.79	41	53	0	6	Some deterioration in water quality	112	Excellent

Table 2.3: Guideline values for estimating water quality condition using SASS4 and ASPT scores (Chutter 1995).

SASS4	ASPT	WATER CONDITION
> 100	> 6	Water quality natural, habitat diversity high.
< 100	> 6	Water quality natural, habitat diversity reduced.
> 100	< 6	Borderline case between water quality natural and some deterioration in water quality, interpretation should be based on the extent the which SASS4 exceed 100 and ASPT is less than 6.
50-100	< 6	Some deterioration in water quality.
< 50	ASPT variable	Major deterioration in water quality.

2.2.3. Test organisms

Few South African aquatic macroinvertebrates are well researched, and little is known of their life histories, biological requirements, environmental importance and tolerance limits to toxicants. As a result many of the guidelines set by the DWAF to protect aquatic ecosystems are based on international databases and there is a need in South Africa to develop a toxicity database for indigenous aquatic organisms. The site-specific indigenous test species chosen for this study were selected on the basis of: their abundance and their suitability to the conditions of the test system. Standard methods (APHA 1992) require a minimum number of twenty individuals of each species at a toxicant concentration, thus the field population must be able to support the collection of many individuals at a time. Hydraulic conditions in the test system are known to influence test organism behaviour and mortality (Lowell *et al.* 1995), and as the Channel system is designed to meeting the needs of invertebrates living in flowing water (Palmer *et al.* 1994), only riffle dwelling organisms were sampled. 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 (Palmer and Scherman 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). These three other potential test organisms were again chosen for their abundance and ease of collection. All the species collected are lotic species, with *C.nilotica* and the Corixidae found near the banks of the river, and the mayfly species in the riffles. While *C.nilotica* were found near the reeds and the Corixidae were often in the fine sediments in the shallows, both species seemed to prefer the slower flowing waters. Baetidae were often found in both fast and slow flowing riffles, with *A.peringueyi* preferring the slower flowing riffles, and *E.elegans* being more abundant in the faster flowing riffles.

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 remain unidentified, but the Baetidae were known to be a complex of species. This is a disadvantage in toxicology as different species of one family may exhibit different sensitivities to the same chemical (Medlyn 1980, Naylor *et al.* 1990, Hickey and Vickers 1992, Hoekstra *et al.* 1993, Schdoma 1994, Rand *et al.* 1995, Kammenga and Riksen 1996, McKinney and Wade 1996), subsequently the concentration-response relationship for the family may not be monotonic. All baetids were identified to species level and percentage frequency Tables prepared (Chapters 4 and 5).

Table 2.4 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 2.4: 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) (Chapter 4)	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 (Chapter 5)	Effluent A1	*C.nilotica*	Emfuleni Park
	Effluent A2	Baetidae	Taaibosspruit
	Effluent A3	Baetidae	Taaibosspruit
	Effluent B	*C.nilotica* Corixidae	Engelbrechtdrift weir

2.2.4. Toxicity test methodology

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. It was found that if temperatures increased much above 20°C, the mayfly species were more prone to emerge into subimagoes and adults, and because only those individuals which were exposed (as nymphs) for the full 96 h (unless they died) were counted, emergence resulted in low end numbers for these species (i.e. below 20). 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 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. In a non-renewal system, nutrient concentrations can increase to toxic levels due to increased metabolites over the test period, which may result in the stress and death of test organisms (APHA 1992). It is therefore important to establish whether mortality is related to increased nutrient levels, physical factors (e.g. temperature) or to the test chemical. Standard methods therefore specify that temperature, electrical conductivity and pH be measured in each experimental system (Channel) daily, as these factors are known to influence the toxicity of metals (APHA 1992). These were measured during the zinc experiments using a Conductivity meter (WTW LF 320), pH meter (WTW pH 320) and thermometer. Conductivity and pH were not recorded daily during effluent experiments as they are a characteristic of the effluent.

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.

2.3. GENERAL METHODOLOGY

2.3.1. End-point selection

The ultimate aim of determining the toxicity of a chemical or effluent is to be able to provide quantitative and meaningful information to managers and regulatory agencies. All experiments in this study were acute, with lethality as the measured end-point. Cairns (1992) argues that lethality is not an acceptable final threshold as an aim of toxicology is to protect aquatic organisms from harm. However, the use of acute toxicity tests, with lethality as an end-point, do provide a fast assessment of an unknown chemical mixture/stressor. Acute tests, although crude, provide important information as to the effects of a stressor and form a vital part of any toxic evaluation (Rand and Petrocelli 1985). Lethality is also clearly defined and easy to measure. If the test animal, when gently prodded, does not respond the animal is regarded as dead.

2.3.2. Effluent collection and toxicant preparation

Effluents were collected at two industries, Industry A in Vereeniging and Industry B in Sasolburg (Figure 2.1). 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. Effluent samples were collected in 20 *l* plastic buckets as close to the industrial outlet as possible. Effluent was used immediately in experiments in order to reduce variability in the effluents' composition over time. Collecting buckets were thoroughly cleaned and acid rinsed before use to avoid contamination of samples. Preservatives were not added (Slabbert *et al.* 1996). 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.

Zinc sulphate was chosen as a reference toxicant (see Section 1.4) and appropriate amounts were measured for each treatment and dissolved in fresh dilution medium (Section 2.2.3). Samples taken at the end of each experiment using indigenous invertebrates were sent for chemical analysis, to determine the actual concentration of zinc the test organisms were exposed to during the experiment. Toxicity data was calculated and expressed in terms of the actual zinc concentration, as opposed to weighed zinc sulphate concentration.

2.2.3. Dilution medium

In toxicity testing a standard synthetic medium (e.g. dechlorinated tap water) or the receiving water, from which the organisms are collected, are recommended as dilution media (APHA 1992). It is argued that receiving water (defined in this study as the water from which test organisms originate or water into which discharge of toxicants occur) is preferable to use as the dilution water when determining the sensitivity of indigenous site-specific organisms to a toxicant or when evaluating the toxicity of a whole effluent (Buikema *et al.* 1982, Guckert 1993). Chemical and physico-chemical characteristics of the receiving water may change the toxicity of the toxicant or whole effluent, e.g. pH may result in the ionization of chemicals within an effluent, and any binding to suspended solids may result in decreased toxicity (Gerhardt 1993, Grothe *et al.* 1996). Therefore, by using the receiving water as the dilution medium, 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 effluent 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.

As it is not always possible to use the receiving water as the dilution medium due to practical constraints (e.g. the transport of large volumes of water to the laboratory may be impractical), dechlorinated tap water has been a suggested alternative (APHA 1992). Both the receiving water (i.e. Vaal Dam water) and tap water were used to compare the differences dilution water may have on the toxicity of a single substance toxicant (i.e. zinc) (Chapter 4). The tap water was bubbled for at least 48 h to ensure dechlorination.

Only Vaal Dam water was used in the indigenous site-specific macroinvertebrate whole effluent experiments (i.e. the relative toxicity was determined), whereas the culture medium was used as the diluent in the *D.pulex* whole effluent experiments (i.e. inherent toxicity) (Chapter 5). Vaal Dam water was not used as the diluent in the *D.pulex* experiments, as the standardised diluent for these tests is the culture medium, and the aim of the study is to compare the sensitivities of different test organisms using standard methodologies.

2.4. EXPERIMENTAL DESIGN OF THE ACUTE TOXICITY EXPERIMENTS

Toxicity tests generally ask two questions: does a chemical produce an adverse effect on a test population and if so, how much of an adverse effect is produced? Experiments are designed to yield as much information as possible to answer these questions (Ellersieck and La Point 1995).

The most important issues in toxicity test design are the number of treatments (concentration or dilution) which should be run and the number of individuals in each treatment (Weber 1991, Hoekstra 1993a). The repeatability and precision of toxicity tests is a function of the number of test organisms used at each toxicant concentration (Weber 1991). Jensen (1972) discussed the relationship between sample size (test organism number) and the standard error of the test, and considered 20 fish per treatment as optimum for Probit Analysis (a toxicological statistical model). APHA (1992) also recommends that 20 individuals be the minimum number of test organisms in each treatment. Therefore in each treatment at least 20 individuals of each selected test organism was used in this study. A tocixity 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.

The two more commonly used experimental designs in aquatic toxicology are the analysis of variance (ANOVA) and linear regression designs, with the major difference being the use of replicates. Both test designs have their advantages and disadvantages. The ANOVA approach, where each treatment is replicated, is robust and can account for the variability in a population's response to a chemical (Stephan and Rogers 1985); i.e. if response around the concentration-response curve is not monotonic, the ANOVA design can incorporate this variability as the method compares the means of the different concentration replicates. The disadvantage of the ANOVA design however is that each concentration must have at least two or more true replicates (APHA 1992), and this may become an issue if test organism availability or space is limited (as it was in this study) or if true replicates are difficult to achieve. Also an ANOVA can only give comparisons between the replicates, thus giving an indication of significant differences between the responses within the different concentrations, but not the relationship between all the concentrations in the experiments. The advantage of the linear regression design is that no replicates are required and each concentration (or treatment) is treated independently (Stephan and Rogers 1985, Weber 1991).

The optimal experimental design depends on the question being addressed and on the required precision of the toxicity estimate (APHA 1992). Hoekstra (1993a) however recommends that a large set of concentrations be given preference over a large number of replicates per concentration, as more data is obtained on the concentration-response curve. 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 (Chapters 3 and 5).
- Laboratory space in which to run the Channel systems was limited (Chapters 4 and 5)
- Precise $ZnSO_4$ replicates were difficult to maintain throughout the experiments (Chapter 4).

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 LC50 increases with an increased number of concentrations (i.e. the linear regression becomes more rigorous with increased dilutions and both Probit and Spearman-Karber statistical models (used in this study)

estimate LC50s based on a linear regression).

2.5. Statistical Analysis

LC50 values (concentration at which 50% of the test population dies) (Hoekstra 1993b) were calculated for each test organism using both Probit and Trimmed Spearman-Karber methods (Gelber *et al.* 1985). Probit is the most widely used parametric statistical method in aquatic toxicology (Hoekstra 1993) and uses the Probit transformation of mortality data in combination with a standard curve-fitting technique to determine the LC50 (APHA 1992). The Probit model requires 0 and 100% mortality data, because, being a parametric method, it can be utilized only when at least 2 "partial kills" are present in the data set (APHA 1992). The model yields a symmetric concentration-response curve and Chi-Square heterogeneity value. If the data fits an asymmetric concentration-response curve, a high Chi-Square value results, suggesting that the model is not appropriate for the data and the LC50 calculated will not be valid.

The non-parametric Trimmed Spearman-Karber method is a widely accepted alternative to the Probit method for analysing mortality data (Hamilton *et al.* 1977). The method does not require data to be symmetric and does not depend on the presence of partial kills. Generally, lack of fit to a predicted model is due to outliers in one or both the tails of the response distribution (Sanathanan *et al.* 1987). The Trimmed Spearman-Karber method "trims" the tails of the concentration-response curve thus restricting the distribution to the more central, approximately linear, portion of the concentration response (Ellersieck and La Point 1995). A percentage trim which is 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 LC50 (Hamilton *et al.* 1977) (i.e. only 60% of the data was used to calculated the LC50). The disadvantage of Trimmed Spearman-Karber is that data must cover the range of 0 to 100% mortality in order to estimate the LC50. If the true concentration-response curve is asymmetric however, Spearman-Karber estimates the mean tolerance (APHA 1992). Hamilton *et al.* (1977) argue that the Trimmed Spearman-Karber method is the better method than Probit because this method always produced useful results and based on their experience, the model is considered more accurate and robust.

In this study, if the data did not fit a model's assumptions, data were analysed using the alternate model. If control mortality exceeds 10%, the test is considered invalid (APHA 1992), thus no data set was used if control mortality exceeded 10% (i.e. recorded mortality of 11% and above).

To determine whether there were any significant differences between two LC50s 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 LC50, i.e. calculated by dividing the upper confidence limit by the LC50. If the ratio (greater LC50)/(lower LC50) exceeds the value for $f_{1.2}$ the LC50s are considered to be significantly different.

If LC50s between experiments were shown not to be significantly different for a particular test organism, the data were pooled and one LC50 calculated; if shown to be significantly different, a LC50 range is discussed.

CHAPTER 3

DIFFERENCES IN ZINC TOLERANCE BETWEEN *Daphnia pulex* CULTURES STARTED FROM SEPARATE POPULATIONS

3.1. INTRODUCTION

The Family Daphniidae (Order Cladocera), including *Daphnia* and *Ceriodaphnia* species, is used extensively in aquatic toxicity testing and include a number of important standardised test species (Nikunen 1985, Fargasova 1994). Daphnid bioassays are among the more cost effective toxicity tests to conduct and are argued to provide the most useful toxicity information per unit effort compared to other freshwater test organisms, such as fish (Eagleson *et al.* 1986). Daphnids are also relatively easy and economical to culture in the laboratory (Leeuwangh 1978, Koivisto 1995). As a result, the *Daphnia magna*, *D.pulex* and *Ceriodaphnia dubia* standardised culturing and test methodologies are endorsed by internationally recognized organizations such as the United States Environmental Protection Agency (USEPA). They are also used in numerous toxicological studies, and in the setting of substance-specific guidelines and whole effluent licences designed to protect the aquatic environment (Persoone and Janssen 1993).

Daphnids have been described as ubiquitous in temperate fresh waters (Berner 1986), and are abundant in lakes, ponds and quiescent sections of streams and rivers (Pennak 1978). They are therefore relatively easy to collect from the field and many laboratories have started their own daphnid cultures from nearby lentic populations. In South Africa, *D. pulex* are among the most widely used freshwater invertebrate test species in routine and experimental toxicity testing at laboratories such as the Department of Water Affairs and Forestry (DWAF) Institute for Water Quality Studies (IWQS) and the Center for Scientific and Industrial Research (CSIR), with both laboratories having initiated their own cultures from local populations.

In South Africa, laboratories have initiated *D.pulex* cultures from females from different field

populations, e.g. the CSIR and IWQS cultures. It can be assumed that genetically these two wild populations are different, as both are isolated from one another spatially, and have experienced different field conditions. Although international standard culturing and testing techniques have been followed by both laboratories for over eight years, slight variations in the culture methodology between the two laboratories were identified (e.g. different feeding regime) (IWQS 1997). The differences in culture methodology and the influence of possible genetic differences on tolerance to toxicants between these two culture is unknown. This chapter therefore investigates the potential impact of these differences. Zinc sulphate was used as a reference toxicant in acute 48 h toxicity tests.

Once a *Daphnia* culture has been established in a laboratory it can be kept indefinitely and no studies were found which indicated whether the time spent under culturing conditions in the laboratory influence the tolerance of the *Daphnia* to toxicants. It is assumed in laboratories that the genetic structure of the culture remains the same through time and that no genetic mutation, which may change the susceptibility to a chemical, occurs (Weider 1993). This study therefore also briefly investigated the assumption that susceptibility of a culture remains constant over time. The acute 48 h tolerance to zinc of a culture, which has been in the laboratory for eight years, was compared to that of cultures which had been in the laboratory for less than a year (both cultures were established from females collected from the same area). The different generations within a culture were also compared to determine if "culture age" (time spent under laboratory conditions) influenced tolerance to zinc.

3.2. Materials and Methods

Acute 48 h *D.pulex* experiments using two mature cultures (originally initiated from different females) were undertaken to determine whether *D.pulex* cultures set up from different field populations showed similar sensitivity to a single-substance toxicant. Mature and immature cultures (see Section 2.1) were compared independently from one another because the influence of time exposed to laboratory culturing on tolerance is unknown (i.e. it is not known if culturing in the laboratory affects the toxicological response to a chemical; cultures may become "adapted/acclimatized" to laboratory conditions. Therefore CSIR culture was not compared to Sewage Works (SW), Grahamstown or

Roodeplaat Dam (RD), Pretoria, but only compared with IWQS culture.

A number of experiments (Table 3.1) were designed to determine whether genetic differences between cultures influence the susceptibility of *D.pulex* to zinc sulphate. Experiments 1-3 were conducted at IWQS (Pretoria, where culture care was the responsibility of IWQS) and Experiments 4-6 were conducted at the Institute for Water Research (IWR) (Grahamstown) using the two mature cultures (i.e. IWQS and CSIR cultures). Both cultures were kept in the same culture room at both laboratories and therefore experienced the same conditions. This increased the confidence that any differences in tolerance could be attributed to genetic differences between the two cultures. There were however slight variations in the culture methodologies between IWQS and IWR laboratories, with IWR preferring to follow methodologies of the Weber (1991). At IWQS, the cultures were fed 5m*l* artificial food three times a week and not on weekends, whereas at IWR the cultures were fed 4m*l* artificial food per day and sometimes on weekends. At both laboratories, the cultures were subjected to the respective feeding regimes for weeks before the experiments and therefore this slight change in feeding regime is unlikely to have influenced results. Air temperature was maintained around 20°C in both laboratories. The experimental protocols and statistical analysis described in Chapter 2 were followed to determine significant differences in zinc tolerance between the cultures.

Three experiments were carried out to investigate the tolerance of a culture to a specific chemical changed with increased time spent cultured in the laboratory (referred to here as "culture age"). IWQS mature cultures were compared with RD immature cultures, as both were set up using a female collected from Roodeplaat Dam. To further compare culture age (time spent cultured in the laboratory) and its potential influence on tolerance to a specific chemical, each generation for both RD and SW cultures was kept separate from one another, and experiments on the earlier and later generations were compared (see Chapter 2).

Experiments 4-6, designed to investigate genetic differences between cultures, simultaneously investigated the potential influence of culture age on the tolerance to zinc sulphate, with both mature and immature cultures being run at the same time. The 48 h LC50s calculated for the mature IWQS

and immature RD cultures were compared; similarly the 48 h LC50s calculated for the different generations within the two immature cultures were compared (i.e. SW and RD). The experimental protocols and statistical analysis, described in Chapter 2, were followed to calculate significant differences in zinc tolerance between the cultures.

The number of concentrations run in each experiment was subject to neonate availability. The minimum number of concentrations considered acceptable in a regression design experiment is five with one control (Cooney 1995). In Experiments 1 and 3, five concentrations and one control were run; in Experiment 2, an additional concentration was run because neonates were available. In Experiment 4, 5 and 6, nine, seven and seven concentrations were run respectively. The zinc sulphate concentration range was chosen to ensure zero and a hundred percent mortality and determined using a geometric series, with each successive concentration being 0.5 of the previous concentration (USEPA 1989). A 0.5 dilution was chosen for its ease of calculation and set up. Initially the concentration range was based on the literature, with a reported 48 h LC50 of between 0.115 and 0.124 mg/*l* for zinc sulphate (Sheedy *et al.* 1991). Thus the concentrations in Experiments 1-3 were 0.01, 0.03, 0.06, 0.12 and 0.25 mg/*l* $ZnSO_4$, with an extra concentration (0.003 mg/*l* $ZnSO_4$) being run in Experiment 2 due to the available neonates. A similar range was chosen for an experiment at IWR, but 12 concentrations were run (0.5 geometric range between 0.007-0.25 mg/*l* $ZnSO_4$), no mortality however was recorded during this experiment, thus the upper concentration was increased arbitrarily to 5.0 mg/*l* $ZnSO_4$ to ensure 100% mortality in Experiments 4-6. Thus the concentrations in Experiments 4-6 included a 0.5 geometric range from 0.009 - 5.0 mg/*l* $ZnSO_4$. One $ZnSO_4$ stock solution was used for Experiments 1-3 and different stock solutions were made up for Experiments 4-6. Due to the large apparent variability between the concentration ranges of $ZnSO_4$ which resulted in acute 48 h mortality, the Zn concentration of each $ZnSO_4$ stock solution was determined on an Atomic Absorption Spectrophotometer at Rhodes (detection window between 0 mg/*l* and 1.5 mg/*l*) and Zn was used in the LC50 determinations. Unfortunately, standard *D.pulex* test methodology does not require chemical analysis to determine toxicant concentration, and because of this, chemical analysis was not preformed on the stock solution used in Experiments 1-3 (i.e. those carried out at IWQS). Rather, the amount of Zn added was calculated and used in the LC50 determinations. Table

3.1 summarizes where each experiment was conducted, its purpose and which concentrations were included.

Table 3.1: This table summarises where the experiment was conducted, cultures used, purpose of the experiment and zinc concentration used in each acute 48 h experiment for both the mature IWQS and CSIR *D.pulex* cultures. Zinc sulphate concentrations were selected according to a 0.5 geometric progression to ensure zero and 100% mortality.

Experiment number	Place of conduct	Cultures used	Purpose of experiment	Toxicant Concentration (mg/*l* Zn)
1	IWQS	CSIR IWQS	genetic population differences	0.000, 0.003, 0.007, 0.014, 0.027, 0.057
2	IWQS	CSIR IWQS	genetic population differences	0.000, 0.0007, 0.003, 0.007, 0.014, 0.027, 0.057
3	IWQS	CSIR IWQS	genetic population differences	0.000, 0.003, 0.007, 0.014, 0.027, 0.057
4	IWR	CSIR IWQS RD SW	genetic population differences & culture age differences	0.000, 0.007, 0.013, 0.027, 0.054, 0.108, 0.215, 0.430, 0.860, 1.72
5	IWR	CSIR IWQS RD SW	genetic population differences & culture age differences	0.000, 0.015, 0.029, 0.058, 0.120, 0.230, 0.470, 0.930
6	IWR	CSIR IWQS RD SW	genetic population differences & culture age differences	0.000, 0.022, 0.043, 0.086, 0.173, 0.345, 0.690, 1.380

Both Probit and Trimmed Spearman-Karber methods were followed to calculate LC50s for each culture in each experiment. The data was better suited to the Trimmed Spearman-Karber method however, because there was not always a monotonic relationship between mortality and increased concentration. A percentage trim which is considered to be unacceptable could not be found in the literature, thus in this study a percentage trim greater than 20% (Trimmed Spearman-Karber method)

was considered to be high, as 20% on both sides of the concentration-response curve had to be trimmed off to calculate the LC50 (i.e. only 60% of the data was used to calculated the LC50). Statistically significant differences between LC50s were determined using the APHA formula (APHA 1992) given in Chapter 2. Differences were determined between experiments (i.e. within one culture) and between cultures.

The co-efficient of variation (CV), used to determine test precision between toxicity experiments, was calculated for a culture, and each experiment within a culture compared to the mean LC50. The CV is typically used when comparing variability in samples from populations with different means, and is the ratio between the standard deviation to the mean (expressed as a percentage by multiplying by 100) (Fowler and Cohen 1993) (e.g. a mean LC50 and CV for CSIR experiments 1-6 was calculated). Rue *et al.* (1988) reviewed intra- and inter-laboratory variability in acute toxicity tests for a variety of effluents. The average intra-laboratory CV for LC50 values was 15.8%. Effluents, due to their chemical composition, are more variable than a single-substance toxicant, thus the variability around an LC50 for a reference toxicant should not the greater than that recorded for effluents. Therefore, in this study a CV greater than 15% is considered to be unacceptable.

3.3. RESULTS

In each experiment the *D.pulex* neonates responded to the zinc sulphate, and the classic concentration-response curves were obtained for each culture. As zinc concentration increased, mortality increased (Figures 3.1-3.13, Sections 3.3.2 and 3.3.3).

3.3.1. Variability in LC50 results between experiments

Within the CSIR and IWQS cultures, experimental variability was high (i.e. greater than 15%), with each culture showing a CV above 130% (Tables 3.2-3.3). In an attempt to characterize the variability within a culture, the mean LC50 for each culture was calculated (despite high CVs) and experimental LC50s for each experiment compared to it. The same pattern of variability can be seen in all

cultures, where LC50 values in Experiments 5 and 6 are greater than those of Experiments 1-3.

The CV around the calculated 48 h LC50s for the CSIR culture was 139%, with the mean LC50 calculated at 354.44 $\mu g/l$ Zn. Experiment 4 showed the highest LC50 value, 89% greater than the mean and was calculated at 1280.00 $\mu g/l$ Zn. In the first three experiments, LC50s fell between 12.10 $\mu g/l$ Zn and 32.16 $\mu g/l$ Zn, with subsequent experiments having LC50s of 320 $\mu g/l$ Zn and 470 $\mu g/l$ Zn respectively. Experiments 5 and 6 showed LC50 values which were the most comparable to the calculated mean. The LC50 for Experiment 5 was calculated as being 10% less than the mean and Experiment 6 showed an LC50 25% greater than the mean.

Table 3.2: The 48 h LC50 values (Trimmed Spearman-Karber) calculated for each CSIR experiment, along with the confidence limits and percentage trim. Due to the high variability between LC50s for each experiment, a mean LC50 value (354.44 $\mu g/l$ Zn) was calculated for the culture and the variation between experiments expressed as a percentage smaller or greater than the mean. The CV around the mean 48 h LC50 was calculated to be 139%.

Experiment	CSIR culture: 48 h LC50 ($\mu g/l$ Zn) (% trim)	% greater/less than the mean 48 h LC50 (354.44 $\mu g/l$ Zn)
1	12.1 (10.64-13.76) (0.0%)	smaller by 96.6%
2	7.15 (6.19-8.25) (12.50%)	smaller by 98.0%
3	37.39 (29.27-47.78) (30%)	smaller by 89.4%
4	1280.00 (1210.00-1350.00) (20.00%)	greater by 72.3%
5	320.00 (290.00-350.00) (0.00%)	smaller by 9.7%
6	470.00 (440.00-510.00) (0.00%)	greater by 24.6%

Similar variability around the end-point (LC50) was experienced in the IWQS culture. The CV around the calculated mean 48 h LC50 was calculated at 132% (Table 3.3). Experiments 1 and 2 showed the lowest LC50 values (18.51 $\mu g/l$ Zn and 10.13 $\mu g/l$ Zn respectively), with Experiment 4 again having an LC50 calculated as being significantly greater than previous and subsequent experiments. The mean LC50 for this culture was calculated at 324.40 $\mu g/l$ Zn; Experiment 1-3

LC50s were between 94% and 97% less than the mean, with Experiment 4 being 70% greater than the mean. Experiment 5 showed an LC50 32% smaller than the mean and Experiment 6 had an LC50 which was 46% greater than the mean.

Table 3.3: The 48 h LC50 values (Trimmed Spearman-Karber) calculated for each IWQS experiment, along with the confidence limits and percentage trim. Due to the high variability between LC50s for each experiment, a mean LC50 value (324.40 $\mu g/l$ Zn) was calculated for the culture and the variation between experiments expressed as a percentage smaller or greater than the mean. The CV around the mean 48 h LC50 for this culture was calculated to be 132%.

Experiment	**IWQS culture: 48 h LC50 ($\mu g/l$ Zn) (% trim)**	**% greater/less than the mean 48 h LC50 (324.40 $\mu g/l$ Zn)**
1	18.51 (16.79-20.42) (0.00%)	smaller by 94.3%
2	10.13 (8.31-12.34) (12.5%)	smaller by 96.9%
3	27.78 (24.28-31.78) (20.00%)	smaller by 91.4%
4	1070.00 (940.00-1230.00) (20.00%)	greater by 69.7%
5	220.00 (200.00-250.00) (0.00%)	smaller by 32.2%
6	600.00 (570.00-640.00) (0.00%)	greater by 45.9%

The immature cultures, RD and SW, also showed significant variability between LC50 values in Experiments 4-6, with the CV around 48 h LC50s calculated at 130% and 134% respectively. For example the RD culture in Experiment 4 showed an LC50 of 1370.00 $\mu g/l$ Zn, which then dropped to LC50s of 350 $\mu g/l$ Zn and 460.00 $\mu g/l$ Zn in Experiments 5 and 6 respectively (Table 3.4). Similarly in the SW culture, Experiment 4 showed an LC50 of 1500 $\mu g/l$ Zn, compared to Experiments 5 and 6 where the LC50s dropped to 450 $\mu g/l$ Zn and 470 $\mu g/l$ Zn respectively (Table 3.5). Both mature and immature cultures were run simultaneously in Experiments 4-6, with all four cultures showing the greatest LC50 in Experiment 4, with the LC50s in the subsequent two

experiments being significantly less (Tables 3.1-3.4).

Table 3.4: The 48 h LC50 values (Trimmed Spearman-Karber) calculated for each RD experiment, along with the confidence limits and percentage trim. Due to the high variability between LC50 for each experiment, a mean LC50 value (726.67 $\mu g/l$ Zn) was calculated for the culture and the variation between experiments expressed as a percentage smaller or greater than the mean. The CV around the mean 48 h LC50 was calculated to be 130%.

Experiment	RD culture: 48 h LC50 ($\mu g/l$ Zn) (% trim)	% greater/less than the mean 48 h LC50 (726.67 $\mu g/l$ Zn)
4	1370.00 (1160.00-1610.00) (40.00%)	greater by 47.0%
5	350.00 (320.00-390.00) (0.00%)	smaller by 51.8%
6	460.00 (430.00-490.00) (0.00%)	smaller by 36.7%

Table 3.5: The 48 h LC50 values (Trimmed Spearman-Karber) calculated for each SW experiment, along with the confidence limits and percentage trim. Due to the high variability between LC50s for each experiment, a mean LC50 value (806.67 $\mu g/l$ Zn) was calculated for the culture and the variation between experiments expressed as a percentage smaller or greater than the mean. The CV around the mean 48 h LC50 was calculated to be 134%.

Experiment	SW culture: 48 h LC50 ($\mu g/l$ Zn) (% trim)	% greater/less than the mean 48 h LC50 (806.67 $\mu g/l$ Zn)
4	1500.00 (1340.00-1670.00) (40.00%)	greater by 46.2%
5	450.00 (410.00-490.00) (5.00%)	smaller by 44.2%
6	470.00 (440.00-510.00) (0.00%)	smaller by 41.7%

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

Due to the significant variability occurring between the LC50s for each experiment within one culture, comparisons made between the cultures is limited to each separate experiment. Therefore a comparison is made between IWQS and CSIR Experiment 1, and treated independently from a comparison made between IWQS and CSIR Experiment 2, etc.

Both IWQS and CSIR cultures were initiated about eight years ago from a single female collected from geographically isolated populations and are assumed to be genetically different. When both the 24 h and 48 h LC50 values were compared in each experiment, the two populations were statistically different using the APHA formula (Table 3.6 and 3.7). For example, the CSIR cultures in Experiment 1 showed a 48 h LC50 value of 12.10 $\mu g/l$ Zn compared to 18.51 $\mu g/l$ Zn calculated for the IWQS culture (Table 3.7).

Table 3.6: The 24 h LC50 values (Trimmed Spearman-Karber), with confidence limits and percentage trim for CSIR and IWQS *D.pulex* experiments. Where low mortality was recorded in the upper concentrations, an LC50 could not be calculated because the percentage trim required was too great. In Experiment 2, 95% confidence intervals were not calculated for the CSIR culture because they were unreliable due to the 50% trim.

Experiment	CSIR culture: 24 h LC50 ($\mu g/l$ Zn) (% trim)	IWQS culture: 24 h LC50 ($\mu g/l$ Zn) (% trim)
1	28.76 (24.98-33.11) (21.00%)	32.16 (27.51-37.58) (22.00%)
2	39.41 (not reliable) (50.00%)	Trim too great
3	Trim too great	Trim too great
4	Trim too great	Trim too great
5	450.00 (410.00-500.00) (5.00%)	580.00 (520.00-640.00) (15.00%)
6	980.00 (830.00-1140.00) (35.00%)	1200.00 (960.00-1510.00) (45.00%)

Table 3.7: The 48 h LC50s (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 (Figures 3.3 and 3.4).

Experiment	CSIR culture: 48 h LC50 ($\mu g/l$ Zn) (% trim)	IWQS culture: 48 h LC50 ($\mu g/l$ Zn) (% trim)
1	12.10 (10.64-13.76) (0.00%)	18.51 (16.79-20.42) (0.00%)
2	7.15 (6.19-8.25) (12.50%)	10.13 (8.31-12.34) (12.00%)
3	37.39 (29.27-47.78) (30.00%)	27.78 (24.28-31.78) (20.00%)
4	1280.00 (1210.00-1350.00) (20.00%)	1070.00 (940.00-1230.00) (20.00%)
5	320.00 (290.00-350.00) (0.00%)	220.00 (200.00-250.00) (0.00%)
6	470.00 (440.00-510.00) (0.00%)	600.00 (570.00-640.00) (0.00%)

The fact that the LC50 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 24 h, the CSIR appeared to be more sensitive, showing a higher percentage mortality at each concentration (Figures 3.1-3.6). However, 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.

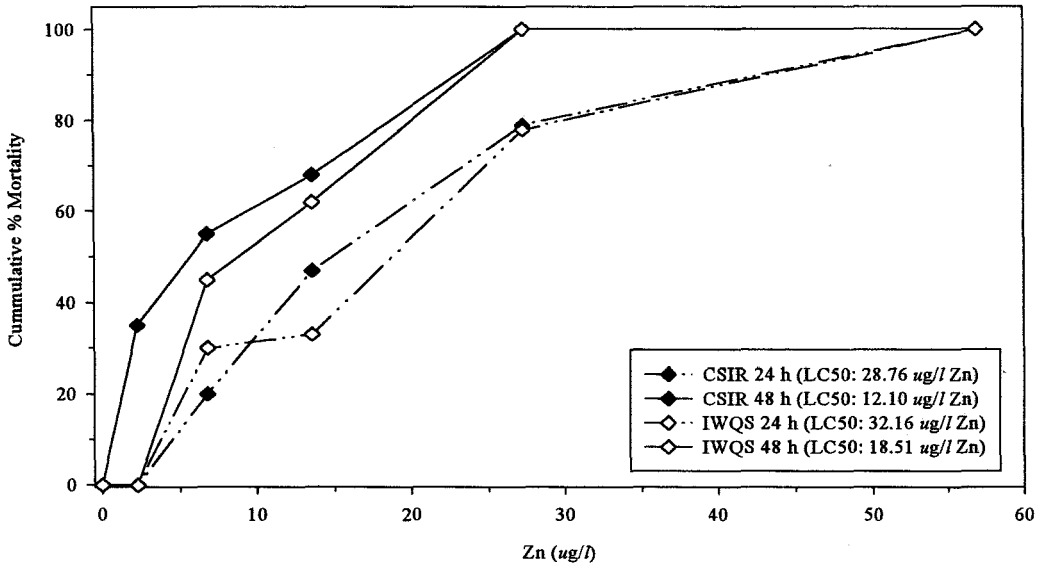

Figure 3.1: The graph plots both the 24 h and 48 h cumulative percentage mortalities against increasing zinc concentration for both the CSIR and IWQS cultures recorded in Experiment 1.

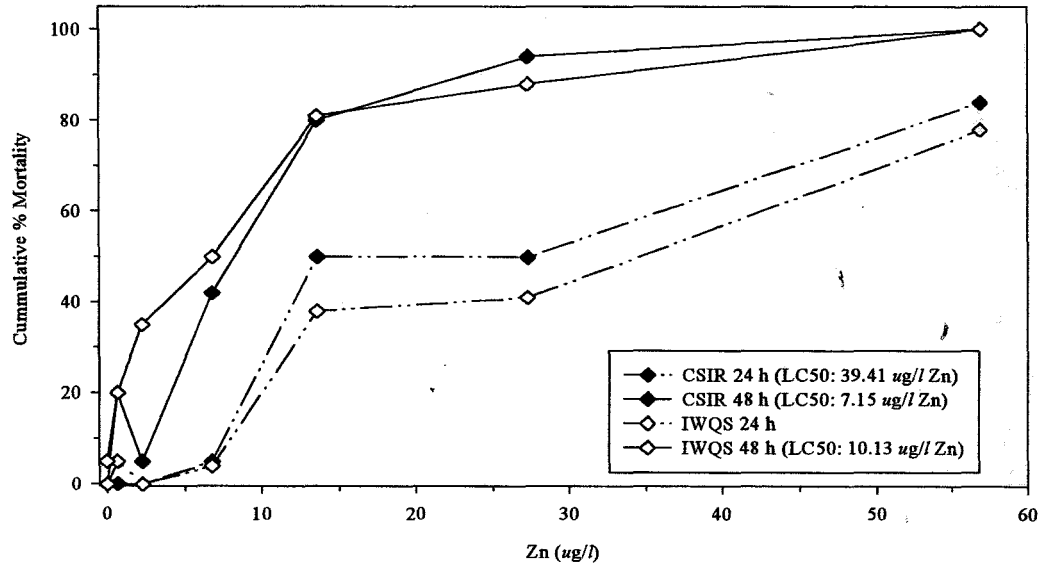

Figure 3.2: The graph plots both the 24 h and 48 h cumulative percentage mortalities against increasing zinc concentration for both the CSIR and IWQS cultures recorded in Experiment 2. Twenty four hour LC50 values are not reliable due to low percentage mortality and hence the high percentage trim.

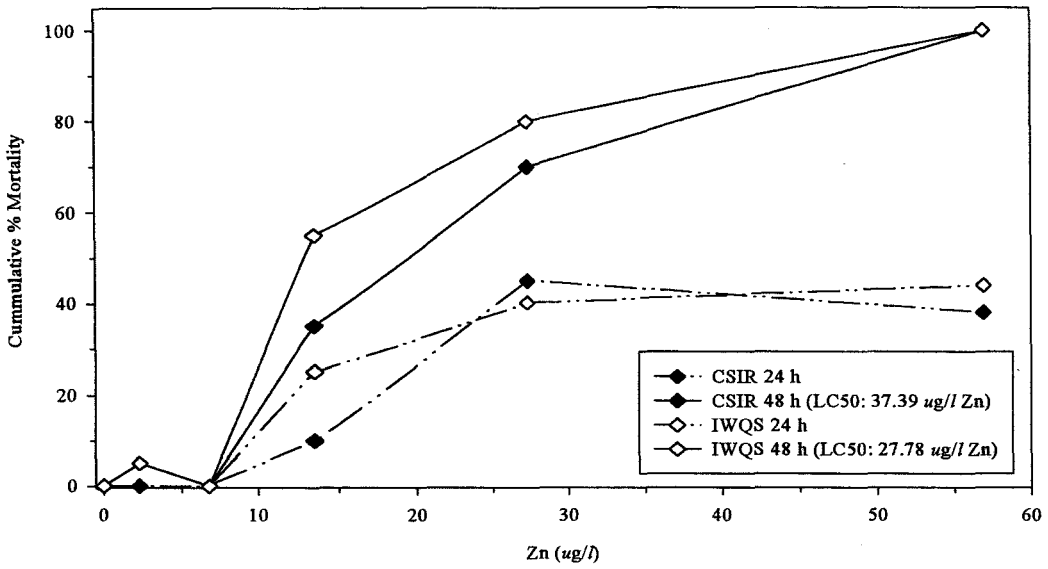

Figure 3.3: The graph plots the 24 h and 48 h cumulative percentage mortalities for both the CSIR and IWQS cultures recorded in Experiment 3. Twenty four hour LC50 values were not calculated because 50% mortality was not reached.

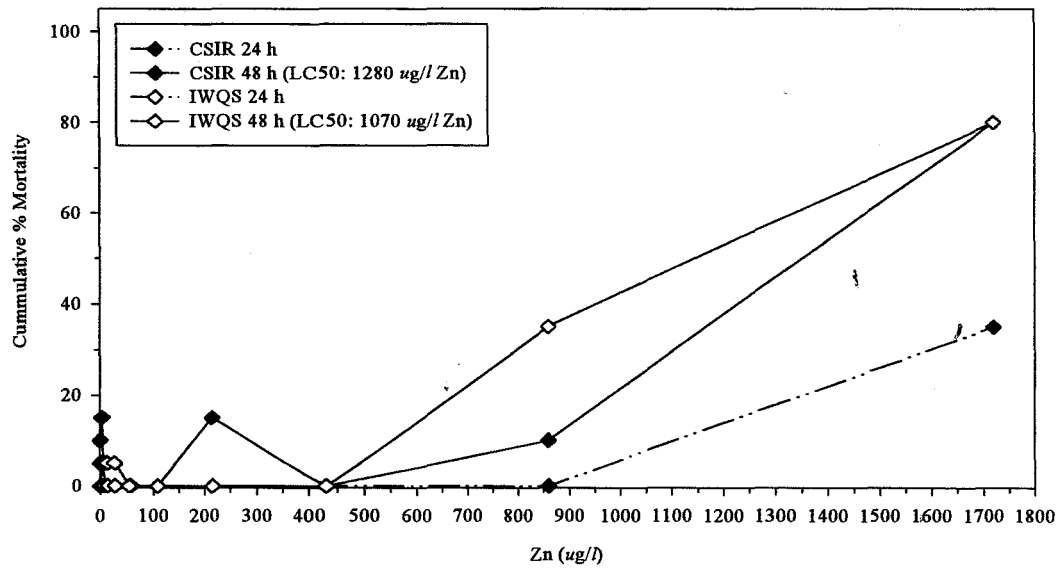

Figure 3.4: The graph plots the 24 h and 48 h cumulative percentage mortalities against increasing zinc concentration for both the CSIR and IWQS cultures recorded in Experiment 4. Twenty four hour LC50 values were not calculated because 50% mortality was not reached.

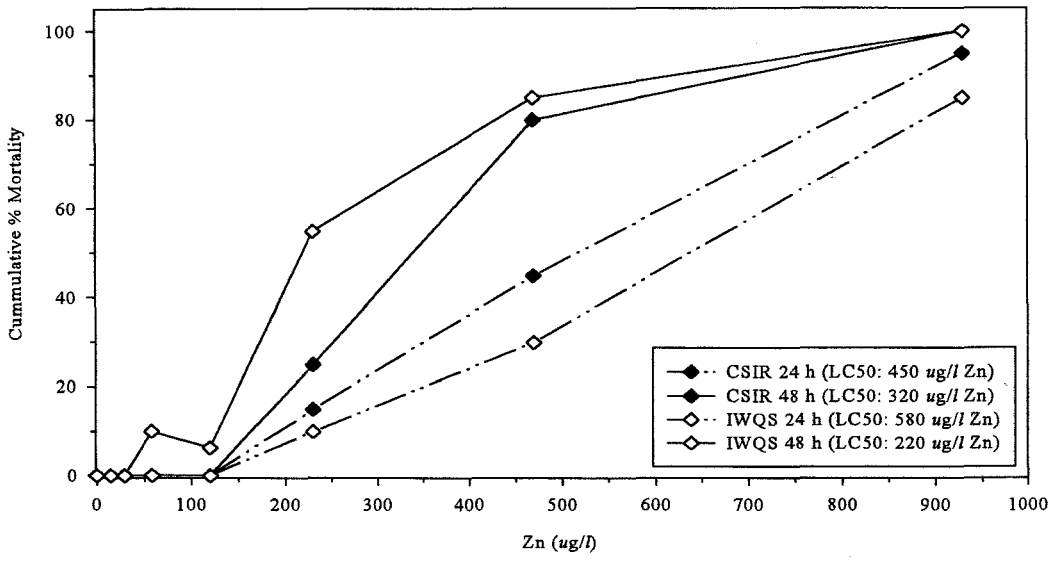

Figure 3.5: The graph plots the 24 h and 48 h cumulative percentage mortalities against increasing zinc concentration for both CSIR and IWQS cultures recorded in Experiment 5.

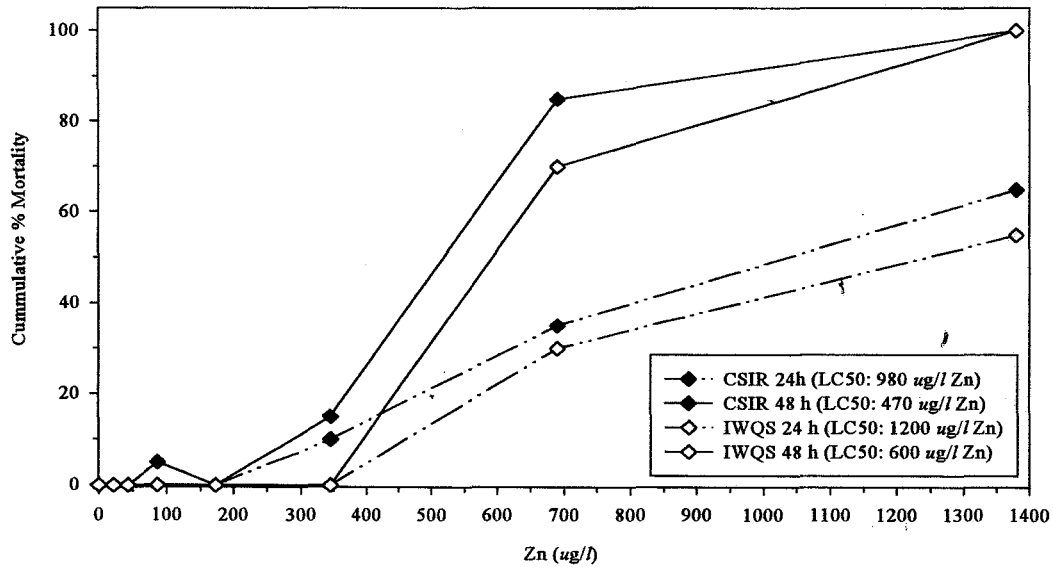

Figure 3.6: The graph plots the 24 h and 48 h cumulative percentage mortalities against increasing zinc concentration for both the CSIR and IWQS cultures in Experiment 6.

3.3.3. 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.

As in the results in the above section (Section 3.3.2), because of the variability experienced between LC50 values within one culture, the comparisons made between cultures are limited to the same experiment. That is, IWQS Experiment 1 is compared only to RD Experiment 1, similarly IWQS Experiment 2 is compared only to RD Experiment 2, etc.

In each experiment, the 24 h and 48 h LC50s calculated for the IWQS culture were significantly different from those calculated for the RD culture (Tables 3.8 and 3.9). For example, the 48 h LC50 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 3.9).

Table 3.8: The 24 h LC50 values (Trimmed Spearman-Karber), with confidence limits and percentage trim for each IWQS and RD *D.pulex* experiment. The high percentage trim associated with IWQS Experiment 6 was due to only 3 partial mortalities being recorded on the regression line (Figure 3.9). Where low mortality was recorded in the upper concentrations, an LC50 could not be calculated because the percentage trim required was too great. The IWQS 24 h LC50 value calculated in Experiment 6 is almost twice that of the LC50 calculated for RD and is therefore significantly different from the RD culture (although the confidence in the data is low due to percentage trim).

Experiment	IWQS culture: 24 h LC50 ($\mu g/l$ Zn) (% trim)	RD culture: 24 h LC50 ($\mu g/l$ Zn) (% trim)
4	Trim too great	Trim too great
5	580.00 (520.00-640.00) (15.00%)	Trim too great
6	1200.00 (960.00-1510.00) (45.00%)	620.00 (570.00-690.00) (5.00%)

Table 3.9: The 48 h LC50 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 (Figure 3.7). IWQS culture LC50 values for each experiment are significantly different from those calculated for the RD culture.

Experiment	IWQS culture: 48 h LC50 ($\mu g/l$ Zn) (% trim)	RD culture: 48 h LC50 ($\mu g/l$ Zn) (% trim)
4	1070.00 (940.00-1230.00) (20.00%)	1370.00 (1160.00-1610.00) (40.00%)
5	220.00 (200.00-250.00) (0.00%)	350.00 (320.00-390.00) (0.00%)
6	600.00 (570.00-640.00) (0.00%)	460.00 (430.00-490.00) (0.00%)

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 (Figures 3.7-3.9). 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.

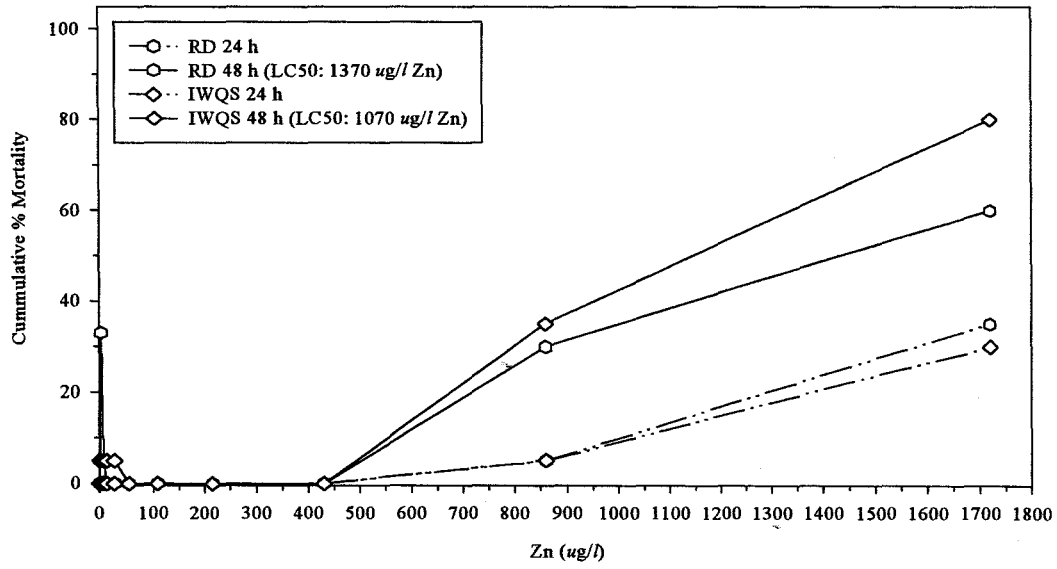

Figure 3.7: The graph plots the cumulative percentage mortalities against increasing zinc concentration for both the RD and IWQS cultures in Experiment 4. Twenty four hour LC50 values were not calculated because 50% mortality was not reached.

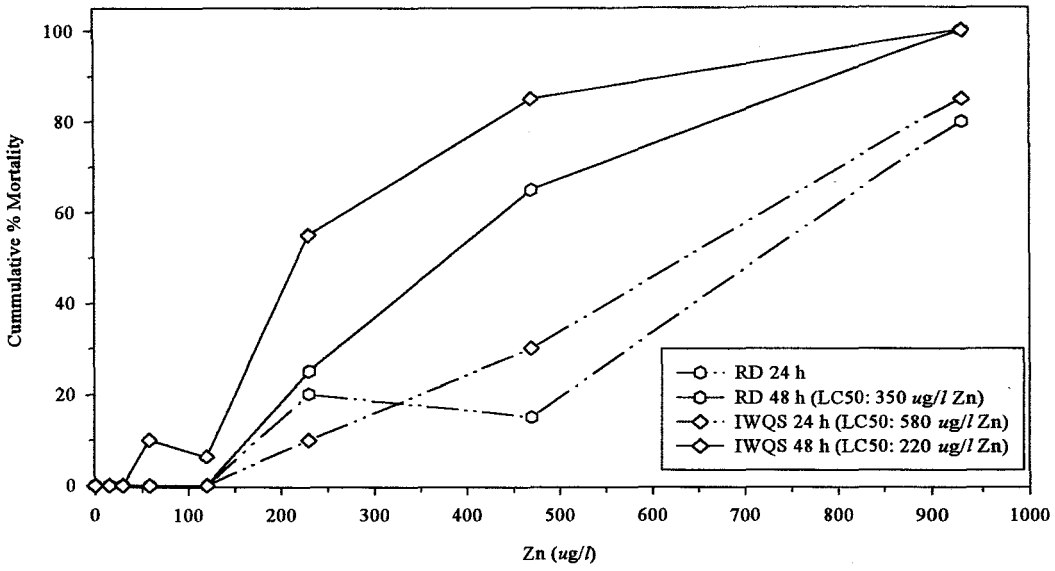

Figure 3.8: The graph plots both the 24 h and 48 h cumulative percentage mortalities against increasing zinc concentration for both the RD and IWQS cultures in Experiment 5. A 24 h LC50 was not calculated for the RD culture because the required trim was too great due to the non-monotonic relationship between mortality and zinc concentration.

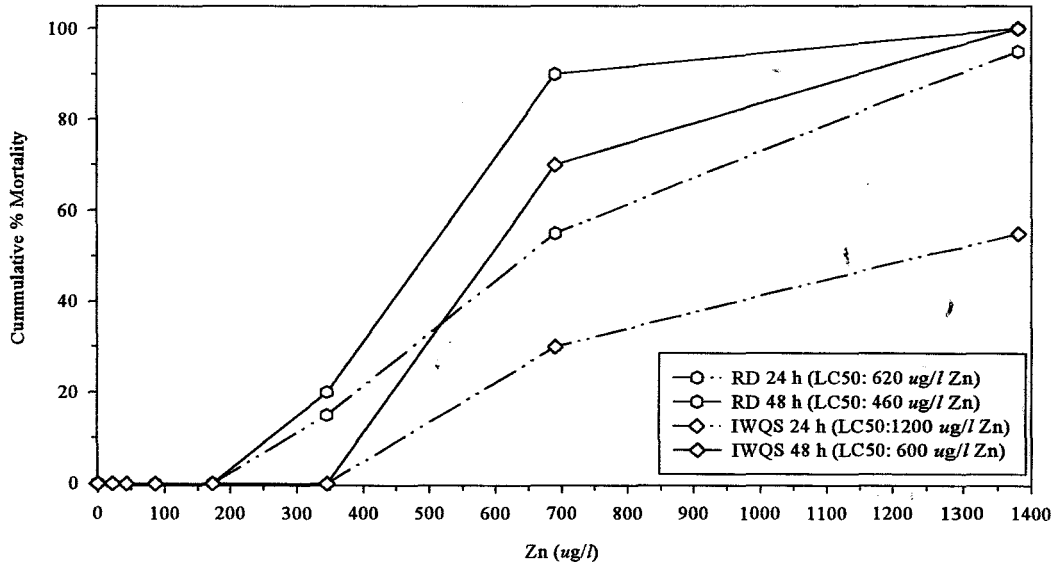

Figure 3.9: The graph plots both the 24 h and 48 h cumulative percentage mortalities against increasing zinc concentration recorded in Experiment 6.

In the two immature cultures set up at the beginning of 1998 (RD and SW cultures), 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 LC50 calculated for the thirteenth generation was significantly greater than that calculated for the sixteenth and seventeenth generations. The sixteenth and seventeenth generation 24 h and 48 h LC50s showed no significant differences from one another (Tables 3.10 and 3.11, Figures 3.10 and 3.11). This trend was not repeated in the RD culture, where all three generations were significantly different in their response to zinc (Tables 3.12 and 3.13, Figures 3.12 and 3.13).

The LC50 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 7) (Table 3.11). 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 3.10: The 24 h LC50s (Trimmed Spearman-Karber), with confidence limits and percentage trim for each SW generation tested. Fifty percent mortality was not reached in the thirteenth generation, thus a 24 h LC50 could not be calculated (Figure 3.10).

Experiment	Generation	SW culture: 24 h LC50 ($\mu g/l$ Zn) (% trim)
4	F13	Trim too great
5	F16	680.00 (630.00-730.00) (25.00%)
6	F17	630.00 (560.00-710.00) (20.00%)

Table 3.11: The 48 h LC50 values (Trimmed Spearman-Karber), with confidence limits and percentage trim for each SW generation tested. The high percentage trim was recorded in Experiment 4 due to the non-monotonic increase in mortality with increasing zinc concentration (Figure 3.11).

Experiment	Generation	SW culture: 48 h LC50 ($\mu g/l$ Zn) (% trim)
4	F13	1500.00 (1340.00-1670.00) (40.00%)
5	F16	450.00 (410.00-490.00) (5.00%)
6	F17	470.00 (440.00-510.00) (0.00%)

Table 3.12: The 24 h LC50s (Trimmed Spearman-Karber), with confidence limits and percentage trim for each RD generation tested. Fifty percent mortality was not reached in the thirteenth generation, therefore a 24 h LC50 could not be calculated. Due to the non-monotonic increase in mortality, the percentage trim required to calculate an LC50 for the sixteenth generation was too great (Figure 3.12).

Experiment	Generation	RD culture: 24 h LC50 ($\mu g/l$ Zn) (% trim)
4	F13	Trim too great
5	F16	Trim too great
6	F17	620.00 (570.00-690.00) (5.00%)

Table 3.13: The 48 h LC50 values (Trimmed Spearman-Karber), with confidence limits and percentage trim for each RD generation tested. The high percentage trim recorded in Experiment 4 was due to the non-monotonic increase in mortality with increasing zinc concentration (Figure 3.13).

Experiment	Generation	RD culture: 48 h LC50 ($\mu g/l$ Zn) (% trim)
4	F13	1370.00 (1160.00-1610.00) (40.00%)
5	F16	350.00 (320/00-390.00) (0.00%)
6	F17	460.00 (430.00-490.00) (0.00%)

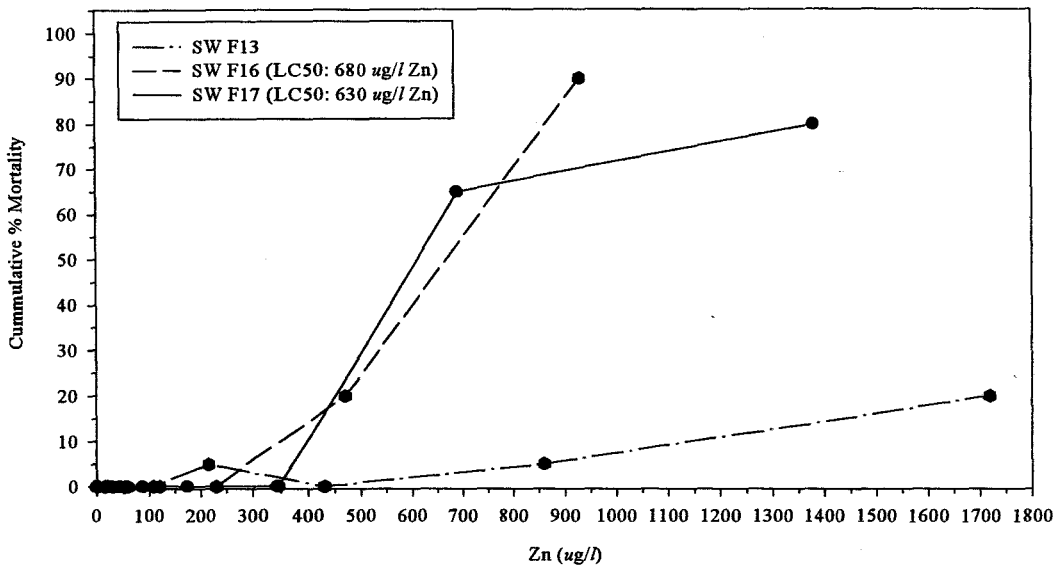

Figure 3.10: The graph plots the 24 h cumulative percentage mortalities for each SW generation tested against increasing zinc concentration. A 24 h LC50 was not calculated for the thirteenth generation because 50% mortality was not reached.

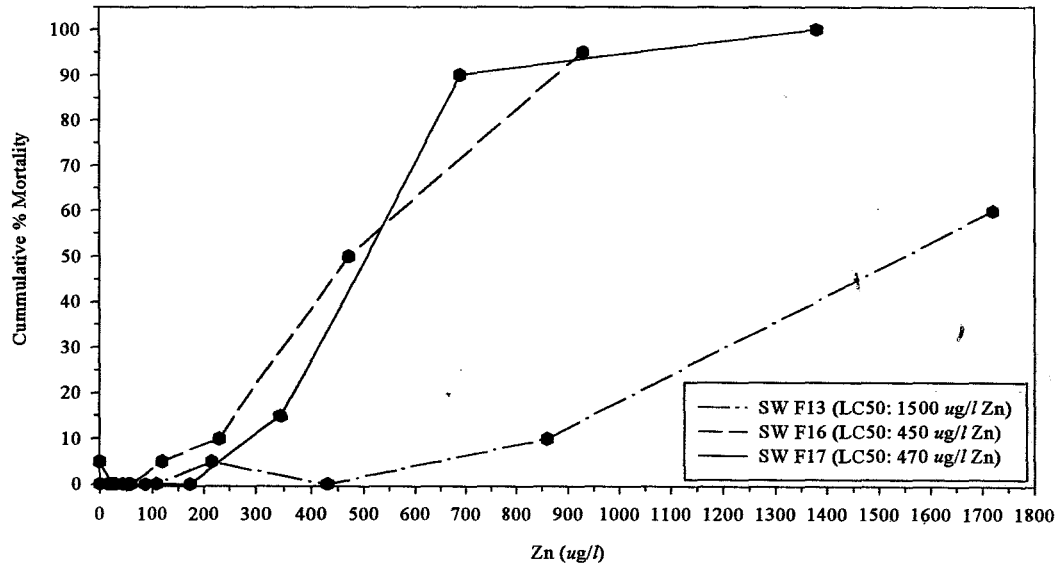

Figure 3.11: The graph plots the 48 h cumulative percentage mortalities for each SW generation tested against increasing zinc concentration.

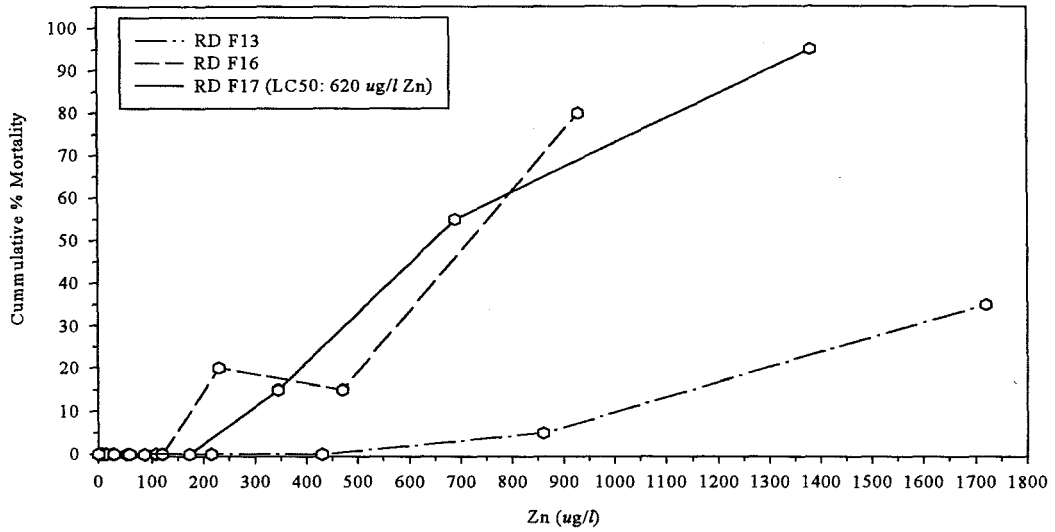

Figure 3.12: The graph plots the 24 h cumulative percentage mortalities for each RD generation tested. Twenty four hour LC50 values were not calculated for generations thirteen and sixteen because the percentage trim required was too great.

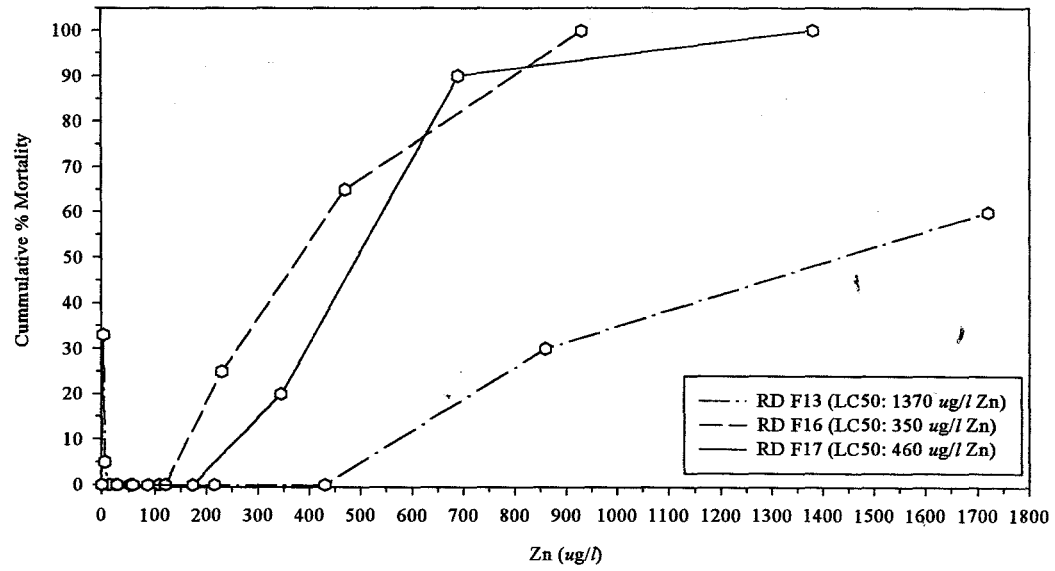

Figure 3.13: The graph plots the 48 h cumulative percentage mortalities against increasing zinc concentration for each RD generation tested.

3.4. DISCUSSION

3.4.1. End-point variability between experiments and implications for aquatic toxicology

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. Acceptable levels of variability around a measured end point are often set and agreed upon within a laboratory (intra-laboratory) and between laboratories (inter-laboratory) comparing data (Rue *et al.* 1988). These levels of variability often vary according to the toxicant and end-point chosen. For example, a round robin study involving a 21-day life-cycle test using sodium bromide showed that, of the 37 laboratories taking part, only 22 returned results which satisfied the validity criteria specified in the protocol (Baird *et al.* 1989). The acceptable variability for a whole effluent inter-laboratory toxicity study tends to be greater than that set for a single-substance toxicity study because of the inherent variability associated with whole effluent (refer to discussion in Chapter 6). The percentage of variation between acute toxicity experiments on one species for a single toxicant has been recorded as being as low as 9% and as high as 120% (Weber 1991). 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%.

The variability around the measured end-point (48 h LC50) in experiments in this study was high (130% to 139%) and the same pattern of variability was noted between different cultures. For example, Experiment 4 consistently showed greater LC50 values than the other experiments in all four cultures. It can be argued that Experiments 3 and 4 should not be included in any analysis because the percentage trim required was above 20% and thus considered to be too high in this study. However, even when disregarding these two experiments, the LC50 values (for both IWQS and CSIR cultures) increase significantly between those experiments conducted at IWQS (i.e. Experiments 1

and 2) and those conducted at IWR (i.e. Experiments 5 and 6). This would suggest a consistent error in the experiments and which may be due to an error in the make up of Zn solutions or an extreme variability around *D.pulex* acute Zn tolerance. Chemical analysis was not carried out on the stock solutions used in Experiments 1-3 and it can therefore be argued that it is possible an error was made when making up the $ZnSO_4$ solution. Despite the uncertainty of the Zn concentration in Experiments 1 and 2, these two experiments were found to be significantly different from one another in both cultures. Similarly, Experiments 5 and 6 were found to be significantly different from one another in both cultures. 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.

A number of variables have been identified in the literature which may have contributed to variability between experiments. These variables can be categorized into physical (e.g. temperature effects and differences in culture technique) and biological variables (e.g. the influence of diet and maternal nutrition, and genetic population) (Falconer 1981, Spraque 1985, Clarke and LaZerte 1987, Baird *et al.* 1989, Cooney 1995), and are discussed in relation to this study.

Ambient temperature is known to have a pronounced effect on the tolerance of a species to a chemical. The sensitivity of *D.pulex* to heavy metals (Cairns *et al.* 1975, Shcherban 1977, Braginskiy and Scherban 1979), herbicides (Cairns *et al.* 1978, Stephenson and Watts 1984) and several other chemicals (Lewis and Horning 1991) has been shown to be temperature sensitive. Lewis and Horning (1991) illustrated that a six degree difference in temperature significantly affected the mortality rate of *D.pulex* to two organic toxicants. Similarly these authors showed that *D.pulex* cadmium sensitivity was also shown to increase 7-fold with a six degree temperature change. Temperature was kept constant around 20°C in both laboratories, but problems were experienced with the temperature control in the constant environment room at IWR, with temperatures fluctuating between 17°C and 24°C overnight. These problems were noted after Experiment 4 had been run and therefore may have contributed to the experimental error in this experiment. Temperatures were kept constant at 20°C over Experiments 1-3, 5 and 6.

A second variable known to influence tolerance to a toxicant is diet and feeding regime (Stephenson and Watts 1984). Maternal reserves have also been reported to play an important role in juvenile daphnid growth (Tessier *et al.* 1983). The sensitivity of *D.magna* neonates has been shown to be largely dependent on maternal nutrition (Enserink *et al* 1990), and Cowgill (1987) suggested that the first daphnid brood be omitted in toxicity testing because they often consist of underweight neonates. Small neonates of well-fed mothers were found to be three times more sensitive to cadmium than large neonates from badly-fed mothers (Enserink *et al* 1990). The diet was the same for both IWQS and IWR laboratories, with only slight variations in the feeding regime (see Section 3.2). The different feeding regime may therefore have influenced maternal reserves and thus neonate sensitivity. However, size differences in neonates were not noted when cultures were moved from IWQS to IWR, nor were they noted during the culturing at either laboratory.

The findings in this chapter 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 (Warren-Hicks and Parkhurst 1992, Parkhurst 1995, Thursby *et al.* 1997). A guideline based on an experimental method in which there is high variability around the end-point is easily questioned. For example, when considering the variability of most whole effluents, a water quality manager must have confidence in the reliability of the test results to enforce a discharge licence. If a licence requirement is exceeded, it must be clear that it is the result of an unacceptably high concentration of the pollutant and not due to method variability (Dhaliwal *et al.* 1995).

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

No two river systems are exactly alike, each is influenced by a unique set of factors which impact on local populations, changing and shaping the local biotic communities. Physiological processes are adapted and modified during exposure to various natural environmental disturbances (Schmidt-Nielsen 1983) and different populations of the same species are influenced by different environmental factors. Thus, when integrated, the responses selected during evolutionary history provide each population with extensive physiological repertoires appropriate to their habitats (Depledge 1982).

It is thought that communities retain information about events in their history (the Community Conditioning Hypothesis) (Matthews *et al.* 1996), and the same may be said of populations. Populations are continually adapting in order to survive and due to the heterogenous nature of river systems, local populations do become isolated (Duan *et al.* 1997), resulting in different genetic populations in different localities. Populations which are separated geographically have shown differences in tolerance to the same chemicals. For example, Crane (1995) found differences in zinc tolerance between different populations of *Gammarus pulex.* Naylor *et al.* (1990) examined the differences in tolerance between upstream and downstream populations of *Asellus aquiticus* and *G. pulex* exposed to zinc pollution and acidic conditions, and found that the downstream populations for both species were more tolerant than the respective unexposed upstream populations. The authors concluded that this difference in tolerance was due to acclimatisation to the local conditions.

Differences in genotypes are therefore 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). 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.

3.4.3. 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). An indication of adverse conditions in a culture is the production of ephippia, a black egg case which protects the sexual egg (embryo), and which is cast off in the next molt. 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.

CHAPTER 4

SINGLE-SUBSTANCE ACUTE TOXICITY TESTING: A COMPARISON BETWEEN SELECTED INDIGENOUS MACROINVERTEBRATES AND *Daphnia pulex*

4.1. INTRODUCTION

Numerous toxicity studies have shown that different aquatic species respond differently to the same chemical (Cairns *et al.* 1975, Sloof 1983, Ewell *et al.* 1986, Hickey and Martin 1995, Toussaint *et al.* 1995, McKinney and Wade 1996, Roseth *et al.* 1996). A primary reason for the United States Environmental Protection Agency (USEPA) restricting routine toxicity testing to a number of standardised test organisms was the variability in response to a chemical by different species (USEPA 1986). The standardised toxicity test organisms chosen include fish, invertebrates and algae. All the USEPA toxicity studies are therefore conducted using the same species, allowing for comparison of data between laboratories and the generation of a database for each standardised test species to a wide variety of toxicants. Laboratory reared organisms are preferred over field-caught species in toxicity testing by the USEPA (Weber 1991) because field caught animals may have been biased for more resistant members of the population due to previous exposure to chemicals. For example, Bodar *et al.* (1990) showed that if exposed to low levels of cadmium, *D.magna* neonates became more resistant to both the acute and chronic effects of cadmium than those neonates previously unexposed. Laboratory cultured organisms are also argued to exhibit less genetic variability than field populations, as a result of reduced genetic input to a laboratory reared culture (Leeuwangh 1978). The advantage of reduced genetic variability within a test population is that variability in response around a predetermined end-point should be reduced. Laboratory cultures allow for better quality assurance and control in toxicity studies, and the age, life-history and existing conditions of the cultures are documented (APHA 1992).

Those species which appeared to be sensitive to most toxicants were chosen to be cultured and used in routine toxicity tests in the United States (USEPA 1986). These species were chosen on the basis that there was sufficient data on their sensitivities to numerous toxicants to assume that

water quality guidelines set using these species would adequately protect other aquatic species (Cairns 1956). An example of one such species is *D.pulex.* In South Africa, it is still necessary to investigate whether water quality guidelines set using standard laboratory reared organisms will be protective of indigenous organisms. A database of indigenous species tolerance to chemical substances (toxicants) is required before appropriate test species can be selected to adequately protect other aquatic species (Palmer and Sherman in press). DWAF are committed to both protecting and ensuring the sustainable use of South Africa's aquatic environments and aim to use the best methods available to them to achieve this goal (Conley 1990, DWAF 1997). To set appropriate water quality guidelines for the protection of the aquatic ecosystem, South African aquatic species' tolerances to toxicants must therefore be investigated.

The aim of this chapter is to compare the tolerance of selected indigenous site-specific macroinvertebrate species to that of cultured *D.pulex* using the reference toxicant, zinc sulphate. Single-substance toxicity tests are ideal for this comparison, as experimental factors can be controlled (e.g. known toxicant concentration). The toxicity of a single-substance can be measured and the concentration-response relationships of different test organisms determined and compared. *D.pulex* is found in the water column and is therefore best suited to a static experimental environment, whereas the selected indigenous invertebrates were better suited to the flowing experimental environment. This therefore resulted in a comparison between a static and flowing test method. Also, because the length of the life-cycle influences the length of the acute test (Janssen and Persoone 1993) and because standard test methods were followed, the LC50 comparisons are made at different time intervals, i.e. 48 h for *D.pulex* and 96 h for indigenous invertebrates.

4.2. MATERIALS AND METHODS

The methods described in Chapter 2, for both indigenous site-specific macroinvertebrate and *D.pulex* acute toxicity tests, were followed. Four acute 96 h experiments on selected indigenous MACROINVERTEBRATES as test species were conducted at Rand Water in the artificial stream systems, the Channels (Figure 2.2). The susceptibility (defined for the indigenous invertebrates as the 96 h LC50) to zinc of the selected invertebrates was compared to that of *D.pulex* (48 h

LC50) determined in experiments initiated during separate field trips to the Institute for Water Quality Studies (IWQS) in Pretoria and the Institute for Water Research (IWR) in Grahamstown (see Chapter 3). The *D.pulex* and indigenous invertebrate experiments could not be run simultaneously as *D.pulex* neonates from Rand Water cultures were not available in sufficient numbers at the time of conducting indigenous invertebrate experiments.

Details of the indigenous site-specific macroinvertebrate and *D.pulex* experiments in this chapter are summarised in Tables 4.1 and 4.2, respectively. Zinc was added in the form of zinc sulphate, the concentrations of which were determined using a geometric progression which would ensure zero and one hundred percent mortality, a requirement of both Probit and Spearman-Karber statistical models (APHA 1992). A 0.5 dilution range was chosen for the zinc sulphate because of the ease of making up the dilutions and the number of treatments run depended primarily on the number of Channel systems which could be set up. Zinc concentrations were determined for each treatment at the end of each experiment by Rand Water analytical laboratories and the measured zinc concentration used to calculate LC50 values. Chemical analysis is not a requirement in standardised *D.pulex* methodology, therefore zinc was not initially measured (i.e. in *D.pulex* Experiments 1-3) (see Section 3.2). Due to high variability experienced in *D.pulex* test results, however, the zinc concentration in stock solutions made for *D.pulex* Experiments 4-6 (i.e. those conducted at IWR) were determined on the Atomic Absorption Spectrophotometer at Rhodes University. Only the results from *D.pulex* Experiments 5 and 6 are used in the comparison with the indigenous organisms in this Chapter (see Chapter 3).

Both Probit and Spearman-Karber statistical models were used to calculate LC50 values, with the model chosen depending on the data (i.e. if Probit model assumptions were not met, the Spearman-Karber model was used to calculate an LC50). A percentage trim greater than 20% was considered to be high. The APHA formula for determining significant differences between LC50s was used to calculated significant differences between the LC50s determined for each species (Chapter 2).

Both dechlorinated tap water and the receiving water (Vaal Dam water) were used as dilution media in the indigenous invertebrate experiments. The culture medium as used for dilution

medium in *D.pulex* experiments (see Section 2.2.3).

Table 4.1: A summary table listing the test organisms, dilution medium and zinc concentrations used for each acute 96 h experiment with selected indigenous site-specific MACROINVERTEBRATES. All invertebrates were collected from Engelbrechtdrift weir (reference site on the Vaal River) and experiments conducted at Rand Water.

Experiment	Test Organisms	Dilution Medium	Concentration (mg/*l* Zn)
1	Baetidae Heptageniidae *(Afronurus peringueyi)* *Caradina nilotica*	Vaal Dam water	0.10, 0.11, 0.12, 0.32, 0.59, 2.10, 6.60, 13.00, 20.00
2	Baetidae Heptageniidae *(Afronurus peringueyi)* Leptophlebiidae *(Euthraulus elegans)*	Vaal Dam water	0.10, 0.33, 0.34, 0.55, 0.67, 0.92, 1.00, 1.14, 1.60, 1.80, 2.00, 2.60, 3.10, 13.00, 16.00, 79.00, 79.01
3	Baetidae Corixidae *Caradina nilotica*	Dechlorinated tap water	0.57, 0.70, 0.72, 1.00, 2.07, 3.86, 9.50, 9.67, 42.00
4	Baetidae Corixidae *Caradina nilotica*	Dechlorinated tap water	0.30, 0.67, 0.71, 0.74, 0.75, 0.88, 0.95, 0.99, 1.10, 1.50, 1.70, 1.71, 1.80, 1.81, 6.90, 8.90, 11.00, 69.00

Table 4.2: The table summarises the zinc concentration used in the acute 48 h expeiment for both the IWQS and CSIR *D.pulex* cultures. LC50 values were calculated using the zinc concentrations presented in the table.

Experiment	Place of Conduct	Concentration (mg/*l* Zn)
5	IWR	0.000, 0.015, 0.029, 0.058, 0.120, 0.230, 0.470, 0.930
6	IWR	0.000, 0.022, 0.043, 0.086, 0.173, 0.345, 0.690, 1.380

Results of chemical analysis of water samples taken at the end of the indigenous invertebrate experiments were compared to chemical data for Engelbrechtdrift weir to give an indication whether concentrations of selected water quality variables recorded during the experiments were to be considered high (see Section 2.2.4).

4.3. RESULTS

Each indigenous invertebrate experiment is discussed, and where the same species was used in all experiments, significant differences between the LC50s determined. If no significant difference was calculated, the results from experiments were pooled. These LC50s were then compared to the *D.pulex* LC50s calculated for IWQS and CSIR cultures.

4.3.1. Experiment 1

Temperature is known to influence the toxicity of a chemical to a test organism (Weber 1991) and should therefore remain stable during an experiment. The temperature within each channel was stable, not varying more than 3°C over the test period (96 h). The average water temperature was between 18.8°C and 20.3°C in each channel, with a minimum of 18.0°C and maximum of 22.0°C recorded. Nutrient concentrations remained below the median value calculated for the reference site (Engelbrechtdrift weir) and well within the guidelines set for the protection of aquatic life (Table 4.3). Nutrients were therefore unlikely to have resulted in mortality during the experiment. The sulphate concentration range recorded during the experiment was greater than the reference site median, but less than the maximum experienced in the field. The target water quality range (TWQR) for sulphate is set at 1400.00 mg/*l* and the highest sulphate concentration in a treatment was recorded at 180 mg/*l*; as compared to 510 mg/*l*, the maximum recorded value at Engelbrechtdrift weir. It is therefore unlikely that the sulphate levels resulted in stress or mortality of the test organisms.

Table 4.3: Summary of the physical and chemical ranges measured in the Channel systems during Experiment 1. The Target Water Quality Range (TWQR) ([1]DWAF 1996g) and Guideline for the Protection of Aquatic Life (GPAL) ([2]Kempster *et al* 1980) are provided to give an indication of acceptable concentrations for each variable (guidelines for nitrite and nitrate have not been specified by DWAF). Chemical data, collected by Rand Water (1992-1998) from the reference site (Engelbrechtdrift weir), is summarised as the median, minimum and maximum recorded values. If values fall below the detection limits for the analysis method used, a "<" is used.

Water Quality variable	TWQR [1]; GPAL[2]	Reference Site: Median (mg/l)	Reference Site: Min-Max (mg/l)	Experiment 1 (mg/l)
Temperature (°C)	Should not vary by more than 2°C over 24h [1]			18.5-20.0°C
Ammonia	<0.01mg/l [1]; 0.02-124.00mg/l[2]	0.08	0.02-1.60	<0.05
Nitrite	-	0.05	0.02-0.25	<0.03
Nitrate	-	0.38	0.05-7.40	0.03-0.41
Total Phosphate	Should not change by more than 15% [1]	0.05	0.05-10.00	<0.03
Sulphate	1400.00mg/l[2]	17.00	5.00-510.00	21.00-180.00
Zinc	<0.002mg/l [1]; 0.03-0.10mg/l[2]	0.05	0.05-0.33	<0.10-13.00

A number of baetid species occur at the reference site and because of the difficulty in speciating organisms in the field, this family was used as a species complex in the experiment (Table 4.4). Each baetid was collected after the experiment and identified to species level by the Albany Museum, Grahamstown. *Baetis harrisoni* made up 75% of the species within this complex, with *Cheleocloeon excisum* making up the next highest proportion (22.5%).

Table 4.4: The percentage proportions of species of the Baetidae complex used in Experiment 1.

Species	Experiment 1
Baetis harrisoni	75
Baetis glaucus	1.0
Baetis quintis	1.0
Cheleocloeon excisum	22.5
Unknown	0.5

Control mortality for the Baetidae, *Afronurus peringueyi* and *Caradina nilotica* remained at zero over the test period, suggesting that the immediate needs (e.g. hydraulic environment) of the test organisms were met by the experimental conditions. The Baetidae, *A.peringueyi* and *C.nilotica* mortalities showed a positive correlation with zinc, with percentage mortality increasing with increased zinc concentration (Figure 4.1). The Baetidae were the more susceptible of the selected indigenous invertebrates to the zinc; with *A.peringeuyi* showing the greatest tolerance and *C.nilotica* a tolerance which fell between the other two test species. For example, baetid mortality occurred in the lower concentrations (0.10-0.32 mg/*l* Zn) after 96 h, whereas no 96 h mortality was recorded for *A.peringueyi* or *C.nilotica* in these treatments (Table 4.5). The baetids were also more quickly affected by the zinc than the other two species, with 82.8% mortality occurring at 20.00 mg/*l* Zn after 24 h, compared to 7.4% and 75.0% 24 h mortality recorded for *A.peringueyi* and *C.nilotica* respectively. A 96 h Spearman-Karber LC50 was calculated at 0.99 (0.67-1.46) mg/*l* Zn (7.14% trim) for the Baetidae, which was significantly more sensitive than the other two test species (Table 4.6). A Spearman-Karber LC50 was calculated at 3.17 (2.39-4.20) mg/*l* Zn (0% trim) for *C.nilotica.* The heptageniid mayfly, *A.peringueyi*, showed a significantly greater tolerance to zinc than the other test organisms. Only 81.4% mortality was recorded in the 20.0mg/*l* Zn treatment after 96 h, compared to the 100% 96 h mortality recorded for the other two test organisms (Table 4.5). *A.peringueyi* showed a 96 h Spearman-Karber LC50 calculated at 8.8 (6.37-12.17) mg/*l* Zn (18.52% trim) (Table 4.6).

Table 4.5: The daily cumulative percentage mortalities are summarised for each indigenous test species in Experiment 1.The rate of mortality within each concentration can be read by reading down a column, and a comparison between concentrations recorded at one time can be made by reading along a row.

		Zn concentration (mg/*l*)									
	Hours	Control	0.10	0.11	0.12	0.32	0.59	2.10	6.60	13.00	20.00
Baetidae	24	0.00	0.00	0.00	0.00	0.00	0.00	7.40	36.00	29.60	82.80
	48	0.00	0.00	0.00	0.00	0.00	4.20	25.90	84.00	77.70	100.00
	72	0.00	0.00	5.30	5.60	11.10	12.50	25.90	92.00	85.10	100.00
	96	0.00	7.10	21.10	11.20	11.10	50.00	55.50	100.00	100.00	100.00
A.peringueyi	24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.30	7.40
	48	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.30	45.80	51.80
	72	0.00	0.00	0.00	0.00	0.00	0.00	0.00	29.10	50.00	53.50
	96	0.00	0.00	0.00	0.00	0.00	0.00	4.20	41.60	58.30	81.40
C.nilotica	24	0.00	0.00	0.00	4.00	0.00	4.00	16.00	12.50	26.90	75.00
	48	0.00	0.00	0.00	4.00	0.00	4.00	28.00	75.00	73.10	100.00
	72	0.00	0.00	0.00	4.00	0.00	4.00	28.00	79.00	73.10	100.00
	96	0.00	0.00	0.00	4.00	0.00	4.00	28.00	91.70	76.90	100.00

Table 4.6: The 96 h Spearman-Karber LC50s, with confidence limits and percentage trim for each indigenous test species used in Experiment 1: The 96 h LC50s are significantly different from one another (as indicated by different letters).

	Baetidae	***A.peringueyi***	***C.nilotica***
LC50 (mg/*l* Zn)	0.99 (0.67 - 1.46)	8.80 (6.37 - 12.17)	3.17 (2.39 - 4.20)
% Trim	7.14%	18.52%	0.00%
Significant Difference	a	b	c

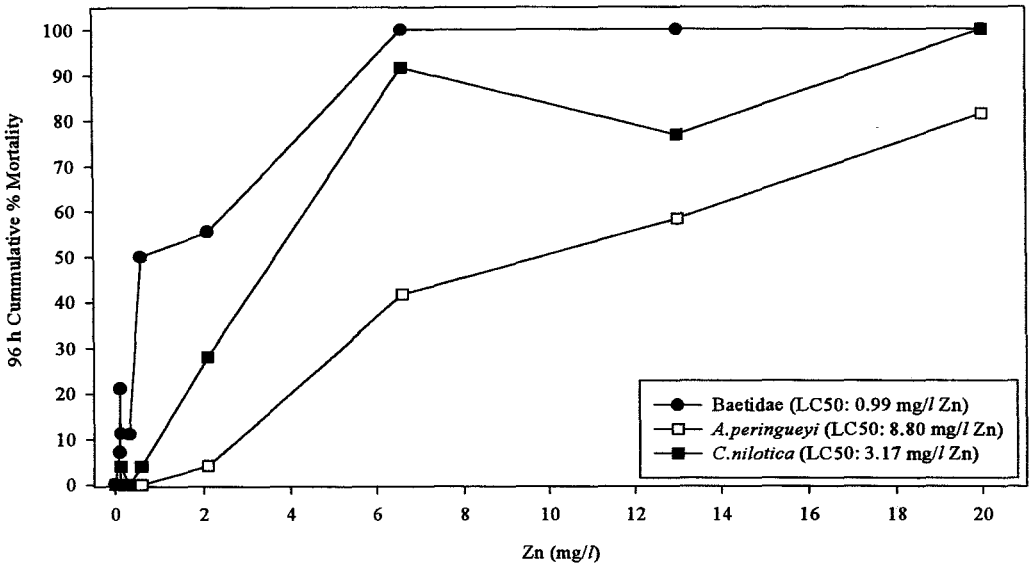

Figure 4.1: The 96 h cumulative percentage mortalities for each indigenous macroinvertebrate test organism used in Experiment 1.

4.3.2. Experiment 2

Temperatures in the channels were again stable, fluctuating by a degree over the 96 h test period. The maximum temperature recorded was 21.0°C and the minimum 18.4°C. Electrical conductivity and pH were constant within a treatment over the test period, but showed an increasing and decreasing trend respectively with increasing zinc concentration. This relationship may be due to increased sulphate concentrations in the higher zinc treatments (Dallas and Day 1993). Nutrient concentrations again remained low, with all the experimental concentrations less than the median values recorded at the reference site (Engelbrechtdrift weir) (Table 4.7). The maximum sulphate concentration recorded in a treatment was 225 mg/*l*, which is just over half the maximum value recorded at the reference site (510 mg/*l*) and significantly lower than the TWQR, which is set at 1400 mg/*l* (DWAF 1996g). It was therefore assumed that observed mortality was due to the added zinc and sulphate and nutrients did not stress the animals in any significant way.

Table 4.7: Summary of the physical and chemical ranges measured in the Channel systems during Experiment 2. The Target Water Quality Range (TWQR)([1]DWAF 1996g) and Guideline for the Protection of Aquatic Life (GPAL) ([2]Kempster *et al* 1980) are provided to give an indication of acceptable concentrations for each variable (guidelines for nitrite and nitrate have not been specified). Chemical data, collected by Rand Water from 1992-1998 at the reference site (Engelbrechtdrift weir), is summarised as the median, minimum and maximum recorded values, to give an indication of the concentrations at which the test organisms were exposed when in the field. If values fall below the detection limits for the analysis method used, a "<" is used.

Water Quality variable	TWQR [1]; GPAL[2]	Reference Site: Median (mg/l)	Reference Site: Min-Max (mg/l)	Experiment 2 (mg/l)
Temperature (°C)	Should not vary by more than 2°C over 24h [1]			18.4-21.0°C
pH	Should not vary more than 0.5 pH units over 24h	7.96	7.3-8.8	7.2-8.4
Ammonia	<7ug/l [1]; 0.02-124mg/l[2]	0.08	0.02-1.60	<0.05-0.69
Nitrite		0.05	0.02-0.25	<0.03
Nitrate		0.38	0.05-7.40	0.18-0.31
Total Phosphate	Should not change by more than 15%[1]	0.05	0.05-10.00	<0.10
Sulphate	1400mg/l[2]	17.00	5.00-510.00	32-255
Zinc	<2ug/l [1]; 0.03-0.1mg/l[2]	0.05	0.05-0.33	<0.10-79

The baetids in this experiment were again made up of a complex of species (Table 4.8). Each baetid was collected after the experiment and identified to species level at the Albany Museum, Grahamstown. *Baetis harrisoni* made up the largest percentage of the complex (47.4%), although the percentage was far lower than in Experiment 1, where they made up 75% of the individuals used (Table 4.4). A greater percentage of *C.excisum* and *B.glaucus* were used in Experiment 2, 32.1% and 15.8% respectively, compared with 22.5% and 1.0% in Experiment 1.

Table 4.8: The percentage proportions of species of the Baetidae complex used in Experiment 2.

Species	Experiment 2
Baetis harrisoni	47.4
Baetis glaucus	15.8
Baetis quintis	3.7
Baetis monticola	0.5
Cheleocloeon excisum	32.1
Unknown	0.5

Control mortality for each test organism was below 10% (Figure 4.2). Each invertebrate species showed a concentration-response relationship between increasing percentage mortality and increasing zinc concentration. The Baetidae and *E.elegans* showed similar 96 h percentage mortalities, and their 96 h LC50s were not significantly different from one another (Table 4.9). *A.peringueyi* was again significantly more tolerant than the other indigenous invertebrates, with an LC50 calculated at 11.67 (6.59-20.50) mg/*l* Zn. This is compared to the 96 h LC50s calculated for the Baetidae and *E.elegans* at 1.02 (0.86-1.12) mg/*l* Zn and 0.98 (0.84-1.14) mg/*l* Zn, respectively. The concentration-response relationship for all three invertebrates was not monotonic (Figure 4.2). The non-monotonic relationship between concentration exposure and 96 h cumulative mortality is especially obvious when reading Table 4.10. Similarly *E.elegans* showed 89.5% mortality at 2.6 mg/*l* Zn after 96 h, but 94.4% and 100% 96 h mortalities were recorded at 2.00 mg/*l* Zn and 3.1 mg/*l* Zn, respectively. This did not however appear to influence the calculation of the *E.elegans* and baetid LC50s , as the percentage trim was 0.0% and 6.67% respectively (Table 4.9).

Table 4.9: The calculated 96 h Spearman-Karber LC50s, with confidence limits and percentage trim for each indigenous test organism in Experiment 2. Significant differences between the series are indicated by different letters, no significant difference is indicated by the same letter. The Baetidae and *E.elegans* LC50s were not significantly different from one another, but both were significantly different to *A.peringueyi*, which showed the greatest tolerance to zinc.

	Baetidae	***A.peringueyi***	***E.elegans***
96 h LC50 (mg/*l* Zn)	1.02 (0.86-1.12)	11.67 (6.59 - 20.50)	0.98 (0.84 - 1.14)
% Trim	6.67%	15.79%	0.00%
Significant Difference	a	b	a

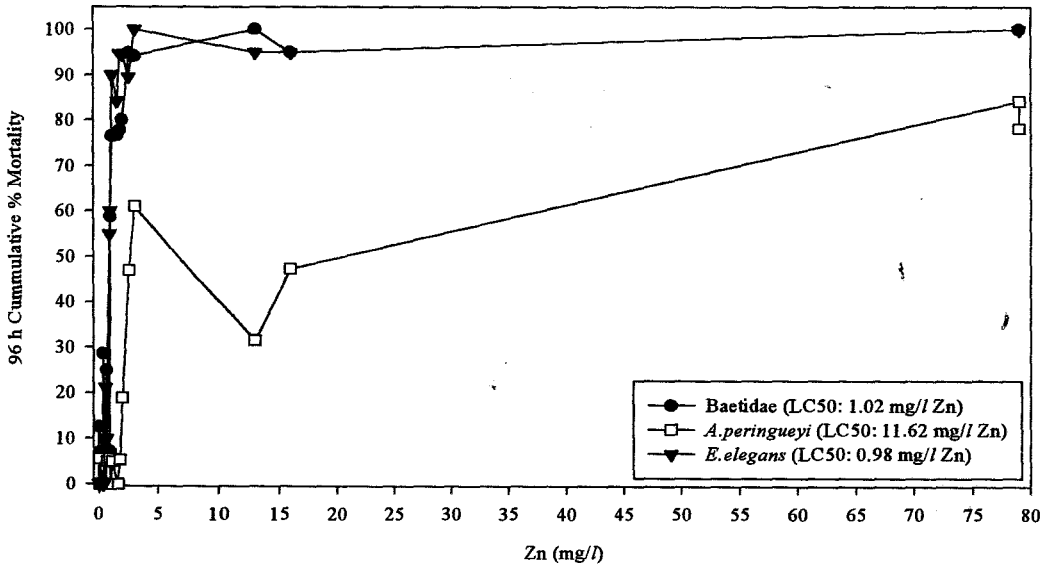

Figure 4.2: The 96 h cumulative percentage mortalities recorded for each indigenous macroinvertebrate test organism used in Experiment 2.

Table 4.10: The daily cumulative percentage mortalities are summarised for each indigenous test invertebrate in Experiment 2.The rate of mortality within each concentration can be read by reading down a column, and a comparison between concentrations recorded at one time can be made by reading along a row.

		Zn concentration (mg/*l*)																
	Hours	Control	0.33	0.34	0.55	0.67	0.92	1.00	1.14	1.60	1.80	2.00	2.60	3.10	13.00	16.00	79.00	79.00
Baetidae	24	0.00	0.00	7.10	0.00	8.30	0.00	17.60	29.40	11.80	38.90	30.00	58.00	64.70	64.70	70.00	90.00	100.00
	48	6.70	0.00	14.20	0.00	25.00	0.00	35.20	52.90	47.10	50.00	60.00	84.30	76.50	82.30	85.00	90.00	100.00
	72	6.70	0.00	21.30	0.00	25.00	7.10	47.00	64.70	53.00	55.60	70.00	94.80	58.30	100.00	90.00	100.00	100.00
	96	9.20	6.70	28.60	0.00	25.00	7.10	58.80	76.50	76.50	77.80	80.00	94.80	94.20	100.00	95.00	100.00	100.00
A.peringueyi	24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.90	11.20	15.80	15.80	21.00	6.70
	48	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.00	0.00	5.30	12.50	17.80	33.40	21.10	31.60	36.80	40.00
	72	0.00	0.00	0.00	0.00	5.00	0.00	0.00	5.00	0.00	5.30	18.80	47.00	50.10	26.30	36.90	47.30	53.30
	96	5.60	0.00	0.00	0.00	5.00	5.60	0.00	5.00	0.00	5.30	18.80	47.00	61.10	31.60	47.40	84.20	78.30
E.elegans	24	0.00	0.00	0.00	5.30	0.00	5.00	20.00	30.00	15.80	63.20	33.30	57.90	66.70	55.00	90.00	75.00	79.00
	48	0.00	0.00	0.00	10.60	5.00	40.00	45.00	70.00	57.90	79.00	77.70	89.50	95.30	70.00	95.00	90.00	89.50
	72	0.00	0.00	0.00	15.90	10.00	50.00	55.00	90.00	84.20	84.30	88.80	89.50	100.00	90.00	95.00	100.00	100.00
	96	0.00	0.00	0.00	21.20	10.00	55.00	60.00	90.00	84.20	94.70	94.40	89.50	100.00	95.00	95.00	100.00	100.00

4.3.3. Experiments 3 and 4

Acclimation mortalities for the Baetidae, *E.elegans*, Corixidae and *C.nilotica* greatly exceeded 5% in both Experiments 3 and 4 (Table 4.11). APHA standard methods (1992) state that if more than 5% of the test organisms die during the 48h acclimation period, the test should be terminated. The Vaal Dam was experiencing algal blooms at the time (pers. obs.) and as a result potable water was heavily chlorinated. However, tap water (used as dilution medium) was bubbled for between 2 and 4 days before experiments were initiated and chloride levels were recorded as being within the TQWR chlorine levels. The high acclimation mortalities indicated that the test organisms were stressed by the dechlorinated tap water and that this water was unacceptable as a dilution medium.

Table 4.11: Acclimation mortalities experienced in dechlorinated tap water during Experiments 3 and 4.

	Baetidae	***E.elegans***	**Corixidae**	***C.nilotica***
Experiment 3	22.3%	-	79.4%	12%
Experiment 4	75.3%	8.7%	-	-

4.3.4. 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 is being made. In this Chapter, the *D.pulex* methodology followed is 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.

The Baetidae LC50s 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 LC50 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 4.3, Table 4.12), with the 48 h daphnid LC50s being significantly less than the 96 h LC50s 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 LC50s 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). 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.

Table 4.12: Calculated Spearman-Karber LC50s (mg/*l* Zn) and the confidence limits for each test organism from Experiments 1 and 2 are presented. The LC50s calculated for the Baetidae (Experiments 1 and 2) and *E.elegans* were not significantly different from one another (denoted by an *). All other LC50s calculated were significantly different from one another.

Baetidae	***A.peringueyi***	***E.elegans***	***C.nilotica***	***D.pulex* (CSIR)**	***D.pulex* (IWQS)**
0.940 (0.860-1.020)* Pooled (Experiment 1 & 2)	17.420 (10.670-28.430) Pooled (Experiment 1 & 2)	0.980 (0.840 - 1.140)* Experiment 2	3.170 (2.390 - 4.200) Experiment 1	0.320 (0.290-0.350) Experiment 5	0.220 (0.200-0.250) Experiment 5
				0.470 (0.440-0.510) Experiment 6	0.600 (0.570-0.640) Experiment 6

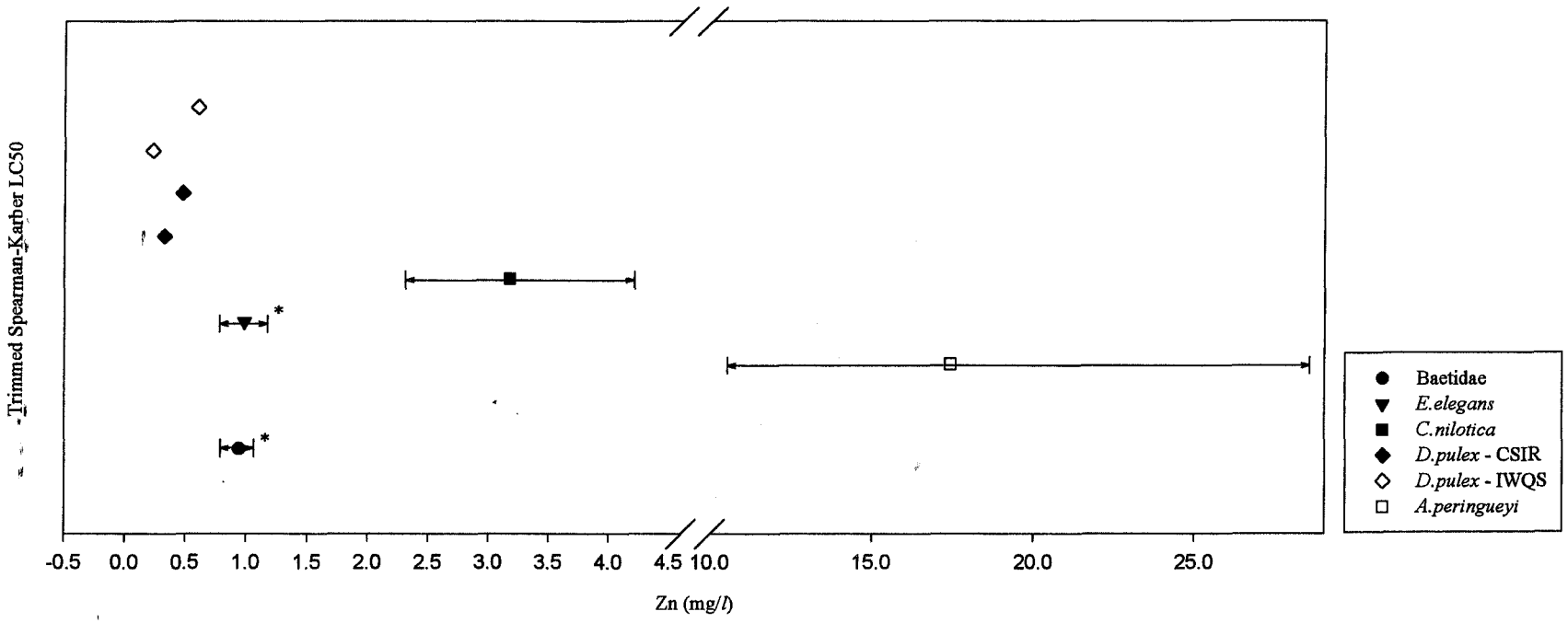

Figure 4.3: The graph summarises all the LC50 values (with 95% confidence limits) calculated for the indigenous site-specific organisms (96 h LC50s) and *D.pulex* (48 h LC50s) (both CSIR and IWQS). All the LC50s were significantly different from one another, except for those calculated for the Baetidae and *E.elegans* (denoted with an *) (see Table 4.12).

4.4. DISCUSSION

Cairns (1980) argues that if one wishes to know the effects of a chemical on living organisms, the effects must be determined by a direct measurement made upon the organisms themselves. Therefore, when determining guidelines aimed at protecting the aquatic ecosystem from pollution, a measurement of the aquatic ecosystem's tolerance to the chemical must be determined. For this reason numerous studies have used aquatic insects in toxicity tests (Phillips 1978, Anderson *et al.* 1980, Hare and Campbell 1992, Brooks *et al.* 1995, Gerhardt and De Bisthoven 1995, Monda *et al.* 1995, Pinel-Alloul *et al.* 1996, Sibley *et al.* 1997), and when reviewed by Maltby and Calow (1989), toxicological data showed that invertebrates were more commonly used in aquatic toxicity tests than fish, which were traditionally the most common toxicity test species.

Ephemeroptera (mayflies), often used as toxicity test organisms (Van Hassel and Gaulke 1986, Diamond *et al.* 1992, Quinn and Hickey 1993, Hickey and Martin 1995), and freshwater shrimp species are considered ecologically important components of the invertebrate community (Hart 1980), and are therefore often used in toxicity testing (Correa 1987, Hatakeyama and Sugaya 1989). Three different mayflies, *A.peringueyi* (Heptageniidae), *E.elegans* (Leptophlebiidae) and Baetidae, and the shrimp *C.nilotica* were selected for use as test organisms in this Chapter. They were chosen as potential test organisms because of their abundance at the reference site and their suitability to the test environment in the recirculating Channels.

Of the indigenous invertebrates selected in this study, the baetids and *E.elegans* appeared to be the most sensitive to zinc. The fact that *E.elegans* appeared to be one of the more sensitive of the indigenous invertebrates used in this study, contrasts with other studies which suggested that the Leptophlebiidae are fairly tolerant to chemicals (Gerhardt 1994). A study comparing the leptophlebiid, *Leptophlebia marignata,* and the baetid, *Baetis rhodani,* found that the leptophlebiid was more tolerant with respect to cadmium and lead than the baetid (Gerhardt 1992). In this study, the tolerance to zinc was not significantly different between the baetids and *E.elegans*. It is difficult to generalise about the sensitivity of a family, as species within a family may show different tolerances and the same species may be tolerant to one chemical but sensitive to another (Toussaint *et al.* 1995, McKinney and Wade 1996, Roseth *et al.* 1996). For example,

Hatakeyama and Sugaya (1989) noted that the shrimp, *Paratya compressa improvisa,* appeared more sensitive to herbicide toxins, but was more tolerant to heavy metal toxins when compared with *D.magna*. Thus a species should not be dismissed as a potential toxicity test species because others within the same family are shown to be tolerant to certain chemicals.

A.peringueyi was the most tolerant of the indigenous invertebrates tested to zinc, in terms of 96 h LC50s, 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. Also, when compared with two other freshwater shrimp species in the literature, *Artemia salina* (Govindarajan *et al.* 1993) and *Mysidopsis bahia* (Lussier *et al.* 1985), *C.nilotica* is more tolerant to zinc and therefore not a good indicator of zinc toxicity.

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. Pontasch and Cairns (1989) experienced a similar problem, finding that nearly 80% of *Baetis* sp. emerged from control streams during their study. 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.

Aquatic toxicology is still a relatively new field in South Africa (DWAF 1998). Although there has been an international trend toward using standardised cultured organisms in the setting of water quality guidelines (DWAF 1996g), the decision as to whether or not standardised organisms are representative of those in the field is still to be made for South African conditions (Scherman and Palmer in press). An aim of this study was therefore to compare the sensitivity of the widely used and standardised *D.pulex* acute toxicity test methodology with that of acute indigenous

invertebrate toxicity testing, using zinc as the reference toxicant.

The *D.pulex* were found to be more sensitive to zinc than the indigenous invertebrates in this study and had lower LC50 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.

A secondary aim of this chapter was to compare the use of different dilution waters in the indigenous macroinvertebrate test methodology. Four experiments, two with Vaal Dam water and two with dechlorinated tap water as dilution media, were run. The results indicated that dechlorinated tap water was unsuitable as a dilution medium. Most of the test organisms died during the acclimation period, indicating that this water was highly toxic to the test organisms. At the time of testing the Vaal Dam was experiencing algal blooms and Rand Water, as a result, was chlorinating the water heavily. Although the water was bubbled for between two and four days, high chlorine levels may have caused recorded mortalities. The significance of this finding is that test organism sensitivity to the dilution water should be investigated prior to the initiation of toxicity testing.

CHAPTER 5

A CASE STUDY: WHOLE EFFLUENT TOXICITY TESTING

5.1. INTRODUCTION

Aquatic toxicology aims to determine at what dosage and under what conditions an organism is influenced adversely by a toxicant, and makes the use of laboratory toxicity tests to investigate concentration-response relationships. In the field, aquatic organisms are exposed to numerous chemicals and rarely to a single chemical or toxicant, and the exposure to a chemical mixture may be sequential (e.g. pulses of different chemicals) or simultaneous (e.g. whole effluent) in time. Exposure to a mixture of substances may result in a biological response which is quantitatively or qualitatively different from that expected from the action of each substance on its own (Rand *et al.* 1995), and the toxic response may be magnified or reduced. For example, an additive effect occurs when the combined effect of two chemicals is equal to the sum of the individual chemicals applied alone. When the combined effect of two chemicals is much greater than the sum of the effects of individual chemicals applied alone, a synergistic effect has occurred. Antagonism occurs when the two chemicals interfere with each others' toxic action, and potentiation occurs when one chemical has a toxic effect only when applied with the other chemical (Rand *et al.* 1995). The toxic relationship between a substance and exposed population is therefore complex and further complicated when a mixture of chemicals (e.g. whole effluent) is discharged into the environment, as these chemicals interact with one another, the surrounding water and with other discharged chemicals in the receiving water system (Filella *et al.* 1995).

Whole effluent toxicity (WET) testing evaluates the toxicity of effluents to aquatic test organisms. The toxicity tests incorporate chemical interactions between the substances within the effluent mixture and their effects on the organism as a whole. The USEPA's 1984 national policy document, the Policy for the Development of Water Quality Based Permit Limitations for Toxic Pollutants, recommends that WET tests be used in toxic management programmes (in Warren-Hicks and Parkhurst 1992). As a result of this recommendation, the USEPA has increasingly promoted the use of WET testing (Warren-Hicks and Parkhurst 1992). In South Africa, the

Department of Water Affairs and Forestry (DWAF) have also identified the need to assess the effects of hazardous effluent on the aquatic environment and have identified WET testing as a tool to evaluate the suitability of hazardous effluents for discharge into receiving waters (Slabbert *et al.* 1998).

With the passing of the new Water Act (Act No. 36, 1998) in South Africa, and the recognition of the importance of toxicity testing, there is a need to establish WET test methodology suited to local conditions. This includes determining which test organisms may be used in WET, either standard laboratory test organisms, indigenous site-specific organisms, or both. A number of WET studies have been initiated by the Institute for Water Research (IWR) (Grahamstown), funded by the Water Research Commission, and have primarily used indigenous site-specific invertebrates as test species. These studies include a doctoral thesis by Davies-Coleman (pers. comm.), which is investigating the use of the freshwater limpet, *Burnupia stenochorias*, as an indicator in WET; and a masters thesis by Zokufa, which aims to develop WET methods using flowing indigenous macroinvertebrates as test organisms (Zokufa 1998).

In this study, Chapter 4 investigated and compared the tolerance and use of selected indigenous invertebrates and *D.pulex*, to a single-substance reference toxicant. The recirculating artificial streams (Channels) used in that chapter (Chapter 4) had yet to be tested using a whole effluent. This Chapter is a first attempt at using the Channel systems and site-specific indigenous invertebrates in acute WET testing. The study involved two six week field trips to the study area, Vereeniging, thus the logistics and practicality of site-specific testing was also investigated. The aim of this Chapter was not specifically to determine the toxicity of the selected industrial effluents to *D.pulex* and selected indigenous invertebrates, but rather a case study to highlight any difficulties in using site-specific invertebrates in WET.

5.2. MATERIALS AND METHODS

The experimental methodology used for toxicity testing with indigenous invertebrates and *D.pulex* experiments is described in Chapters 2 and 4. Acute 96 h whole effluent experiments using indigenous invertebrates as test species were run simultaneously with acute 48 h *D.pulex*

experiments using the same effluents. WET experiments were conducted at the Rand Water Hydrobiology laboratories. Indigenous invertebrates were collected from two different reference sites in the Vaal River catchment (see Chapter 2 for details) and experiments were run in the artificial stream systems, the Channels (Figure 2.2). Rand Water *D.pulex* cultures were used and standardised 48 h acute experiments were set up using the same effluent samples as those used to setup the indigenous invertebrate experiments. Culture media used was dilution media for *D.pulex* experiments (as stipulated in standard methodology), and Vaal Dam water (receiving water) for indigenous invertebrate experiments.

A number of industrial effluents, from industries in the industrial areas of Vereeniging and Sasolburg, were selected for WET tests. Forty eight hour acute *D.pulex* screening tests were conducted to determine which of the industries produced the more acutely toxic effluent. Industries were chosen primarily because they discharge their effluent either directly, or indirectly via an effluent canal, into the Vaal Barrage. The selected industries were close to Rand Water laboratories. Two industries, Industry A and B, were chosen for WET testing based on the results from the acute *D.pulex* screening tests. Industry A discharges a variable effluent (Table 5.7) into the Rietspruit via an effluent canal. The effluent is known to consist primarily of heavy metals, and three experiments were conducted using this effluent. The first experiment (Experiment A1) was conducted approximately 10 and 12 weeks prior to the second (Experiment A2) and third (Experiment A3) experiments respectively. Industry B discharges a highly variable effluent, consisting mainly of PVC and nutrients, into the Taaibosspruit via an effluent canal. One experiment was carried out using this effluent.

No modification was made to either *D.pulex* or site-specific indigenous invertebrate methodologies described in Chapter 2. Only data sets where control mortalities were below 10%, and therefore acceptable according to standard methods (APHA 1992), were used in analysis. Effluent dilutions were chosen to include a control and 100% effluent. Numbers of dilutions were determined primarily by the number of Channel systems which could be set up in the laboratory. The range of dilutions in Experiments A1-3 were chosen on a 0.5 dilution range, with the addition of a 75% treatment (i.e. eight treatments were run). The range in Experiment B was chosen on a 0.7 dilution range because two more Channel systems could be set up in this experiment (i.e.

ten treatments). See Tables 5.1 and 5.2 for a summary of each experiment.

Table 5.1: A summary listing the indigenous test organism, the reference site from which they were collected and the effluent dilutions used for each acute 96 h WET experiment.

Experiment	Test organism	Reference site	Effluent Dilution (%)
A1	*C.nilotica*	Emfuleni Park	0.00, 3.12, 6.25, 12.50, 25.00, 50.00, 75.00, 100.00
A2	Baetidae	Taaibosspruit	0.00, 3.12, 6.25, 12.50, 25.00, 50.00, 75.00, 100.00
A3	Baetidae	Taaibosspruit	0.00, 3.12, 6.25, 12.50, 25.00, 50.00, 75.00, 100.00
B	*C.nilotica* Corixidae	Engelbrechtdrift weir	0.00, 6.00, 8.500, 12.00, 17.00, 24.00, 34.50, 49.00, 70.00, 100.00

Table 5.2: A summary listing the effluent dilutions for each acute 48 h *D.pulex* experiment.

Experiment	Effluent Dilution (%)
A1	0.00, 12.50, 25.00, 50.00, 75.00, 100.00
A2	0.00, 3.12, 6.25, 12.50, 25.00, 50.00, 75.00, 100.00
A3	0.00, 3.12, 6.25, 12.50, 25.00, 50.00, 75.00, 100.00
B	0.00, 6.00, 8.500, 12.00, 17.00, 24.00, 34.50, 49.00, 70.00, 100.00

In a non-renewal system, nutrient concentrations can increase to toxic levels (APHA 1992) and should therefore be determined in each treatment at the end of an experiment. Standard 48 h acute *D.pulex* experiments do not require this precaution (see Chapter 4). Due to cost constraints, and because the chemical composition of the effluent was unknown and variable, it was assumed that the nutrient concentrations were a characteristic of the effluent itself and concentrations were not measured (it was therefore also assumed that the organisms did not significantly increase nutrient concentrations through the duration of the experiment). This assumption was also based on

previous experience (Chapter 4) where the nutrient concentrations remained within acceptable levels over the experimental period.

An effluent sample was sent for chemical analysis at the start of each Industry A experiment in order to provide information on the effluent's composition, and to determine the variability in the measured chemical variables between experiments. It was accepted that the chemical analysis could not be definitive, because not all chemicals can be tested for (Roux *et al.* 1993, Heber *et al.* 1996). For example, some chemicals within the effluent may have been below detection limits and the analysis for organic compounds is very expensive (Hunt *et al.* 1992). A chemical sample was sent for analysis for Industry B, but results are not yet available.

To provide an assessment of the chemical conditions experienced by the selected indigenous site-specific organisms in the field, chemical data from Engelbrechtdrift weir was obtained from Rand Water. Data for this reference site was collected monthly from 1992 to 1998, and summarised as the median, maximum and minimum recorded concentrations for each presented chemical variable. Effluent concentrations were compared to those calculated for the reference site, the Target Water Quality Range (TWQR), the Guideline for the Protection of Aquatic Life (GPAL) and Acute Effect Value (AEV) to give an indication whether the values recorded in each effluent sample were to be considered high.

5.3. RESULTS

5.3.1. Industry A

A1 effluent appeared to be the most toxic of the three effluents tested. After 48 h exposure, *D.pulex* responded with the only monotonic concentration-response curve recorded; no concentration-mortality relationship was recorded for the other two Industry A samples (Tables 5.3, 5.5 and 5.6 and Figures 5.1-5.3). The *D.pulex* mortality in Experiments A2 and A3 appeared to be unrelated to increasing effluent dilution, and neonates did not show a concentration response to either effluent samples (Figures 5.2 and 5.3).

D.pulex was more sensitive to Effluent A1 than *C.nilotica*. A 48 h *D.pulex* LC50 was calculated at 39.1 (32.5-47.0)% A1 Effluent, whereas no 96 h *C.nilotica* LC50 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 5.3). *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 5.3: 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 LC50 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	0.0	0.0	0.0
	48	14.3	9.8	2.0	0.0	0.0	2.0	0.0	0.0
	72	18.4	11.8	2.0	0.0	0.0	2.0	0.0	0.0
	96	22.5	11.8	2.0	0.0	0.0	2.0	0.0	0.0
D.pulex	24	93.3	46.7	33.3	6.7	0.0	-	-	0.0
	48	100.0	86.7	73.3	13.3	0.0	-	-	0.0

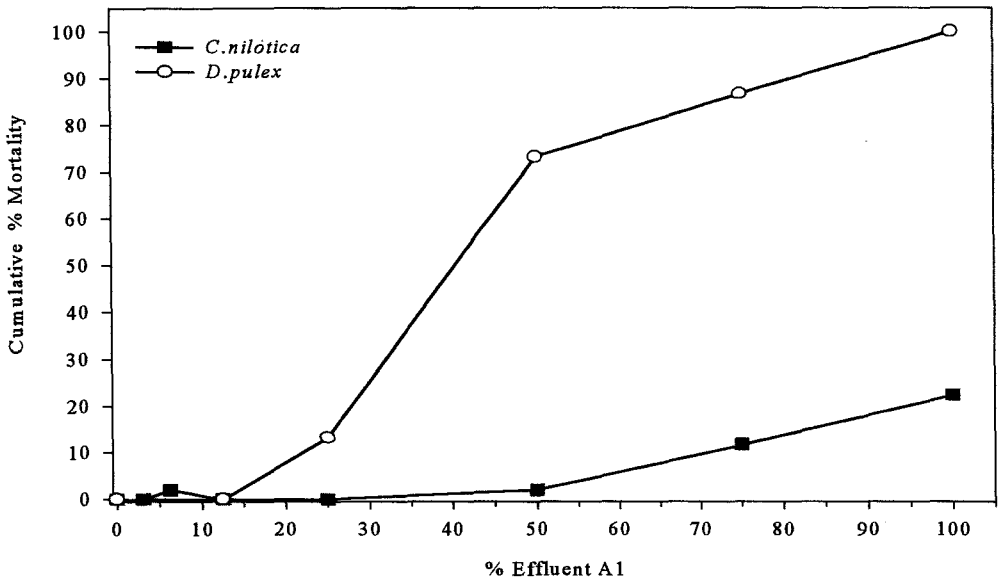

Figure 5.1: The 96 h *C.nilotica* and 48 h *D.pulex* percentage mortalities recorded in response to Effluent A1. The Spearman-Karber LC50 was at 39.1% (32.5%-47.0%) A1 Effluent. No LC50 was calculated for *C.nilotica.*

The Baetidae are a complex of species, with each species being identified after use in Experiments A2 and A3 by the Albany Museum, Grahamstown. The relative proportions of each species within the test complexes are listed in Table 5.4. The two most common species in both Experiments A2 and A3 were *Baetis glaucus* and *Cheleocloeon excisum*, with *B.quintus* making up a high percentage (24%) of the complex in Experiment A2. The response of each species within a treatment was not determined because of the low number per species within a treatment. Baetidae were therefore treated as a family for data analysis and equal tolerance assumed within the family complex.

Table 5.4: Relative percentage proportions of each species within the Baetidae complex used in experiments with Effluents A2 and A3.

Species	Effluent A2	Effluent A3
Baetis glaucus	38.5	45.0
Baetis quintus	24.0	1.7
Baetis harrisoni	3.1	3.3
Cheleocloeon excisum	32.3	46.7
Unknown	2.1	3.3

Neither the Baetidae nor *D.pulex* showed a concentration-response relationship to the A2 and A3 effluents (Tables 5.5. and 5.6, Figures 5.2 and 5.3). *D.pulex* also did not appear to be acutely effected by Effluents A2 and A3, and mortality appeared at random. For example, mortality in the 50% A2 effluent treatment was double that recorded in the 100% A2 effluent treatment (Figure 5.3). Recorded baetid mortalities also appeared random. For example, in A2 effluent the Baetidae showed mortality in the 6.25%, 12.5%, 25% and 75% effluent treatments, but no mortality occurred in the 50% or 100% effluent treatments (Table 5.5). High baetid emergence rates occurred during the experiments, resulting in low test numbers and may have contributed to the resultant non-monotonic response shown by the baetids in both Experiments A2 and A3. It is also likely however, that Effluents A2 and A3 were not acutely toxic to the baetids (Tables 5.5. and 5.6). Due to the lack of an acute toxic effect, natural variability in response to the effluents became evident.

Table 5.5: The cumulative percentage mortalities recorded at the end of each experiment for Baetidae (96 h) and *D.pulex* (48 h) in response to Industry A2 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 A2							
	Hours	100%	75%	50%	25%	12.5%	6.25%	3.12%	Control
Baetidae	24	0.0	0.0	0.0	6.7	10.0	5.9	6.3	0.0
	48	0.0	0.0	0.0	13.3	10.0	5.9	6.3	0.0
	72	0.0	21.4	0.0	26.7	20.0	17.6	6.3	0.0
	96	0.0	21.4	0.0	26.7	20.0	23.5	6.3	0.0
D.pulex	24	5.0	10.0	15.0	0.0	4.8	0.0	0.0	0.0
	48	5.0	10.0	15.0	0.0	9.5	0.0	0.0	0.0

Table 5.6: The cumulative percentage mortalities recorded at the end of each experiment for Baetidae (96 h) and *D.pulex* (48 h) in response to Industry A3 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 A3							
	Hours	100%	75%	50%	25%	12.5%	6.25%	3.12%	Control
Baetidae	24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	48	6.7	4.2	0.0	0.0	0.0	0.0	0.0	0.0
	72	6.7	4.2	0.0	0.0	0.0	0.0	0.0	0.0
	96	6.7	4.2	0.0	0.0	0.0	0.0	26.3	0.0
D.pulex	24	0.0	5.0	10.0	0.0	5.0	10.5	0.0	0.0
	48	10.0	5.0	20.0	5.0	5.0	15.8	0.0	5.0

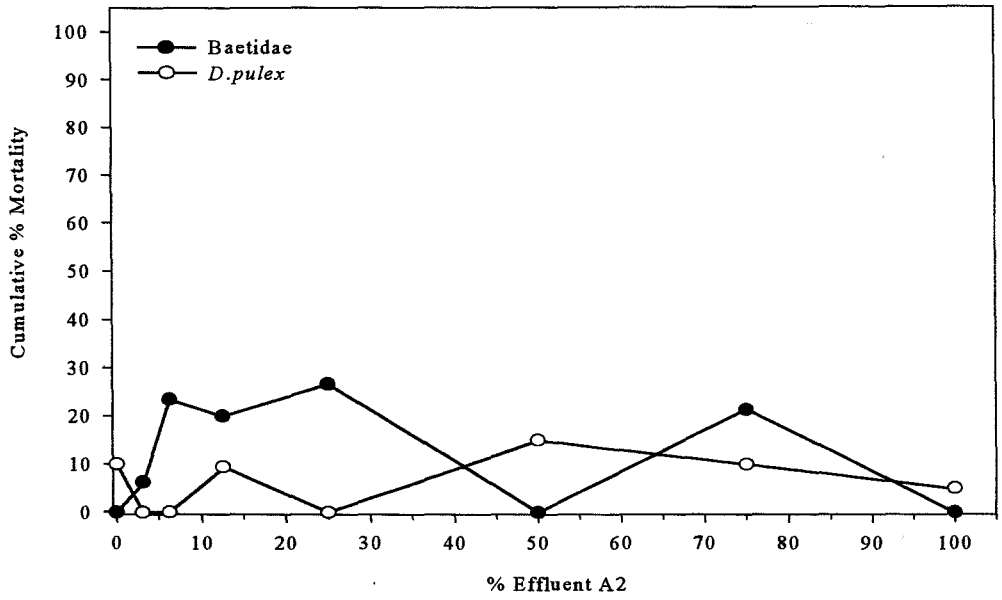

Figure 5.2: The 96 h Baetidae and 48 h *D.pulex* percentage mortalities recorded in response to Effluent A2. LC50s could not be calculated.

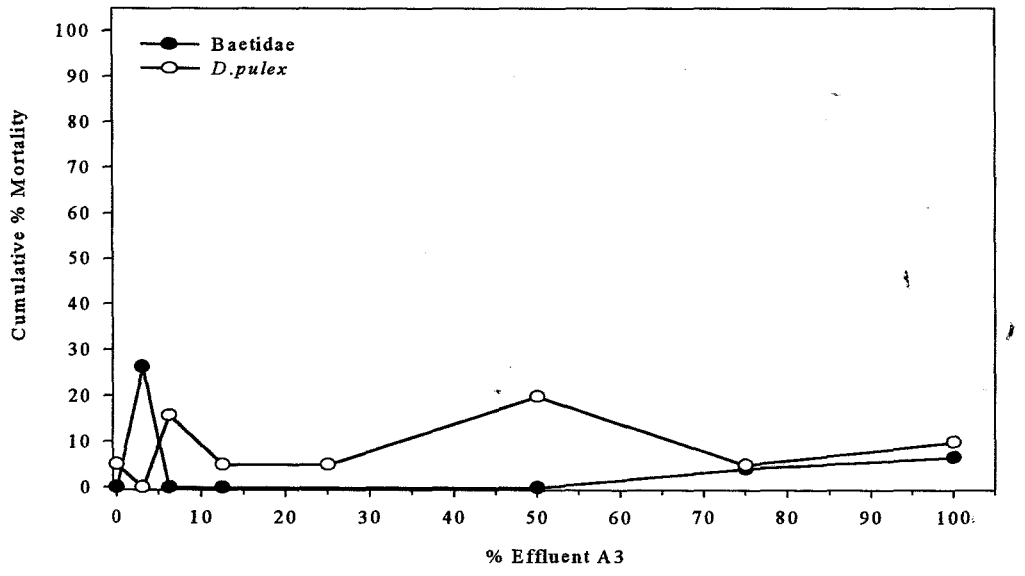

Figure 5.3: The 96 h Baetidae percentage mortality and 48 h *D.pulex* percentage mortality recorded in response to Effluent A3. No LC50s could be calculated.

Most chemical variables in Effluent A1 were greater than those measured in Effluents A2 and A3 (Table 5.7). 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 (see Table 5.7 for list of variables), 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) (DWAF 1996g), Guideline for the Protection of Aquatic Life (GPAL) (Kempster *et al.* 1980) values or both in all three effluent samples (Figures 5.4-5.7). 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.

Table 5.7: Summary of the physical and chemical variables measured for each effluent and compared to the average and maximum values recorded at Engelbrechtdrift weir and the Acute Effect Value (AEV) and the Target Water Quality Range (TWQR) and Guideline for the Protection of Aquatic Life (GPAL). Those values in bold exceed the AEV, those with a * exceed the TWQR or GPAL and those values which are italicized exceed the reference site median. ([1]DWAF 1996g, [2]Kempster *et al.* 1980).

Quality variable (mg/l)	TWQR[1]; GPAL[2]	AEV [1]	Reference Site median	Reference Site min-max	Effluent A1	Effluent A2	Effluent A3
PHYSICAL AND CHEMICAL CHARACTERISTICS							
Conductivity at 25°C (mS/m)			18.00	8.80-50.00	*400.00*	*212.00*	*220.00*
Hardness as $CaCO_3$			60.00	31.00-180.00	*920.00*		
Total Dissolved Solids	Should not change by more than 15%[1]		135.00	58.00-570.00			*1490.00*
pH	Should not vary by more than 0.5 pH units over 24 h[1]		7.90	7.30-8.80	*8.00*	*8.80*	*8.60*
Alkalinity as $CaCO_3$	>20.00				130.00	81.00	91.00
NUTRIENTS							
Ammonia	<0.01[1]; 0.02-124.00[2]	0.1[1]	0.08	0.02-1.60	***25.00****	***9.50****	***8.10****
Nitrite			0.05	0.02-0.25	*0.74*	*1.45*	*0.34*
Nitrate			0.38	0.05-7.40	*6.40*	*3.95*	*8.06*
Ortho Phosphate					<0.03	<0.05	<0.05
Total Phosphate	Should not change by more than 15%[1]		0.05	0.05-10.00	*<0.30*	*0.10*	*0.14*
MACRO-ELEMENTS							
Calcium	1000.00[2]		13.50	6.90-45.00	*250.00*	*189.00*	*228.00*
Magnesium	1500.00[2]		6.40	3.10-28.00	*71.00*	*29.80*	*32.00*
Sodium	500.00[2]		11.00	2.20-55.00	*340.00*	*200.00*	*160.00*
Potassium	50.00[2]		3.90	1.60-8.60	*100.00**	*36.00*	*35.00*
Cadmium	<0.01[1]	<0.01[1]	0.03	0.03-0.03	*<0.05*	<0.01	<0.01

Quality variable (mg/l)	TWQR[1]; GPAL[2]	AEV [1]	Reference Site median	Reference Site min-max	Effluent A1	Effluent A2	Effluent A3
Chromium	<0.01[1]; 0.01-0.10[2]	0.20[1]	0.03	0.03-0.07	*<0.05*	0.02	<0.01
Cobalt	1.00[2]		0.05	0.05-0.05	*<0.10*	0.02	<0.01
Copper	<0.01[1]; 0.01-0.20[2]	<0.01[1]	0.05	0.05-0.39	*<0.10*	<0.02	<0.01
Manganese	0.18[1]; 0.10-1.00[2]	1.30[1]	0.15	0.05-0.15	***2.00****	*0.42**	*0.38**
Lead	<0.01[1]; 0.02-0.10[2]	<0.01[1]	0.15	0.05-0.16	<0.10*	<0.13*	<0.09*
Zinc	<0.01[1]; 0.03-0.10[2]	<0.04[1]	0.05	0.05-0.33	***0.24****	***0.24****	***0.09****
Nickel	0.03-0.05[2]		0.05	0.05-0.10	*<0.10**	<0.05	0.03
Aluminium	<0.01[1] 0.10-1.50[2]	0.15[1]	0.05	27.00	*<0.10*	*0.08*	*0.06*
Boron	1.50-5.00[2]		0.05	0.05-0.23	*0.49*	*0.18*	*0.19*
Vanadium	0.50[2]		0.05	0.05-0.24	<0.01	<0.01	<0.01
Molybdenum	0.10[2]		0.25	0.03-0.14	<0.01	0.04	0.05
Active silica as SiO_2			11.00	0.00-19.00	*12.00*	11.00	9.80
Iron	Should not vary by more than 10%[1] 0.20-1.00[2]		1.10	0.03	<0.05	*2.15*	*1.35*
Arsenic	<0.01[1] 0.01-1.00[2]	0.13[1]	0.50	0.50-1.00		0.01	<0.01
Mercury	<0.01[1] 0.01[2]	<0.01[1]	0.50	0.50-0.50		<0.01	<0.01
Selenium	<0.01[1] 0.01[2]	0.03[1]	0.50	0.50-0.50		<0.01	0.01
Cyanide	<0.01[1] 0.01-5.00[2]	0.11[1]	0.02	0.02-0.11		*<0.05*	*<0.05*
Bromide	0.10[2]		0.39	0.25-3.50		<0.20	<0.20
Sulphide						<0.10	<0.10
Total Silica	50.00[2]		18.00	0.50-98.00	13.00	11.00	9.00
Sulphate	1400.00[2]		17.00	5.00-150.00		*414.00*	*420.00*
Chloride	50.00-400.00[2]		5.00	5.00-25.00	*200.00*	*358.00*	*406.00*
Fluoride	<0.75[1] 1.5[2]	2.54[1]	0.19	0.10-0.37	***3.90****	***2.81****	*2.45**
Sulphur			4.20	0.50-67.00		*151.10*	*140.00*

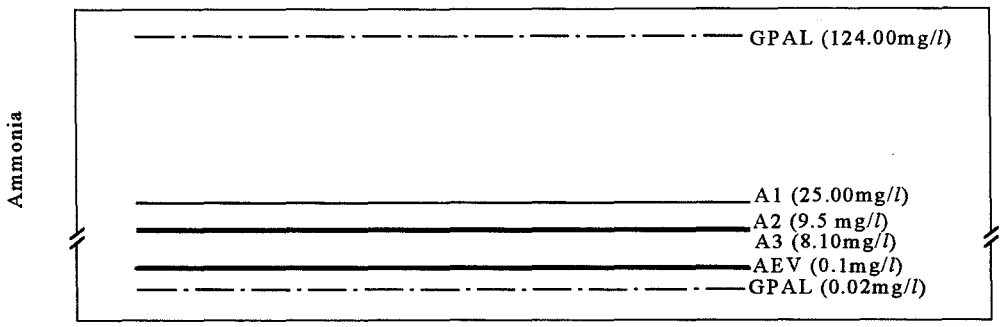

Figure 5.4: A graphic representation showing the ammonia concentrations recorded in Effluents A1, A2 and A3 in relation to the Acute Effect Value (AEV) (DWAF 1996g) and Guideline for the Protection of Aquatic Life (GPAL) (Kempster *et al.* 1980). (The y-axis plots the concentration of zinc and is to scale (note the break in the axis), but for ease of reading, the actual concentrations are plotted in the legend). All three effluents exceeded the AEV value for ammonia, but fell within the GPAL range.

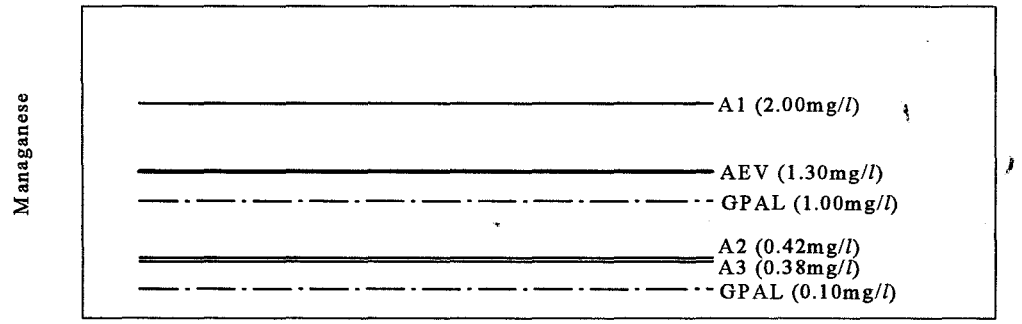

Figure 5.5: A graphic representation showing the manganese concentrations recorded in Effluents A1, A2 and A3 in relation to the Acute Effect Value (AEV) (DWAF 1996g) and Guideline for the Protection of Aquatic Life (GPAL) (Kempster *et al.* 1980). (The y-axis plots the concentration of manganese and is to scale, but for ease of reading, the actual concentrations are plotted in the legend). Only Effluent A1 exceeded the AEV value, the other two effluents (A2 and A3) fell within the GPAL and below the AEV.

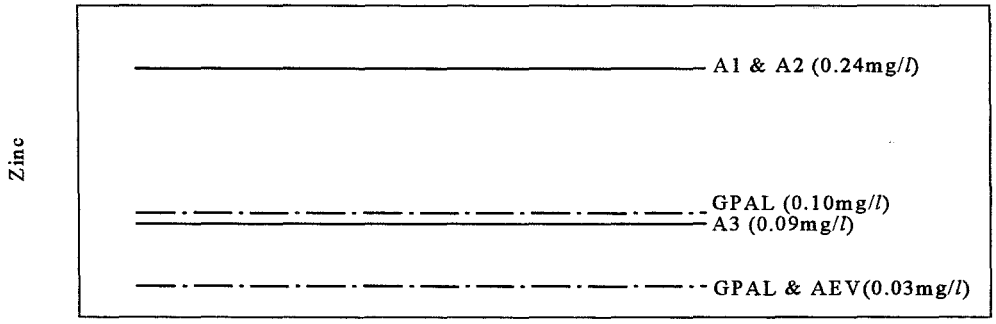

Figure 5.6: A graphic representation showing the zinc concentrations recorded in Effluents A1, A2 and A3 in relation to the Acute Effect Value (AEV) (DWAF 1996g) and Guideline for the Protection of Aquatic Life (GPAL) (Kempster *et al.* 1980). (The y-axis plots zinc concentration and is to scale, but for ease of reading, the actual concentrations are plotted in the legend). Effluents A1 and A2 exceed both the GPAL and AEV values. Effluent A3 exceeds the AEV, but falls within the GPAL range.

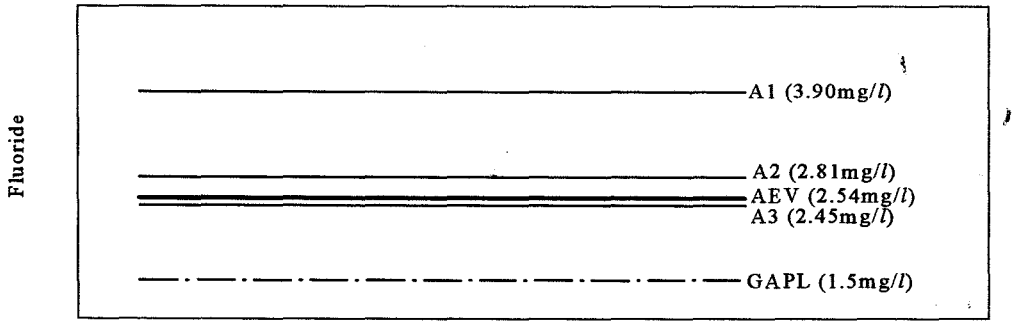

Figure 5.7: A graphic representation showing the fluoride concentrations recorded in Effluents A1, A2 and A3 in relation to the Acute Effect Value (AEV) (DWAF 1996g) and Guideline for the Protection of Aquatic Life (GPAL) (Kempster *et al.* 1980). (The y-axis plots the concentration of fluoride and is to scale, but for ease of reading, the actual concentrations are plotted in the legend). Effluents A1 and A2 exceed both the AEV and GPAL. Effluent A3 exceeds the GPAL.

5.3.2. Industry B

This effluent did not result in any significant *C.nilotica*, Corixidae or *D.pulex* mortality (Table 5.8, Figure 5.8). 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 5.8). 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 5.8: 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	0.0	0.0	0.0	0.0	0.0
	48	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	72	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	96	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Corixidae	24	0.0	20.0	10.0	0.0	0.0	0.0	20.0	0.0	0.0	0.0
	48	0.0	20.0	20.0	0.0	0.0	0.0	20.0	0.0	10.0	11.1
	72	0.0	30.0	20.0	20.0	0.0	10.0	20.0	0.0	10.0	11.1
	96	10.0	40.0	40.0	40.0	20.0	40.0	40.0	30.0	50.0	11.1
D.pulex	24	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.0
	48	5.0	5.0	0.0	0.0	15.0	5.0	0.0	0.0	0.0	10.0

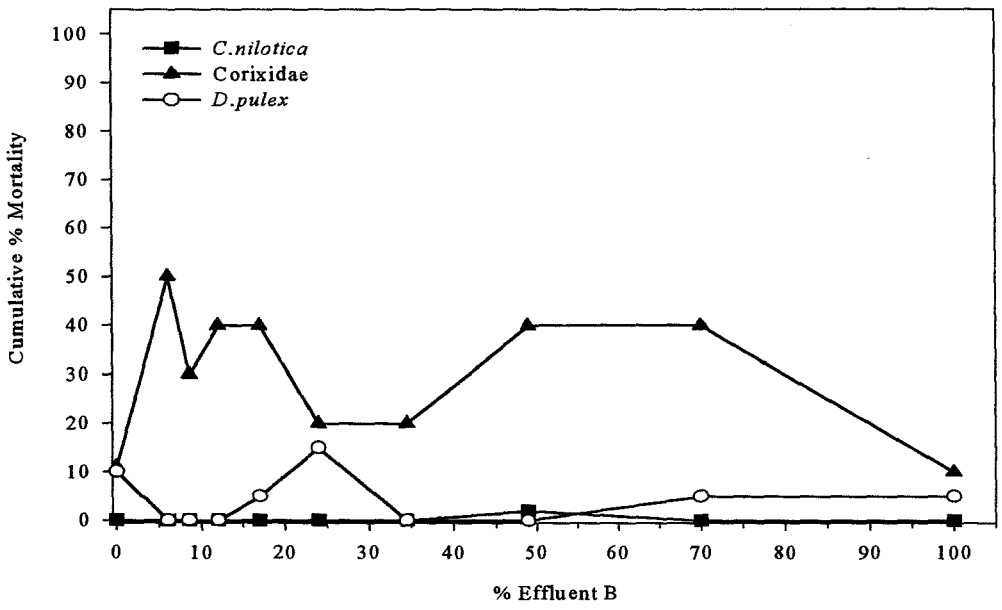

Figure 5.8: The 96 h *C.nilotica,* Corixidae and 48 h *D.pulex* percentage mortalities in response to Effluent B. LC50s could not be calculated.

5.4. DISCUSSION

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). This natural variability is expected in a field-collected population, but is expected to be reduced in a homogenous population, such as the laboratory cultured *D.pulex* (Woods *et al.* 1989). However, substantial differences do exist among individuals within a cultured population (e.g. *D.pulex*) due to differences in the condition of individuals (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.

These experiments highlight a limitation of this study. When determining the toxicity of an effluent, chronic or long-term toxicity tests should be carried out, as the effluent may not be acutely toxic. Chronic toxicity testing was, however, beyond the scope of this project and it is recommended by this study that chronic whole effluent methodology be investigated (using both *D.pulex* and indigenous invertebrates).

Although chemical samples for each effluent were sent for analysis and a chemical profile examined, little information was gained in terms of the effluent's potential toxicity. Chemical profiles cannot predict the interactions between effluent components, nor the effect of these

interactions on the toxicity of the effluent (Cairns and Neiderlehner 1990). Chemical speciation models can be used to predict what ionic forms (or species) of an inorganic chemical will take when present in a water body (MacKay *et al.* 1995) and are important in the understanding of metal toxicity (Filella *et al.* 1995). Speciation models are however reliant on extensive chemical knowledge of the receiving water and toxicant. Full chemical profiles of a whole effluent are expensive and often not comprehensive (e.g. organic chemicals are difficult to detect), thus chemical speciation is not always possible or practical. A chemical profile is however important if an effluent is to be monitored over time or if the effluent is to be broken down in an attempt to identify the toxic component (i.e. In Toxicity Identification Evaluation (TIE) and Toxicity Reduction Evaluation (TRE) processes) (Burkhard and Ankley 1989, Doi and Grothe 1989, Chapman 1995).

In South Africa, single-substance and WET testing are new tools in the evaluation and management of toxicants (Van Vliet 1996). The IWR initiated the use of indigenous macroinvertebrates as test species, and consequently the use of the Channels as test systems, in response to the highlighted need for research into the tolerance of South Africa's indigenous aquatic organisms to toxicants (Moore 1990, Palmer *et al.* 1996). Methodology for the use of indigenous invertebrates was developed for the evaluation of single-substance toxicants, such as metals, and this study was the first to investigate the site-specific application of this method for the toxicity of whole effluents. The usefulness of site-specific indigenous invertebrates as test species in WET testing could not however be properly evaluated due to the effluents not resulting in an acute toxic effect. The advantages and limitations of the methodology are discussed in Chapter 6. An aim of this Chapter was to use WET to further highlight any difficulties in using site-specific indigenous invertebrates as toxicity test species, and these, together with those noted in Chapter 4, are discussed in Chapter 6.

CHAPTER 6

THE PREDICTIVE VALUE OF TOXICITY TESTS AND CONSIDERATIONS FOR THE SETTING OF WATER QUALITY GUIDELINES FOR THE PROTECTION OF AQUATIC ECOSYSTEMS

In South Africa water resources are limited (Schulze 1986), and it is recognised that there is a limit to the degree of utilisation by man that is sustainable by aquatic systems (Ashton *et al.* 1995, DWAF 1997). The resilience of aquatic ecosystems to disturbance, such as that caused by pollution, depends on maintaining a certain level of ecological integrity and function, and it is this level which is termed the Resource Base (MacKay 1998). Without protecting the Resource Base, the long term sustainable use of water resources by man, for domestic, agricultural and other uses, will not be possible. The South African Bill of Rights gives all South Africans the right to an environment that is protected for the benefit of present and future generations (DWAF 1997). To ensure that this right is achieved, the needs of the environment, which will ensure the maintenance of the Resource Base, must be determined, quantified and enforced.

The first step toward the quantification of the needs of the aquatic environment was the publication of the South African Water Quality Guidelines for the Aquatic Ecosystem by the Department of Water Affairs and Forestry (DWAF 1996g). The information contained within these guidelines provides a starting point for management decisions, but these guidelines are by no means complete and those water quality constituents included were selected according to the availability of cause-effect toxicity data and/or local knowledge. Where local data was unavailable, international databases were used and safety factors included (DWAF 1996g). Due to limited information on the water quality requirements of South Africa's aquatic environments, there is a need for South Africa to develop a database quantifying the toxic effects of substances on the aquatic system (Moore 1990). Toxicity tests are an invaluable tool in the determination of toxic effects to aquatic organisms (Richardson and Martin 1994) and can therefore be used as a tool in the assessment of toxic effects of chemicals on South Africa's aquatic environments. The use of aquatic toxicology is relatively new in South Africa (DWAF 1998) and the choice of appropriate test species (field-caught vs laboratory-cultured) is still under investigation. This

study investigated the use of a cultured organism, *Daphnia pulex*, and selected indigenous invertebrates for use in aquatic toxicity testing. The findings and limitations of each method are discussed in the context of the predictive value of toxicity tests to determine in-stream toxic effects, and in the use of toxicity test results in setting water quality guidelines for the aquatic environment.

6.1. 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 is their convenience (Leeuwangh 1978). In South Africa, aquatic toxicology is relatively new and because standardised methodology exists for laboratory cultured species (USEPA 1979, APHA 1992), the use of these organisms is more extensive than that of field caught indigenous organisms. As a result, the sensitivities of indigenous invertebrates is not yet well documented in South Africa (Moore 1990). The suitability of these cultured organisms (e.g. *D.pulex*) for estimating in-stream toxic effects is however still under investigation. Thus an aim of this study was to investigate the advantages and limitations of using *D.pulex* and indigenous site-specific invertebrates in toxicity testing. These and recommendations concerning the use of *D.pulex* and indigenous site-specific invertebrates in aquatic toxicity testing in South Africa are discussed below.

6.1.1. 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). For example, if the experimental system is static, only species which live in still waters can be used as test organisms. The IWR have developed artificial streams suitable for toxicity testing using riffle-dwelling organisms (Palmer

et al. 1994). These systems, the Channels, were used in this study thus allowing the choice of species adapted to dwelling in flowing environments. As flowing water environments have been characterised as one of the more dominant aquatic environments in South Africa (DWAF 1997) this was 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. Eekhout *et al.* (1996) defined a reference site as a relatively unimpacted site that can contribute toward a database used to define the best available physical habitat, water quality and biological parameters for a river. In other words the 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. Distance between the reference site and laboratory is also an important consideration, as test animals need to be transported back to the laboratory relatively quickly in order to minimise stress. It may therefore be necessary to settle for the best available site, which may not necessarily reflect the "most natural" conditions. Reliability of the reference site became an important consideration in this study. One site was subjected to changes in flow for example, and changes in community composition resulted in test species being 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 reliability may be a factor to consider in the choice of test species.

Reference site, species abundance and availability, and suitability to experimental systems were all important considerations in this study with regards to the use of indigenous invertebrate species as test organisms, and it is recommended that these be thoroughly investigated before using indigenous invertebrates in aquatic toxicity tests. Although the above factors may make the use of indigenous invertebrates impractical, 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).

6.1.2. 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. Chapter 3 highlighted the importance of controlling and monitoring abiotic factors, such as temperature, which may influence toxicity results. 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.

A disadvantage of laboratory cultured populations is that they may not accurately reflect the susceptibility of indigenous organism populations. It is therefore important to compare the susceptibility of laboratory species to that of indigenous invertebrates. In the comparisons made

between *D.pulex* and the selected indigenous invertebrates (Chapter 4), *D.pulex* was more sensitive than the selected indigenous invertebrates to the reference toxicant zinc. These results suggested that guidelines set for zinc according to the tolerance of *D.pulex* would protect the indigenous invertebrates selected in this study. However, it is important to note that inherent toxicity was determined for *D.pulex*, whereas relative toxicity was determined for the selected indigenous invertebrates. Also the indigenous invertebrates selected were not chosen according to their sensitivities, but for their abundance and may therefore be among the more tolerant of the indigenous aquatic community.

The frequency of toxicity testing and sampling have obvious consequences if toxic evaluations of a whole effluent are to be conducted, and therefore has important implications on test design and in the choice of test species. *D.pulex* acute and chronic experiments are quicker to conduct and are therefore more attractive as a potential test species for whole effluent characterisation and compliance monitoring (Leeuwangh 1978, Janssen and Persoone 1993). This is advantageous when determining compliance to a whole effluent discharge licence for example, because if conditions stipulated in the licence are not being met, corrective action by the regulatory agent can be enforced quickly. The potential for environmental damage to occur is greater when waiting for the results from a 96 h acute toxicity test (e.g. mayfly acute toxicity test), vs a 48 h *Daphnia* test.

6.1.3. 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, DWAF 1996g), as different species are known to exhibit different susceptibilities to the same chemical (Cairns *et al.* 1975, Sloof 1983, Ewell *et al.* 1986, Hickey and Martin 1995, Toussaint *et al.* 1995, 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.

6.2. TOXICITY TESTS

It is assumed that aquatic laboratory toxicity tests (both single substance and whole effluent) are predictive of effects in receiving waters (Denton and Norberg-King 1996, Dorn 1996). That is, it is assumed that laboratory test results (using both site-specific field-caught and standard laboratory test species) are reflective of the toxic impacts experienced in the field, and that an end-point determined in the laboratory can be extrapolated to the field. The USEPA, in an attempt to determine if WET tests were predictive of in-stream effects, analysed data from approximately 60 industrial and municipal water treatment plant discharges at eight sites across the USA (Wall and Hanmer 1987). It was concluded that toxicity tests conducted on a minimum of three to five test species from a range of taxonomic groups, do predict whether or not effluents will adversely effect a receiving water. The toxicity tests included standard laboratory species, such as the fathead minnow, *Pimephales promelas*, *Ceriodaphnia dubia* and the plant species, *Selenastrum capricornutum*. These predicted effects were confirmed by in-stream biosurvey data suggesting that laboratory toxicity test end-points can be extrapolated to the field (Wall and Hanmer 1987). The USEPA's 1984 national policy document, the Policy for the Development of Water Quality Based Permit Limitations for Toxic Pollutants, recommended that toxicity tests be used in toxic management programmes. As a result of this recommendation, the USEPA has increasingly promoted the use of single substance toxicity and WET testing (Warren-Hicks and Parkhurst 1992).

The fundamental assumption behind the use of single species toxicity tests is that by searching for and testing the "most sensitive species" and setting standards to protect this species, all other species and activities at higher levels of biological organisation will also be protected (Cairns

1986). The growing appreciation of the complexity of ecosystems has, however, resulted in the questioning of the "most sensitive species approach" and on the exclusive reliance on single-species toxicity tests to determine guidelines for the protection of aquatic communities from industrial (and other) sources of pollution (Pontasch *et al.* 1989a, Roux 1994). Some of the disadvantages and criticisms against single-species toxicity tests are listed below, the relevance and the findings of this study, and the application of toxicology in South Africa is discussed.

6.2.1. Sources of experimental variability

Experimental variability due to test organism

The greatest criticism of single species toxicity tests is the fact that test results with one toxicant can be highly variable within a species (Cairns 1983). Variability in both physical and biological parameters are kept to a minimum in laboratory toxicity tests. Genetically homogenous laboratory cultured organisms, such as *D.pulex,* are therefore popular test species in aquatic toxicology, because it is assumed that biological variability will be reduced due to genetic homogeneity between individuals (Pontasch *et al.* 1989a). These cultures however lack the adaptive capability of natural heterogenous populations (Hickey and Vickers 1992), thus the realism/validity of end-points using these cultures can be questioned. It has therefore been argued that indigenous field caught organisms should be used as test species instead of cultured laboratory species, because they are representative of the affected aquatic system. However, indigenous populations obviously show greater variability in tolerance around a measured end-point than a genetically homogenous population (Woods *et al.* 1989). In the past, the primary reason behind the choice of a test species was its ease and robustness to culturing conditions, rather than its suitability as an indicator of the effected ecosystem, its sensitivity to pollutants or ability to indicate subtle ecological effects (Gray 1989). Laboratory cultures are able to provide test species at the time of the test, the organisms are of a known age and background, and because the life-history and existing culture conditions are well documented, there is better control and quality assurance. As the responses of the cultured organisms are expected to be more consistent between tests (Leeuwangh 1978, Janssen and Persoone 1993, Koivisto 1995), laboratory cultures are more extensively used in aquatic toxicology than field caught indigenous invertebrates.

This study and others (Rue *et al.* 1988, Baird *et al.* 1989) have shown that unexplained variation can occur using laboratory cultured organisms, even though a methodology has been standardised (Chapter 2). The variability in cultured populations, such as *D.pulex*, may be due to genetic differences, culture technique (e.g. diet) or physical factors (e.g. temperature) (Weber 1991). In this study it was highlighted that if factors, such as temperature, are not kept constant, the result/impact on toxicity test end-points can be significant. For example, in Experiment 4 (Chapter 3), LC50s for all four *D.pulex* cultures were significantly greater than subsequent experiments and a suspected reason for this was the temperature fluctuations experienced during the experiment. Other studies have also highlighted the significant influence of temperature on tolerance to chemicals (Cairns *et al.* 1975, Shcherban 1977, Cairns *et al.* 1978, Braginskiy and Shcherban 1979, Stephenson and Watts 1984, Lewis and Horning 1991). Strict control over abiotic factors must be exercised if their contribution to experimental variability is to be reduced. This therefore requires appropriate laboratory facilities and continual monitoring.

It is assumed that because *D.pulex* cultures are genetically homogenous, variability around the measured end-point (e.g. LC50) should be minimal (Woods *et al.* 1989). However, it is unlikely that cultures remain homogenous over time. If a culture is stressed in any way, through the build up of metabolic waste for example, males are produced, thus creating the potential for sexual recombination and genetic changes within the culture. Mutations may also result in a genetic change (Baird *et al.* 1989). Thus, over time, variability around an end-point may be attributed to genetic changes within a culture. This study was unable to investigate this hypothesis due to confounding factors experienced during the experiments, however it is suspected that genetic changes over time play a role in the variability experienced between experiments within a culture. It has been shown in other studies that the difference between two cultures in their tolerance to a chemical was due to the collection of *D.pulex* from different localities (i.e. cultures were initiated with females collected from different populations) (Buikema 1983, Woods *et al.* 1989). For example, one of the major factors identified as contributing to the measured differences in LC50s between *D.pulex* cultures to the same chemical was genetic variability between cultures, with a number of laboratories having obtained cultures directly from different wild populations (Baird *et al.* 1989). In other words, cultures were set up from a female caught in a nearby location and not subcultured from a pre-existing culture. It can be assumed that these cultures were

different from one another genetically, with the implication that the difference in genetic make-up between the cultures may cause different expressed susceptibilities to the same toxicant. Laboratories in South Africa have initiated *D.pulex* cultures with females from different populations. Experiments in this study were confounded by other factors (e.g. temperature) (Chapter 3), and it can therefore not be stated with confidence that the noted differences in zinc tolerance between cultures was due to population differences or culture age differences. However, the differences in genetics between these cultures may have contributed to the differences measured in the LC50s and if these cultures are used in interlaboratory studies their genetic differences may contribute to inter-laboratory variability. A recommendation would therefore be that cultures are subcultured from the same female.

Regular testing with a reference toxicant is recommended in order to establish if a laboratory culture undergoes significant changes in tolerance due to possible genetic changes within the culture over time. A database can also be generated to determine baseline tolerance to the reference chemical, thus providing a measure of variability over time.The influence of age (Blaise *et al.* 1988, Hoekstra *et al.* 1993), nutritional status and pre-exposure to the chemical (Naylor *et al.* 1990, Crane 1995), factors known to influence the tolerance to a chemical, can be controlled in laboratory reared test organisms. For example, *D.pulex* neonates, less than a day old, are hatched specifically the day before the experiment, and they are not fed before the test is initiated (APHA 1992).

The variability associated with field caught organisms however can be more difficult to control. It is for example, not practically possible to age indigenous invertebrates without detailed life history data. However, in an attempt to control for age, individuals of a similar size are chosen for the experiment (APHA 1992) (Chapter 2). Also during acclimation, it is hoped that the test organisms will reach the same nutritional status, as they are not fed before or during the acute experiment (APHA 1992).

Another factor known to significantly influence experimental variability is genetic differences between field populations (Naylor *et al.* 1990, Crane 1995). It is therefore recommended that a field-caught test population be collected from one reference site only, as this may help reduce a

potential source of organism variability. The choice of the reference site is therefore important, as it must be able to provide enough test organisms for testing. In this study, variability between experiments (i.e. LC50 variability over time) was not tested for as the indigenous invertebrate species chosen largely depended on their availability at the time of testing. As a result the same species were not used in all experiments (Chapters 4 and 5). This illustrates a practical disadvantage of using field collected organisms.

Field populations are also subject to natural conditions and may therefore not be available when experiments need to be conducted. Different species are also known to exhibit different tolerances to the same chemical (Cairns *et al.* 1975, Sloof 1983, Ewell *et al.* 1986, Hickey and Martin 1995, Toussaint *et al.* 1995, McKinney and Wade 1996, Roseth *et al.* 1996). It is therefore important to ensure the test population is one species and not a complex of species, as it is difficult to speciate a population such as the baetids at the onset of the experiment. The differences in species tolerance within a species complex may contribute to variability between experiments (Chapters 4 and 5). It is also difficult to speciate a species complex at the onset of the experiment. A recommendation is that all individuals are identified to species level to confirm a single-species population.

It has been argued that the variability around measured end-points (e.g. LC50 values) between experiments should not be viewed as a limitation (Depledge 1990). A measure of individual variation in tolerance to a chemical within a test population is often missing or suppressed in toxicity studies and it has been argued that consideration of this biological variability should be incorporated into the determination of a populations' tolerance to a chemical or toxicant (Hallam *et al.* 1990). Guidelines for protecting ecosystems may be more realistic with the incorporation of biological variability, experienced at the population and ecosystem scales. However, it is also argued that variability around LC50 values (or other toxicity test end-point) reduces the confidence in the test method (Warren-Hicks and Parkhurst 1992). The question of what level of variability around a measured end-point is due to individual variability (i.e. biological variability) and what may be a reflection of experimental error (e.g. due to the method used), remains undefined, i.e. biological significance is difficult to define and toxicologists rely on statistics (Giesy and Alfred 1985, Thursby *et al.* 1997). In this study for example, the within variability (i.e. the

variability (around LC50s) between experiments within one population) was greater than the variability measured between populations (i.e. the variability between cultures) (Chapter 3). The CSIR *D.pulex* culture in Experiment 5, for example, showed an LC50 of 0.32 mg/*l* Zn which, according to the APHA formula (Chapter 2), was significantly different to that measured for the IWQS culture (0.22 mg/*l* Zn), similar results were obtained in Experiment 6. The difference between the two LC50 values equalled 0.10 mg/*l* Zn in Experiment 5 and 0.13 mg/*l* Zn in Experiment 6. This difference is less than that recorded between Experiments 5 and 6 within one culture (i.e. a difference of 0.15 mg/*l* Zn was recorded between Experiments 5 and 6 for the CSIR culture, similarly a difference of 0.38 mg/*l* Zn was recorded between experiments in the IWQS culture) (Experiment 6: CSIR LC50 = 0.47 mg/*l* Zn; IWQS = 0.60 mg/*l* Zn). In other words, the experimental variability within a culture, which was assumed to be genetically homogenous, was greater than that recorded between two different populations assumed to be genetically different. Can the difference between the CSIR and IWQS cultures therefore be considered biologically significant, although calculated as being statistically different, when the difference within one population was greater than that calculated between populations? Skalski (1981) has argued that the differences measured among two or more biological populations, although statistically different, may not be biologically significant. There is therefore a need for further research to determine the differences between biological and statistical differences and what level of variability between end-points can be considered biologically significant.

Experimental variability due to the toxicant

A further source of variability between experiments (other than those attributed to the test organism) may be associated with the toxicant. Errors may occur in the making up of single-substance toxicant stock solutions and it is therefore important to be able to quantify the exact amount of toxicant test organisms are exposed to. The variability between experiments in Chapter 3 illustrates this point clearly. Chemical analysis was not carried out on the stock solutions made up for the first three experiments because standard *D.pulex* methodology did not require chemical analysis. After a high magnitude of variation between *D.pulex* Experiments 1-3 and Experiment 4, chemical analysis on the new stock solution was carried out to determine the exact concentration of zinc. Therefore it became impossible to compare the first three experiments with

subsequent experiments. If exact exposure concentrations are known, differences in stock solutions become accounted for and can potentially be eliminated as a source of variability between experiments. Contaminants may also influence stock solutions of a single-substance toxicant (Rand *et al.* 1995). It is suggested therefore that chemical profiles on all stock solutions are carried out, to confirm exposure concentration and to identify contaminants.

Acceptable levels of variability between single-substance toxicity experiments need to be determined and the use of an uncertainty estimate, such as the application of safety factors, are included in single-substance guidelines to help compensate for the inherent variability experienced between tests (Dorn 1996, DWAF 1996g).

Minimising the variability between end-points over time in WET tests is more difficult than in single-substance toxicity tests because of the continuously changing nature of the effluent (Rand *et al.* 1995). Effluents are made up of numerous chemicals and are thus complex in composition. They can also be highly variable over time, making toxic evaluations difficult (Warren-Hicks and Parkhurst 1992, Dorn 1996). No established criteria exist stipulating the acceptable levels of variability in a WET test (Parkhurst and Mount 1991) because of the inherent variability in effluent composition experienced over time. Average intra-laboratory coefficients of variation for acute WET tests have been recorded as 17%, with as much as 135% variability being recorded between experiments (Parkhurst and Mount 1991). Such high variability between experiments can be expected if the composition of the effluent is highly variable, thus understanding the effluent's variability may be more important than evaluating one toxic result as a significant toxic event (Lave and Gruenspecht 1991, Roux 1994, Grothe *et al.* 1996). It may therefore be important to incorporate a series of WET tests over time before setting a discharge licence. The presumption of predicting receiving water effects based on one or two WET laboratory tests is inappropriate unless a significant pattern of toxicity can be established (Dorn 1996).

The sampling and testing regime are therefore important in establishing the toxic potential of an effluent. A number of different sampling methods have been proposed by which to characterise effluent variability (Slabbert *et al.* 1996), although it is recognised that the frequency of sampling is largely dependent on the effluent in question (Heber *et al.* 1996). Slabbert *et al.* (1996) suggest

a sampling protocol which involves the monthly testing of an effluent over 12 months in order to characterise the effluent's variability and toxic effects. Highly variable effluents may have to be sampled daily (maybe even hourly) in order to be confident that the most toxic plug is sampled and tested. Some effluents are sampled on the spot (grab samples), i.e. the samples are characteristic of the effluent only at the time of sampling; whereas composite sampling involves the collection of equal volumes of effluent over a set period and the mixing of these samples into one effluent, thus attempting to characterize the effluent over the period sampled (Grothe *et al.* 1996). In this study, spot effluent samples were taken because the aim of WET tests in this study was not to characterise the selected effluent's toxicities, but to highlight potential difficulties in WET testing using indigenous invertebrates and *D.pulex*. It is however recommended by this study that both spot and composite samples of an effluent are evaluated, to accurately characterize the effluent's toxicity potential. This study also recommends the use of chronic testing when establishing the toxic potential of an effluent, as experiments in Chapter 5 illustrated that not all whole effluent is acutely toxic.

6.2.2. Toxicity test design

There are two basic approaches to toxicity test design, the unreplicated regression design and the replicated ANOVA design. The regression design is argued to allow more treatments to be tested, thus including more "partial kills" on a concentration-response curve and allowing for more rigorous end-point (e.g. LC50) determination (Gerhardt pers comm.). The ANOVA design requires at least three replicates (APHA 1992), thus if space or test population numbers are a constraint (as they were in this study), fewer treatments can be run. Replicates do however provide some characterisation of test population variability within a treatment (Stephan and Rogers 1985).

Preference was given to the regression design in this study. Both *D.pulex* neonate availability (Chapters 3 and 5) and space in which to run the experimental systems for the indigenous invertebrates (Chapters 4 and 5) were limiting factors. It was decided that running more (unreplicated) treatments was preferable to running only a few replicated treatments. Unfortunately, the sacrifice of an unreplicated design is that there is no measure of variability

is envisaged by DWAF that an integrated management strategy be implemented, which takes advantage of a number of methodologies to determine and monitor water quality in our rivers (Conley 1990). Biological assessment methods (e.g. SASS), for example, can be used to assess the integrity of the affected aquatic community and monitor the changes taking place within a community in response to a released toxicant. They therefore compensate for some of the inability of toxicity tests (both single-substance and whole effluent) and chemical specific programmes to predict actual receiving water impacts (Heber *et al.* 1996). It is also important, once having determined the impact of a chemical/toxin on the environment, to try and reduce its effect, particularly in South Africa where waste water is required by law to be returned to the stream of origin (Act 54, 1956). Numerous methods, such as Toxicity Identification Evaluations (TIE), help identify the toxic components of an effluent (Burkhard and Ankley 1989, Chapman 1995), enabling the industry/polluter to reduce the toxicity of their effluent (i.e. Toxicity Reduction Evaluation (TRE)) (Doi and Grothe 1989, Goodfellow *et al.* 1989, Schimmel *et al.* 1989, Chapman 1995).

6.4. CONCLUSIONS AND RECOMMENDATIONS

It is recommended by this study that the setting of discharge licences and environmental water quality guidelines should therefore 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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4.4. DISCUSSION

Cairns (1980) argues that if one wishes to know the effects of a chemical on living organisms, the effects must be determined by a direct measurement made upon the organisms themselves. Therefore, when determining guidelines aimed at protecting the aquatic ecosystem from pollution, a measurement of the aquatic ecosystem's tolerance to the chemical must be determined. For this reason numerous studies have used aquatic insects in toxicity tests (Phillips 1978, Anderson *et al.* 1980, Hare and Campbell 1992, Brooks *et al.* 1995, Gerhardt and De Bisthoven 1995, Monda *et al.* 1995, Pinel-Alloul *et al.* 1996, Sibley *et al.* 1997), and when reviewed by Maltby and Calow (1989), toxicological data showed that invertebrates were more commonly used in aquatic toxicity tests than fish, which were traditionally the most common toxicity test species.

Ephemeroptera (mayflies), often used as toxicity test organisms (Van Hassel and Gaulke 1986, Diamond *et al.* 1992, Quinn and Hickey 1993, Hickey and Martin 1995), and freshwater shrimp species are considered ecologically important components of the invertebrate community (Hart 1980), and are therefore often used in toxicity testing (Correa 1987, Hatakeyama and Sugaya 1989). Three different mayflies, *A.peringueyi* (Heptageniidae), *E.elegans* (Leptophlebiidae) and Baetidae, and the shrimp *C.nilotica* were selected for use as test organisms in this Chapter. They were chosen as potential test organisms because of their abundance at the reference site and their suitability to the test environment in the recirculating Channels.

Of the indigenous invertebrates selected in this study, the baetids and *E.elegans* appeared to be the most sensitive to zinc. The fact that *E.elegans* appeared to be one of the more sensitive of the indigenous invertebrates used in this study, contrasts with other studies which suggested that the Leptophlebiidae are fairly tolerant to chemicals (Gerhardt 1994). A study comparing the leptophlebiid, *Leptophlebia marignata,* and the baetid, *Baetis rhodani,* found that the leptophlebiid was more tolerant with respect to cadmium and lead than the baetid (Gerhardt 1992). In this study, the tolerance to zinc was not significantly different between the baetids and *E.elegans.* It is difficult to generalise about the sensitivity of a family, as species within a family may show different tolerances and the same species may be tolerant to one chemical but sensitive to another (Toussaint *et al.* 1995, McKinney and Wade 1996, Roseth *et al.* 1996). For example,

Hatakeyama and Sugaya (1989) noted that the shrimp, *Paratya compressa improvisa,* appeared more sensitive to herbicide toxins, but was more tolerant to heavy metal toxins when compared with *D.magna*. Thus a species should not be dismissed as a potential toxicity test species because others within the same family are shown to be tolerant to certain chemicals.

A.peringueyi was the most tolerant of the indigenous invertebrates tested to zinc, in terms of 96 h LC50s, 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. Also, when compared with two other freshwater shrimp species in the literature, *Artemia salina* (Govindarajan *et al.* 1993) and *Mysidopsis bahia* (Lussier *et al.* 1985), *C.nilotica* is more tolerant to zinc and therefore not a good indicator of zinc toxicity.

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. Pontasch and Cairns (1989) experienced a similar problem, finding that nearly 80% of *Baetis* sp. emerged from control streams during their study. 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.

Aquatic toxicology is still a relatively new field in South Africa (DWAF 1998). Although there has been an international trend toward using standardised cultured organisms in the setting of water quality guidelines (DWAF 1996g), the decision as to whether or not standardised organisms are representative of those in the field is still to be made for South African conditions (Scherman and Palmer in press). An aim of this study was therefore to compare the sensitivity of the widely used and standardised *D.pulex* acute toxicity test methodology with that of acute indigenous

invertebrate toxicity testing, using zinc as the reference toxicant.

The *D.pulex* were found to be more sensitive to zinc than the indigenous invertebrates in this study and had lower LC50 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.

A secondary aim of this chapter was to compare the use of different dilution waters in the indigenous macroinvertebrate test methodology. Four experiments, two with Vaal Dam water and two with dechlorinated tap water as dilution media, were run. The results indicated that dechlorinated tap water was unsuitable as a dilution medium. Most of the test organisms died during the acclimation period, indicating that this water was highly toxic to the test organisms. At the time of testing the Vaal Dam was experiencing algal blooms and Rand Water, as a result, was chlorinating the water heavily. Although the water was bubbled for between two and four days, high chlorine levels may have caused recorded mortalities. The significance of this finding is that test organism sensitivity to the dilution water should be investigated prior to the initiation of toxicity testing.

CHAPTER 5

A CASE STUDY: WHOLE EFFLUENT TOXICITY TESTING

5.1. INTRODUCTION

Aquatic toxicology aims to determine at what dosage and under what conditions an organism is influenced adversely by a toxicant, and makes the use of laboratory toxicity tests to investigate concentration-response relationships. In the field, aquatic organisms are exposed to numerous chemicals and rarely to a single chemical or toxicant, and the exposure to a chemical mixture may be sequential (e.g. pulses of different chemicals) or simultaneous (e.g. whole effluent) in time. Exposure to a mixture of substances may result in a biological response which is quantitatively or qualitatively different from that expected from the action of each substance on its own (Rand *et al.* 1995), and the toxic response may be magnified or reduced. For example, an additive effect occurs when the combined effect of two chemicals is equal to the sum of the individual chemicals applied alone. When the combined effect of two chemicals is much greater than the sum of the effects of individual chemicals applied alone, a synergistic effect has occurred. Antagonism occurs when the two chemicals interfere with each others' toxic action, and potentiation occurs when one chemical has a toxic effect only when applied with the other chemical (Rand *et al.* 1995). The toxic relationship between a substance and exposed population is therefore complex and further complicated when a mixture of chemicals (e.g. whole effluent) is discharged into the environment, as these chemicals interact with one another, the surrounding water and with other discharged chemicals in the receiving water system (Filella *et al.* 1995).

Whole effluent toxicity (WET) testing evaluates the toxicity of effluents to aquatic test organisms. The toxicity tests incorporate chemical interactions between the substances within the effluent mixture and their effects on the organism as a whole. The USEPA's 1984 national policy document, the Policy for the Development of Water Quality Based Permit Limitations for Toxic Pollutants, recommends that WET tests be used in toxic management programmes (in Warren-Hicks and Parkhurst 1992). As a result of this recommendation, the USEPA has increasingly promoted the use of WET testing (Warren-Hicks and Parkhurst 1992). In South Africa, the

Department of Water Affairs and Forestry (DWAF) have also identified the need to assess the effects of hazardous effluent on the aquatic environment and have identified WET testing as a tool to evaluate the suitability of hazardous effluents for discharge into receiving waters (Slabbert *et al.* 1998).

With the passing of the new Water Act (Act No. 36, 1998) in South Africa, and the recognition of the importance of toxicity testing, there is a need to establish WET test methodology suited to local conditions. This includes determining which test organisms may be used in WET, either standard laboratory test organisms, indigenous site-specific organisms, or both. A number of WET studies have been initiated by the Institute for Water Research (IWR) (Grahamstown), funded by the Water Research Commission, and have primarily used indigenous site-specific invertebrates as test species. These studies include a doctoral thesis by Davies-Coleman (pers. comm.), which is investigating the use of the freshwater limpet, *Burnupia stenochorias*, as an indicator in WET; and a masters thesis by Zokufa, which aims to develop WET methods using flowing indigenous macroinvertebrates as test organisms (Zokufa 1998).

In this study, Chapter 4 investigated and compared the tolerance and use of selected indigenous invertebrates and *D.pulex*, to a single-substance reference toxicant. The recirculating artificial streams (Channels) used in that chapter (Chapter 4) had yet to be tested using a whole effluent. This Chapter is a first attempt at using the Channel systems and site-specific indigenous invertebrates in acute WET testing. The study involved two six week field trips to the study area, Vereeniging, thus the logistics and practicality of site-specific testing was also investigated. The aim of this Chapter was not specifically to determine the toxicity of the selected industrial effluents to *D.pulex* and selected indigenous invertebrates, but rather a case study to highlight any difficulties in using site-specific invertebrates in WET.

5.2. Materials and Methods

The experimental methodology used for toxicity testing with indigenous invertebrates and *D.pulex* experiments is described in Chapters 2 and 4. Acute 96 h whole effluent experiments using indigenous invertebrates as test species were run simultaneously with acute 48 h *D.pulex*

experiments using the same effluents. WET experiments were conducted at the Rand Water Hydrobiology laboratories. Indigenous invertebrates were collected from two different reference sites in the Vaal River catchment (see Chapter 2 for details) and experiments were run in the artificial stream systems, the Channels (Figure 2.2). Rand Water *D.pulex* cultures were used and standardised 48 h acute experiments were set up using the same effluent samples as those used to setup the indigenous invertebrate experiments. Culture media used was dilution media for *D.pulex* experiments (as stipulated in standard methodology), and Vaal Dam water (receiving water) for indigenous invertebrate experiments.

A number of industrial effluents, from industries in the industrial areas of Vereeniging and Sasolburg, were selected for WET tests. Forty eight hour acute *D.pulex* screening tests were conducted to determine which of the industries produced the more acutely toxic effluent. Industries were chosen primarily because they discharge their effluent either directly, or indirectly via an effluent canal, into the Vaal Barrage. The selected industries were close to Rand Water laboratories. Two industries, Industry A and B, were chosen for WET testing based on the results from the acute *D.pulex* screening tests. Industry A discharges a variable effluent (Table 5.7) into the Rietspruit via an effluent canal. The effluent is known to consist primarily of heavy metals, and three experiments were conducted using this effluent. The first experiment (Experiment A1) was conducted approximately 10 and 12 weeks prior to the second (Experiment A2) and third (Experiment A3) experiments respectively. Industry B discharges a highly variable effluent, consisting mainly of PVC and nutrients, into the Taaibosspruit via an effluent canal. One experiment was carried out using this effluent.

No modification was made to either *D.pulex* or site-specific indigenous invertebrate methodologies described in Chapter 2. Only data sets where control mortalities were below 10%, and therefore acceptable according to standard methods (APHA 1992), were used in analysis. Effluent dilutions were chosen to include a control and 100% effluent. Numbers of dilutions were determined primarily by the number of Channel systems which could be set up in the laboratory. The range of dilutions in Experiments A1-3 were chosen on a 0.5 dilution range, with the addition of a 75% treatment (i.e. eight treatments were run). The range in Experiment B was chosen on a 0.7 dilution range because two more Channel systems could be set up in this experiment (i.e.

ten treatments). See Tables 5.1 and 5.2 for a summary of each experiment.

Table 5.1: A summary listing the indigenous test organism, the reference site from which they were collected and the effluent dilutions used for each acute 96 h WET experiment.

Experiment	Test organism	Reference site	Effluent Dilution (%)
A1	*C.nilotica*	Emfuleni Park	0.00, 3.12, 6.25, 12.50, 25.00, 50.00, 75.00, 100.00
A2	Baetidae	Taaibosspruit	0.00, 3.12, 6.25, 12.50, 25.00, 50.00, 75.00, 100.00
A3	Baetidae	Taaibosspruit	0.00, 3.12, 6.25, 12.50, 25.00, 50.00, 75.00, 100.00
B	*C.nilotica* Corixidae	Engelbrechtdrift weir	0.00, 6.00, 8.500, 12.00, 17.00, 24.00, 34.50, 49.00, 70.00, 100.00

Table 5.2: A summary listing the effluent dilutions for each acute 48 h *D.pulex* experiment.

Experiment	Effluent Dilution (%)
A1	0.00, 12.50, 25.00, 50.00, 75.00, 100.00
A2	0.00, 3.12, 6.25, 12.50, 25.00, 50.00, 75.00, 100.00
A3	0.00, 3.12, 6.25, 12.50, 25.00, 50.00, 75.00, 100.00
B	0.00, 6.00, 8.500, 12.00, 17.00, 24.00, 34.50, 49.00, 70.00, 100.00

In a non-renewal system, nutrient concentrations can increase to toxic levels (APHA 1992) and should therefore be determined in each treatment at the end of an experiment. Standard 48 h acute *D.pulex* experiments do not require this precaution (see Chapter 4). Due to cost constraints, and because the chemical composition of the effluent was unknown and variable, it was assumed that the nutrient concentrations were a characteristic of the effluent itself and concentrations were not measured (it was therefore also assumed that the organisms did not significantly increase nutrient concentrations through the duration of the experiment). This assumption was also based on

previous experience (Chapter 4) where the nutrient concentrations remained within acceptable levels over the experimental period.

An effluent sample was sent for chemical analysis at the start of each Industry A experiment in order to provide information on the effluent's composition, and to determine the variability in the measured chemical variables between experiments. It was accepted that the chemical analysis could not be definitive, because not all chemicals can be tested for (Roux *et al.* 1993, Heber *et al.* 1996). For example, some chemicals within the effluent may have been below detection limits and the analysis for organic compounds is very expensive (Hunt *et al.* 1992). A chemical sample was sent for analysis for Industry B, but results are not yet available.

To provide an assessment of the chemical conditions experienced by the selected indigenous site-specific organisms in the field, chemical data from Engelbrechtdrift weir was obtained from Rand Water. Data for this reference site was collected monthly from 1992 to 1998, and summarised as the median, maximum and minimum recorded concentrations for each presented chemical variable. Effluent concentrations were compared to those calculated for the reference site, the Target Water Quality Range (TWQR), the Guideline for the Protection of Aquatic Life (GPAL) and Acute Effect Value (AEV) to give an indication whether the values recorded in each effluent sample were to be considered high.

5.3. RESULTS

5.3.1. Industry A

A1 effluent appeared to be the most toxic of the three effluents tested. After 48 h exposure, *D.pulex* responded with the only monotonic concentration-response curve recorded; no concentration-mortality relationship was recorded for the other two Industry A samples (Tables 5.3, 5.5 and 5.6 and Figures 5.1-5.3). The *D.pulex* mortality in Experiments A2 and A3 appeared to be unrelated to increasing effluent dilution, and neonates did not show a concentration response to either effluent samples (Figures 5.2 and 5.3).

D.pulex was more sensitive to Effluent A1 than *C.nilotica*. A 48 h *D.pulex* LC50 was calculated at 39.1 (32.5-47.0)% A1 Effluent, whereas no 96 h *C.nilotica* LC50 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 5.3). *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 5.3: 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 LC50 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	0.0	0.0	0.0
	48	14.3	9.8	2.0	0.0	0.0	2.0	0.0	0.0
	72	18.4	11.8	2.0	0.0	0.0	2.0	0.0	0.0
	96	22.5	11.8	2.0	0.0	0.0	2.0	0.0	0.0
D.pulex	24	93.3	46.7	33.3	6.7	0.0	-	-	0.0
	48	100.0	86.7	73.3	13.3	0.0	-	-	0.0

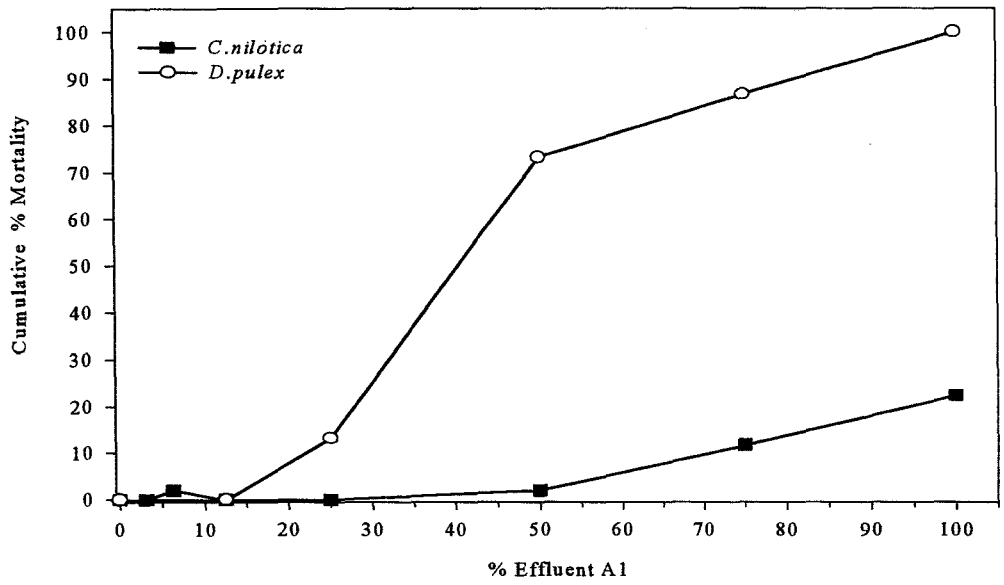

Figure 5.1: The 96 h *C.nilotica* and 48 h *D.pulex* percentage mortalities recorded in response to Effluent A1. The Spearman-Karber LC50 was at 39.1% (32.5%-47.0%) A1 Effluent. No LC50 was calculated for *C.nilotica.*

The Baetidae are a complex of species, with each species being identified after use in Experiments A2 and A3 by the Albany Museum, Grahamstown. The relative proportions of each species within the test complexes are listed in Table 5.4. The two most common species in both Experiments A2 and A3 were *Baetis glaucus* and *Cheleocloeon excisum*, with *B.quintus* making up a high percentage (24%) of the complex in Experiment A2. The response of each species within a treatment was not determined because of the low number per species within a treatment. Baetidae were therefore treated as a family for data analysis and equal tolerance assumed within the family complex.

Table 5.4: Relative percentage proportions of each species within the Baetidae complex used in experiments with Effluents A2 and A3.

Species	Effluent A2	Effluent A3
Baetis glaucus	38.5	45.0
Baetis quintus	24.0	1.7
Baetis harrisoni	3.1	3.3
Cheleocloeon excisum	32.3	46.7
Unknown	2.1	3.3

Neither the Baetidae nor *D.pulex* showed a concentration-response relationship to the A2 and A3 effluents (Tables 5.5. and 5.6, Figures 5.2 and 5.3). *D.pulex* also did not appear to be acutely effected by Effluents A2 and A3, and mortality appeared at random. For example, mortality in the 50% A2 effluent treatment was double that recorded in the 100% A2 effluent treatment (Figure 5.3). Recorded baetid mortalities also appeared random. For example, in A2 effluent the Baetidae showed mortality in the 6.25%, 12.5%, 25% and 75% effluent treatments, but no mortality occurred in the 50% or 100% effluent treatments (Table 5.5). High baetid emergence rates occurred during the experiments, resulting in low test numbers and may have contributed to the resultant non-monotonic response shown by the baetids in both Experiments A2 and A3. It is also likely however, that Effluents A2 and A3 were not acutely toxic to the baetids (Tables 5.5. and 5.6). Due to the lack of an acute toxic effect, natural variability in response to the effluents became evident.

Table 5.5: The cumulative percentage mortalities recorded at the end of each experiment for Baetidae (96 h) and *D.pulex* (48 h) in response to Industry A2 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 A2							
	Hours	100%	75%	50%	25%	12.5%	6.25%	3.12%	Control
Baetidae	24	0.0	0.0	0.0	6.7	10.0	5.9	6.3	0.0
	48	0.0	0.0	0.0	13.3	10.0	5.9	6.3	0.0
	72	0.0	21.4	0.0	26.7	20.0	17.6	6.3	0.0
	96	0.0	21.4	0.0	26.7	20.0	23.5	6.3	0.0
D.pulex	24	5.0	10.0	15.0	0.0	4.8	0.0	0.0	0.0
	48	5.0	10.0	15.0	0.0	9.5	0.0	0.0	0.0

Table 5.6: The cumulative percentage mortalities recorded at the end of each experiment for Baetidae (96 h) and *D.pulex* (48 h) in response to Industry A3 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 A3							
	Hours	100%	75%	50%	25%	12.5%	6.25%	3.12%	Control
Baetidae	24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	48	6.7	4.2	0.0	0.0	0.0	0.0	0.0	0.0
	72	6.7	4.2	0.0	0.0	0.0	0.0	0.0	0.0
	96	6.7	4.2	0.0	0.0	0.0	0.0	26.3	0.0
D.pulex	24	0.0	5.0	10.0	0.0	5.0	10.5	0.0	0.0
	48	10.0	5.0	20.0	5.0	5.0	15.8	0.0	5.0

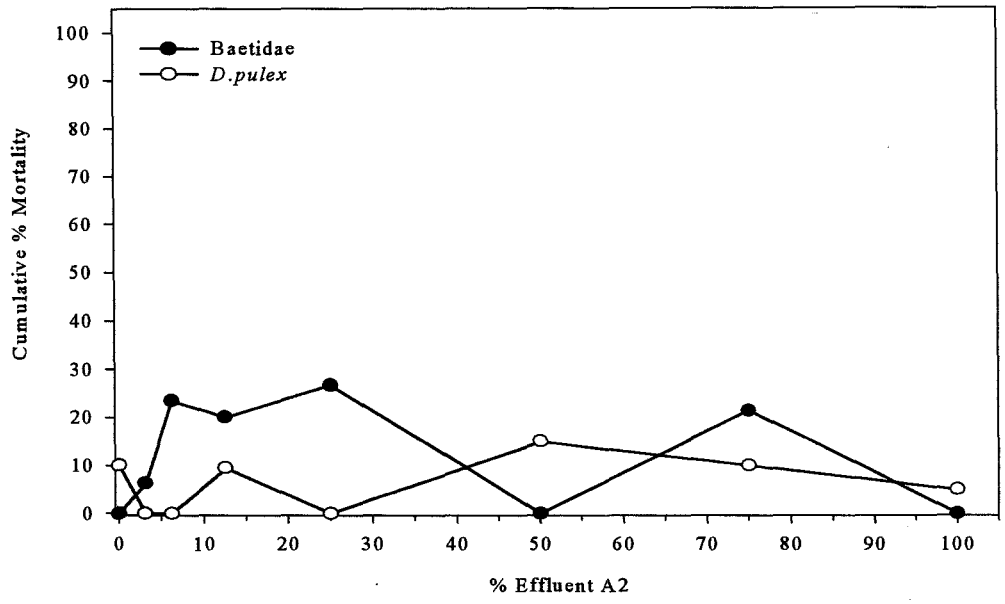

Figure 5.2: The 96 h Baetidae and 48 h *D.pulex* percentage mortalities recorded in response to Effluent A2. LC50s could not be calculated.

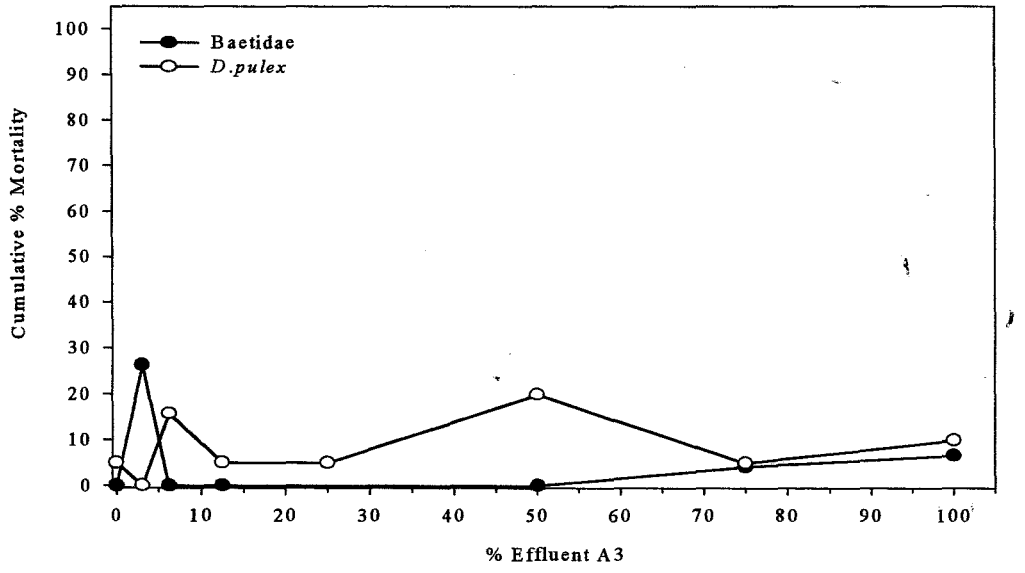

Figure 5.3: The 96 h Baetidae percentage mortality and 48 h *D.pulex* percentage mortality recorded in response to Effluent A3. No LC50s could be calculated.

Most chemical variables in Effluent A1 were greater than those measured in Effluents A2 and A3 (Table 5.7). 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 (see Table 5.7 for list of variables), 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) (DWAF 1996g), Guideline for the Protection of Aquatic Life (GPAL) (Kempster *et al.* 1980) values or both in all three effluent samples (Figures 5.4-5.7). 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.

Table 5.7: Summary of the physical and chemical variables measured for each effluent and compared to the average and maximum values recorded at Engelbrechtdrift weir and the Acute Effect Value (AEV) and the Target Water Quality Range (TWQR) and Guideline for the Protection of Aquatic Life (GPAL). Those values in bold exceed the AEV, those with a * exceed the TWQR or GPAL and those values which are italicized exceed the reference site median. ([1]DWAF 1996g, [2]Kempster *et al.* 1980).

Quality variable (mg/*l*)	TWQR[1]; GPAL[2]	AEV [1]	Reference Site median	Reference Site min-max	Effluent A1	Effluent A2	Effluent A3
PHYSICAL AND CHEMICAL CHARACTERISTICS							
Conductivity at 25°C (mS/m)			18.00	8.80-50.00	*400.00*	*212.00*	*220.00*
Hardness as $CaCO_3$			60.00	31.00-180.00	*920.00*		
Total Dissolved Solids	Should not change by more than 15%[1]		135.00	58.00-570.00			*1490.00*
pH	Should not vary by more than 0.5 pH units over 24 h[1]		7.90	7.30-8.80	*8.00*	*8.80*	*8.60*
Alkalinity as $CaCO_3$	>20.00				130.00	81.00	91.00
NUTRIENTS							
Ammonia	<0.01[1]; 0.02-124.00[2]	0.1[1]	0.08	0.02-1.60	***25.00****	***9.50****	***8.10****
Nitrite			0.05	0.02-0.25	*0.74*	*1.45*	*0.34*
Nitrate			0.38	0.05-7.40	*6.40*	*3.95*	*8.06*
Ortho Phosphate					<0.03	<0.05	<0.05
Total Phosphate	Should not change by more than 15%[1]		0.05	0.05-10.00	*<0.30*	*0.10*	*0.14*
MACRO-ELEMENTS							
Calcium	1000.00[2]		13.50	6.90-45.00	*250.00*	*189.00*	*228.00*
Magnesium	1500.00[2]		6.40	3.10-28.00	*71.00*	*29.80*	*32.00*
Sodium	500.00[2]		11.00	2.20-55.00	*340.00*	*200.00*	*160.00*
Potassium	50.00[2]		3.90	1.60-8.60	*100.00**	*36.00*	*35.00*
Cadmium	<0.01[1]	<0.01[1]	0.03	0.03-0.03	*<0.05*	<0.01	<0.01

Quality variable (mg/*l*)	TWQR[1]; GPAL[2]	AEV [1]	Reference Site median	Reference Site min-max	Effluent A1	Effluent A2	Effluent A3
Chromium	<0.01[1]; 0.01-0.10[2]	0.20[1]	0.03	0.03-0.07	*<0.05*	0.02	<0.01
Cobalt	1.00[2]		0.05	0.05-0.05	*<0.10*	0.02	<0.01
Copper	<0.01[1]; 0.01-0.20[2]	<0.01[1]	0.05	0.05-0.39	*<0.10*	<0.02	<0.01
Manganese	0.18[1]; 0.10-1.00[2]	1.30[1]	0.15	0.05-0.15	***2.00****	*0.42**	*0.38**
Lead	<0.01[1]; 0.02-0.10[2]	<0.01[1]	0.15	0.05-0.16	<0.10*	<0.13*	<0.09*
Zinc	<0.01[1]; 0.03-0.10[2]	<0.04[1]	0.05	0.05-0.33	***0.24****	***0.24****	***0.09****
Nickel	0.03-0.05[2]		0.05	0.05-0.10	*<0.10**	<0.05	0.03
Aluminium	<0.01[1] 0.10-1.50[2]	0.15[1]	0.05	27.00	*<0.10*	*0.08*	*0.06*
Boron	1.50-5.00[2]		0.05	0.05-0.23	*0.49*	*0.18*	*0.19*
Vanadium	0.50[2]		0.05	0.05-0.24	<0.01	<0.01	<0.01
Molybdenum	0.10[2]		0.25	0.03-0.14	<0.01	0.04	0.05
Active silica as SiO_2			11.00	0.00-19.00	*12.00*	11.00	9.80
Iron	Should not vary by more than 10%[1] 0.20-1.00[2]		1.10	0.03	<0.05	*2.15*	*1.35*
Arsenic	<0.01[1] 0.01-1.00[2]	0.13[1]	0.50	0.50-1.00		0.01	<0.01
Mercury	<0.01[1] 0.01[2]	<0.01[1]	0.50	0.50-0.50		<0.01	<0.01
Selenium	<0.01[1] 0.01[2]	0.03[1]	0.50	0.50-0.50		<0.01	0.01
Cyanide	<0.01[1] 0.01-5.00[2]	0.11[1]	0.02	0.02-0.11		*<0.05*	*<0.05*
Bromide	0.10[2]		0.39	0.25-3.50		<0.20	<0.20
Sulphide						<0.10	<0.10
Total Silica	50.00[2]		18.00	0.50-98.00	13.00	11.00	9.00
Sulphate	1400.00[2]		17.00	5.00-150.00		*414.00*	*420.00*
Chloride	50.00-400.00[2]		5.00	5.00-25.00	*200.00*	*358.00*	*406.00*
Fluoride	<0.75[1] 1.5[2]	2.54[1]	0.19	0.10-0.37	***3.90****	***2.81****	*2.45**
Sulphur			4.20	0.50-67.00		*151.10*	*140.00*

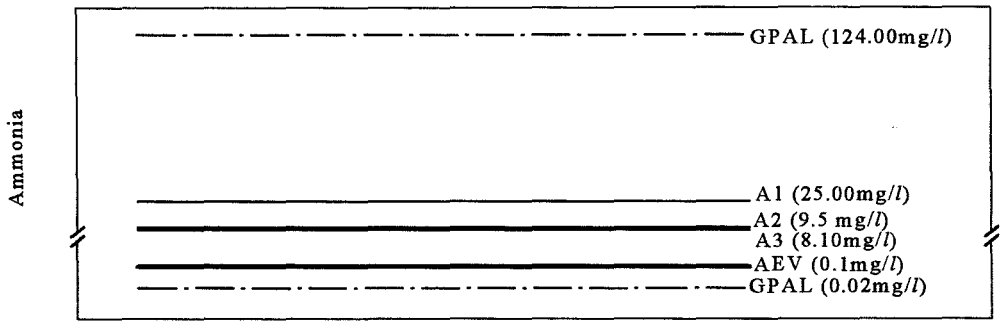

Figure 5.4: A graphic representation showing the ammonia concentrations recorded in Effluents A1, A2 and A3 in relation to the Acute Effect Value (AEV) (DWAF 1996g) and Guideline for the Protection of Aquatic Life (GPAL) (Kempster *et al.* 1980). (The y-axis plots the concentration of zinc and is to scale (note the break in the axis), but for ease of reading, the actual concentrations are plotted in the legend). All three effluents exceeded the AEV value for ammonia, but fell within the GPAL range.

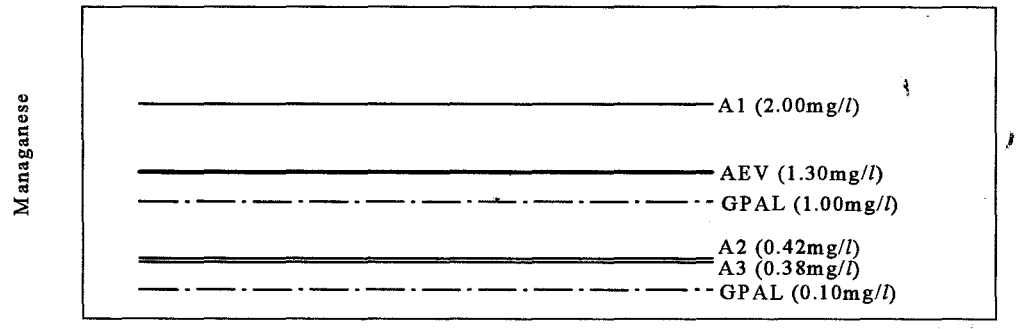

Figure 5.5: A graphic representation showing the manganese concentrations recorded in Effluents A1, A2 and A3 in relation to the Acute Effect Value (AEV) (DWAF 1996g) and Guideline for the Protection of Aquatic Life (GPAL) (Kempster *et al.* 1980). (The y-axis plots the concentration of manganese and is to scale, but for ease of reading, the actual concentrations are plotted in the legend). Only Effluent A1 exceeded the AEV value, the other two effluents (A2 and A3) fell within the GPAL and below the AEV.

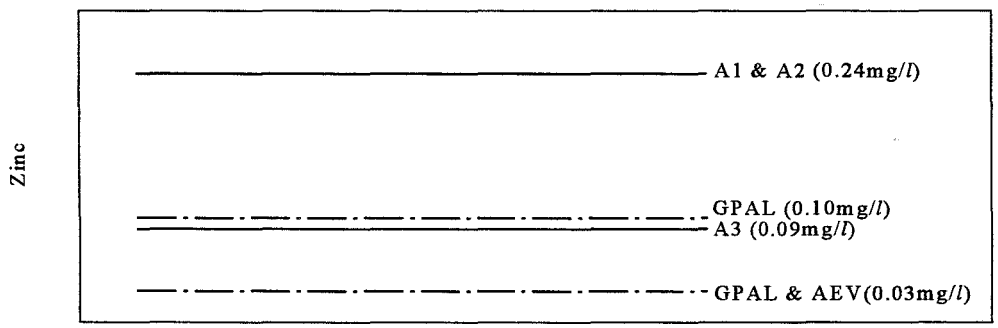

Figure 5.6: A graphic representation showing the zinc concentrations recorded in Effluents A1, A2 and A3 in relation to the Acute Effect Value (AEV) (DWAF 1996g) and Guideline for the Protection of Aquatic Life (GPAL) (Kempster *et al.* 1980). (The y-axis plots zinc concentration and is to scale, but for ease of reading, the actual concentrations are plotted in the legend). Effluents A1 and A2 exceed both the GPAL and AEV values. Effluent A3 exceeds the AEV, but falls within the GPAL range.

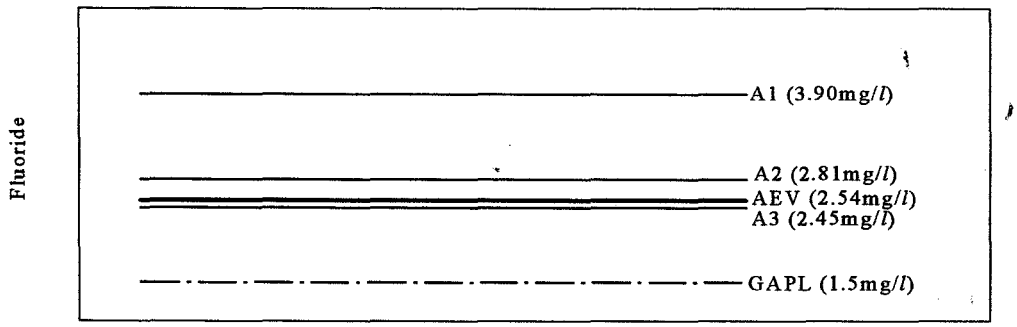

Figure 5.7: A graphic representation showing the fluoride concentrations recorded in Effluents A1, A2 and A3 in relation to the Acute Effect Value (AEV) (DWAF 1996g) and Guideline for the Protection of Aquatic Life (GPAL) (Kempster *et al.* 1980). (The y-axis plots the concentration of fluoride and is to scale, but for ease of reading, the actual concentrations are plotted in the legend). Effluents A1 and A2 exceed both the AEV and GPAL. Effluent A3 exceeds the GPAL.

5.3.2. Industry B

This effluent did not result in any significant *C.nilotica*, Corixidae or *D.pulex* mortality (Table 5.8, Figure 5.8). 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 5.8). 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 5.8: 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	0.0	0.0	0.0	0.0	0.0
	48	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	72	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	96	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Corixidae	24	0.0	20.0	10.0	0.0	0.0	0.0	20.0	0.0	0.0	0.0
	48	0.0	20.0	20.0	0.0	0.0	0.0	20.0	0.0	10.0	11.1
	72	0.0	30.0	20.0	20.0	0.0	10.0	20.0	0.0	10.0	11.1
	96	10.0	40.0	40.0	40.0	20.0	40.0	40.0	30.0	50.0	11.1
D.pulex	24	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.0
	48	5.0	5.0	0.0	0.0	15.0	5.0	0.0	0.0	0.0	10.0

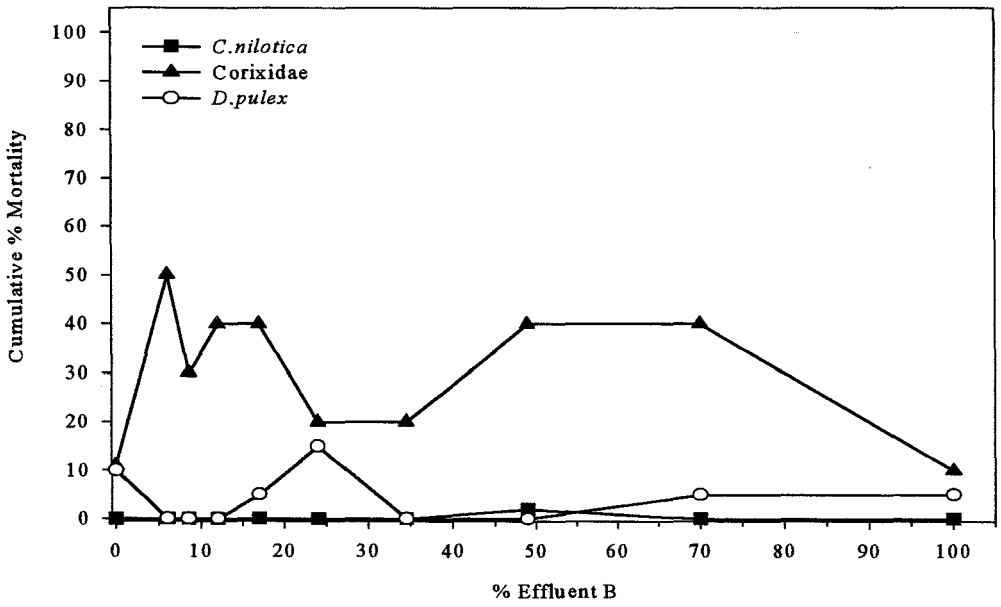

Figure 5.8: The 96 h *C.nilotica,* Corixidae and 48 h *D.pulex* percentage mortalities in response to Effluent B. LC50s could not be calculated.

5.4. DISCUSSION

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). This natural variability is expected in a field-collected population, but is expected to be reduced in a homogenous population, such as the laboratory cultured *D.pulex* (Woods *et al.* 1989). However, substantial differences do exist among individuals within a cultured population (e.g. *D.pulex*) due to differences in the condition of individuals (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.

These experiments highlight a limitation of this study. When determining the toxicity of an effluent, chronic or long-term toxicity tests should be carried out, as the effluent may not be acutely toxic. Chronic toxicity testing was, however, beyond the scope of this project and it is recommended by this study that chronic whole effluent methodology be investigated (using both *D.pulex* and indigenous invertebrates).

Although chemical samples for each effluent were sent for analysis and a chemical profile examined, little information was gained in terms of the effluent's potential toxicity. Chemical profiles cannot predict the interactions between effluent components, nor the effect of these

interactions on the toxicity of the effluent (Cairns and Neiderlehner 1990). Chemical speciation models can be used to predict what ionic forms (or species) of an inorganic chemical will take when present in a water body (MacKay *et al.* 1995) and are important in the understanding of metal toxicity (Filella *et al.* 1995). Speciation models are however reliant on extensive chemical knowledge of the receiving water and toxicant. Full chemical profiles of a whole effluent are expensive and often not comprehensive (e.g. organic chemicals are difficult to detect), thus chemical speciation is not always possible or practical. A chemical profile is however important if an effluent is to be monitored over time or if the effluent is to be broken down in an attempt to identify the toxic component (i.e. In Toxicity Identification Evaluation (TIE) and Toxicity Reduction Evaluation (TRE) processes) (Burkhard and Ankley 1989, Doi and Grothe 1989, Chapman 1995).

In South Africa, single-substance and WET testing are new tools in the evaluation and management of toxicants (Van Vliet 1996). The IWR initiated the use of indigenous macroinvertebrates as test species, and consequently the use of the Channels as test systems, in response to the highlighted need for research into the tolerance of South Africa's indigenous aquatic organisms to toxicants (Moore 1990, Palmer *et al.* 1996). Methodology for the use of indigenous invertebrates was developed for the evaluation of single-substance toxicants, such as metals, and this study was the first to investigate the site-specific application of this method for the toxicity of whole effluents. The usefulness of site-specific indigenous invertebrates as test species in WET testing could not however be properly evaluated due to the effluents not resulting in an acute toxic effect. The advantages and limitations of the methodology are discussed in Chapter 6. An aim of this Chapter was to use WET to further highlight any difficulties in using site-specific indigenous invertebrates as toxicity test species, and these, together with those noted in Chapter 4, are discussed in Chapter 6.

CHAPTER 6

THE PREDICTIVE VALUE OF TOXICITY TESTS AND CONSIDERATIONS FOR THE SETTING OF WATER QUALITY GUIDELINES FOR THE PROTECTION OF AQUATIC ECOSYSTEMS

In South Africa water resources are limited (Schulze 1986), and it is recognised that there is a limit to the degree of utilisation by man that is sustainable by aquatic systems (Ashton *et al.* 1995, DWAF 1997). The resilience of aquatic ecosystems to disturbance, such as that caused by pollution, depends on maintaining a certain level of ecological integrity and function, and it is this level which is termed the Resource Base (MacKay 1998). Without protecting the Resource Base, the long term sustainable use of water resources by man, for domestic, agricultural and other uses, will not be possible. The South African Bill of Rights gives all South Africans the right to an environment that is protected for the benefit of present and future generations (DWAF 1997). To ensure that this right is achieved, the needs of the environment, which will ensure the maintenance of the Resource Base, must be determined, quantified and enforced.

The first step toward the quantification of the needs of the aquatic environment was the publication of the South African Water Quality Guidelines for the Aquatic Ecosystem by the Department of Water Affairs and Forestry (DWAF 1996g). The information contained within these guidelines provides a starting point for management decisions, but these guidelines are by no means complete and those water quality constituents included were selected according to the availability of cause-effect toxicity data and/or local knowledge. Where local data was unavailable, international databases were used and safety factors included (DWAF 1996g). Due to limited information on the water quality requirements of South Africa's aquatic environments, there is a need for South Africa to develop a database quantifying the toxic effects of substances on the aquatic system (Moore 1990). Toxicity tests are an invaluable tool in the determination of toxic effects to aquatic organisms (Richardson and Martin 1994) and can therefore be used as a tool in the assessment of toxic effects of chemicals on South Africa's aquatic environments. The use of aquatic toxicology is relatively new in South Africa (DWAF 1998) and the choice of appropriate test species (field-caught vs laboratory-cultured) is still under investigation. This

study investigated the use of a cultured organism, *Daphnia pulex*, and selected indigenous invertebrates for use in aquatic toxicity testing. The findings and limitations of each method are discussed in the context of the predictive value of toxicity tests to determine in-stream toxic effects, and in the use of toxicity test results in setting water quality guidelines for the aquatic environment.

6.1. 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 is their convenience (Leeuwangh 1978). In South Africa, aquatic toxicology is relatively new and because standardised methodology exists for laboratory cultured species (USEPA 1979, APHA 1992), the use of these organisms is more extensive than that of field caught indigenous organisms. As a result, the sensitivities of indigenous invertebrates is not yet well documented in South Africa (Moore 1990). The suitability of these cultured organisms (e.g. *D.pulex*) for estimating in-stream toxic effects is however still under investigation. Thus an aim of this study was to investigate the advantages and limitations of using *D.pulex* and indigenous site-specific invertebrates in toxicity testing. These and recommendations concerning the use of *D.pulex* and indigenous site-specific invertebrates in aquatic toxicity testing in South Africa are discussed below.

6.1.1. 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). For example, if the experimental system is static, only species which live in still waters can be used as test organisms. The IWR have developed artificial streams suitable for toxicity testing using riffle-dwelling organisms (Palmer

et al. 1994). These systems, the Channels, were used in this study thus allowing the choice of species adapted to dwelling in flowing environments. As flowing water environments have been characterised as one of the more dominant aquatic environments in South Africa (DWAF 1997) this was 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. Eekhout *et al.* (1996) defined a reference site as a relatively unimpacted site that can contribute toward a database used to define the best available physical habitat, water quality and biological parameters for a river. In other words the 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. Distance between the reference site and laboratory is also an important consideration, as test animals need to be transported back to the laboratory relatively quickly in order to minimise stress. It may therefore be necessary to settle for the best available site, which may not necessarily reflect the "most natural" conditions. Reliability of the reference site became an important consideration in this study. One site was subjected to changes in flow for example, and changes in community composition resulted in test species being 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 reliability may be a factor to consider in the choice of test species.

Reference site, species abundance and availability, and suitability to experimental systems were all important considerations in this study with regards to the use of indigenous invertebrate species as test organisms, and it is recommended that these be thoroughly investigated before using indigenous invertebrates in aquatic toxicity tests. Although the above factors may make the use of indigenous invertebrates impractical, 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).

6.1.2. 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. Chapter 3 highlighted the importance of controlling and monitoring abiotic factors, such as temperature, which may influence toxicity results. 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.

A disadvantage of laboratory cultured populations is that they may not accurately reflect the susceptibility of indigenous organism populations. It is therefore important to compare the susceptibility of laboratory species to that of indigenous invertebrates. In the comparisons made

between *D.pulex* and the selected indigenous invertebrates (Chapter 4), *D.pulex* was more sensitive than the selected indigenous invertebrates to the reference toxicant zinc. These results suggested that guidelines set for zinc according to the tolerance of *D.pulex* would protect the indigenous invertebrates selected in this study. However, it is important to note that inherent toxicity was determined for *D.pulex*, whereas relative toxicity was determined for the selected indigenous invertebrates. Also the indigenous invertebrates selected were not chosen according to their sensitivities, but for their abundance and may therefore be among the more tolerant of the indigenous aquatic community.

The frequency of toxicity testing and sampling have obvious consequences if toxic evaluations of a whole effluent are to be conducted, and therefore has important implications on test design and in the choice of test species. *D.pulex* acute and chronic experiments are quicker to conduct and are therefore more attractive as a potential test species for whole effluent characterisation and compliance monitoring (Leeuwangh 1978, Janssen and Persoone 1993). This is advantageous when determining compliance to a whole effluent discharge licence for example, because if conditions stipulated in the licence are not being met, corrective action by the regulatory agent can be enforced quickly. The potential for environmental damage to occur is greater when waiting for the results from a 96 h acute toxicity test (e.g. mayfly acute toxicity test), vs a 48 h *Daphnia* test.

6.1.3. 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, DWAF 1996g), as different species are known to exhibit different susceptibilities to the same chemical (Cairns *et al.* 1975, Sloof 1983, Ewell *et al.* 1986, Hickey and Martin 1995, Toussaint *et al.* 1995, 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.

6.2. TOXICITY TESTS

It is assumed that aquatic laboratory toxicity tests (both single substance and whole effluent) are predictive of effects in receiving waters (Denton and Norberg-King 1996, Dorn 1996). That is, it is assumed that laboratory test results (using both site-specific field-caught and standard laboratory test species) are reflective of the toxic impacts experienced in the field, and that an end-point determined in the laboratory can be extrapolated to the field. The USEPA, in an attempt to determine if WET tests were predictive of in-stream effects, analysed data from approximately 60 industrial and municipal water treatment plant discharges at eight sites across the USA (Wall and Hanmer 1987). It was concluded that toxicity tests conducted on a minimum of three to five test species from a range of taxonomic groups, do predict whether or not effluents will adversely effect a receiving water. The toxicity tests included standard laboratory species, such as the fathead minnow, *Pimephales promelas*, *Ceriodaphnia dubia* and the plant species, *Selenastrum capricornutum*. These predicted effects were confirmed by in-stream biosurvey data suggesting that laboratory toxicity test end-points can be extrapolated to the field (Wall and Hanmer 1987). The USEPA's 1984 national policy document, the Policy for the Development of Water Quality Based Permit Limitations for Toxic Pollutants, recommended that toxicity tests be used in toxic management programmes. As a result of this recommendation, the USEPA has increasingly promoted the use of single substance toxicity and WET testing (Warren-Hicks and Parkhurst 1992).

The fundamental assumption behind the use of single species toxicity tests is that by searching for and testing the "most sensitive species" and setting standards to protect this species, all other species and activities at higher levels of biological organisation will also be protected (Cairns

1986). The growing appreciation of the complexity of ecosystems has, however, resulted in the questioning of the "most sensitive species approach" and on the exclusive reliance on single-species toxicity tests to determine guidelines for the protection of aquatic communities from industrial (and other) sources of pollution (Pontasch *et al.* 1989a, Roux 1994). Some of the disadvantages and criticisms against single-species toxicity tests are listed below, the relevance and the findings of this study, and the application of toxicology in South Africa is discussed.

6.2.1. Sources of experimental variability

Experimental variability due to test organism

The greatest criticism of single species toxicity tests is the fact that test results with one toxicant can be highly variable within a species (Cairns 1983). Variability in both physical and biological parameters are kept to a minimum in laboratory toxicity tests. Genetically homogenous laboratory cultured organisms, such as *D.pulex,* are therefore popular test species in aquatic toxicology, because it is assumed that biological variability will be reduced due to genetic homogeneity between individuals (Pontasch *et al.* 1989a). These cultures however lack the adaptive capability of natural heterogenous populations (Hickey and Vickers 1992), thus the realism/validity of end-points using these cultures can be questioned. It has therefore been argued that indigenous field caught organisms should be used as test species instead of cultured laboratory species, because they are representative of the affected aquatic system. However, indigenous populations obviously show greater variability in tolerance around a measured end-point than a genetically homogenous population (Woods *et al.* 1989). In the past, the primary reason behind the choice of a test species was its ease and robustness to culturing conditions, rather than its suitability as an indicator of the effected ecosystem, its sensitivity to pollutants or ability to indicate subtle ecological effects (Gray 1989). Laboratory cultures are able to provide test species at the time of the test, the organisms are of a known age and background, and because the life-history and existing culture conditions are well documented, there is better control and quality assurance. As the responses of the cultured organisms are expected to be more consistent between tests (Leeuwangh 1978, Janssen and Persoone 1993, Koivisto 1995), laboratory cultures are more extensively used in aquatic toxicology than field caught indigenous invertebrates.

This study and others (Rue *et al.* 1988, Baird *et al.* 1989) have shown that unexplained variation can occur using laboratory cultured organisms, even though a methodology has been standardised (Chapter 2). The variability in cultured populations, such as *D.pulex*, may be due to genetic differences, culture technique (e.g. diet) or physical factors (e.g. temperature) (Weber 1991). In this study it was highlighted that if factors, such as temperature, are not kept constant, the result/impact on toxicity test end-points can be significant. For example, in Experiment 4 (Chapter 3), LC50s for all four *D.pulex* cultures were significantly greater than subsequent experiments and a suspected reason for this was the temperature fluctuations experienced during the experiment. Other studies have also highlighted the significant influence of temperature on tolerance to chemicals (Cairns *et al.* 1975, Shcherban 1977, Cairns *et al.* 1978, Braginskiy and Shcherban 1979, Stephenson and Watts 1984, Lewis and Horning 1991). Strict control over abiotic factors must be exercised if their contribution to experimental variability is to be reduced. This therefore requires appropriate laboratory facilities and continual monitoring.

It is assumed that because *D.pulex* cultures are genetically homogenous, variability around the measured end-point (e.g. LC50) should be minimal (Woods *et al.* 1989). However, it is unlikely that cultures remain homogenous over time. If a culture is stressed in any way, through the build up of metabolic waste for example, males are produced, thus creating the potential for sexual recombination and genetic changes within the culture. Mutations may also result in a genetic change (Baird *et al.* 1989). Thus, over time, variability around an end-point may be attributed to genetic changes within a culture. This study was unable to investigate this hypothesis due to confounding factors experienced during the experiments, however it is suspected that genetic changes over time play a role in the variability experienced between experiments within a culture. It has been shown in other studies that the difference between two cultures in their tolerance to a chemical was due to the collection of *D.pulex* from different localities (i.e. cultures were initiated with females collected from different populations) (Buikema 1983, Woods *et al.* 1989). For example, one of the major factors identified as contributing to the measured differences in LC50s between *D.pulex* cultures to the same chemical was genetic variability between cultures, with a number of laboratories having obtained cultures directly from different wild populations (Baird *et al.* 1989). In other words, cultures were set up from a female caught in a nearby location and not subcultured from a pre-existing culture. It can be assumed that these cultures were

different from one another genetically, with the implication that the difference in genetic make-up between the cultures may cause different expressed susceptibilities to the same toxicant. Laboratories in South Africa have initiated *D.pulex* cultures with females from different populations. Experiments in this study were confounded by other factors (e.g. temperature) (Chapter 3), and it can therefore not be stated with confidence that the noted differences in zinc tolerance between cultures was due to population differences or culture age differences. However, the differences in genetics between these cultures may have contributed to the differences measured in the LC50s and if these cultures are used in interlaboratory studies their genetic differences may contribute to inter-laboratory variability. A recommendation would therefore be that cultures are subcultured from the same female.

Regular testing with a reference toxicant is recommended in order to establish if a laboratory culture undergoes significant changes in tolerance due to possible genetic changes within the culture over time. A database can also be generated to determine baseline tolerance to the reference chemical, thus providing a measure of variability over time.The influence of age (Blaise *et al.* 1988, Hoekstra *et al.* 1993), nutritional status and pre-exposure to the chemical (Naylor *et al.* 1990, Crane 1995), factors known to influence the tolerance to a chemical, can be controlled in laboratory reared test organisms. For example, *D.pulex* neonates, less than a day old, are hatched specifically the day before the experiment, and they are not fed before the test is initiated (APHA 1992).

The variability associated with field caught organisms however can be more difficult to control. It is for example, not practically possible to age indigenous invertebrates without detailed life history data. However, in an attempt to control for age, individuals of a similar size are chosen for the experiment (APHA 1992) (Chapter 2). Also during acclimation, it is hoped that the test organisms will reach the same nutritional status, as they are not fed before or during the acute experiment (APHA 1992).

Another factor known to significantly influence experimental variability is genetic differences between field populations (Naylor *et al.* 1990, Crane 1995). It is therefore recommended that a field-caught test population be collected from one reference site only, as this may help reduce a

potential source of organism variability. The choice of the reference site is therefore important, as it must be able to provide enough test organisms for testing. In this study, variability between experiments (i.e. LC50 variability over time) was not tested for as the indigenous invertebrate species chosen largely depended on their availability at the time of testing. As a result the same species were not used in all experiments (Chapters 4 and 5). This illustrates a practical disadvantage of using field collected organisms.

Field populations are also subject to natural conditions and may therefore not be available when experiments need to be conducted. Different species are also known to exhibit different tolerances to the same chemical (Cairns *et al.* 1975, Sloof 1983, Ewell *et al.* 1986, Hickey and Martin 1995, Toussaint *et al.* 1995, McKinney and Wade 1996, Roseth *et al.* 1996). It is therefore important to ensure the test population is one species and not a complex of species, as it is difficult to speciate a population such as the baetids at the onset of the experiment. The differences in species tolerance within a species complex may contribute to variability between experiments (Chapters 4 and 5). It is also difficult to speciate a species complex at the onset of the experiment. A recommendation is that all individuals are identified to species level to confirm a single-species population.

It has been argued that the variability around measured end-points (e.g. LC50 values) between experiments should not be viewed as a limitation (Depledge 1990). A measure of individual variation in tolerance to a chemical within a test population is often missing or suppressed in toxicity studies and it has been argued that consideration of this biological variability should be incorporated into the determination of a populations' tolerance to a chemical or toxicant (Hallam *et al.* 1990). Guidelines for protecting ecosystems may be more realistic with the incorporation of biological variability, experienced at the population and ecosystem scales. However, it is also argued that variability around LC50 values (or other toxicity test end-point) reduces the confidence in the test method (Warren-Hicks and Parkhurst 1992). The question of what level of variability around a measured end-point is due to individual variability (i.e. biological variability) and what may be a reflection of experimental error (e.g. due to the method used), remains undefined, i.e. biological significance is difficult to define and toxicologists rely on statistics (Giesy and Alfred 1985, Thursby *et al.* 1997). In this study for example, the within variability (i.e. the

variability (around LC50s) between experiments within one population) was greater than the variability measured between populations (i.e. the variability between cultures) (Chapter 3). The CSIR *D.pulex* culture in Experiment 5, for example, showed an LC50 of 0.32 mg/*l* Zn which, according to the APHA formula (Chapter 2), was significantly different to that measured for the IWQS culture (0.22 mg/*l* Zn), similar results were obtained in Experiment 6. The difference between the two LC50 values equalled 0.10 mg/*l* Zn in Experiment 5 and 0.13 mg/*l* Zn in Experiment 6. This difference is less than that recorded between Experiments 5 and 6 within one culture (i.e. a difference of 0.15 mg/*l* Zn was recorded between Experiments 5 and 6 for the CSIR culture, similarly a difference of 0.38 mg/*l* Zn was recorded between experiments in the IWQS culture) (Experiment 6: CSIR LC50 = 0.47 mg/*l* Zn; IWQS = 0.60 mg/*l* Zn). In other words, the experimental variability within a culture, which was assumed to be genetically homogenous, was greater than that recorded between two different populations assumed to be genetically different. Can the difference between the CSIR and IWQS cultures therefore be considered biologically significant, although calculated as being statistically different, when the difference within one population was greater than that calculated between populations? Skalski (1981) has argued that the differences measured among two or more biological populations, although statistically different, may not be biologically significant. There is therefore a need for further research to determine the differences between biological and statistical differences and what level of variability between end-points can be considered biologically significant.

Experimental variability due to the toxicant

A further source of variability between experiments (other than those attributed to the test organism) may be associated with the toxicant. Errors may occur in the making up of single-substance toxicant stock solutions and it is therefore important to be able to quantify the exact amount of toxicant test organisms are exposed to. The variability between experiments in Chapter 3 illustrates this point clearly. Chemical analysis was not carried out on the stock solutions made up for the first three experiments because standard *D.pulex* methodology did not require chemical analysis. After a high magnitude of variation between *D.pulex* Experiments 1-3 and Experiment 4, chemical analysis on the new stock solution was carried out to determine the exact concentration of zinc. Therefore it became impossible to compare the first three experiments with

subsequent experiments. If exact exposure concentrations are known, differences in stock solutions become accounted for and can potentially be eliminated as a source of variability between experiments. Contaminants may also influence stock solutions of a single-substance toxicant (Rand *et al.* 1995). It is suggested therefore that chemical profiles on all stock solutions are carried out, to confirm exposure concentration and to identify contaminants.

Acceptable levels of variability between single-substance toxicity experiments need to be determined and the use of an uncertainty estimate, such as the application of safety factors, are included in single-substance guidelines to help compensate for the inherent variability experienced between tests (Dorn 1996, DWAF 1996g).

Minimising the variability between end-points over time in WET tests is more difficult than in single-substance toxicity tests because of the continuously changing nature of the effluent (Rand *et al.* 1995). Effluents are made up of numerous chemicals and are thus complex in composition. They can also be highly variable over time, making toxic evaluations difficult (Warren-Hicks and Parkhurst 1992, Dorn 1996). No established criteria exist stipulating the acceptable levels of variability in a WET test (Parkhurst and Mount 1991) because of the inherent variability in effluent composition experienced over time. Average intra-laboratory coefficients of variation for acute WET tests have been recorded as 17%, with as much as 135% variability being recorded between experiments (Parkhurst and Mount 1991). Such high variability between experiments can be expected if the composition of the effluent is highly variable, thus understanding the effluent's variability may be more important than evaluating one toxic result as a significant toxic event (Lave and Gruenspecht 1991, Roux 1994, Grothe *et al.* 1996). It may therefore be important to incorporate a series of WET tests over time before setting a discharge licence. The presumption of predicting receiving water effects based on one or two WET laboratory tests is inappropriate unless a significant pattern of toxicity can be established (Dorn 1996).

The sampling and testing regime are therefore important in establishing the toxic potential of an effluent. A number of different sampling methods have been proposed by which to characterise effluent variability (Slabbert *et al.* 1996), although it is recognised that the frequency of sampling is largely dependent on the effluent in question (Heber *et al.* 1996). Slabbert *et al.* (1996) suggest

a sampling protocol which involves the monthly testing of an effluent over 12 months in order to characterise the effluent's variability and toxic effects. Highly variable effluents may have to be sampled daily (maybe even hourly) in order to be confident that the most toxic plug is sampled and tested. Some effluents are sampled on the spot (grab samples), i.e. the samples are characteristic of the effluent only at the time of sampling; whereas composite sampling involves the collection of equal volumes of effluent over a set period and the mixing of these samples into one effluent, thus attempting to characterize the effluent over the period sampled (Grothe *et al.* 1996). In this study, spot effluent samples were taken because the aim of WET tests in this study was not to characterise the selected effluent's toxicities, but to highlight potential difficulties in WET testing using indigenous invertebrates and *D.pulex*. It is however recommended by this study that both spot and composite samples of an effluent are evaluated, to accurately characterize the effluent's toxicity potential. This study also recommends the use of chronic testing when establishing the toxic potential of an effluent, as experiments in Chapter 5 illustrated that not all whole effluent is acutely toxic.

6.2.2. Toxicity test design

There are two basic approaches to toxicity test design, the unreplicated regression design and the replicated ANOVA design. The regression design is argued to allow more treatments to be tested, thus including more "partial kills" on a concentration-response curve and allowing for more rigorous end-point (e.g. LC50) determination (Gerhardt pers comm.). The ANOVA design requires at least three replicates (APHA 1992), thus if space or test population numbers are a constraint (as they were in this study), fewer treatments can be run. Replicates do however provide some characterisation of test population variability within a treatment (Stephan and Rogers 1985).

Preference was given to the regression design in this study. Both *D.pulex* neonate availability (Chapters 3 and 5) and space in which to run the experimental systems for the indigenous invertebrates (Chapters 4 and 5) were limiting factors. It was decided that running more (unreplicated) treatments was preferable to running only a few replicated treatments. Unfortunately, the sacrifice of an unreplicated design is that there is no measure of variability

within a treatment. When treatments are replicated, more confidence can be placed in the test results (Hoekstra 1993a).

The nature of the question being asked should dictate which statistical design should be used (Hoekstra 1993b). If it is important to be able to characterise variability within a test population, then replicates are essential. If it is more important to determine the toxicity of a toxicant, a large number of treatments may be more advantageous, as this experimental design provides more information around the concentration-response curve than does a replicated design (Weber 1991).

6.3. THE PREDICTIVE VALUE OF LABORATORY TOXICITY TESTS AND THE MOVE TO POLLUTION CONTROL USING AN INTEGRATED MANAGEMENT APPROACH

Authors (Cairns 1983, Cairns and Niederlehner 1987) have questioned the scientific justification of using single-species toxicity tests to predict changes in competition, predation, community function and ecosystem energy flow due to the input of a toxin to the aquatic system. Aquatic toxicity tests are also conducted under physical and chemical conditions which lack similarity to natural habitats, and authors question the choice of criteria used to validate laboratory predictions in real world field situations (Pontasch *et al.* 1989b).

In an attempt to overcome some of the above criticisms, some authors recommend the use of multi-species toxicity tests. A multi-species toxicity test often involves "laboratory microcosms" or "model ecosystems" (Pontasch and Cairns 1989, Rand *et al.* 1995). These laboratory microcosms are small-scale enclosures containing samples from the natural ecosystem (water, sediment, plants and invertebrates). The advantage of these systems is that effects beyond single-species can be identified (Rand *et al.* 1995). Thus the most obvious advantage of using multi-species as opposed to single-species tests is their ability to measure the interactions of component species (Pontasch *et al.* 1989b). The cost and maintenance of these systems is however high (Mount 1985), and therefore not suitable for guideline setting or compliance monitoring.

The validity of single-species toxicity tests for determining the effects of toxicants to aquatic communities is still under debate (Cairns 1983, Cairns 1986, Cairns and Neiderlehner 1987,

Parkhurst 1995). A study by Frithsen *et al.* (1989) compared the use of single-species and whole ecosystem tests to characterise the toxicity of a sewage treatment plant effluent and found that both approaches were useful in assessing toxicity. Single-species tests were best utilized to characterise the magnitude and persistence of toxic components, whereas the mesocosm experiments identified sensitive communities and processes. Pontasch *et al.* (1989a) compared the predictive capacity of five conventional single-species tests and two types of multi-species tests. Predictions from these tests with a complex effluent where compared to effects observed in the receiving system. They found that the acute single-species tests predicted harmful effects at far higher effluent concentrations than microcosm experiments or field monitoring suggested and populations in the field would not have been protected if values from these tests were used in isolation to determine a discharge permit. Pratt *et al.* (1989) concluded that to be effective in predicting in-stream effects, the design of intensive toxicity studies/surveys should make every effort to mimic local conditions. Suitable test methods (see Section 6.3) and appropriate test species (see Section 6.1) should therefore be chosen and safety factors applied to guidelines and discharge licences.

The fate and effect of toxicants at the point of entry, their potential to persist, and the consequence of increased exposure concentrations and bioaccumulation should also be taken into consideration when evaluating the toxic potential of a chemical or effluent (Cowan *et al.* 1995). Most aquatic toxicity tests are far removed from natural conditions and it is therefore important to conduct in-stream surveys for signs of impact (Pratt *et al.* 1989). Aquatic organisms are constantly exposed to pollutants entering the river and biotic indices/assessments try to integrate the impacts of pollutants over a period of time by sampling the resident aquatic community (Roux *et al.* 1993).

The challenge of managing a complex system, such as the aquatic environment, is to provide the strongest scientific support in the absence of a full understanding of properties and interactions of the system being assessed (Herricks and Schaeffer 1985). It has been argued that risk assessment approaches should incorporate data from several methodologies (Karr 1990, Kelly and Harwell 1990) such as chemical and biological monitoring, as well as toxicity data, as each, if used on its own, cannot adequately determine the effects of a toxicant on the environment. It

is envisaged by DWAF that an integrated management strategy be implemented, which takes advantage of a number of methodologies to determine and monitor water quality in our rivers (Conley 1990). Biological assessment methods (e.g. SASS), for example, can be used to assess the integrity of the affected aquatic community and monitor the changes taking place within a community in response to a released toxicant. They therefore compensate for some of the inability of toxicity tests (both single-substance and whole effluent) and chemical specific programmes to predict actual receiving water impacts (Heber *et al.* 1996). It is also important, once having determined the impact of a chemical/toxin on the environment, to try and reduce its effect, particularly in South Africa where waste water is required by law to be returned to the stream of origin (Act 54, 1956). Numerous methods, such as Toxicity Identification Evaluations (TIE), help identify the toxic components of an effluent (Burkhard and Ankley 1989, Chapman 1995), enabling the industry/polluter to reduce the toxicity of their effluent (i.e. Toxicity Reduction Evaluation (TRE)) (Doi and Grothe 1989, Goodfellow *et al.* 1989, Schimmel *et al.* 1989, Chapman 1995).

6.4. CONCLUSIONS AND RECOMMENDATIONS

It is recommended by this study that the setting of discharge licences and environmental water quality guidelines should therefore 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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