DEVELOPMENT OF CHRONIC TOXICITY TEST METHODS FOR SELECTED INDIGENOUS RIVERINE MACROINVERTEBRATES

Report to the
WATER RESEARCH COMMISSION

by

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This is the third and final report to the Water Research Commission for a project titled: *Development of chronic toxicity test methods for indigenous riverine macroinvertebrates for use in single substance and whole effluent toxicity testing to contribute to resource directed measures (RDM) and source directed controls (SDC)* (WRC Project No. K5/1313).

The other reports in the series are:

*Methods for the direct estimation of ecological effect potential (**DEEEP**) manual* (WRC Report No 1313/1/04), and
Development of a Research Programme on the Application of Aquatic Ecotoxicology to Water Resource Management (WRC Report No 1313/2/08).

EXECUTIVE SUMMARY

Toxicity tests are accepted world-wide as suitable for managing environmental water quality within water resources management and in South Africa this trend has been followed. Water quality guidelines are most often derived from acute toxicity test (short-term exposures with lethal end-points) results, and from using results derived from a range of species from different trophic levels in freshwater ecosystems. Although data on lethal concentrations of chemicals in short-term tests have been found to accurately predict mortalities in the field, it is generally accepted that acute data cannot be used to indicate safe, long-term exposure concentrations of a particular toxicant. The importance of chronic toxicity data for developing water quality guidelines has been stressed because water quality guidelines should protect organisms from long term exposure to toxicants. Chronic toxicity data are more indicative of the effects of long-term toxicant exposure and are therefore, potentially, more useful than acute toxicity data. However there is a scarcity of both chronic data and chronic test procedures. It is this gap that this study addresses.

ENVIRONMENTAL REALISM OF TOXICITY TESTS

There are concerns that most ecotoxicological tests are too simplistic to reflect real life dose-response relationships, since most ecotoxicological tests have evolved more from the field of toxicology than ecology. Ecosystems are characterised by complex interactions between many species, however the majority of toxicity tests to date have been single species tests. The aim of using single species toxicity tests has been to standardise environmental factors so as to ensure experimental reproducibility. This has been at the expense of environmental realism (Cairns, 1983).

At the Unilever Centre for Environmental Water Quality, effort has been made in several aspects of experimental design to approach environmental realism. These include: the use of river water as the toxicant diluents; the use of indigenous, South African test taxa; the retention of a range of trophic levels among test taxa; the use of flowing water test systems (artificial streams) for the exposure of rheophilic taxa; and exposures to whole effluents rather than single substances. This study seeks to add chronic toxicity testing (longer exposures and sub-lethal end-points) to this array of more ecologically realistic design characteristics.

The project aims listed in the contract were therefore to:

1. Develop chronic toxicity test methods for indigenous riverine invertebrates using non-lethal experimental end-point assessment criteria such as stress proteins, measures of growth and fecundity and morphometric analyses for both nymph and adult stages (Chapters 2 and 3);
2. Use the newly developed chronic toxicity test methods for indigenous invertebrates to evaluate the current method of determining water quality criteria for the ecological Reserve, which uses single-substances and mortality as an end-point (Chapter 4);
3. Assess effluent standards by testing the newly developed chronic toxicity test method using whole effluent (Chapters 2 and 3, and data reported in Appendix B);
4. Undertake a scoping study of the use of stress proteins and morphometric analyses. Assess how these approaches could be used in combination with biomonitoring and water chemistry information in the evaluation of non-point source pollution and pesticide contamination. (Reported fully in Gordon et al., 2009)

5. Compare results of chronic toxicity tests using indigenous invertebrates to those of *D. pulex* in an ongoing benchmark assessment of the use of *D. pulex* to determine water quality criteria for South African rivers (Chapter 4 and data reported in Appendix A).

AN INDIGENOUS TEST ORGANISM: *CARIDINA NILOTICA*

The freshwater shrimp, C*aridina nilotica* proved to be a valuable test organism on the development of chronic test procedures. *C. nilotica* have been reared for routine use in experiments, but culture crashes suggest that maintenance conditions still require refinement.

Initial research work undertaken by Postdoctoral Research Fellow Emily Okuthe (2003/2004) on developmental aspects of *C. nilotica* yielded potentially interesting and promising results for a further chronic toxicity test. The species undergoes a sex change at approximately 3 months of age, changing from male to female (hence the need for a mixed size range in culture tanks). It was not established whether this was an age- or size-triggered switch, but suggested potential as a suitable test method specifically for those toxicants that are known endocrine disruptors. However, further biological investigations were not undertaken and a test method was never developed or refined due to time and funding constraints.

Toxicants tested were limited to NaCl, Na_2SO_4 and $CdCl_2$. In order to adequately test the methods developed, a more diverse group of toxicants with differing toxicological effects, will need to be tested. Salinity tolerance information for indigenous species is still important for setting salinity water quality guidelines in South Africa. The no observed effect concentrations (NOECs) found in this study were compared with NOEC values of different test methods, to give an indication of the sensitivity of the test method developed in this study. Based on results from this study, *C. nilotica* is an excellent indigenous organism to use for chronic toxicity tests where growth of embryos as a biological response is measured, although further testing of other chemicals is required to verify this.

The method developed to measure effects on growth and reproduction suggested good potential for further testing with other toxicants. Potentially, an effect on growth in juveniles can be measured within 20 days, which is significantly shorter than a full life cycle test, although effects of reproduction will then be missed. The results suggest that the embryonic development of *C. nilotica* can be a valuable indicator of contamination in freshwaters by aging the dead eggs and comparing their morphometry to the experimental values. Morphometric measurements of the developmental features could be an indication of an effect caused by chemicals. However, it would be necessary to extend this study to other pollutants to achieve more generalized conclusions useful for water quality monitoring. Embryonic development of *C. nilotica* could possibly be used in future studies in which the toxicity of complex effluents to the developing embryo is assessed. A preliminary experiment was undertaken during this study, exposing the eggs of *C. nilotica* to whole effluent collected from a textile industry. Results from this preliminary assessment suggest that the method developed in this study may be useful in the toxicity assessment of complex effluents.

Comparisons between laboratory-reared and field-caught organisms will need to be undertaken, as differences in sensitivity between these two have been found in other crustacean, and this may have significant ramifications for environmental realism and using results to extrapolate from laboratory to field predictions and developing water quality guidelines.

The results of this study represent a stimulus for further studies of the effects of chemicals on developing embryos, including the establishment of teratogenic effects of aquatic pollutants on *C. nilotica* embryos. The results presented in this study will contribute to the development of chronic toxicity data for *C. nilotica* in the UCEWQ database, and contributes to national and international data available for the development of environmentally realistic water quality guidelines for aquatic ecosystems.

Although the investigation on the usefulness of sub-lethal biomarkers in environmental water quality management in water resource management was commenced under this project, it was in fact superseded by WRC Project 1484/1/09 (Gordon et al., 2009 and references therein). Therefore a brief summary is provided as part of this report.

THE USE OF BIOMARKERS AS SUB-LETHAL TOXICITY INDICATORS

Toxicity test methods that provide reliable and good quality chronic toxicity data which can be used for the derivation and refinement of water quality guidelines are needed and it is thought that biochemical biomarkers will provide appropriate sublethal endpoints for toxicity tests.

Gordon et al. (2009) investigated the advantages and limitations of biochemical endpoints in the derivation of water quality guidelines. Although there has been criticism of the use of biochemical biomarkers for regulatory purposes (including the derivation of water quality guidelines), there are many advantages that potentially outweigh the criticisms. Although there currently is no evidence that links biomarker endpoints from individual organisms to community and population level responses, biomarkers have been shown to be highly sensitive responders to environmental stressors and they can be found after short exposure durations. This suggests that these endpoints may provide the capacity for more accurate environmental protection. In addition, the tests utilise fewer test organisms and are of shorter duration, thus reducing costs associated with lengthy and complex toxicity tests that provide other chronic data.

In the ensuing experiments undertaken by Gordon et al. (2009), it was demonstrated that the resultant lowest observed effect concentrations (LOEC), and subsequent NOEC, for the different biochemical biomarker methods selected varied widely. When these observed results were incorporated into the currently used water quality guideline derivation method for South Africa, the resultant guideline value which would ensure long-term protection of ecosystem was marginally less than similar guideline values for Australia and New Zealand as well as those obtained from complex mesocosm studies. The report concluded that biochemical biomarkers could be used as sensitive and accurate endpoints for the derivation of water quality guidelines but cautioned that quality assurance of these data are vital for their inclusion within an environmental water quality regulatory framework. A number of issues were identified for further investigation, including establishing the relationship between individual organism biomarker result, health of the organism, and the subsequent risk to ecosystem health and integrity. It was also suggested that a suite of biochemical markers, rather than only a single biomarker, would be more useful.

In addition to these substantive results the study provides details in two appendices about toxicity testing using the standard laboratory organism, D*aphnia pulex*, and the responses of various organisms to complex effluents. The study provides a clear basis for work to continue on the development and use of chronic toxicity tests in water quality management in South Africa.

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GLOSSARY AND ABBREVIATIONS

Acute toxicity test	A toxicity test where mortality is the response measured.
Bioassay	An experiment using living organisms.
Biomarker	A measurable physiological, biochemical or histological change as an indicator of exposure to a stressor.
Chronic toxicity test	A toxicity test where a sublethal response is measured.
DWAF	Department of Water Affairs and Forestry
EC_x	Effective concentration, i.e. the concentration of the toxicant at which a specified endpoint is measured in x% of the population.
Endpoint	The biological response that is measured in a toxicity test.
EWQ	Environmental water quality
Indigenous species	Species living or growing naturally in a particular area, but not naturally confined only to that area.
IWR	Institute for Water Research
LC_x	Lethal concentration, i.e. the concentration of the toxicant at which x% of the population dies.
LOEC	Lowest observed effect concentration
NOEC	No observed effect concentration
RDM	Resource directed measures
RQO	Resource quality objective
Sublethal	A concentration less than that which causes mortality, and resulting in subtle effects on behaviour, biochemistry and other physiological functions.
Toxicant	A substance capable of producing an adverse response in organisms, causing injury and / or death.
UCEWQ	Unilever Centre for Environmental Water Quality
WQG	Water quality guideline
WRC	Water Research Commission

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Parts of this research were undertaken towards fulfilment of requirements for two Master of Science degrees, Mr Andrew Slaughter (degree was awarded in 2005) and Ms Nosi Ketse (degree was awarded in 2007), and a Doctor of Philosophy (Zoology), Mrs Heather Davies-Coleman (degree awarded 2002).

SLAUGHTER AR (2005) The refinement of protective salinity guidelines for South African fresh water resources. MSc Degree, Rhodes University, Grahamstown, South Africa.

KETSE N (2007) The effects of selected reference toxicants on embryonic development of the freshwater shrimp *Caridina nilotica* (Decapopa: Atyidae). MSc Degree, Rhodes University, Grahamstown, South Africa.

DAVIES-COLEMAN (2002) The growth and reproduction of the freshwater limpet *Burnupia stenochorias* (Pulmonata, Ancylidae), and an evaluation of its use as an ecotoxicological indicator in whole effluent testing. PhD (Zoology), Rhodes University, Grahamstown, South Africa.

CHAPTER 1

APPLICATION OF TOXICITY TESTING FOR ENVIRONMENTAL WATER QUALITY MANAGEMENT

1.1 INTRODUCTION

Toxicity tests are accepted world-wide as suitable for managing environmental water quality within water resources management, and in South Africa, this trend is also being followed. Water quality guidelines are derived from, mostly, acute toxicity test results, using results derived from species from different trophic levels in freshwater ecology. Persoone et al. (1990) cited a number of papers indicating that data on lethal concentrations of chemicals in short-term tests can accurately predict mortalities in the field. Nevertheless, acute data cannot be used to indicate safe, long-term exposure concentrations of a particular toxicant (Warne, 1998). Warne (1998) stresses the importance of chronic toxicity data for developing water quality guidelines as water quality guidelines should protect organisms from long term exposure to toxicants. Chronic toxicity data are more indicative of the effects of long-term toxicant exposure and are therefore, potentially, more useful than acute toxicity data. For example, Heming et al. (1989) showed that the organochlorine pesticide methoxychlor displayed a delayed toxic effect to certain fish species. Therefore an acute test may have underestimated the toxic effect of this particular pesticide.

1.1.1 ENVIRONMENTAL REALISM OF TOXICITY TESTS

There are concerns that ecotoxicological tests are too simplistic to reflect real life dose – response relationships since ecotoxicological tests have evolved more from the field of toxicology than ecology (Calow, 2003). Ecosystems are characterised by complex interactions between many species, however the majority of toxicity tests to date have been single species tests. The aim of using single species toxicity tests has been to standardise environmental factors so as to make experiments reproducible (Warne, 1998) but this has been at the expense of environmental realism (Cairns, 1983). A common theme in the literature is the need for toxicity tests that recognise a higher ecological level than just single species tests (Cairns, 1983). Results of single species toxicity tests run under constant conditions are assumed to reflect tolerance in field situations where many species co-exist and conditions are changing over time and space (Cairns, 1983). In reality, toxicity of a particular toxicant may be altered by other water quality parameters in the same environment (Cairns, 1983). For example, the toxicity of certain metals such as lead is affected by water hardness (Warne, 2001). Ecological characteristics such as ongoing functioning of ecosystems, despite the loss of individual species, are not addressed in simple single species toxicity tests. A USEPA study found that single species tests were not a good predictor of effect of a toxicant at the community level, although there was a relationship at a coarse level (Marcus and McDonald, 1992). Additionally, there have been claims that using water quality guidelines derived from single species tests have resulted in no disasters (Cairns, 1983).

Within single species toxicity testing, chronic toxicity tests will generally give more environmentally accurate tolerance information than acute toxicity tests. This is reflected by the ANZECC and ARMCANZ (2000) water quality guidelines which chose chronic toxicity data in preference to acute toxicity data to compile water quality guidelines (Warne, 2001).

Organisms in the field are unlikely to be exposed to toxicants for only 96 hours or less and are more likely to be exposed for extended periods and may suffer sub-lethal effects such as inhibition of reproduction or growth. The lower concentration range and longer exposure time tested for in single species chronic toxicity tests therefore provide more environmentally realistic tolerance information. Multi-species toxicity test data using mesocosm or microcosm systems have been chosen in preference to single species chronic toxicity test data in the generation of the ANZECC and ARMCANZ (2000) water quality guidelines (Warne, 2001), indicating that the greatest deal of confidence of environmental reality is placed with this type of toxicity testing. Unfortunately multi-species toxicity testing is complex and is still in a pioneering stage.

Cairns (1983;1986) notes that a common argument justifying the use of single species tests is that the use of apparently sensitive species will result in guidelines that will protect most of the species in a community, and further comments that this approach is based on the assumption that the biological response chosen will be the most sensitive possible, money and time saved from using a single species toxicity tests will be greater than expenses incurred from rehabilitation of the environment after a bad management decision, and that the species chosen for sensitivity to a particular toxicant will show similar sensitivity to most other toxicants. However, these assumptions are refuted by Cairns (1983, 1986), since only a few species are suitable for laboratory tests and they can hardly be termed the most sensitive species. Although it is reasonable to assume that safe levels of a particular toxicant for a sensitive organism will probably also be safe for most other organisms, it must first be proved that a particular test organism is markedly more sensitive to a particular toxicant than most other organisms. It is unreasonable to assume that endpoints chosen for test species are the most sensitive endpoints possible. In fact, most endpoints in toxicity tests are chosen for reasons of replicability and ease of measurement (Cairns, 1986). In addition, a test species chosen for sensitivity from past results with other toxicants may not display the same sensitivity to the toxicant being studied. In a study of interspecies variability in sensitivity to toxicants, Sloof et al. (1983) concluded that it is not possible to predict the sensitivity of a particular species to one toxicant based on previous toxicity testing of the same species using a different toxicant. Other outcomes of the Sloof et al. (1983) study included the conclusion that the sensitivity of standard test organisms are not greater than the sensitivity of all other test organisms, indicating that safe levels of toxicants for standard test organisms will not necessarily protect all other biota. The results of single species toxicity tests may also be too sensitive as certain toxicants may not have the same degree of bio-availability in the field as they would in the laboratory (Cairns, 1983).

Although single species toxicity tests have obvious limitations, they remain a valuable method of determining safe toxicant concentrations for aquatic organisms and should be used until protocols for toxicity tests at a higher ecological level are developed such as multi-species tests using mesocosms and microcosms. It is however bad practice to extract an unreasonable amount of management information from single species tests (Cairns, 1986). New methods of guideline development, such as species sensitivity distributions, take the sensitivity of many organisms into account, and not just those that are most sensitive (Warne, 1998). Therefore, all tolerance information for a particular toxicant is valuable, regardless of the sensitivity of the test organism.

Many test species are not suitable for full lifecycle toxicity tests (Hutchinson et al., 1998). The preliminary study for this chapter for example, found that young *Caridina nilotica* juveniles have a high natural attrition rate and therefore full lifecycle tests cannot be

performed using this animal. Therefore partial lifecycle tests must be used to predict full lifecycle exposure results. Although literature exists on the relative sensitivity of different life stages in fish, there is a lack of knowledge on the relative sensitivity of life stages of aquatic invertebrates (Hutchinson et al., 1998). This may be because many aquatic invertebrates have a short lifecycle and in many cases the aquatic stage is only part of the lifecycle. However some literature does exist in this respect. Hutchinson et al. (1998) found that according to available chronic data on the ECETOC database, the sensitivity of invertebrate juveniles is greater than or equal to the sensitivity of invertebrate adults in 91% of cases. McHohan et al. (1989) found that younger instars of the larvae of a caddisfly species were less tolerant than the older instars to cadmium. Kefford et al. (2004a) showed that the young juvenile stages of a species of stonefly and a species of caddisfly from the Barwon River in Australia are more sensitive to sea salt than older juvenile stages. Short-term chronic tests run on sensitive early life stages of a test organism may give an indication of lifecycle tolerance levels (McKim, 1977; Giedy and Graney, 1989). McKim (1977) demonstrated that the tolerance of early juvenile stages of certain fish species were comparable to those of full lifecycle tests within a factor of two. It has been suggested that individuals in a younger life stage would have a greater surface area to volume ratio thereby possibly making them more vulnerable to a toxin (Kefford et al., 2004a).

There are as many aspects to 'environmental realism" as there are aspects to ecosystems. One aspect is incorporating into toxicity testing the responses of different levels of biological organisation from the sub-cellular (for example using bio-markers such as Acetylcholinesterase (Gordon et al., 2009) through the organism level species tests reported in this study, to population and community level responses which are rarer (Horrigan et al., 2005, 2007; Kefford 2006.). In this study the focus was on developing sub-lethal responses tests that were robust and gave reliable results, with an emphasis on the use of indigenous South African species.

1.1.2 PROJECT AIMS

The project aims listed in the contract were to:
1. Develop chronic toxicity test methods for indigenous riverine invertebrates using non-lethal experimental end-point assessment criteria such as stress proteins, measures of growth and fecundity and morphometric analyses for both nymph and adult stages (Chapters 2 and 3);
2. Use the newly developed chronic toxicity test methods for indigenous invertebrates to evaluate the current method of determining water quality criteria for the ecological Reserve, which uses single-substances and mortality as an end-point (Chapter 4);
3. Assess effluent standards by testing the newly developed chronic toxicity test method using whole effluent (Chapters 2 and 3, and data reported in Appendix B);
4. Undertake a scoping study of the use of stress proteins and morphometric analyses. Assess how these approaches could be used in combination with biomonitoring and water chemistry information in the evaluation of non-point source pollution and pesticide contamination.
5. Compare results of chronic toxicity tests using indigenous invertebrates to those of *D. pulex* in an ongoing benchmark assessment of the use of *D. pulex* to determine water quality criteria for South African rivers (Chapter 4 and data reported in Appendix A).

Aim 4 was largely superseded by WRC Project 1484, and a summary of findings is presented in Chapter 4.

CHAPTER 2

DEVELOPING ORGANISM-LEVEL CHRONIC TOXICITY TEST METHODS FOR FRESHWATER SHRIMP, *Caridina nilotica*

2.1 INTRODUCTION

The Atyidae is a major family of freshwater decapod shrimps with the genus *Caridina* as the only member of the Atyid shrimps in southern Africa (Hart, 1983). Species within the genus *Caridina* are recognized as being ecologically important (Hart, 1980), playing substantial roles in the food web by clearing debris and epiphytic microalgae from the leaves and roots of submerged macrophytes, and featuring in the diet of fish (Oh et al, 2003), Nile crocodiles, certain birds, including various herons, lake terns, and perhaps also jacana's (Hart et al*.,* 2001). Their ecological importance has stimulated investigations into the reproduction and ecology of atyid shrimps (Oh et al*.*, 2003; Coetzee,, 1988; Hart, 1980; Hart, 2001). Their feeding behaviour is largely directed toward carnivorous or scavenger habits (Schram, 1986), feeding on periphyton scraped from aquatic hydrophytes and on plant detritus, and scavenging on the corpses of animals such as fish, insects and shrimps (Hart et al., 2001).

Caridina nilotica occur in the rivers and lakes of southern Africa (Hart, 1981) and scattered observations suggest that it is widespread elsewhere in warm waters at least in the eastern Central and North Africa (Hart et al., 2001). According to Barnard (1950) (quoted by Hart (1983)), the distribution of *C. nilotica* in South Africa extends as far south as the Umzimvubu River and as far west as Lake Ngami. Specimens of *C. nilotica* have been collected in the Keiskamma and Bushmans River (Eastern Cape), representing a southerly extension of its distribution (Hart, 1983). In 1984, a further southerly extension to *C. nilotica* distribution was determined when specimens were found in the Gamtoos River (Coetzee, 1985). In most crustaceans, the relationship between embryonic duration and temperature appears to be a negative curvilinear function (García-Guerrero et al*.*, 2003; Hancock, 1998, Hart, 1980; Sutcliffe and Carrick, 1981; Yamaguchi, 2001). Hart (1983) found that *C. nilotica* have a temperature activity range of 11.5 to 31.5˚C and concluded that temperature tolerance may be a significant factor affecting this organism's distribution. In southern Africa, *C. nilotica* are ecologically important as herbivores and scavengers (Hart, 1981). *C. nilotica* have a specialised feeding system and feed as a scraper of surfaces of organic macrophytes and other substrates, feeding on microbiota (Hart, 1980). As scavengers, the species may enhance the production of submerged macrophytes by removing debris and epiphytic microphlora (Hart, 1981). These shrimp may also be an important source of food for small fish (Hughes, 1992). *C. nilotica* comprise a significant proportion of zooplankton in Lake Victoria (Hart, 2001, quoting Lehman et al*.*, 1996). The species time from 'egg to egg' takes approximately three months (personal observation and Muller, pers. comm., 2004) and individuals transform from male to female with increasing age (Okuthe et al., 2004).

Most carideans have separate sexes (Schram, 1986), and protandrous hermaphroditism is assumed to be a typical trait in the atyids (Hart et al*.,* 2001, Schram, 1986); the shrimps start out as males and then at a later stage they change into females. The androgenic gland, the site of a factor that controls development of male characteristics, degenerates (Landau and Laufer, 1999). Oh et al*.* (2003) stated that temperate atyids have a single defined breeding

period, whereas tropical and subtropical atyids breed throughout the year. This is also supported by Hart (1981) when he determined that *C. nilotica* bred continually throughout the year. Reproduction in decapods is exclusively sexual, with no resting stages evident in the life cycle. Mating occurs immediately or soon after the female moults and spawning occurs within a few hours after mating. Further mountings by other males can occur, but the female holds her pleopods forward to prevent other spermatophores from being implaced on her last thoracic or sometimes first abdominal sternites. Females in breeding state have extra setae on the posterior thoracic sternites to hold the spermatophore and direct the eggs as they exit the genital opening. Before the actual spawning and all through the brooding period, the female grooms the pleopods and egg masses using specialized setae on the thoracic appendages (Schram, 1986).

The success of reproduction in most crustaceans may be due to the fact that the female carries the fertilized eggs during embryogenesis until hatching, resulting in a high survival rate for the eggs (Hart et al., 2001; Landau and Laufer; 1999; Schram, 1986). In decapods the female carries the eggs in her brood chamber made of a complex of setae on the pleopods and pulpates and turns them over regularly for aeration until hatching, the eggs contain large quantities of yolk and are attached to the pleopods with thin filamentous membrane (Landau and Laufer, 1999; Nazari et al., 2000; Schram, 1986). Protection and ventilation are two important functions of crustacean maternal care (Adiyodi and Adiyodi, 1994).

Based on their wide distribution, and ability to withstand laboratory conditions (see Section 2.2), *C.* nilotica was considered a potentially suitable test organism for the development of chronic toxicity test methods using a local species.

Many test species are not suitable for full lifecycle toxicity tests (Hutchinson et al., 1998), therefore partial lifecycle tests must be used to predict full lifecycle exposure results. Literature exists on the relative sensitivity of different life stages in fish, However, there is a general lack of knowledge on the relative sensitivity of life stages of aquatic invertebrates (Hutchinson et al., 1998). This may be because many aquatic invertebrates have a short lifecycle and in many cases the aquatic stage is only part of the lifecycle. Hutchinson et al. (1998) noted that according to available chronic data on the ECETOC database, the sensitivity of invertebrate juveniles is greater than or equal to the sensitivity of invertebrate adults in 91% of cases. McCahon et al. (1989) found that younger instars of the larvae of a caddisfly species were less tolerant than the older instars to cadmium. Kefford et al. (2004) showed that the young juvenile stages of a species of stonefly and a species of caddisfly from the Barwon River in Australia are more sensitive to sea salt than older juvenile stages. Short-term chronic tests run on sensitive early life stages of a test organism may give an indication of lifecycle tolerance levels (McKim, 1977; Giesy and Graney, 1989). McKim (1977) demonstrated that the tolerance of early juvenile stages of certain fish species were comparable to those of full lifecycle tests within a factor of two. It has been suggested that individuals in a younger life stage would have a greater surface area to volume ratio thereby possibly making them more vulnerable to a toxin (Kefford et al., 2004). For these reasons, it was decided to focus separately on two developmental phases of *C. nilotica* (Section 2.4.1 and 2.4.2).

2.2 LABORATORY CULTURES OF *Caridina nilotica*

Caridina nilotica were collected from a reference site in the Mpsini stream (Richards Bay, KwaZulu-Natal) in 1999 and a laboratory culture was initiated.

Developing a laboratory culture for *C. nilotica* was largely a result of trial and error as little is known about the biology of the species. The methods, equipment and feeding described here are still being refined, but are currently in use in the Unilever Centre for Environmental Water Quality laboratories to obtain test organisms for use in acute and chronic toxicity tests.

We have found that *C. nilotica* can be used within toxicity tests for a range of toxicants (single substance toxicity tests, effluent (industrial and sewage) toxicity tests and receiving (surface) waters) and using different life-history stages. Different life-history stages have been recognised for use in both acute and chronic toxicity tests, and the breeding method described allows for obtaining the specific life-history stage for a specified test. For example, acute toxicity tests can be undertaken using embryos, neonates (defined as <7 days), juveniles (defined as 20-30 days old) and adults (defined as >40 days). Chronic toxicity tests have been developed for the embryo and juvenile life-history stages, but the development of full life-cycle (approximately 3 months from egg to egg) tests has not been precluded although not yet attempted.

2.2.1 EQUIPMENT, REAGENTS AND OTHER MATERIALS

In order to establish a laboratory culture of *C. nilotica* for the production of organisms for acute and chronic toxicity tests, the basic equipment and materials required are listed in Table 2.1. The laboratory breeding facility must be free of vapours, odours and dust that may be toxic to the shrimps, and ideally the test facility is kept separate from the culture facility.

All glassware, test vessels and non-disposal-non-glass containers used for breeding and test purposes is cleaned according to the following protocol:

- Rinse with tap water
- Soak in phosphate free soap (5% solution) for 15-30 minutes.
- Rinse three times with dechlorinated water
- Soak in 10% hydrochloric acid solution for 30 minutes
- Rinse three times with deionised water
- Rinse three times with dechlorinated water
- Air dry glassware
- All washed glassware, test vessels and containers should be stored in a dust free environment
- All glassware and equipment must be rinsed thoroughly with dilution water immediately before usage.

The necessary reagents are prepared as follows:

- Phosphate free soap, 5% solution: prepare by adding 1 l to 20 l dechlorinated tap water. The solution is stored in a polyethylene container between 15°C and 25°C and is stable for three months.
- Hydrochloric acid, 10% solution: prepare by adding 6.25 l, 32% HCl to 20 l dechlorinated tap water. The solution is stored in a polyethylene container between 15°C and 25°C and is stable for three months.

- Ethanol, 70% solution: prepare by adding 729.2 mL, 96% Ethanol to 1 l deionised tap water. The solution is stored in a polyethylene container between 15°C and 25°C and is stable for three months.

TABLE 2.1 Basic equipment necessary to establish a breeding culture of *Caridina nilotica*.

Equipment	
Breeding tanks (50 l)	Rearing tanks (10 l)
Air pumps and air tubing (5 mm)	Tubing connecters, splitters and regulators
Air stones (round)	Kaolin stones and/or tiles
Aquarium heaters (100W and 50W)	Thermometers
Power filters and under gravel filters	Medium shell grit and gravel
Webbing	Perspex and/or dowling rods
Black plastic (thick ±5-7 mm)	Fine netting
Buckets (approximately 20 l-25 l)	Polyethylene water container with tap (25 l)
Mini fish nets	Breeding nets (10cm x 10cm x 10cm net constructed from fine mesh netting)
Marine silicone (clear) and PVC weld	Dechlorination filter unit and filter
Activated bone charcoal	Plastic jugs (5 l)
Glass pipettes, 5 mL and 10 mL	Minimum/maximum thermometer
Breeding laboratory equipped with a light timer	Biolux and fluorescent light tubes
Toothbrushes	pH meter
Conductivity meter	Dissolved oxygen meter
Nitrate strips (dipstick tests)	Chlorine strips/Lovibond comparator
Glass microscope slides (scrapers)	Datasheets
Reagents (all reagents must be analytical grade)	
Hydrochloric acid (HCl, 32%)	Deionised water
Ethanol (EtOH, 96%)	Appropriate medicine, as and if required
Phosphate free soap (Extran)	
Food	
Spirulina flakes and Tetramin® flakes	Hikari wafers (17 mm)

2.2.2 CULTURE MAINTENANCE AND BREEDING

Breeding tanks are used to maintain the females and males in culture. Mixed cultures are necessary as the species reproduces sexually. Gravid females are removed from the culture on a routine basis and placed in **rearing tanks**. As the breeding cultures are checked (at least) twice weekly for the appearance of gravid females, the approximate ages of embryos are known (±∀3 days), and can be used to predict the appearance of neonates (or older individuals) so toxicity tests can be planned accordingly. More frequent checking of cultures for gravid females will result in greater accuracy of aging the embryos, necessary for the embryo tests (see section 2.4.2).

Breeding tanks undergo routine daily, weekly and longer term maintenance, described in Table 2.2, and are set up as follows:

- Each tank is given a unique number, noted on the data sheet.
- The breeding (50 l) tank is thoroughly rinsed with dechlorinated tap water. Do not use any soap (even phosphate free) or detergent.
- Fill the breeding tank with dechlorinated tap water to approximately 5cm from the top of the tank.
- Place the power filter on the side of the tank and fill with dechlorinated tap water.
- Place an aquarium heater with temperature control into the breeding tank and insert a thermometer. The temperature in the tanks is maintained between 26-27°C, ±1°C.
- Add webbing (attached to a dowel, for vertical substrate surface), kaolin stones and tiles into the breeding tank.
- Add air stones, air tubing and an air pump to the breeding tank.
- Allow the tank to settle for one week before introducing shrimps.

TABLE 2.2 Routine *Caridina nilotica* breeding tank maintenance

Daily maintenance (Monday to Friday)	▪ Remove dead shrimps from the tanks and record ▪ Record temperatures (mornings and afternoons) and adjust aquarium heaters as required ▪ Ensure that aerators, air pumps and filters are working. ▪ Add dechlorinated water to the tanks to compensate for evaporation ▪ Feed shrimp once / twice daily. Food should be carefully controlled to prevent accumulation of debris. Only feed a second time if all the food has been eaten by late afternoon
Weekly maintenance	▪ Siphon excess food, debris and waste from tank. Be careful not to remove all faecal pellets, for the shrimps scrape them for food ▪ Measure pH, conductivity and dissolved oxygen (DO): record ▪ Dilute with deionised water if conductivity >60 mS/m ▪ pH should be between 6 and 8.5 ▪ DO should be >70% of saturation ▪ Check breeding tanks for gravid females on Monday and Thursday afternoons. Remove and place in rearing tanks ▪ Clean filter netting as required
Long-term maintenance	▪ Scrape tank walls to remove excessive build-up of algal growth as necessary ▪ Scrape kaolin stones and tiles to remove excessive build-up of algal growth as necessary ▪ Clean filters and shell grit in filters as required ▪ Clean webbing and air stones as necessary ▪ Clean aquarium heaters and thermometers from algal growth as required ▪ Measure nitrate and chlorine levels at least once a month

Shrimps of a range of different sizes are kept in each breeding tank. This is because larger individuals are females while smaller individuals are males (Okuthe et al., 2004) and since reproduction is sexual, it is important to maintain a variety of sizes in each of the tanks. Individuals not used in toxicity tanks are randomly placed in the breeding tanks, ensuring a range of different sized individuals in each tank for breeding purposes. Apart from the maintenance checks, the breeding tanks are checked twice weekly (to minimize disturbance to the shrimps) for gravid (those carrying eggs) females. These gravid females are removed carefully using a mini fish net, place in a plastic beaker with water collected from the tanks. Once all the tanks have been checked, all gravid females are released simultaneously into their specifically allocated rearing tanks (Section 2.2.3). Females are left in the rearing tanks until eggs/neonates are released from the brood pouch after which they are caught and randomly assigned to breeding tanks to minimize in-breeding (the genetic variability of the breeding stock will have to be assessed in a future research project).

2.2.3 OBTAINING TEST ORGANISMS FOR TOXICITY TESTS

Rearing tanks are used to provide a constant environment for gravid females, where they are less likely to be disturbed by other individuals which may result in possible loss of eggs. In addition, females that are no longer gravid are easily removed from these smaller tanks and neonates and juveniles are maintained under adult-free conditions until they are required for toxicity tests or introduced to culture tanks for breeding purposes. In the Unilever Centre for Environmental Water Quality, eight rearing tanks have been set-up to ensure that the neonates are old enough after hatching to be used for toxicity tests and removed from the tanks before the next set of gravid females are placed into the rearing tanks.

Rearing tanks undergo routine daily, weekly and longer term maintenance, described in Table 2.3, and are set up as follows:

- Each tank is given a unique number and noted on the data sheet.
- The rearing (10 l) tank is thoroughly rinsed with dechlorinated tap water. Do not use any soap (even phosphate free soap) or detergent.
- Rinse the gravel and medium shell grit with dechlorinated tap water until all dust is removed and the water runs clear.
- Place the under gravel filter in the tank, place the rinsed gravel shell grit mixture at the bottom of the tank to cover the under gravel filter completely.
- Fill the rearing tank with dechlorinated tap water to approximately 5cm from the top of the tank.
- Place an aquarium heater with temperature control into the rearing tank and insert a thermometer.
- Add webbing, for vertical substrate, to the rearing tank.
- Add air stones, air tubing and an air pump to the rearing tank.
- Allow the tank to settle for one week before introducing the shrimps.

TABLE 2.3 Routine *Caridina nilotica* rearing tank maintenance

Daily maintenance (Monday to Friday)	▪ Remove dead shrimps from the tanks and record ▪ Record temperatures (mornings and afternoons) and adjust aquarium heaters as required ▪ Ensure that aerators and air pumps are working ▪ Add dechlorinated water to the tanks to compensate for evaporation ▪ Feed shrimp once daily. Food should be carefully controlled to prevent overfeeding and accumulation of debris
Weekly maintenance	▪ Siphon excess food, debris and waste from tank. Be careful not to remove all faecal pellets, for the shrimps scrape them for food ▪ Measure pH, conductivity and dissolved oxygen (DO) twice weekly on Tuesday and Thursday ▪ Dilute with deionised water if conductivity >60 mS/m ▪ pH should be between 6 and 8.5 ▪ DO should be >70% of saturation ▪ Remove female shrimps from rearing tanks as the neonates are released and place back into the breeding tanks
Long-term maintenance	▪ Scrape tank walls to remove excessive build-up of algal growth as necessary ▪ Scrape kaolin stones and tiles to remove excessive build-up of algal growth as necessary ▪ Cleaning of shell grit at the bottom of tank as necessary ▪ Cleaning of webbing and air stones as necessary ▪ Clean aquarium heaters and thermometers from algal growth as required ▪ Measure nitrate and chlorine levels at least once a month

To collect test organisms (independent of age) from the rearing tanks, the organisms are first transferred to a holding net. Neonates and juveniles are gently collected in a mini fish net (extra fine netting): the net should not breach the surface of the water. A 5 mL or 10 mL pipette (depending on the size and age of the shrimps that are being collected) is used with gentle suction to collect the organisms out of the net and placed into the larger breeding net which is placed in a 2 l round bottomed container filled with tank water. The test organisms should not come into contact with air as this causes stress to the animals. The container with breeding net is then placed in the relevant test laboratory so that the organisms can acclimate to the new temperature before commencement of the specific toxicity test.

To transfer test organisms from the breeding net to the test container, a 5 mL or 10 mL pipette (depending on the size and age of the shrimps that are being collected) is used with gentle suction to collect the test organisms and release them into the different test containers. Again, the test organisms should not come into contact with air as this causes them stress. The pipette should be rinsed (using dechlorinated tap water) between every transfer, especially between concentrations, before taking the next neonate from the breeding (holding) net. Dilution of the test sample is prevented by not releasing the pressure on the pipette when the test organisms are released but rather waiting for them to swim out of the pipette. If more than the required number of organisms is accidentally released into the test

beaker, do not attempt to remove the extra animals, rather take the total number of test organisms into account when analysing results.

It has been noted that a certain level of skill is required to handle the organisms. The younger individuals (neonates) in particular appear to be extremely sensitive to handling and great care and a gentle touch is required to transfer test organisms between tanks, holding vessels and test vessels. Older test organisms (>40 days) appear robust and are easily transferred using a small fish net with fine mesh, although care must be taken to ensure that individuals are not kept out of water for extended periods of time.

2.3 ACUTE TOXICITY TEST METHODS FOR *Caridina nilotica*

Acute toxicity tests have been developed for neonates (<7 days old), juveniles (20-30 days old) and adult (>40 days old) *C. nilotica*. These age groupings were selected on the basis of their different sensitivities to handling (in particular the neonates and juveniles) balanced against the mortality rates in the rearing tanks (in particular the neonates) while still providing sufficient test organisms to undertake an acute toxicity test. Further refinement of conditions in the breeding and rearing tanks as a result of further knowledge of the species' biology may result in reduction in rearing tank mortality rates and further refinement of the ages of the neonate and juvenile groupings. All tests are undertaken in a temperature controlled facility, with ambient temperature not varying by more than 2°C during the test. Ambient laboratory illumination (artificial and/or day light) is used, with a photoperiod of 12hrs light:12hrs dark.

Adult *C. nilotica* (i.e. individuals older than 40 days) have been used successfully in artificial streams, following the standard protocol for using riffle-dwelling organisms (DWAF, 2000). However, the flowing water environment created in the recirculating streams is not appropriate for testing younger individuals (<30 days) and it is recommended that static tests are used to undertake acute toxicity tests for neonates and young juveniles.

The procedures for undertaking static toxicity tests for *C. nilotica* are the same as those described in USEPA (1985) for *Daphnia pulex* 48 hour acute toxicity test and fish 96 hour acute toxicity test. The *D. pulex* 48 hour acute toxicity test protocol is used for *C. nilotica* neonates (test organisms less than 7 days) and the fish 96 hour acute toxicity test protocol is used for *C. nilotica* juveniles (20-30 days old, although older individuals can also be used) (Table 2.4). The test end point is either mortality, or when no movement of the body or appendages is noted when the test individuals are prodded gently.

TABLE 2.4 Summary of test conditions for different ages of *Caridina nilotica* in static toxicity tests.

Test parameters	Condition for different ages of test organisms	
	< 7days	**20-30 days / > 40 days**
Water temperature	21°C ± 2°C	21°C ± 2°C
Laboratory illumination	Biolux and Fluorescent	Biolux and Fluorescent
Photoperiod	12hrs dark: 12hrs light	12hrs dark: 12hrs light
Oxygen concentration	As obtained by diluent	As obtained by diluent
pH	As obtained by diluent	As obtained by diluent
Feeding regime	None	None
Size of test container	50 mL	600 mL
Volume of test sample	25 mL	250 mL
Number of organisms per container	5	10
Number of containers	4	2
Total number of organisms per test	20	20
Control and diluent water	Dechlorinated tap water	Dechlorinated tap water
Test duration	48hrs	96hrs
Frequency of monitoring	30 min, 1, 2, 4, 8, 24 and 48 hours	12 hourly intervals until 96 hours

2.4 CHRONIC TOXICITY TEST METHODS FOR *Caridina nilotica*

2.4.1 *CARIDINA NILOTICA* GROWTH, REPRODUCTION AND MORTALITY AS CHRONIC (LONG-TERM) TOXICITY TEST ENDPOINTS

A chronic (long-term) test method using *Caridina nilotica* growth, reproduction and mortality as test endpoints was developed as part of an MSc degree (Slaughter, 2005). The toxicants used to develop the method were NaCl and Na_2SO_4, but further research is required using other toxicants to adequately assess growth as a suitable experimental endpoint for exposure studies using *C. nilotica*.

2.4.1.1 Experimental method

Preliminary experiments in 2003 were used to develop and refine a successful protocol for chronic testing using *C. nilotica* and as range finding tests for determining a tolerance range for NaCl and Na_2SO_4. The outcomes of these experiments included the realisation that young *C. nilotica* juveniles appear to have a high natural attrition rate, leading to unacceptable control mortalities if used in chronic tests. These initial experiments also helped to determine a suitable test concentration range for NaCl, and the development of an appropriate feeding protocol. A method for measuring growth as a biological response of this species was refined. The methods and results of these preliminary tests are not recorded here as the tests were reproduced using the more refined protocol in 2004 and subsequently yielded better results.

Life stages used and biological endpoints measured

The chronic toxicity test developed using *C. nilotica* is essentially a partial life-cycle test. At test initialisation, juveniles of approximately 45 days in age are used. The exposure period

ends when females become gravid and is approximately 80 days as most individuals start developing eggs within 90 days of age (Muller, pers. comm.). At the end of the test, individuals are approximately 125 days of age. A separate method for chronic tests using *C. nilotica* embryos was undertaken (see Section 2.4.2). No acclimation periods were implemented as test organisms were obtained from a laboratory culture which was maintained under similar temperature and light regimes.

Ecologically important biological responses such as survival, growth and reproduction should be measured in a chronic toxicity test (Warne, 1998; Giesy and Graney, 1989). It was decided to focus on survival, growth and reproduction as measurable biological responses in this study.

Survival was measured by counting and recording dead individuals in the treatments daily and removing them from the experimental chambers.

The effects of the salts on growth rate of *C. nilotica* may be as a result of energy within individuals being spent on detoxification of the toxicant rather than growth (Giesy and Graney, 1989; Lanno *et a1.,* 1989). Carapace length measurements as an indication of growth were made by measuring the distance laterally from the anterior margin of the carapace behind the insertion of the eyestalk to the most posterior margin of the carapace (Hart, 1980). At set intervals, 20 individuals (or as many as were available if less than 20 remained in the experimental vessels) were removed from the experimental vessels and their carapace lengths measured. All individuals from a particular treatment were placed in a petri-dish with some of the original treatment solution. The petri-dish was placed into a shallow container containing ice-cold water and finely crushed ice. The container was placed under a microscope fitted with a micrometer. As individuals ceased movement due to the cold temperature, carapace lengths were readily measured, and the individuals returned to the relevant treatment vessel within the experiment. This method of measurement is non-destructive and non-intrusive, as indicated by low control mortalities within the chronic tests. Carapace length measurements occurred at set intervals, namely on days 0, 4, 10, 24, 38, 52, 66 and 80. These intervals were chosen arbitrarily, with shorter intervals used in the earlier stages of the test because of the higher growth rates in young juveniles (Hart, 1980).

In addition to carapace length measures, growth was estimated by measuring dry weights of individuals. Dry weights were taken at the end of experiments only, as the method is destructive. Dry weights of individuals were undertaken by oven drying the organisms for 48 hours at 105 ˚C. Brower et al. (1990) suggested drying at 105˚C until the weights of the samples stabilise. However, since the chronic test concerns relative dry weights of individuals and not absolute biomass measures, a constant drying period of 48 hours was used. Females were removed from the experiment at the first sign of being gravid, and their carapace length and dry weight measured. It is unlikely that females will experience significant growth after becoming gravid as the formation and maintenance of the eggs will consume the majority of growth energy available (Hart, 1980).

Reproductive endpoints were also measured. Females were removed from the experiment at first sign of carrying eggs. The eggs were removed from the female using a soft paintbrush and overall number of eggs per female counted.

Experimental setup

Experimental vessels used were rectangular glass tanks of approximately 14 litres in volume with 12 litres of diluent placed in each tank. The number of shrimp released into the tanks for each exposure experiment was dependent on availability of juveniles from the rearing tanks and thus varied between replicates and toxicants (n=15-45). The shrimp were kept free swimming in these vessels. Netting was suspended within each tank as substrate for the neonates and to prevent individuals from being washed around the vessel. A ceramic tile was also placed in each vessel as an attachment surface for the neonates. Cling wrap was placed over the tank top openings to prevent excessive evaporation and to prevent individuals from jumping out of the tanks.

Temperature of the laboratory was controlled by air conditioning maintained at approximately 22°C (±2°C). Temperature of the vessels was maintained at approximately 24°C (±1°C) using aquarium heaters. A light-dark regime of 12/12 hours was used. No filters were used within the vessels but concentrations were renewed once a week, following a static-renewal protocol as defined by Rand et al. (1995). All vessels were aerated using air stones attached to aquarium air pumps with rubber tubing.

Cooney (1995) recommends that a specific feeding protocol outlining food type, ration and frequency of feeding be used in chronic tests. The selected feeding regime can affect the metabolism of the test animals, which may in turn affect the rate of uptake, absorption and depuration of the toxicant (Lanno et al., 1989). The selected feeding regime may also affect the biochemical composition of the test organism, which could affect their response to a toxicant (Lanno et al., 1989). *C. nilotica* individuals were fed algal flakes ground to a fine powder. Faecal pellets were left in the tanks as algae or bacteria growing on the pellets are a source of food that the shrimp continually scrape (Hart, 1980). Additionally, a ceramic tile seeded with algae was placed in each experimental vessel.

It was decided to use the '*feeding ad libitum*' feeding protocol method (Lanno et al., 1989). This is basically 'feeding to satiety' i.e. enough food for the test organisms to consume until satisfaction. A method to ensure that '*feeding ad libitum*' is occurring as suggested by Muller (pers. comm., 2004) is to examine the transparent feeding canal of the animals on a daily basis. If the test organisms are indeed feeding to satiety then food will be visible in the alimentary canal. To determine whether the individuals were being provided with enough food, the alimentary canals of the shrimp were observed daily during mortality readings. If it appeared that many of the individuals had empty alimentary canals, the amount of food added to the experimental vessel was increased. Animals in the test were fed every day after mortality observations. A small amount of ground algal flakes was placed into each vessel using forceps, ensuring that the food sank to the bottom.

Temperature of the water within the vessels was measured every day using thermometers during the daily mortality readings. The minimum and maximum air temperatures of the laboratory were also recorded daily. Treatment concentrations were changed on a weekly basis. Before and after each water change, Electrical Conductivity and Total Dissolved Solids (Cyberscan 200 conductivity meter), Dissolved Oxygen (Wissenschaftlich Technische Werkstätten OXI 92 dissolved oxygen meter), pH (Cyberscan pH 10 meter) and light intensity were measured. Water samples were taken periodically for measurement of pH, alkalinity, ammonium, nitrate and nitrite, fluoride, sodium, magnesium, silicon, phosphate, sulphate, chloride, potassium, calcium, Electrical Conductivity and Total Dissolved Solids,

barium, boron, vanadium, lead, cadmium, molybdenum, strontium, zinc, copper, nickel, iron, manganese, chromium and aluminium in order to check that water quality parameters, other than the selected toxicants, remained consistent between treatments and replicates. Water samples were analysed by the Department of Water Affairs and Forestry Resource Quality Services (RQS), Pretoria, South Africa.

2.4.1.2 Results

NaCl Experiment

Mortality

Generally, the chronic survival response of *C. nilotica* to NaCl over the full 80 days exposure period was similar for all three replicates (Table 2.5). All treatments under 2700 mg/L showed low mortality. It appears that the 2700 mg/L treatment may be a threshold where increased mortality begins to appear, with the relatively similar 2500 mg/L treatment showing low mortality. The means of mortality response categorised by treatment over the exposure period for all replicates grouped are plotted with standard deviations indicated as whisker-plots around the means (Figure 2.1). It is obvious that the 2700 mg/L treatment showed significantly higher mortality than the control over the entire exposure period. Grouped mortality data for all three replicates at 80 days were analysed for statistical differences between treatments. The log transformed mortality data showed the highest normality in distribution (Shapiro-Wilk $W=0.85$, $p<0.05$). None of the transformations used produced homogeneous data as indicated by the Levene's Test ($p<0.05$). A non-parametric Kruskal-Wallis Test showed no significant differences among the treatments ($p>0.05$). It was evident however from Figure 2.1 that the 2700 mg/L treatment was significantly different from the control. The LOEC for mortality was therefore the 2700 mg/L treatment and the NOEC was the 1900 mg/L treatment.

TABLE 2.5 Control mortalities and LC_{50} values determined using regression models in the NaCl chronic toxicity test. '*' – Could not be calculated.

Replicate	LC_{50} (mg/L)	95% Confidence limits		% Control mortality
		Upper	Lower	
1	2425	*	1700	0
2	2490	3200	2120	13.3
3	2637	2665	2598	33.3

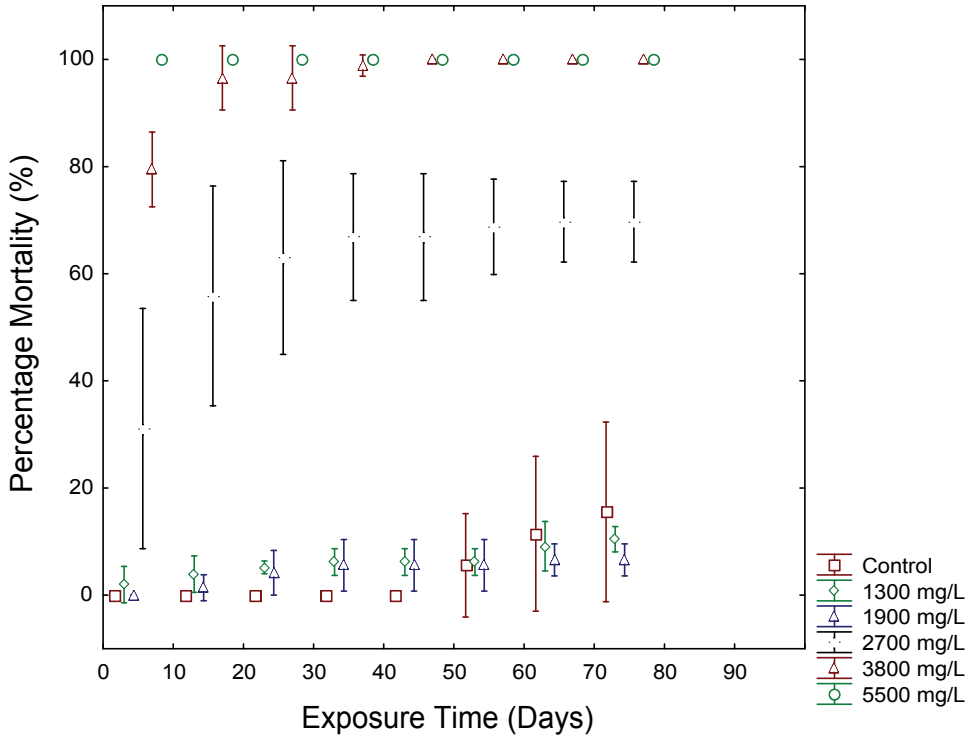

FIGURE 2.1 Means and standard deviations of the grouped mortality results of all replicated concentration ranges in the NaCl chronic toxicity test.

Mortality results for each replicate at 80 days exposure period were analysed using the Probit (USEPA, 1993) model. The Probit model was not suitable for the first two replicates as indicated by a chi^2 value. The mortality data was therefore analysed using least square linear regression (Figure 2.2). The LC_{50} for the first replicate was estimated to be 2425 mg/L. Linear regression did not fit the mortality results of the first replicate particularly well with an r^2 of 0.57. Since a positive relationship between NaCl concentration and mortality was only evident from the 1900 mg/L treatment, a straight line was fitted to the results of the 1300 to 3300 mg/L treatment, thereby ignoring the mortality results in the control (Figure 2.2B).The LC_{50} for the second replicate was estimated to be 2490 mg/L. The mortality results of the third replicate fitted the Probit model (Figure 2.3) as indicated by a chi^2 value. The LC_{50} as estimated by the Probit model was 2637 mg/L. Control mortalities were acceptable for all replicated concentration ranges except that of replicate three (Table 2.5), if the boundary of 20% control mortality for chronic toxicity tests (Cooney, 1995) is accepted. Control mortality in replicate 3 started after day 40 (Figure 2.1); the reason for mortality in the control of replicate 3 could not be established.

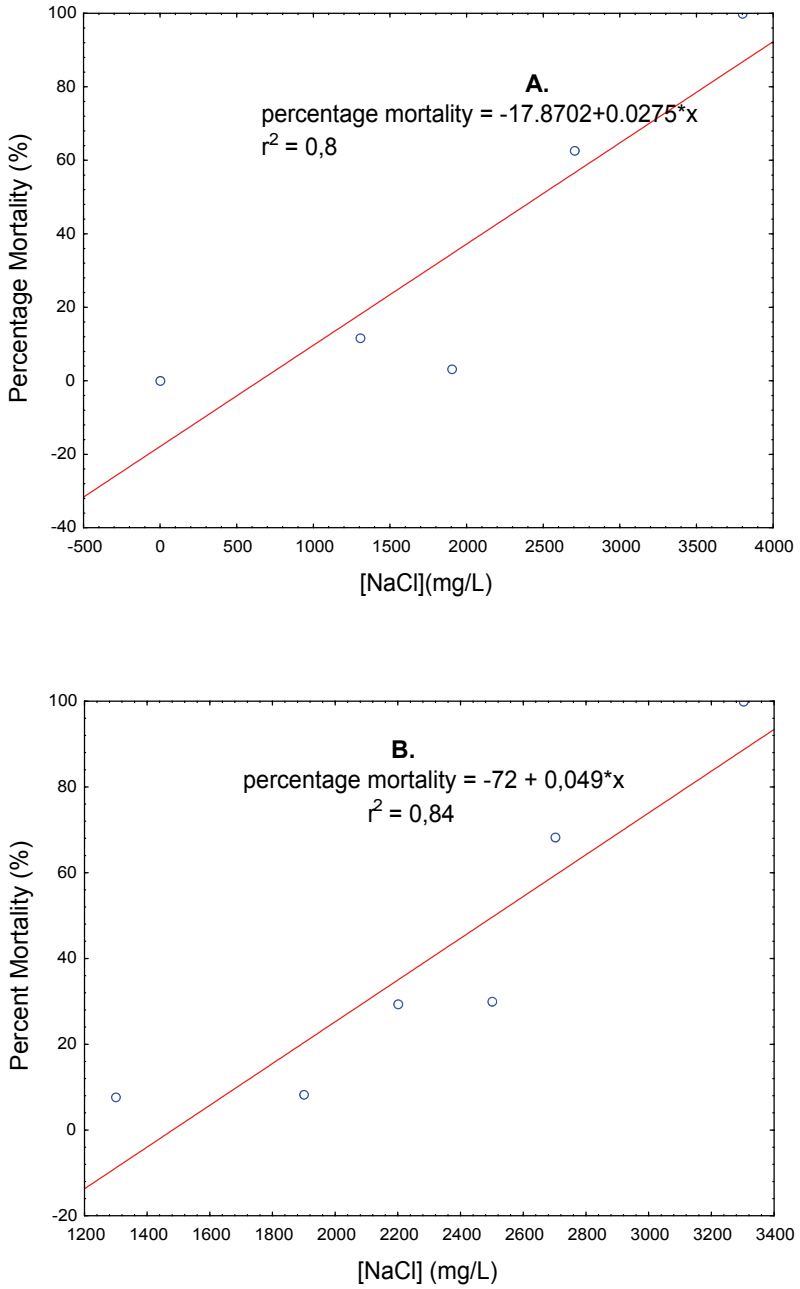

FIGURE 2.2 Regression lines fitted to accumulated mortality response over time for two replicated concentration ranges in the NaCl chronic experiment. Regression equations as well as goodness of fit (r^2) are indicated (Figure A – replicate 1 and Figure B – replicate 2).

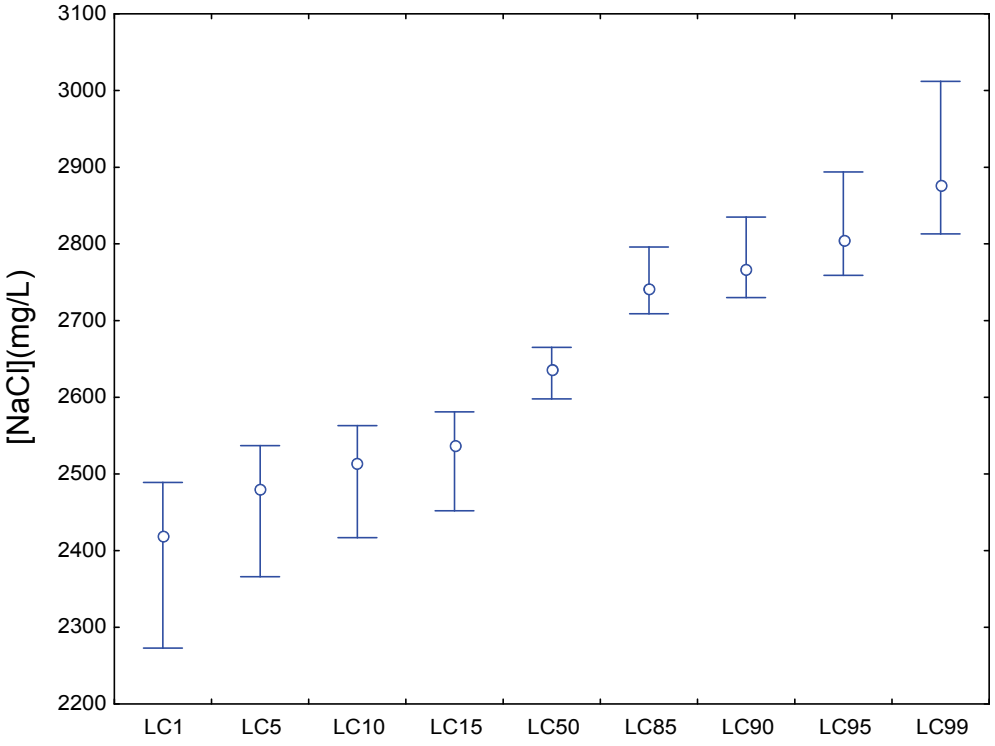

FIGURE 2.3 The LC_x and 95% confidence intervals of the third replicated concentration range mortality results of the NaCl chronic toxicity test analysed using the Probit model (Probit Program Version 1.5).

Growth

Growth results are shown in Figure 2.4 with means and standard deviations of carapace length analysed separately for each replicate. Carapace lengths for all three replicates were not grouped for analyses, as there were inter-replicate differences in the size of the young juveniles at the start of each replicate (Figure 2.4A-C), which may have affected the results of statistical analysis. Differences between treatments were tested for at exposure durations where the standard deviations of the treatments did not appear to overlap (Figure 2.4A-C), although differences between treatments at the full 80 days duration were tested for if non-overlapping of standard deviations persisted for the full exposure duration. No carapace growth measurements were possible for treatments where there was 100% mortality.

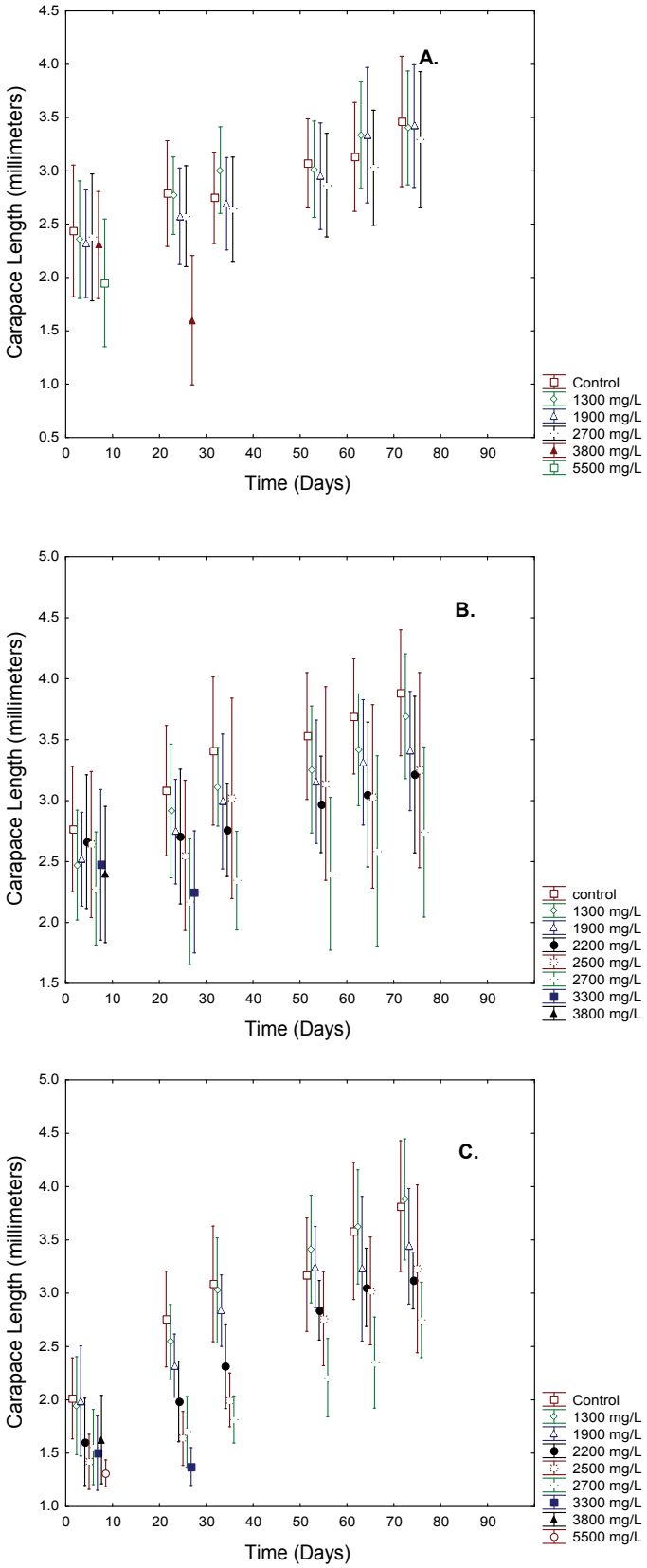

FIGURE 2.4 Means and standard deviations of carapace length results at each of the sampling periods for the three replicated concentration ranges of the NaCl chronic toxicity experiment (Figure A – replicate 1, Figure B – replicate 2 and Figure C – replicate 3).

There were no significant differences between treatments in the first replicate (Figure 2.4A). Replicate 2 showed a significant decrease in mean carapace length with increasing salt concentration. However, carapace length data between treatments appears to show significant difference only on day 80 (Figure 2.4B) and therefore day 80 data were analysed for significant differences between treatments. The untransformed carapace length data showed the highest degree of normality in distribution (Shapiro-Wilk $W=0.96$, $p<0.05$) but none of the data transformations used produced homogeneous data as determined using the Levene's test ($p<0.05$). A non-parametric Kruskal-Wallis test showed that the 2700 mg/L treatment was significantly different from the control on day 80 ($p<0.05$). Therefore the LOEC for carapace length of replicate 2 was the 2700 mg/L treatment and the NOEC was the 2500 mg/L treatment. Replicate three also showed a trend of decreasing carapace length with increasing NaCl concentration, but the trend appears significant from much earlier in the test. Statistical differences between treatments on day 80 were investigated. The log transformation showed the highest degree of normality in distribution (Shapiro-Wilk $W=0.96$, $p<0.05$) but none of the transformations produced homogeneous data as indicated by the Levene's Test ($p<0.05$). A non-parametric Kruskal-Wallis Test ($p<0.05$) showed that all treatments from 2200 mg/L and higher were significantly different from the control on day 80. The LOEC for carapace length of replicate 3 was therefore the 2200 mg/L treatment and the NOEC was the 1900 mg/L treatment.

The means and standard deviations of dry weight results for the three replicates are represented in Figure 2.5. There did not appear to be any obviously significant differences between treatments for replicate 1 (Figure 2.5A) and 3 (Figure 2.5C). Figure 2.5 B did however indicate significant differences among treatments in replicate 2. A Levene's test ($p<0,05$) indicated that the untransformed data had equal variance. The untransformed data has the closest distribution to normality (Shapiro-Wilk $W=0.98$, $p<0.05$). An ANOVA indicated the presence of a significant difference between treatments ($p<0.05$) and a parametric Tukey test indicated that the 2200 mg/L treatment and all higher treatments were significantly different ($p<0.05$) from the control. Therefore the 2200 mg/L treatment was taken as the LOEC and the 1900 mg/L treatment as the NOEC for the dry weight results in replicate 2.

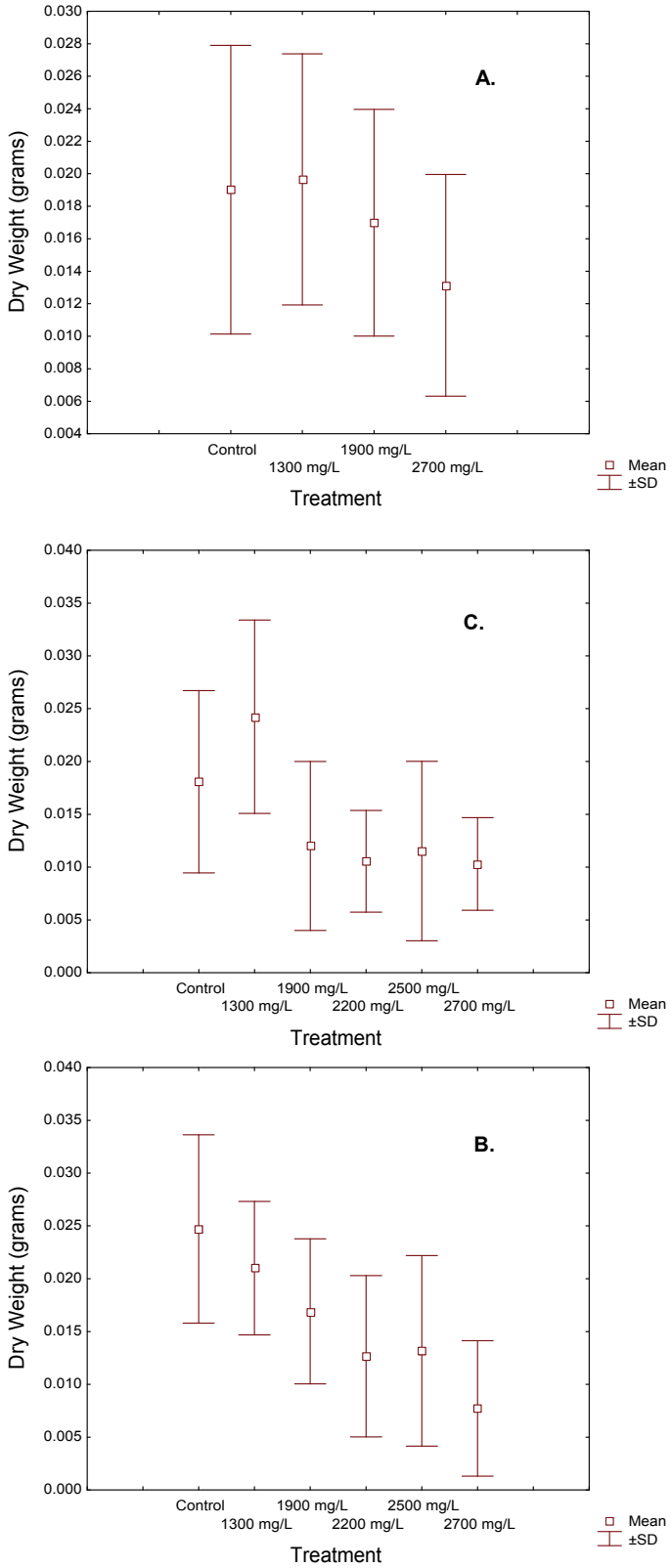

FIGURE 2.5 Means and standard deviations of growth (dry weight in grams) results for the three replicated concentration ranges from the NaCl chronic toxicity experiment (Figure A – replicate 1, Figure B – replicate 2 and Figure C – replicate 3).

Reproduction

The number of gravid females appearing in a treatment was taken as a percentage of total survivors per treatment. The results from all three replicated concentration ranges collected showing the mean and standard deviations of the percentage of the survivors that were gravid is depicted in Figure 2.6. Figure 2.6 shows that the 2700 mg/L treatment had significantly fewer gravid females compared to the control. The log -transformed data had a distribution closest to normality (Shapiro-Wilk $W=0.96$, $p<0.05$). A Levene's test ($p<0.05$) indicated that the log-transformed and untransformed data among treatments had equal variance. An ANOVA indicated that there was a significant difference among treatments ($p<0.05$). A parametric Tukey test ($p<0.05$) found that the 2700 mg/L treatment was the only treatment that was significantly different from the control using the untransformed data. The LOEC for these data was the 2700 mg/L treatment while the NOEC was 1900 mg/L.

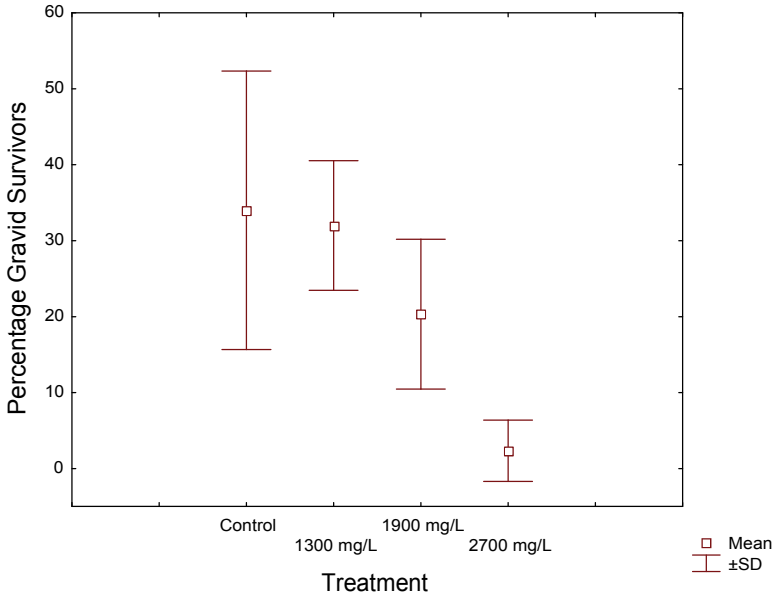

FIGURE 2.6 Means and standard deviations of reproduction (percentage gravid survivors) of the grouped results from all three replicated concentration ranges from the NaCl chronic toxicity experiment.

The mean number of eggs per female is shown in Figure 2.7, where the means and standard deviations are plotted separately for each replicate. No obvious differences were apparent although a decreasing egg number with increasing concentration was apparent. Differences between treatments for the three grouped replicates were also tested for. Figure 2.8 shows the means and standard deviations of number of eggs per female for the three replicates grouped. Here there were also no apparent significant differences. No eggs were found in the highest concentrations, either because of 100% mortality or because no gravid females were evident in the highest concentrations.

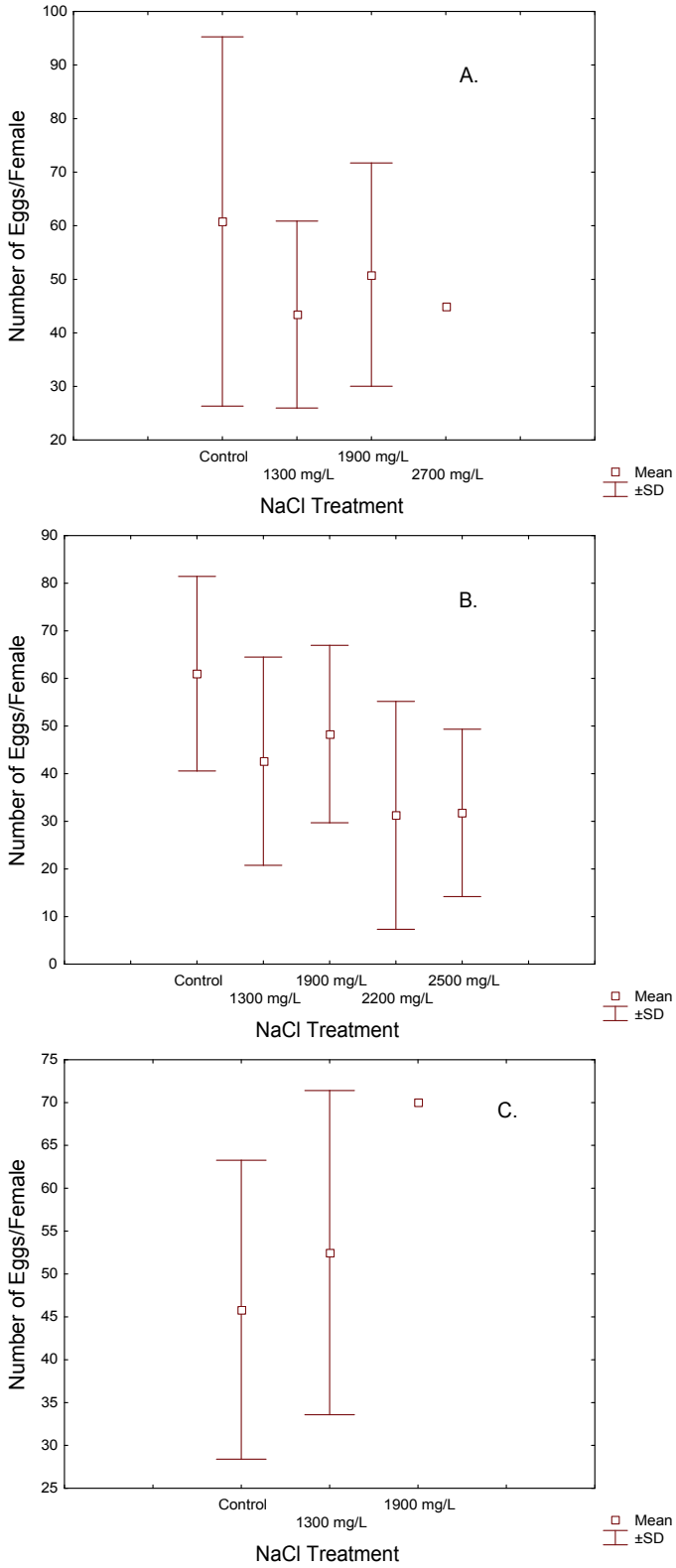

FIGURE 2.7 Means and standard deviations of reproduction (number of eggs per individual) results for the three replicated concentration ranges from the NaCl chronic toxicity experiment (Figure A – represents replicate 1, Figure B – replicate 2 and Figure C – replicate 3).

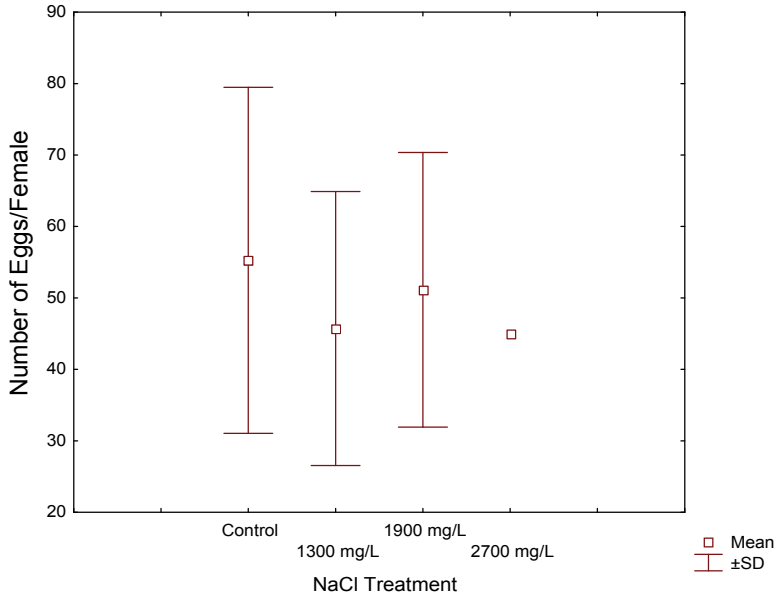

FIGURE 2.8 Means and standard deviations of reproduction (number of eggs) results for the collected results of all three replicated concentration ranges from the NaCl chronic toxicity experiment.

Na_2SO_4 experiment

Mortality

Unfortunately a heater burst during a weekly renewal of test mediums on day 30 causing all remaining individuals in the 2530 mg/L treatment in replicate 3 to perish; and the experiment was not repeated. The percentage mortality on day 30 in this treatment has been treated as the final mortality reading with the assumption that mortality would have stabilised to a large degree by this time. This is supported by the results of the preliminary method development chronic toxicity testing in 2003 where mortality generally stabilised after 15 days duration and the NaCl chronic toxicity test (Figure 2.1) where mortality stabilised by 20-30 days.

Mortality results from all replicated concentration ranges were similar. Figure 2.9 depicts the collected mean and standard deviations of mortality results from all three replicated concentration ranges in the Na_2SO_4 chronic experiment. The 2530 mg/L treatment appears to be significantly different from the control in terms of the mortality response. Mortality data on day 80 was tested for significant differences between treatments. The untransformed data as well as all the data transformations could not produce homogeneous data as indicated by the Levene's Test ($p<0.05$). A non-parametric Kruskal-Wallis test did not find any significant differences among treatments ($p>0.05$) but it is evident (Figure 2.9) that the 2530 mg/L treatment is significantly different from the control. The mortality LOEC for these data was the 2530 mg/L treatment and the NOEC was the 1900 mg/L treatment.

None of the replicated concentration ranges produced data that fitted the Probit (USEPA, 1993) model. Linear regression of untransformed data produced the regression lines depicted in Figure 2.10. The first replicated concentration range produced an LC_{50} value of 2531.3,

the second produced a LC_{50} value of 2802.9 and the third produced an LC_{50} value of 2669.5. LC_{50} values and control mortalities are depicted in Table 2.6.

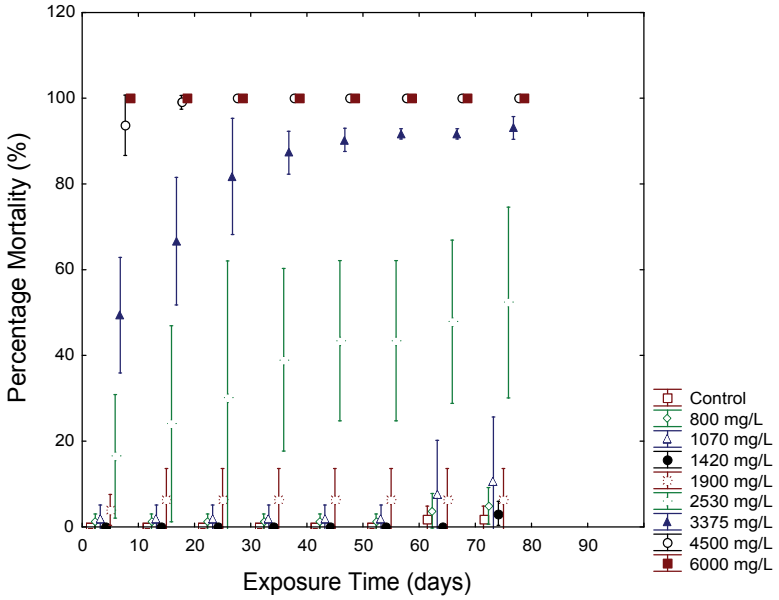

FIGURE 2.9 Means and standard deviations for the collected mortality results of the three replicated concentration ranges for the Na_2SO_4 chronic toxicity experiment.

TABLE 2.6 Control mortalities and LC_{50} values determined using regression models in the Na_2SO_4 chronic toxicity test.

Replicate	LC_{50} mg/L	95% Confidence limits		% Control mortality
		Upper	Lower	
1	2531	3800	1900	5.3
2	2803	4000	2200	0
3	2670	3500	2000	0

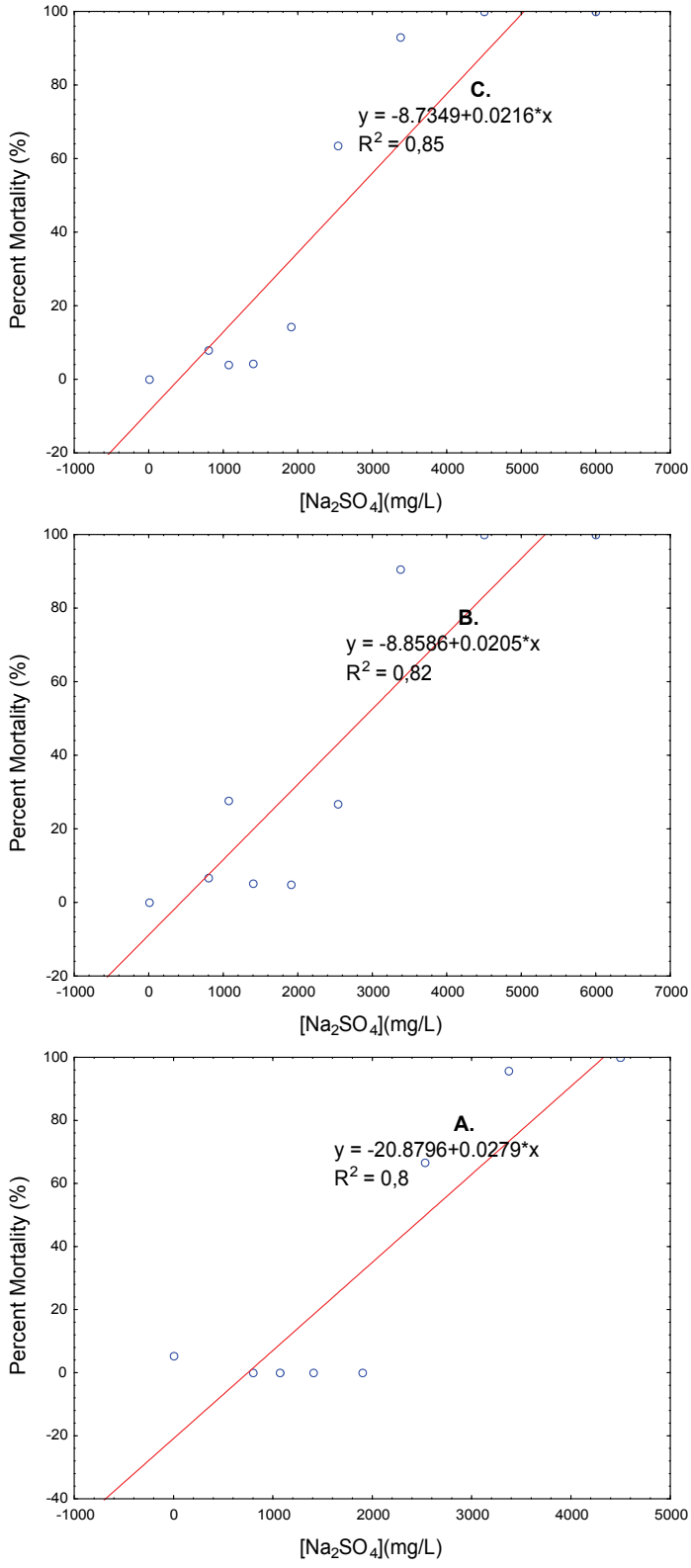

FIGURE 2.10 Regression lines fitted to accumulated mortality response over time for three replicated concentration ranges in the Na_2SO_4 chronic experiment. Regression equations as well as goodness of fit are indicated (Figure A – replicate 1, Figure B – replicate 2 and Figure C – replicate 3.

Growth

Since there were inter-replicate differences in the size of young juveniles at the start of each replicate (Figure 2.11A-C), carapace lengths for all three replicates were not grouped together for analyses, but rather analysed separately for differences between treatments. Differences between treatments were tested for at exposure durations where the standard deviations of the treatments did not appear to overlap (Figure 2.11A-C), although differences between treatments at the full 80 days duration were tested for if non-overlapping of standard deviations persisted for the full exposure duration. No carapace growth measurements were possible on treatments where there were 100% mortality.

Growth differences between treatments as shown by carapace length measurements were not as obvious as occurred in the NaCl experiment (Figure 2.11). It appears that the 3375 mg/L treatment may be significantly different from the control after day 30 in replicate 1 (Figure 2.11A). Statistical differences between treatments according to carapace length measures on day 39 were explored. The log-transformed data had a distribution that was closest to normal (Shapiro-Wilk $W=0.82$, $p<0.05$). However the log transformation did not produce homogeneous data as indicated by a Levene's Test ($p<0.05$). A non-parametric Kruskal-Wallis test indicated that the 3375 mg/L treatment was significantly different from the control ($p<0.05$). The LOEC for carapace length data in replicate 1 was the 3375 mg/L treatment while the NOEC was the 2530 mg/L treatment.

None of the treatments in replicate 2 appear at any stage to be significantly different from the control (Figure 2.11B).

It was unfortunate that the 2530 mg/L treatment in replicate 3 had to be abandoned after day 30 as reduced growth by day 20-30 (Figure 2.11C) was evident in this treatment. Significant differences between treatments in terms of carapace length on day 23 were tested for, so as to include the 2530 mg/L treatment. Untransformed data showed a distribution closest to normality (Shapiro-Wilk $W=0.97$, $p<0.05$). A Levene's test showed that the untransformed data was homogeneous ($p<0.05$). An ANOVA indicated a significant difference among treatments ($p<0.05$), and a Tukey test indicated that the 1900 mg/L treatment and all higher treatments were significantly different from the control ($p<0.05$). The LOEC for these data was the 1900 mg/L treatment and the NOEC was the 1400 mg/L treatment.

In some cases no standard deviations could be calculated for some of the treatments (Figure 2.11A-C). This occurred in very high concentration treatments where only one test animal was available for carapace measurement.

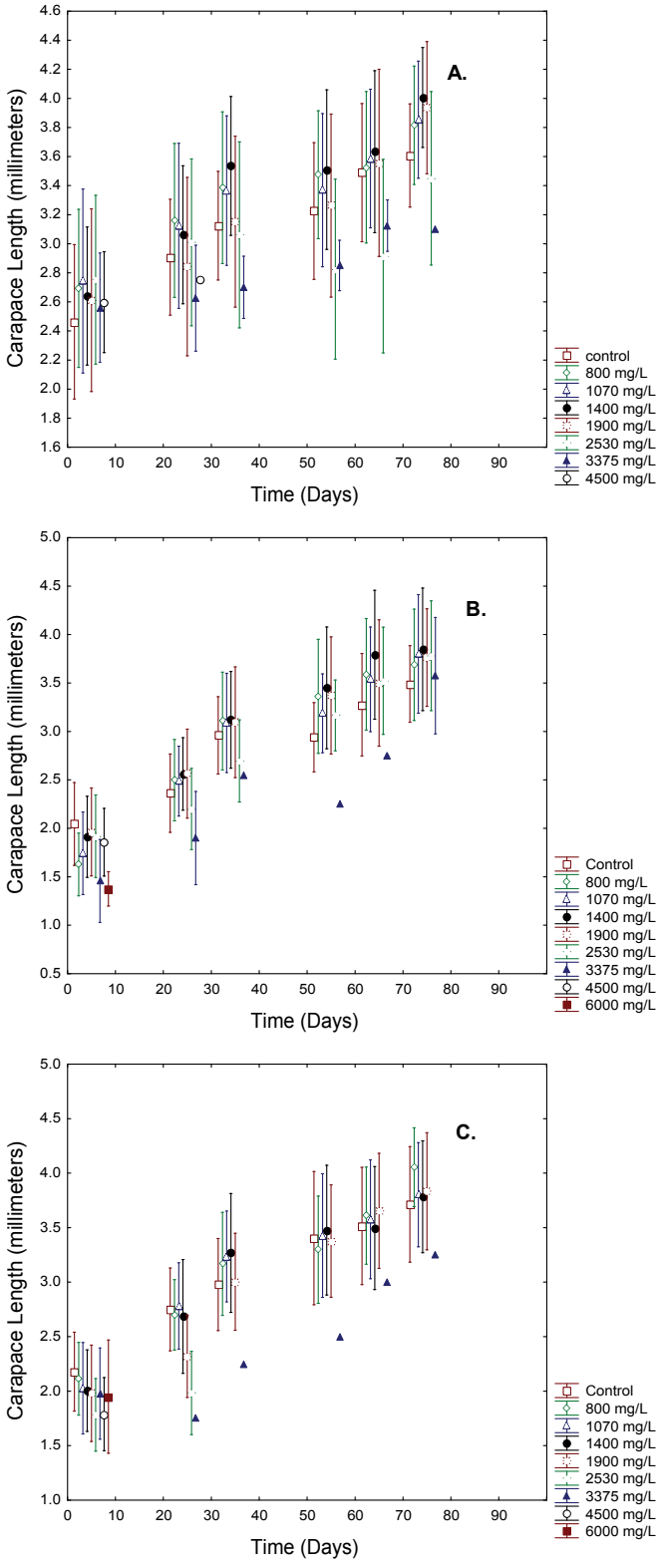

FIGURE 2.11 Means and standard deviations of growth (carapace length) results for the three replicated concentration ranges from the Na_2SO_4 chronic toxicity experiment (Figure A – replicate 1, Figure B – replicate 2 and Figure C – replicate 3).

Untransformed dry weight data of the first replicate (Figure 2.12A) showed a normal distribution (Shapiro-Wilk $W=0.99$, $p<0.05$). A Levene's test indicated that the untransformed data was homogeneous ($p<0.05$). An ANOVA indicated a significant difference among treatments ($p<0.05$) but a Tukey test indicated that the 1400 mg/L treatment was positively statistically different from the control and therefore was not used as a Lowest Observed Effect Concentration (LOEC). A non-parametric Kruskal-Wallis test however found that the 2530 mg/L and 3375 mg/L treatments were significantly different from the control ($p<0.05$). The LOEC of these data was the 2530 mg/L treatment while the NOEC was the 1900 mg/L treatment.

Untransformed dry weight data from replicate 2 (Figure 2.12B) appeared to follow a normal distribution (Shapiro-Wilk $W=0.93$, $p<0.05$) and a Levene's test indicated that the untransformed data was homogeneous. An ANOVA indicated a significant difference among treatments ($p<0.05$) but a Tukey test found no treatments significantly different from the control ($p>0.05$). A non-parametric Kruskal-Wallis test indicated that the 1070 mg/L treatment was positively significantly different to the control while the 2530 mg/L and 3375 mg/L treatments were negatively significantly different from the control ($p<0.05$). The LOEC from these data was the 2530 mg/L treatment while the NOEC was the 1900 mg/L treatment.

There did not appear to be any significant differences between treatments in replicate 3 (Figure 2.12C). However, the 2530 mg/L treatment was abandoned in the replicate, and if this treatment had followed through to the end of the experiment when animals were dry weighed, it is possible that a significant difference would have been found. The single point with no standard deviations at the 3375 mg/L treatment indicates the dry weight measurement of a single animal.

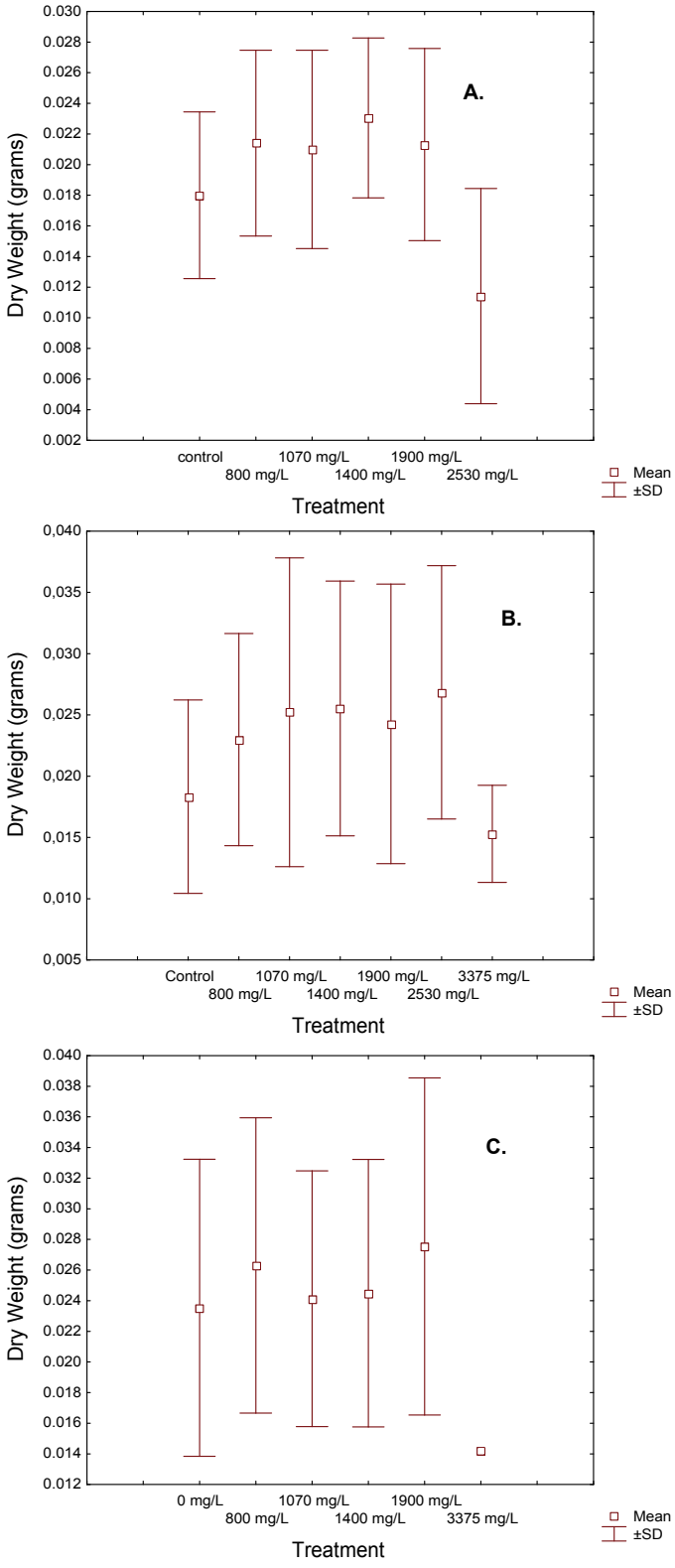

FIGURE 2.12 Means and standard deviations of growth (dry weight) results for the three replicated concentration ranges from the Na_2SO_4 chronic toxicity experiment (Figure A – replicate 1, Figure B – replicate 2 and Figure C – replicate 3).

Reproduction

There did not appear to be any negative effect on reproduction by exposure to Na_2SO_4. The proportion of survivors that were gravid appears to have increased with increasing Na_2SO_4 exposure (Figure 2.13). Parametric and non-parametric tests however found no significant positive differences. Significant differences between the 2530 mg/L treatment and the control could not be tested for as all individuals in this treatment in replicate 3 perished before any individuals could reach sexual maturity and treatment data from all three replicates were needed to test for significant differences.

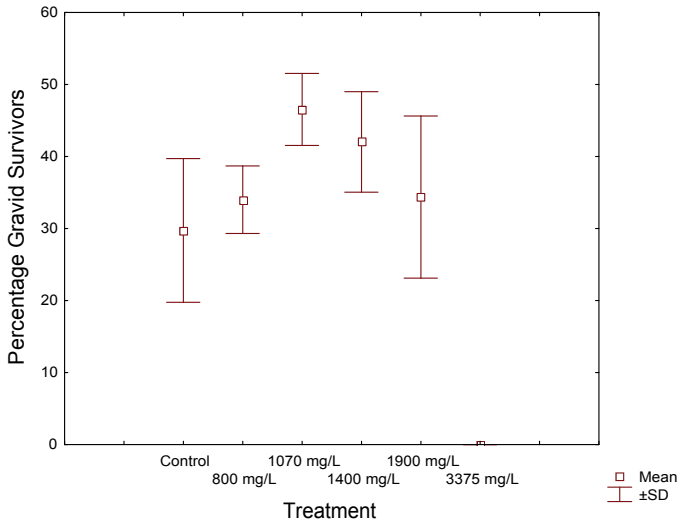

FIGURE 2.13 Means and standard deviations of the grouped reproduction (percentage gravid survivors) results for the three replicated concentration ranges from the Na_2SO_4 chronic toxicity experiment.

There did not appear to be significant differences among treatments in terms of the number of eggs per female in replicate 1 and 3 (Figure 2.14). This was however tested for replicate 2. The data distribution of untransformed data was normal (Shapiro-Wilk $W = 0,94$, $p < 0,05$) but the data was not homogeneous as indicted by the Levene's Test ($p < 0,05$). A non-parametric Kruskal-Wallis found indicated that the 2530 mg/L treatment was significantly different from all other treatments. The LOEC for these data was the 2530 mg/L treatment while the NOEC was the 1900 mg/L treatment.

The grouped results of all replicated concentration ranges did not seem to show significant differences between treatments (Figure 2.15).

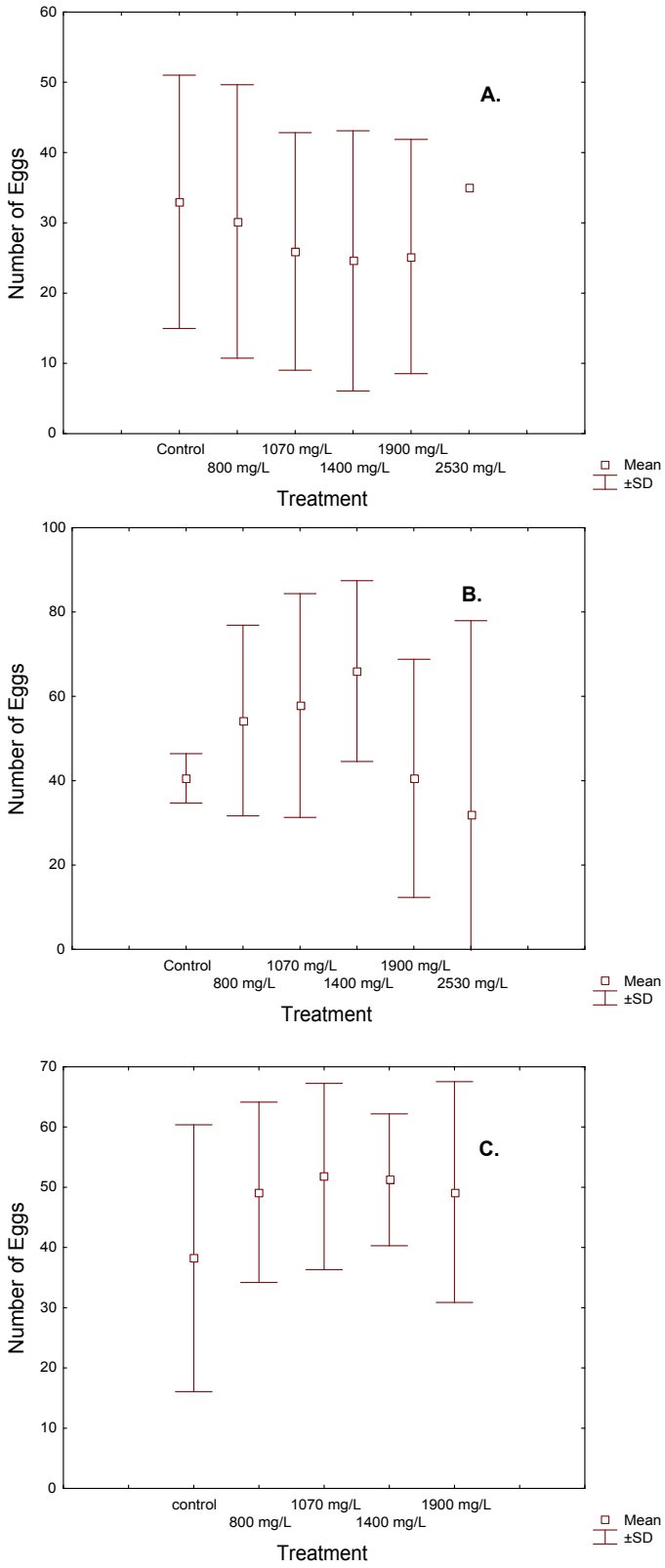

FIGURE 2.14 Means and standard deviations of reproduction (number of eggs) results for the three replicates from the Na_2SO_4 chronic toxicity experiment (Figure A – replicate 1, Figure B – replicate 2 and Figure C – replicate 3).

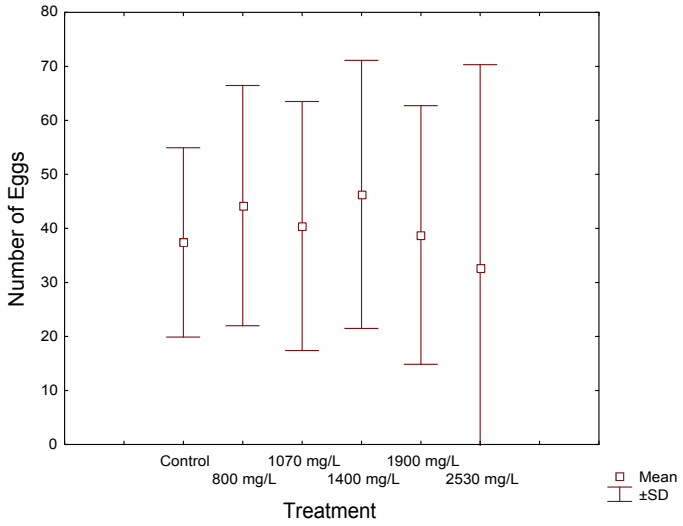

FIGURE 2.15 Means and standard deviations of reproduction (number of eggs) results for the three replicates grouped from the Na_2SO_4 chronic toxicity experiment.

2.4.1.3 Discussion of experimental endpoints for the *Caridina nilotica* growth, reproduction and mortality experiments

A summary of toxicity measures taken in the chronic toxicity tests for both salts is provided in Appendix B.

The chronic exposure of *C. nilotica* to NaCl and Na_2SO_4 yielded sub-lethal toxicity data. It is surprising that the NOECs estimated were the same for NaCl and Na_2SO_4, as one would expect sulphate to be more toxic than chloride, especially since the chloride ion is associated more with natural salinisation (Scherman et al., 2003). Jooste and Rossouw's (2002) benchmark values and salt model rank Na_2SO_4 as being more toxic than NaCl. The fact that in most cases, the different biological responses measured produced the same value of NOEC is also surprising. It is reasonable to assume that measured sub-lethal biological responses would produce lower NOEC values than lethality measures, but this was not the case here. Compared to the salinity tolerance of many macroinvertebrates, the experimental NOEC values estimated here for *C. nilotica* were not particularly sensitive (S. Browne *pers. comm.*, 1994). This may be explained by the fact that *C. nilotica* probably had a marine ancestry and these shrimp have been found in waters of elevated salinity and therefore may be tolerant of elevated salinity. Included in *C. nilotica*'s distribution is Lake Sibaya, which is characterised by high salinity (Hart, 1981). Specimens have also been found in a salinity of 4 parts/thousand in the Keiskamma River and 6 parts/thousand in the Bushman's River (Hart, 1983). Unfortunately, since parts/thousand measures are not salt specific, they cannot be compared to the NOEC values determined in the chronic toxicity tests. Looking at the results here, one could even go further and suggest that a low level of dissolved salt is probably beneficial for the growth and development of these shrimp. Mortality results were often less than the control in the lower salt treatments (Figure 2.1 and 2.9), growth seems to be elevated in low salt treatments (Figures 2.5, 2.11 and 2.12) and the same could even be said for reproduction (Figures 2.13 and 2.14). Chapman (2002) defines hormesis as a toxicological phenomenon that occurs when a low concentration toxicant treatment has a positive effect on

a biological response (mortality, fecundity etc) that is 30-60% higher than that of the control, and the toxicant concentration that has this effect is in the range of ten times less than the NOEC concentration. Dry weight response in replicate 2 of the Na_2SO_4 chronic experiment showed a mean response of 0.019 g in the control compared to 0.025 g in the 1070 mg/L treatment. This is 32% higher dry weight than the control but the 1070 mg/L treatment is not ten times less than the calculated NOEC of 1900 mg/L. This is one of the more pronounced examples from this study of low toxicant concentrations having a positive effect compared to the control, therefore according to Chapman's (2002) definition, no hormetic response was evident in this study.

There were limited numbers of *C. nilotica* individuals available for the chronic toxicity tests, and therefore sample sizes for measurement of significant differences were small in some cases. Ideally, at least 20 individuals per treatment for carapace measures would have preferable (Muller pers. comm., 2003). In high salt concentration treatments this was very often impossible because very few individuals survived these treatments.

The ANZECC and ARMCANZ (2000) guidelines only used chronic toxicity data, either experimental or extrapolated, to derive trigger values (Warne, 2001). Experimental chronic toxicity data were used to derive high reliability trigger values while acute data were used to derive medium or low reliability trigger values (Warne, 2001). This highlights the importance of chronic toxicity data and in particular experimental chronic toxicity data. A working protocol for using *C. nilotica* in chronic toxicity testing is therefore important in this regard. The protocol provides a method of deriving chronic tolerance information for toxicants for an additional species. This will allow the use of the Species Sensitivity Distribution (SSD) method for more toxicants as there are minimum data requirements for using this method (Warne, 2001), as well provide an extra data point in existing SSDs. The chronic protocol is very important in the South African context as *C. nilotica* is indigenous South Africa, and tolerance information for this species will contribute to the development of future water quality guidelines.

Some of the results of the chronic tests indicate that one may be able to detect significant sub-lethal affects within 20 days. This would appear reasonable for growth (Figures 2.4 B and C, Figure 2.11 C). Mortality definitely begins to show the significant differences between treatments (that persist until the end of the test) by day 20 (Figures 2.1 and 2.9). Therefore significant mortality responses could possibly be measured in a short-term chronic toxicity test of 20 days. Unfortunately, starting off with young juveniles and running a test until day 20, will not allow any individuals to reach female sexual maturity, and therefore reproductive endpoints (number of eggs etc) cannot be measured. It is likely that young juveniles of macroinvertebrates are the most sensitive to toxicants of any life stages in the lifecycle (Hutchinson et al., 1998). A study of the relative sensitivities of different life stages of organisms according to toxicity data on the ECETOC database showed that sensitivity of aquatic macroinvertebrates to toxicants decreased with age (Hutchinson et al., 1998). Although there were not enough data for macroinvertebrates, fish larvae appeared to be more sensitive than the embryo stage, probably because eggs are protected by a tegument (Hutchinson et al., 1998). This could very well be the case for macroinvertebrates as well. There are acute toxicity data for both *C. nilotica* juveniles and adults exposed to NaCl and Na_2SO_4 on the UCEWQ Ecotoxicology database. There are four acute NaCl toxicity tests of 48 hours using *C. nilotica* juveniles of less than seven days old and one NaCl 96 hour acute toxicity tests using *C. nilotica* adults (Refer to UCEWQ Ecotoxicology database, IWR, RU). The 48 hour LC_{50} measures for the 4 toxicity tests using juveniles are: 5955 mg/L, 4450

mg/L, 5979 mg/L and 5487 mg/L with a geometric mean of 5430 mg/L. The 48 hour LC_{50} of the 96 hour *C. nilotica* adult toxicity test is 14693 mg/L. It therefore appears that *C. nilotica* adults are up to three times as tolerant of NaCl as juveniles of less than seven days. There are also four acute Na_2SO_4 toxicity tests of 48 hours using *C. nilotica* juveniles of less than seven days old and one Na_2SO_4 96 hour acute toxicity tests using *C. nilotica* adults (Refer to UCEWQ Ecotoxicology database, IWR, RU). The geometric mean of the juvenile toxicity tests is 6024 mg/L, with individual 48 hour LC_{50} values for the four tests being: 5989 mg/L, 7002 mg/L, 5734 mg/L and 5477 mg/L. In contrast, the 48 hour LC_{50} value of the Na_2SO_4 adult toxicity test is 11194 mg/L. This is almost twice as high as that of the juvenile toxicity test LC_{50}s.

The mortality LC_5 value for NaCl was close to the NOEC estimated via hypothesis testing (Appendix B). The LC_5 of these data equated to 2480 mg/L. This is slightly less conservative than the calculated NOEC of 1900 mg/L. The mortality data obtained from the Na_2SO_4 chronic experiment did fit the Probit model (Appendix B). The lethality LC_5 value estimated from the model at 80 days duration was close to the lethality NOEC at 80 days duration, suggesting that using an LC_5 value as indicative of an NOEC proposed by Warne (1998), is justified. However, since the NOEC values being compared to LC_x values are essentially mortality NOEC values, this is hardly surprising. But since most sub-lethal NOEC measures in the chronic toxicity tests were of the same value as the mortality NOECs, the use of an LC_5 as being indicative of an NOEC is justified.

The NOEC concentrations estimated for NaCl and Na_2SO_4 are not statistically significantly different from the control. Biological significance of these estimates however needs to be considered. It has been shown that the NOEC value for Na_2SO_4 for lethality is equivalent to the death of 5% of test animals. The long-term implications of death of 5% of a population of this species in a particular ecosystem is unlikely to be biologically significant. The regression line fitted to the NaCl lethality data produced a lethality percentage of <1%. Therefore it is unlikely that the mortality NOEC would have a biologically significant affect. The biological significance of the measured NOECs should additionally be considered in terms of sub-lethal affects for both salts. In the NaCl chronic experiment, mean carapace length measures per replicate in the 1900 mg/L treatment ranged from a negligible amount to 13% less than mean growth in the control (Figure 2.4 A-C). Mean dry weight measures per replicate in the NaCl chronic test 1900 mg/L treatment ranged from a negligible amount to 36% less than dry weight in the control (Figure 2.5 A-C). The mean amount of gravid survivors in the 1900 mg/L treatment was 41% less than in the control (Figure 2.6). The mean number of eggs per replicate in the 1900 mg/L treatment was about 17% less than that of the control (Figure 2.7 A-C). There are therefore some large affects on sub-lethal biological responses by the 1900 mg/L treatment compared to the control for NaCl, despite the fact that the 1900 mg/L treatment was in most cases deemed statistically insignificantly different from the control. The measured NOEC for NaCL may therefore not be biologically insignificant, and the next lowest treatment of 1300 mg/L may be more protective as an NOEC. This however needs to be investigated further. The mean carapace length per replicate for the Na_2SO_4 chronic test 1900 mg/L treatment ranged from a negligible amount to 8% more than the control (Figure 2.11 A-C). Mean dry weight measured per replicate in the 1900 mg/L treatment ranged from 16 to 33% more that that measured in the control (Figure 2.12 A-C). The mean number of gravid survivors in the 1900 mg/L treatment was 26% less than that of the control (Figure 2.13) while the mean number of eggs per replicate for the 1900 mg/L treatment ranged from an insignificant amount to 32% more than that of the control

(Figure 2.14). The measured NOEC for Na_2SO_4 is therefore unlikely to be negatively biologically significantly different from the control.

Jones (1981) found a seasonal shift in salinity tolerance in the estuarine crab *Helice crassa* related to temperature. The tests run with *C. nilotica* were done under controlled conditions with particular emphasis on controlled water and air temperature and controlled light. Therefore the assumption has been made that there has been no seasonal shift in tolerance, although tests have been running throughout the year. In addition, the laboratory cultures of *C. nilotica* have been running for 6 years under controlled temperature and light conditions and seasonal responses are probably less pronounced than would occur in wild populations.

Generally mortality in the NaCl chronic experiment stabilized after 20 days (Figure 2.1). This is consistent with the theory behind Two-Step Linear Regression (Mayer et al., 1994) and Multi-Factor Probit Analysis (Lee et al., 1995). These methods attempt to predict time independent tolerance of organisms to a particular toxicant. Mortality in the Na_2SO_4 chronic experiment however did not show obvious stabilisation.

In many cases, the LOEC values measured in the chronic toxicity tests, for both NaCl and Na_2SO_4, are greater than the LC_{50} values measured for the salts via regression analysis (Appendix B). It is evident that for both salts, the NOEC represents a threshold where all concentrations lower or equal to the NOEC cause very little mortality and mortality sharply rises at all concentrations higher than the NOEC. The fact that the mortality NOEC is equivalent to the LC_5 and less than the LC_1 for Na_2SO_4 and NaCl respectively and the LOEC is greater than the LC_{50} for NaCl and similar to the LC_{50} for Na_2SO_4, testifies to the fact that *C. nilotica* is subject to a very sharply defined threshold of toxicity to both salts. For NaCl the LOEC is greater than the NOEC by 800 mg/L while the LOEC for Na_2SO_4 is greater than the NOEC by 630 mg/L. Considering the concentration ranges used in the tests these are not great differences.

2.4.2 EMBRYONIC DEVELOPMENT OF *CARIDINA NILOTICA* AS A CHRONIC (LONG-TERM) TOXICITY TEST METHOD

Embryonic development of *C. nilotica* was studied by Ketse (2007) towards an MSc degree. The toxicants used to develop the method are NaCl, Na_2SO_4 and CdCl, but further research is required using other toxicants. A preliminary study using a textile effluent has also been successfully undertaken.

Embryonic development among vertebrates and invertebrates is similar in many respects. For example, embryonic development in both groups undergoes sequential stage cleavage, gastrulation and organogenesis (Kast-Hutcheson et al., 2001). In crustaceans embryos can hatch into a nauplius larval stage, whereas others, often those with larger eggs, have embryonized the nauplius and begin their larval development in a post naupliar form (Landau and Laufer, 1999; Schram, 1986).

Atyid shrimps hatch from the egg superficially resembling diminutive adults, sometimes termed post-larvae. The degree of larval suppression depends primarily on the magnitude of lipid reserves invested in the egg, and is related to the degree of independence from water exhibited by the various species (García-Guerrero et al., 2003; Hart et al., 2001). In general, marine and brackish water species produce numerous small eggs and have extended larval development. Lowland and lentic freshwater species produce eggs of an intermediate size and

number and the number of larval stages is reduced, while headwater stream species produce a small number of large eggs and larval development is abbreviated (Hancock, 1998; Landau and Laufer, 1999; Nair, 1949).

Using organism embryonic development as an experimental endpoint in toxicity tests has been successfully applied to various organisms (*e.g.* frog embryo teratogenesis assay-*Xenopus* (FETAX) (Rand et al., 1995) and is currently being considered for *C. nilotica*. However, the normal embryonic development of this species is not yet known in detail. Hart (1980) described the embryonic duration and post-embryonic development rates of *C. nilotica* under laboratory and experimental field conditions; he did not look at the developmental features from the day of laying until hatching.

2.4.2.1 Developing an experimental method

It was found that eggs could successfully be removed from gravid females, without injury to either females or the developing embryo. Based on this, an experimental chamber and method was designed.

Glass chambers were designed (Figure 2.16) for as experimental chambers (B), a tube with a micropipette on its end was inserted through the bottom of the glass chambers and connected to an air pump through a set of valves which regulated the air flow (E). The chambers were sealed so that no water could enter or move out of the chamber; this prevented mixing of bath water and the water in the chambers. To prevent loss of embryos, a fine mesh gauze was used as a placement for the embryos (F). A water bath (H) was filled with standard laboratory water, and it was maintained at 24ºC (±0.5ºC) using a thermostat (A), and air bubblers were connected to circulate the heat. Holes were made in a Perspex sheet (G), which were approximately 4.5cm in diameter with the distance between the holes 6cm, the sheet acting as an experimental chamber platform. The glass chambers were inserted inside the water bath, and the heat was transferred from the water bath to the chambers by making certain that there was enough water in the water bath surrounding the chambers.

C. nilotica carry their eggs in their pleopods until they hatch (Schram, 1986). Embryonic development could be followed by removing eggs from gravid females and placing them in the chambers. In order to obtain embryos of known ages, eggs were removed from ovigerous females which had been gravid for less than 24 hours. Gravid females were collected from tanks in late afternoon and therefore gravid females collected early the following day would have been less than 24 hours old. The newly spawned eggs were then extracted from the females using a soft paintbrush, and eggs from a single female were placed into each container accordingly.

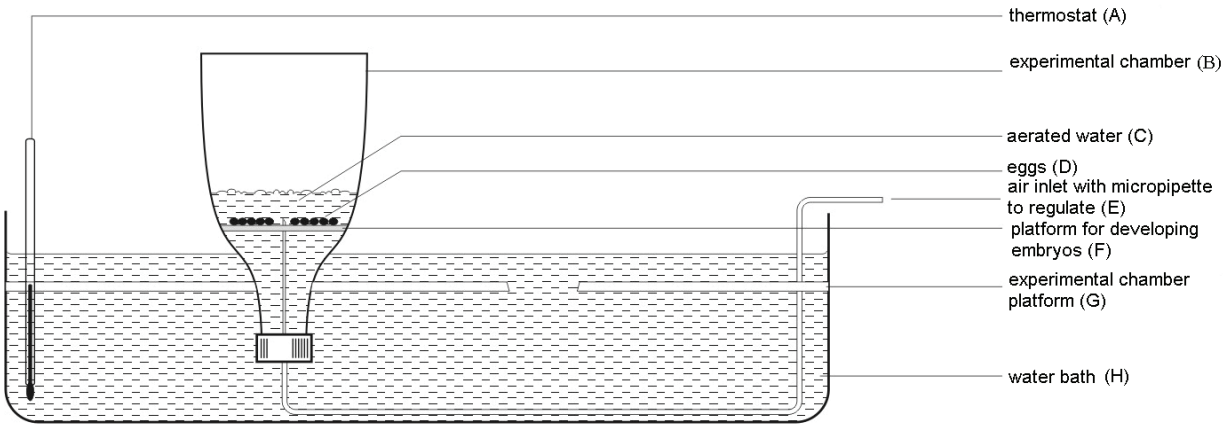

FIGURE 2.16 Diagram showing the experimental chamber in which the developing embryos are kept during exposure experiments

Experimental measurements

Eggs from each container are removed, placed in a Petri dish with water and live specimens examined under a dissecting microscope (12x magnification). These are then fixed in 4% formalin and developmental stages recorded with an Olympus 4040 Zoom digital camera (Olympus American Inc., USA) attached to a light microscope and linked to a computer for image analysis. Measurements were undertaken using AnalySIS by Soft Imaging Systems (SIS). The stage of the embryonic development was determined by stereomicroscopic examination, noting the shape of the egg, the quantity of yolk, the space occupied by the embryo, and the first appearance and subsequent growth of the eyes, appendages and other structures.

2.4.2.2 Describing embryonic development

The complete embryonic development of *Caridina nilotica* was described based on external morphology, described in definable stages as listed in Table 2.7. Biological variability is known to confound interpretation of toxicity experiments, therefore, once these features had been identified, it was necessary to establish whether variability of the developing embryos could compromise or confound the use of embryonic development as an experimental endpoint in toxicity tests. A comparative study was undertaken to compare variability in embryonic development of embryos from the same female (intravariability) as well as those from different females (intervariability). Statistically ($p<0.05$), no significant difference between embryos of (i) the same female and (ii) between embryos of different females of the same age could be established.

In a further set of experiments where eggs from different females were randomly assigned to different experimental chambers, it was established that the random variability in the development of embryos was no different from that established through intervariability and intravariability between embryos. It was therefore considered that embryonic development would be a useful experimental endpoint for toxicity tests.

TABLE 2.7 Embryonic development stages which can be identified in *C. nilotica*.

Day post hatching	Description of embryonic development
Day 1	At spawning, no embryonic structures can be seen and the yolk mass is regularly distributed throughout the egg, with the chorion covering and protecting the egg. The yolk shrinks slightly before cleavage sets in and then it undergoes cleavage into different blastomere stages.
Day 2	Compared to the previous day, numerous, smaller blastomeres can be observed over the entire surface of the egg.
Day 3	The rudiments of the embryonic region are differentiated. The antennae, antennule, optic rudiment and mandibles start to develop.
Day 4	The thoracico-abdominal lobe has increased in size and all the appendage buds also increased in their size. The optic rudiment is clearly differentiated.
Day 6	The antennae and antennules have become long, pointed and well developed. A few additional buds developed namely maxilla I and maxilla II. The caudal papilla gives slight jerks at regular intervals.
Day 9	All the appendages show slight movements. The whole embryo gives periodical jerks. The carapace of the developing embryo is developed. The pigmentation of the eye is visible.
Day 12	The pulsations of the heart are seen clearly. Small setae are developed at the tips of the thoracic appendages. Pleopods are visible.
Day 14	All the external characteristics of the embryo are visible, the development is complete and it becomes larger. The yolk of the embryo is absorbed completely. Heartbeat and movements of the embryo are faster than compared to 12 days.
Day 17	The larvae are released into the water by this day. The newly hatched animals have all the features of the adult shrimp but smaller.

The study allowed morphological characterization of the embryonic structures from the time the eggs were laid until the time they hatched. Females produce between 19 and 60 eggs at spawning. Under the experimental conditions (temperature 24°C), the time from laying to hatching in *C nilotica* was 17 days. The average measurement of egg size was 1.9x0.65 mm (day 1) and 2.3x0.84 mm (day 16). The size of the egg increased continuously throughout embryonic development: it increased from the time of laying until the time the neonate hatched, which is a general occurrence in decapods (Chinnayya, 1974; Lavarias et al*.,* 2002; Nair 1949; Oh et al., 2004; Oh et al., 2003; Nazari et al., 2000; Saito and Koya, 2001; Thatje et al*.,* 2004; Yamaguchi, 2001) although Lizardo-Daudt and Bond-Buckup (2003) found that not to be the case with *Aegla platensis*.

The egg is oblong in shape, and greenish brown or greenish yellow in colour throughout development, similar to *Caridina laevis* (Nair, 1949). The egg of *C. nilotica* resembles that of the large majority of the higher crustacean such as the *Caridina laevis* Heller; *Palaemonetes argentinus* Nobili, 1901; *Macrobrachium olfersi,* Wiegman, 1836; *Macrobrachium potiuna,* Müller, 1880 and *Palaemon pandaliformis*, Stimpson, 1871 in being typically centrolecithal (Nair, 1949; Nazari et al*.,* 2000; Müller et al*.,* 2004), i.e. the yolk food is at the centre of the egg. It was noticed that when the chorion was removed the eggs possessed only a single envelope.

This study further demonstrated that embryos developed normally when they were removed from the brood chambers of parental organisms and incubated in experimental chambers, this may be because they receive no nutrients from the parent but from the yolk until they hatch, but they do need constant aeration. All yolk variables decreased during development of *C. nilotica,* García-Guerrero et al. (2003) said that it was due to the utilization of yolk as energy source. The cleavage furrows pass right through the yolk formed; the blastomeres round off giving the embryo a characteristic grape-bunch appearance as can also be observed in the *Caridina laevis* Heller (Nair, 1949). The eyes appear at around 9 days of development, but become more visible and conspicuous later, becoming more spherical before hatching.

The changes noted from day to day did not occur with the same rate in every specimen, which indicates differences in developmental rhythms during embryogenesis as in *Palaemonetes argentinus* Nobili, 1901 (Nazari et al., 2000). *C. nilotica* hatch out in an advanced stage of development, exhibiting direct development seen in other crustaceans. All stages are retained in an embryonic form, which means direct development to hatching as a post-embryo, as is also seen in *Caridina laevis*, Heller; *Paratya australiensis*, Kemp; *Aegla platensis* (Hancock, 1998; Nair, 1949; Lizardo-Daudt and Bond-Buckup, 2003).

In subsequent days there is an intense internal organization of the embryonic structures that are understood mainly as organogenetic processes, making the various systems functioning. Given that these processes are occurring internally, the exterior aspect does not change so much, giving the impression that they are slow (Balon, 2001), and the changes which were noted from day to day did not occur with the same intensity in every embryo, as in *Palaemonetes argentinus* Nobili, 1901, this indicates that there are differences in developmental rhythms during embryogenesis (Nazari et al., 2000).

Therefore, based on measurements of features that were identified, the following stages were used as experimental endpoints:
(i) Size of the egg
(ii) Size of the yolk
(iii) Antennae and antennule
(iv) Caudal papilla
(v) Eye
(vi) Heartbeat and pleopods, and
(vii) Hatching

2.4.2.3 The effects of selected reference toxicants on embryonic development

Sodium chloride and sodium sulphate have been identified by Davies and Day (1998) as major inorganic salts involved in salinisation of aquatic ecosystems in South Africa. Sodium chloride (NaCl) is linked to natural and agriculturally caused salinisation while sodium sulphate (Na_2SO_4) is linked to mining and other industrially caused salinisation (Scherman et al., 2003). In a study by Kefford et al. (2002) it was shown that although salts are natural physiological stressors, organisms exposed to salts at a high concentration show classic toxicant concentration – response curves. This is also evident in the database of toxicological tests run by the UCEWQ using NaCl and Na_2SO_4 (Palmer et al., 2004), as much of the concentration response data for salts fit the routinely used toxicological analysis models such as Probit. Cadmium chloride ($CdCl_2$) is an important reference toxicant used by the South African Proficiency Test Scheme (for *Daphnia pulex*), of which the UCEWQ is a participant (Muller, pers. comm.).

Salts commonly found in freshwater systems, in decreasing order of toxicity, are magnesium sulphate, sodium sulphate, calcium chloride and sodium chloride (Jooste and Rossouw, 2002). Although the South African Aquatic Ecosystem Water Quality Guidelines do not currently treat salts as toxicants (DWAF, 1996), the current ecological Reserve methods for assessing present state for water quality and determining resource quality do (Jooste and Rossouw, 2002). The concentrations defining the boundaries between different levels of ecosystem protection (ecological categories and management classes) are referred to as ecospecs. Ecospecs for inorganic salts have been determined for each of the ecological categories and management classes using toxicity test results (Jooste and Rossouw, 2002). There is considerable uncertainty about the effect of the increase of salts on aquatic biota and detailed investigations of salinity tolerance are therefore needed.

The UCEWQ database contains acute and short-term chronic toxicity data for a range of indigenous aquatic invertebrates exposed to a range of toxicants (Scherman et al., 2002; Palmer et al., 2004). The short-term chronic toxicity tests in the UCEWQ toxicity database measure survival and have exposure duration of 10 days. The database was examined for data to the selected inorganic salt (NaCl and Na_2SO_4) in which the test species was *Caridina nilotica*.

Table 2.8 shows the test results for *C. nilotica* adults and organisms which were less than 7 days old, from both wild-caught populations (Mpisini stream, KwaZulu-Natal) and laboratory-reared populations. The chemicals used were NaCl, Na_2SO_4 and $CdCl_2$. Having mortality results for adults and neonates provides no indication of potential effects on (possibly) sensitive early life- history stages such as embryonic development, thus providing motivation for this study. Table 2.9 shows the partial life-cycle test results of No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values for NaCl and Na_2SO_4 for *C. nilotica* from Slaughter (2005). The growth was determined by measuring the carapace length and reproduction was determined by the number of eggs produced. The NOEC is determined by Analysis of Variance statistical techniques and is the highest concentration within a toxicity test concentration range that does not have a significant adverse effect on the test organisms when compared to the control (Rand, 1995). The data provided in Tables 2.8 and 2.9 were used to select toxicant test concentrations for NaCl, Na_2SO_4 and $CdCl_2$. The highest concentration was selected to be less than the LC50 except for $CdCl_2$. As no chronic toxicity data were available for $CdCl_2$, it was necessary to undertake a range-finding test using *C. nilotica*.

The aim of the series of experiments was to assess whether *C. nilotica* embryonic development can be used as experimental endpoints in toxicity tests by determining the effects of the selected reference toxicants on embryonic development. This was done by assessing the effects of three toxicants (NaCl, Na_2SO_4 and $CdCl_2$) on the following features of the developing embryo: size, rate of development and mortality of developing embryos.

TABLE 2.8 Selected data from the UCEWQ database: the results reported show NaCl, Na_2SO_4 and $CdCl_2$ toxicity to *Caridina nilotica* juveniles. Results reported provide experimental details (organism source (Mpisini stream or LC), exposure mode (R or S), and exposure duration) and experimental endpoints (LC_{50}). Experimental endpoints are expressed as mg/L nominal concentration per toxicant (with lower and upper confidence limits, LCL and UCL, respectively). All experiments were undertaken using dechlorinated tap water as diluent with the test endpoint being mortality. (LC- Laboratory culture, R- Rercirculating, S- static).

Toxicant	River system	Mode of exposure	Test duration	LC50 (mg/L)	LCL (mg/L)	UCL (mg/L)
Na_2SO_4	Mpisini	R	240 (h)	1752	1522	2006
Na_2SO_4	Mpisini	R	240 (h)	1765	1492	2088
Na_2SO_4	LC	R	240 (h)	3149	2755	3511
Na_2SO_4	LC	R	240 (h)	3249	2349	3685
Na_2SO_4	LC	S	48 (h)	5989	4874	7181
Na_2SO_4	LC	S	48 (h)	7002	4710	9024
Na_2SO_4	LC	S	48 (h)	5734	5084	6614
Na_2SO_4	LC	S	48 (h)	5477	4840	6007
NaCl	LC	S	48 (h)	5979	4823	7059
NaCl	LC	S	48 (h)	5955	5100	6752
NaCl	LC	S	48 (h)	4450	3709	5196
NaCl	LC	S	48 (h)	5487	4528	6446
$CdCl_2$	LC	S	48 (h)	0.05	0.02	0.08
$CdCl_2$	LC	S	48 (h)	0.07	0.04	0.1
$CdCl_2$	LC	S	48 (h)	0.09	0.04	0.14

TABLE 2.9 The LOEC and NOEC values for NaCl and Na_2SO_4 on the growth and reproduction of *C. nilotica* (Slaughter, 2005).

Salt	Endpoint	LOEC (mg/L)	NOEC (mg/L)
NaCl	Mortality	2700	1900
	Growth	2200	1900
	Reproduction	2700	1900
Na_2SO_4	Mortality	2530	1900
	Growth	2530	1900
	Reproduction	2530	1900

Materials and methods

In order to obtain embryos of known ages, eggs were removed from gravid females which had been gravid (females carrying extruded eggs) for less than 24 hours; the eggs had to be newly spawned to follow the embryology from spawning until hatching. Gravid females were collected from all the culture tanks in the afternoon prior to commencement of each test and the gravid females collected early the following day would have eggs less than 24 hours old.

The newly spawned eggs were then extracted from the females using a soft paintbrush, and eggs were randomly distributed to experimental brood chambers.

Experimental design

Five concentrations of each of the toxicants were prepared. The control was dechlorinated tap water; each concentration was run in three replicates to assess variability in embryo development (Mackay et al. in Rand, 1995). Various water quality parameters were measured in each chamber. Temperature and conductivity were measured every day using a thermometer and a conductivity meter respectively, while pH and dissolved oxygen were measured using the pH and DO meters respectively, every third day. Exposure solutions were also changed every 3 days to prevent fungal growth on the developing eggs. All experiments were undertaken at a temperature of 24ºC±0.5ºC.

When the embryonic development of *C. nilotica* was determined (Chapter 3), numerous morphological features were identified. However, seven ages were identified as possible sub-lethal endpoint measures, because the morphological features at these ages were easily identified and could be measured (Chapter 3, Figs. 3.1-3.6). The age of development at specified ages of eggs was noted and the sizes of the various appendages were measured (length, width, length:width ratio and area). The developmental ages are represented in Table 2.7.

Since there were 7 developmental ages to monitor, and each required a sample size of 10 eggs, a total of 70 eggs was required for each brood chamber. At the predetermined developmental stages, 10 eggs were removed from each chamber and placed in a Petri dish with dechlorinated tap water and the live specimens were examined under the dissecting microscope (12x magnification). They were then fixed in 4% formalin and the developmental stages were photographed using an Olympus 4040 Zoom digital camera (Olympus American Inc., USA) attached to a light microscope and the images saved to a computer. The identified features, mortality and abnormalities were recorded, and morphological measures undertaken using AnalySIS by Soft Imaging Systems (SIS).

Exposure concentrations

The concentrations provided in Tables 2.8 and 2.9 guided the choice of concentrations used in this study. Nominal exposure concentrations were used to calculate the NOEC and LOEC values.

Sodium sulphate: 1000 mg/L, 2000 mg/L, 3000 mg/L, 4000 mg/L and 5000 mg/L and a control using dechlorinated tap water.

Sodium chloride: 1000 mg/L, 2000 mg/L, 3000 mg/L, 4000 mg/L and 6000 mg/L and a control using dechlorinated tap water.

Cadmium chloride: Cadmium chloride ($CdCl_2$) used was provided in a stock solution of 10 mg/L as provided by the Proficiency Test Scheme for *Daphnia pulex* (De Kock, pers. comm.). The concentrations were selected based on the results of acute and chronic tests from the UCEWQ and USEPA databases (http://www.epa.gov/ecotox/ , accessed 17/10/2005) for *C. nilotica* and *D. pulex* respectively. Selected test concentrations of 0.31 mg/L, 0.63 mg/L, 1,25 mg/L, 2,5 mg/L and 5 mg/L were prepared by serial dilutions of the standard 10 mg/L stock solution (Rand, 1995).

Statistical Analysis

A one-way Analysis of Variance (ANOVA) test was used to undertake comparisons of features between embryos in the control and the different concentrations (StatSoft, 2002). The results for all three replicates within each experiment were analysed using ANOVA in Statistica Version 6 (StatSoft, 2002), followed by a parametric post-hoc test (Tukey honest significant difference (HSD)) to determine which concentrations were statistically different from the control. The Lowest Observed Effect Concentration (LOEC) was determined as the lowest concentration tested that was statistically significantly different in one of length, width, length:width ratio or area of the measured feature relative to the control using Tukey HSD test. The No Observed Effect Concentration (NOEC) was determined as the highest concentration tested that did not result in a statistically significant different effect in length, width, length:width ratio and area of all features relative to the control.

Results

The pH ranged from 6.2 to7.6 and the DO ranged from 7.5 mg/L to 8.3 mg/L. Differences in length, width, length:width ratio and area of eggs between the concentrations were tested and the results showed that there were no significant differences between the replicates. Since there were no differences between the replicates, data from the replicates were combined. There were significant differences between concentrations over time for length, width, length: width ratios and area. However, as the same trends were displayed for each of the measured features, only the results for length are graphically represented in this chapter, while graphs for width, length:width ratios and area are reported in Appendix B (Figures A-N).

Sodium sulphate

Eggs hatched only in the control and the lowest test concentration of 1000 mg/L. The controls in all replicates had 0% mortality, while all the other concentrations the eggs did not hatch. Mortality started at 24 hours in the concentration of 5000 mg/L. By 336 hours, there was 100% mortality of eggs at 4000 mg/L and 5000 mgL and, at 408 hours (where hatching occurred in the control and 1000 mg/L), there was 100% mortality of eggs at 2000 mg/L and 3000 mg/L as well (Figure 2.17). At 1000 mg/L, 89% of the eggs hatched at 408 hours and all the eggs in the control hatched (Figure 2.18).

From the time the experiment commenced until 72 hours there was an increase in length, width and area of egg in all concentrations. These increases were not significantly different from the control and between concentrations (Figure 2.17 and 2.18). At 144 hours, the time when the caudal papilla appeared in the control, the first observed differences in the length, width and area of all the features were noted. These differences were statistically significantly different when compared to the control for each of the concentrations, except at 1000 mg/L for all features ($p>0.05$) and at 2000 mg/L for egg length, antennae length and antennule length (Figures 2.19-2.25). By this time (144 hours), there was 7% mortality in 3000 mg/L, 11% mortality in 4000 mg/L and 15% mortality in 5000 mg/L (Figure 2.17).

At 216 hours, the eye was not visible as expected at 2000 mg/l, 3000 mg/l, 4000 mg/L and 5000 mg/L. However, in the eggs in the control and those at 1000 mg/L, the eye was visible. At 288 hours the palpitations of the heart can be seen at 1000 mg/L, 2000 mg/L and 3000 mg/L, but not at 4000 mg/l and 5000 mg/L. At 336 hours at 1000 mg/L, 2000 mg/L and 3000

mg/L all the features were developed. The length, width and area were smaller and statistically significantly different from the control except at 1000 mg/L. The LOEC value at 144 hours was 2000 mg/L, the NOEC value was 1000 mg/L (Table 2.10).

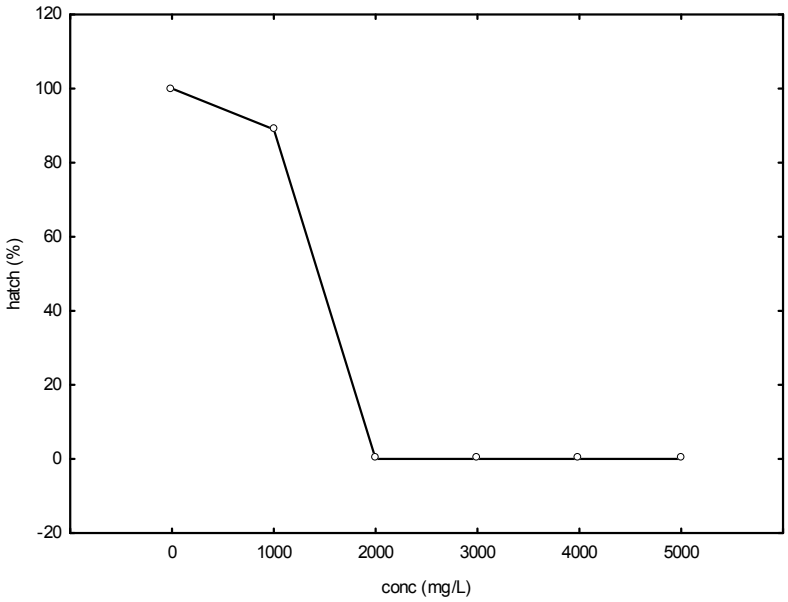

FIGURE 2.17 Total hatching success (%) of *C. nilotica* embryos after 408 hours exposure to Na_2SO_4 at different concentrations

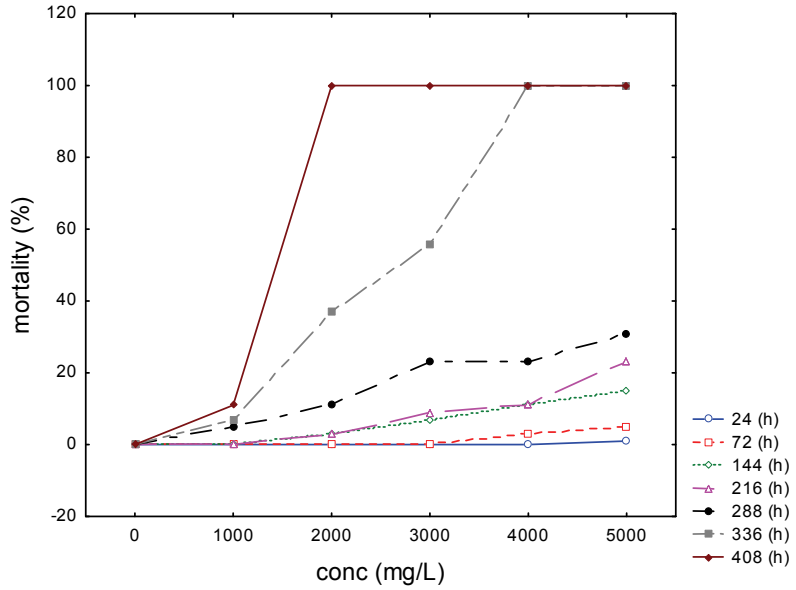

FIGURE 2.18 Total mortality (%) of *C. nilotica* embryos exposed to increasing Na_2SO_4 concentrations. Each line represents a different exposure period. Data for the replicates were combined for each concentration

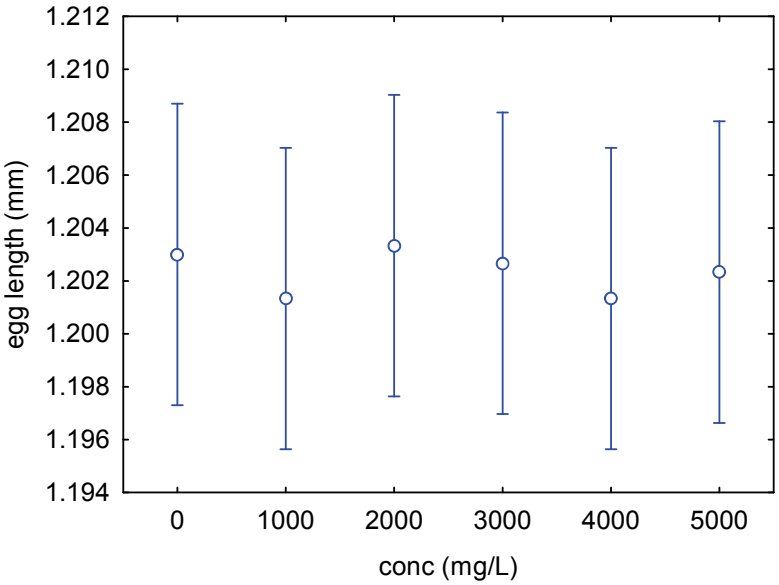

FIGURE 2.19 **Mean and 95% confidence intervals of egg length (mm) of *C. nilotica* embryos at 72 hours. The egg lengths are not statistically significantly different**

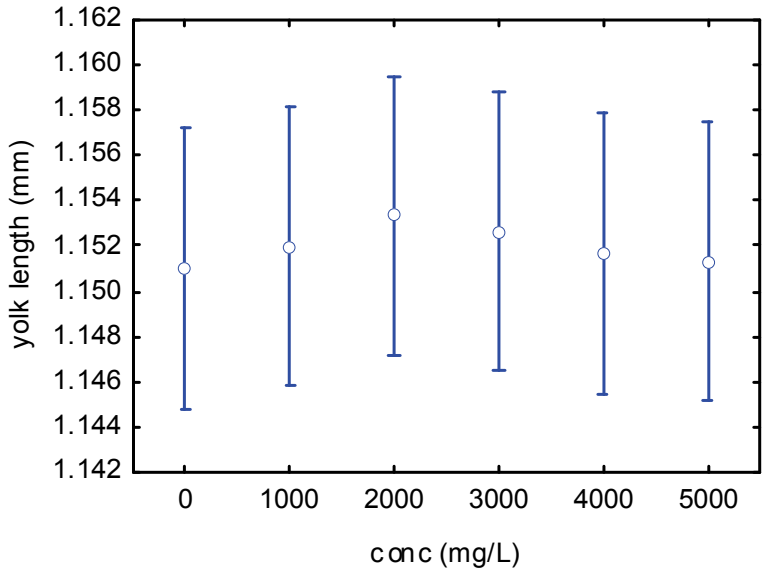

FIGURE 2.20 **Mean and 95% confidence intervals of yolk length (mm) of *C. nilotica* embryos at 72 hours. The yolk lengths are not statistically significantly different**

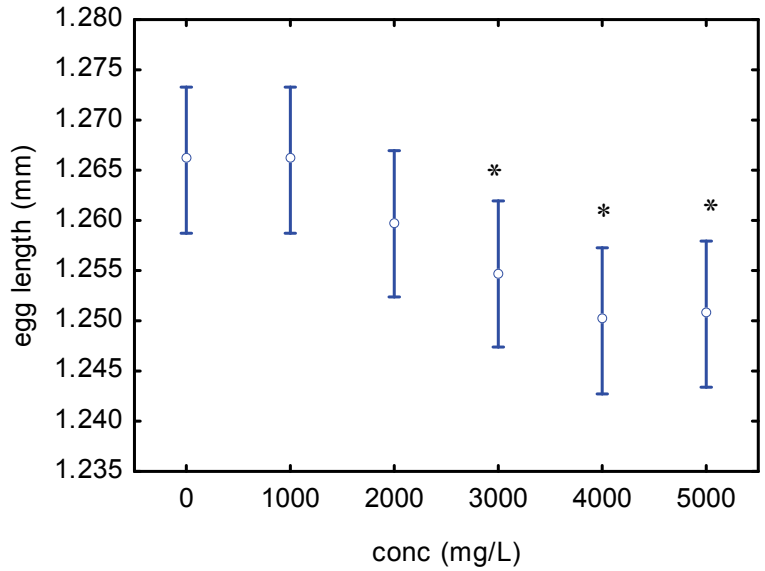

FIGURE 2.21 **Mean and 95% confidence intervals of egg length (mm) of *C. nilotica* embryos at 144 hours. Eggs were significantly smaller at and above Na_2SO_4 concentrations of 3000 mg/L than at 200 mg/L and below. The * indicates the concentrations where the egg length was significantly different from the control**

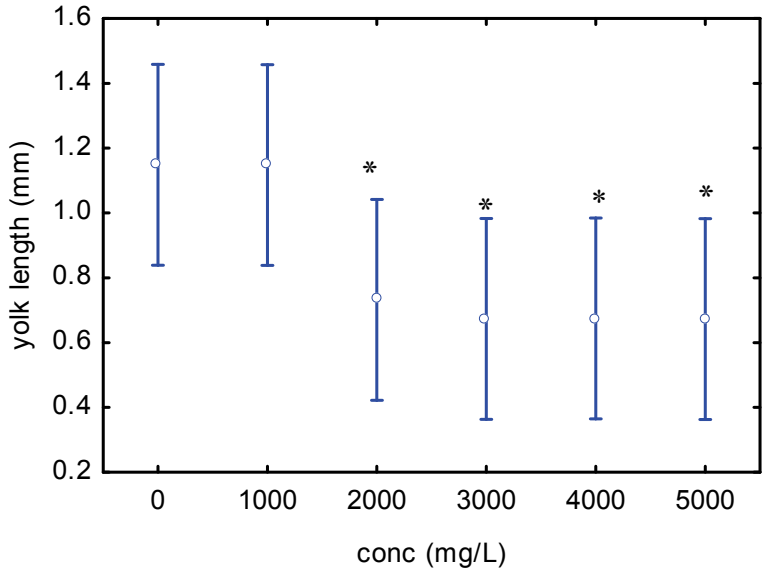

FIGURE 2.22 **Mean and 95% confidence intervals of yolk length (mm) of *C. nilotica* embryos at 144 hours. Yolks were significantly smaller at and above Na_2SO_4 concentrations of 2000 mg/L than at 100 mg/L and below. The * indicates the concentrations where the yolk length was significantly different from the control**

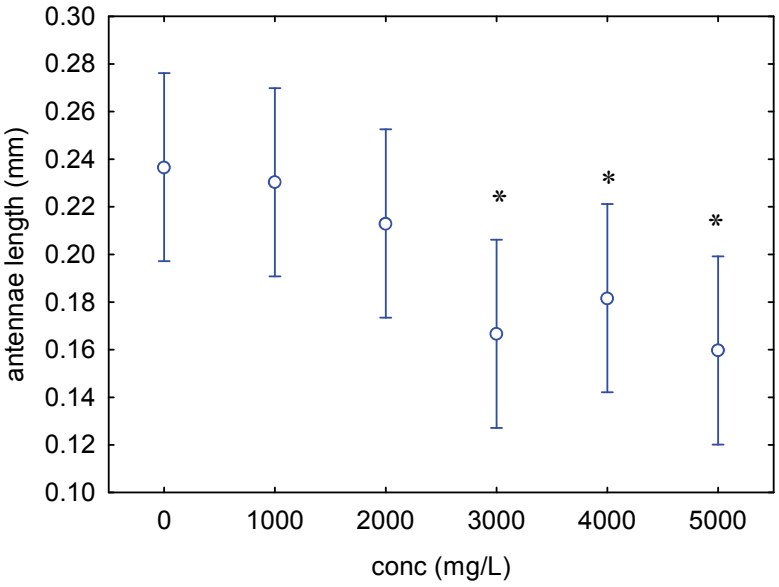

FIGURE 2.23 **Mean and 95% confidence intervals of antennal length (mm) of *C. nilotica* embryos at 144 hours. Antennaes were significantly smaller at and above Na_2SO_4 concentrations of 3000 mg/L than at 200 mg/L and below. The * indicates the concentrations where the antennae length was significantly different from the control**

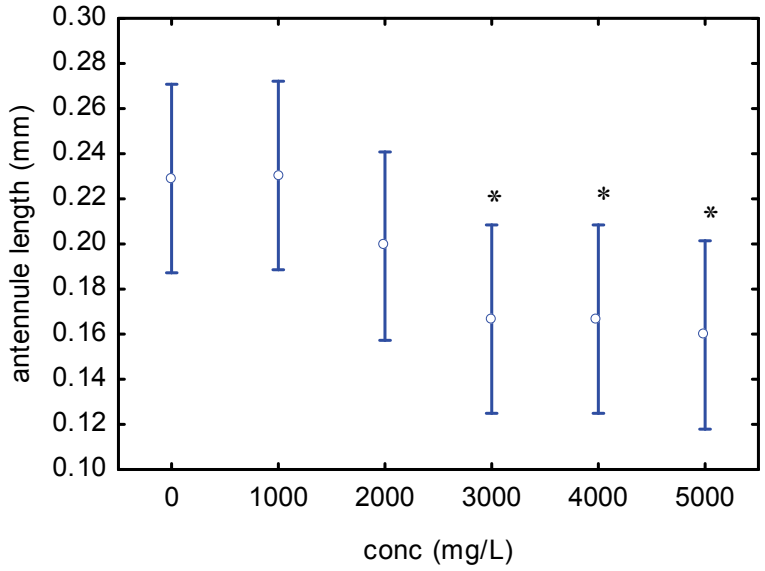

FIGURE 2.24 **Mean and 95% confidence intervals of antennule length (mm) of *C. nilotica* embryos at 144 hours. Antennules were significantly smaller at and above Na_2SO_4 concentrations of 3000 mg/L than at 200 mg/L and below. The * indicates the concentrations where the antennule length was significantly different from the control**

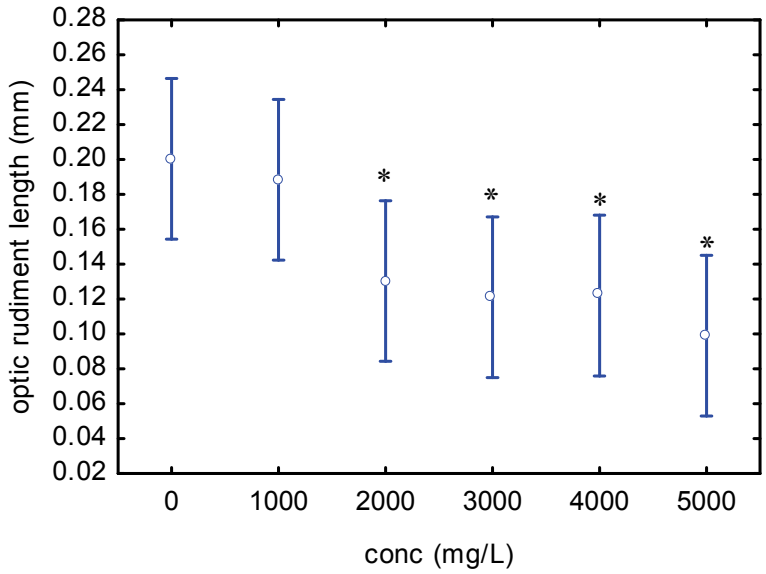

FIGURE 2.25 **Mean and 95% confidence intervals of optic rudiment length (mm) of *C. nilotica* embryos at 144 hours. Optic rudiments were significantly smaller at and above Na_2SO_4 concentrations of 2000 mg/L than at 100 mg/L and below. The * indicates the concentrations where the optic rudiment length was significantly different from the control**

Sodium chloride

All control replicates had 0% mortality and 100% hatching success. At 1000 mg/L, 93% of the eggs hatched, in 2000 mg/L 49% of the eggs hatched and in 3000 mg/L 23% of the eggs hatched (Figure 2.26). There was mortality in 4000 mg/L concentration of NaCl within 72 hours. At 408 hours there was 100% mortality at 4000 mg/L and 6000 mg/L (Figure 2.27).

The length, width and area of the egg, yolk, antennule, antennae and optic rudiment, increased gradually in all concentrations until 144 hours. The increases were not significantly different from the control and between concentrations (Figures 2.28-2.32). At 144 hours the appearance of the caudal papilla was observed as expected in all concentrations. From 3000 mg/L to 6000 mg/L at 216 hours, where the eye developed, the length, width and area of the features were smaller when compared with the controls (Figures 2.33-2.36). At 288 hours the heart palpitations were seen at 1000 mg/l and 2000 mg/L, and the measured features continued to be smaller in length, width and area when compared to the control (Figures 2.37-2.41). At 336 hours all the developmental features can be observed in all the concentrations but at 3000 mg/L to 6000 mg/L the features were significantly smaller when compared to the control.

The LOEC observed was at 3000 mg/l based on the lowest concentration which had a statistically different effect from the control and the NOEC was at 2000 mg/l based on the highest concentration which did not have a statistically different effect from the control (Table 2.10). The length of the egg, yolk, antennae, antennule and optic rudiment at 216 hours were the morphological features which allowed the identification of the LOEC/NOEC values.

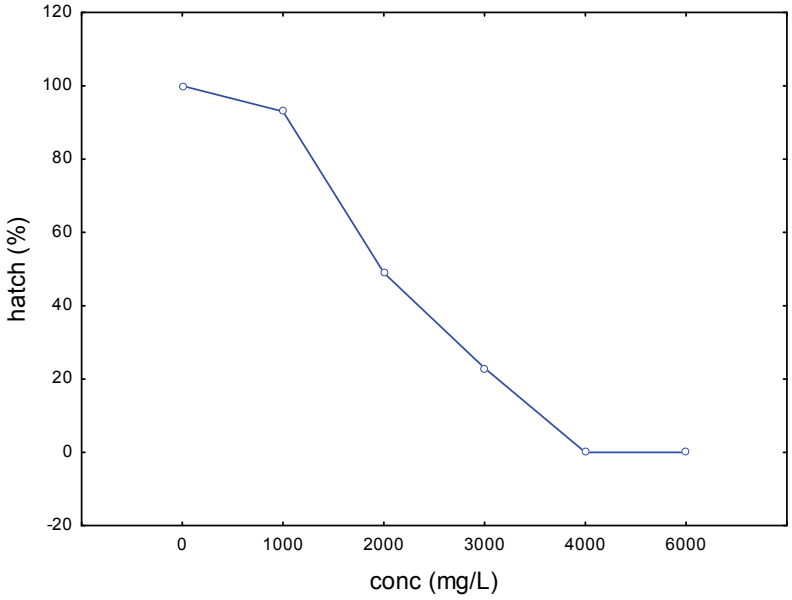

FIGURE 2.26 **Total hatching success (%) of *C. nilotica* embryos after 408 hours exposure to NaCl at different concentrations**

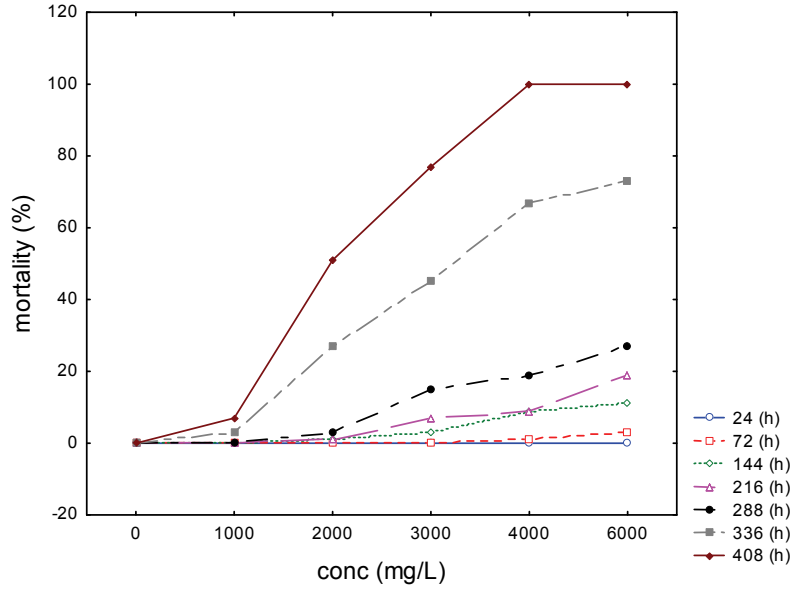

FIGURE 2.27 **Total mortality (%) of *C. nilotica* embryos exposed to increasing NaCl concentrations. Each line represents a different exposure period. Data for the replicates were combined for each concentration**

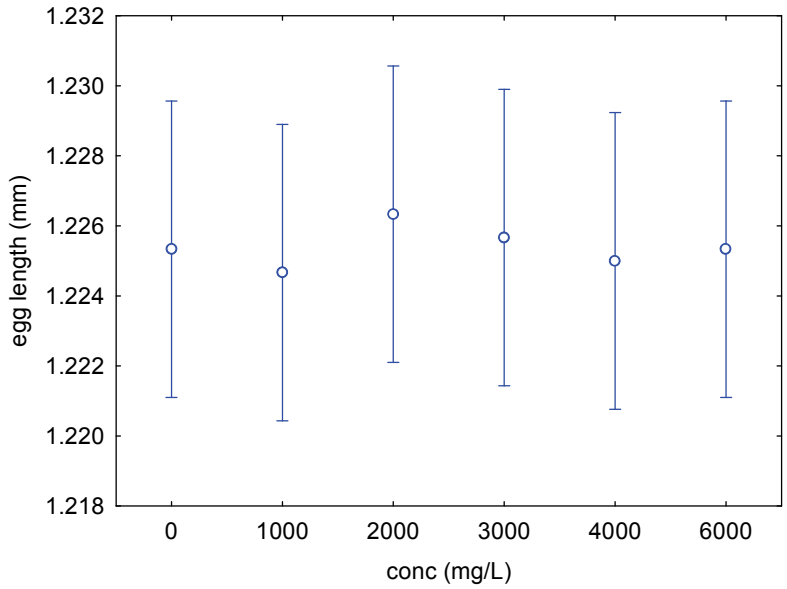

FIGURE 2.28 **Mean and 95% confidence intervals of egg length (mm) of *C. nilotica* embryos at 144 hours. The egg lengths are not statistically significantly different**

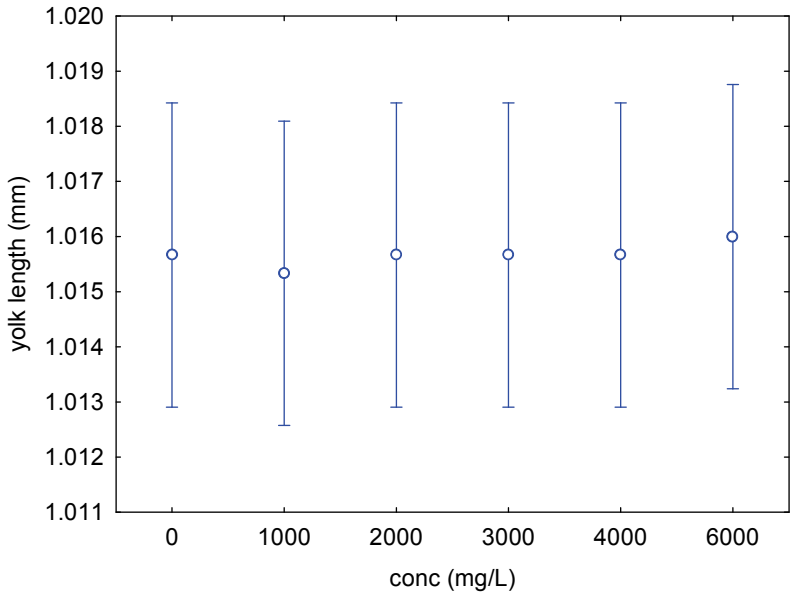

FIGURE 2.29 **Mean and 95% confidence intervals of yolk length (mm) of *C. nilotica* embryos at 144 hours. The yolk lengths are not statistically significantly different**

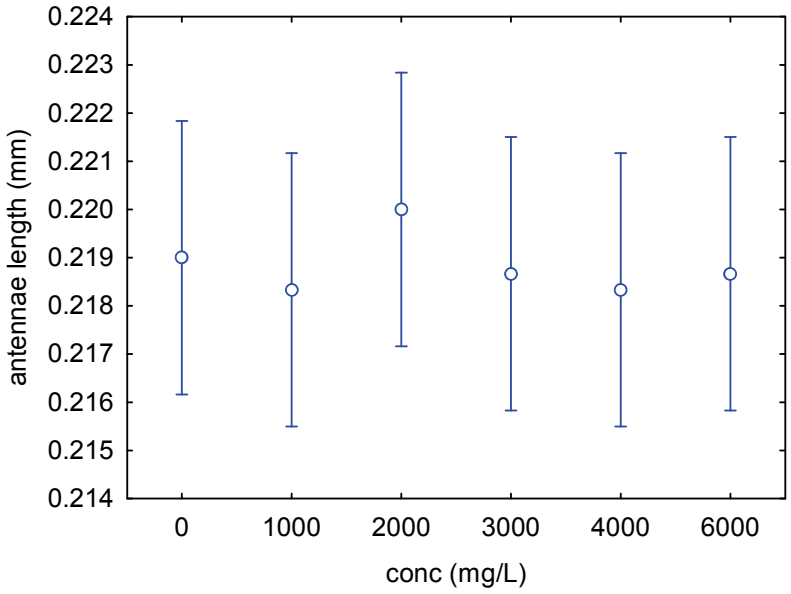

FIGURE 2.30 **Mean and 95% confidence intervals of antennal length (mm) of *C. nilotica* embryos at 144 hours. The antennal lengths are not statistically significantly different**

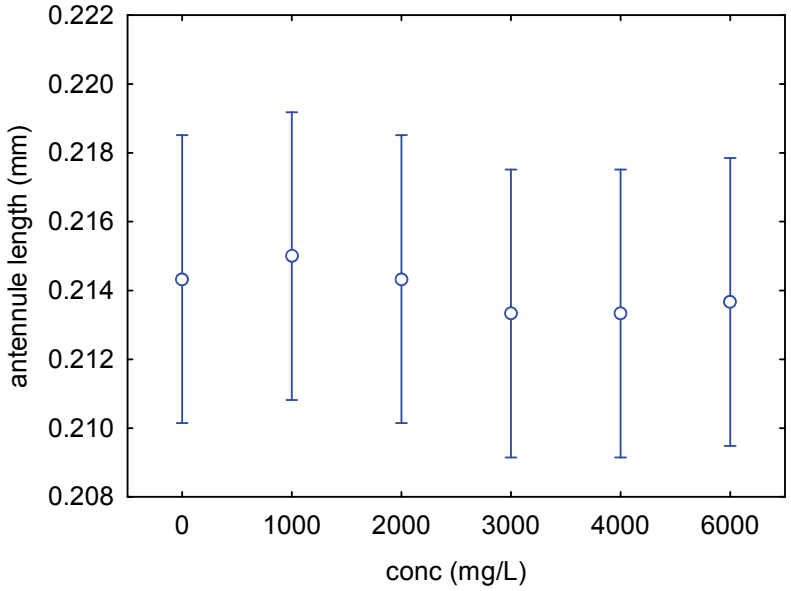

FIGURE 2.31 **Mean and 95% confidence intervals of antennule length (mm) of *C. nilotica* embryos at 144 hours. The antennule lengths are not statistically significantly different**

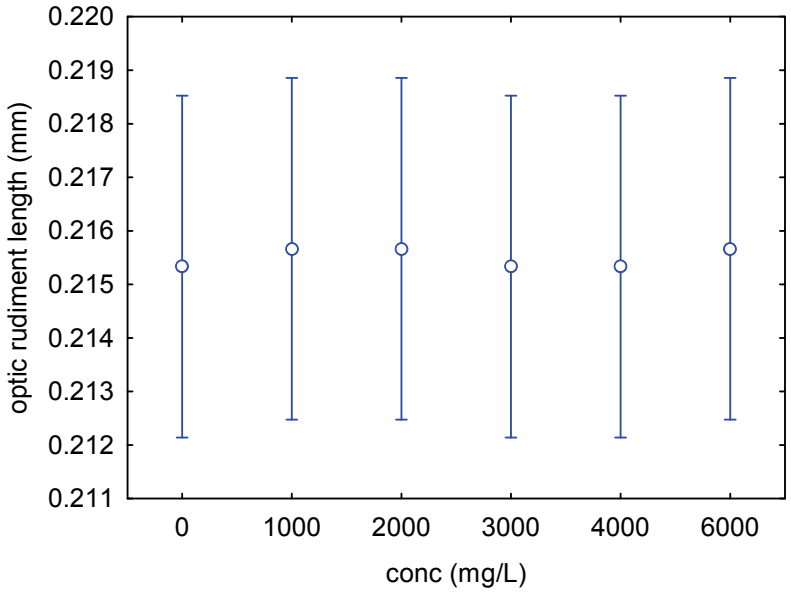

FIGURE 2.32 **Mean and 95% confidence intervals of optic rudiment length (mm) of *C. nilotica* embryos at 144 hours. The optic rudiment lengths are not statistically significantly different**

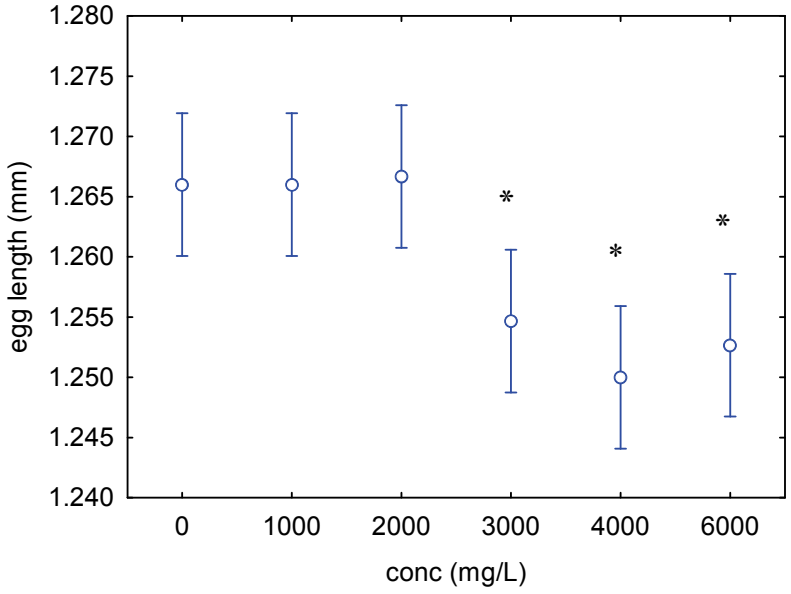

FIGURE 2.33 **Mean and 95% confidence intervals of egg length (mm) of *C. nilotica* embryos at 216 hours. Eggs were significantly smaller at and above NaCl concentrations of 3000 mg/L than at 200 mg/L and below. The * indicates the concentrations where the egg length was significantly different from the control**

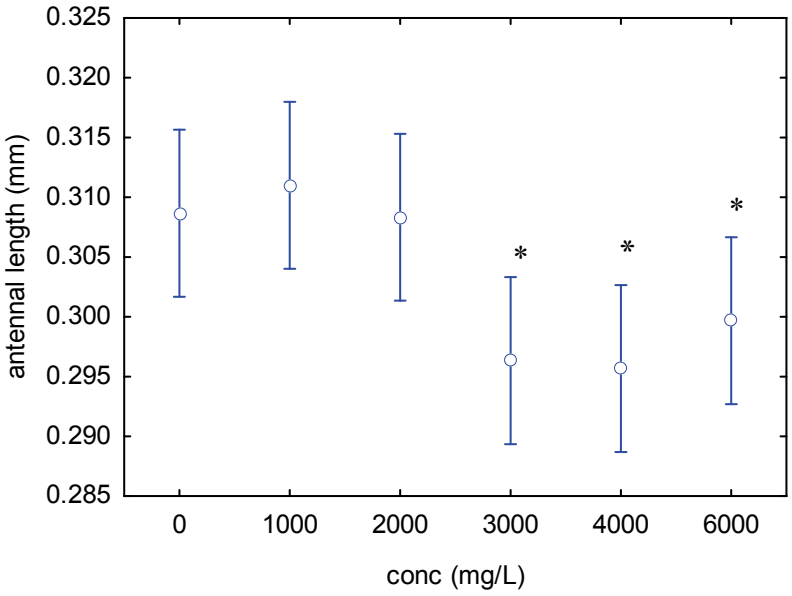

FIGURE 2.34 **Mean and 95% confidence intervals of antennal length (mm) of *C. nilotica* embryos at 216 hours. Antennaes were significantly smaller at and above NaCl concentrations of 3000 mg/L than at 200 mg/L and below. The * indicates the concentrations where the antennal length was significantly different from the control**

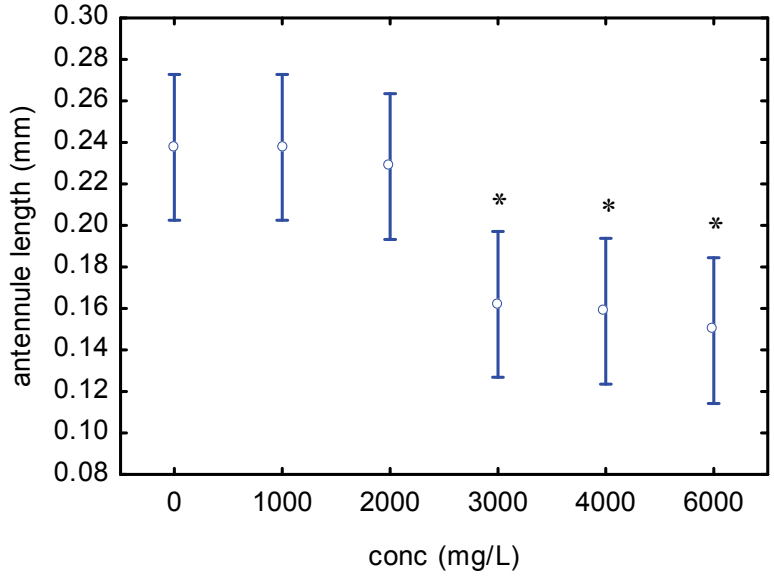

FIGURE 2.35 **Mean and 95% confidence intervals of antennule length (mm) of *C. nilotica* embryos at 216 hours. Antennules were significantly smaller at and above NaCl concentrations of 3000 mg/L than at 200 mg/L and below. The * indicates the concentrations where the antennule length was significantly different from the control**

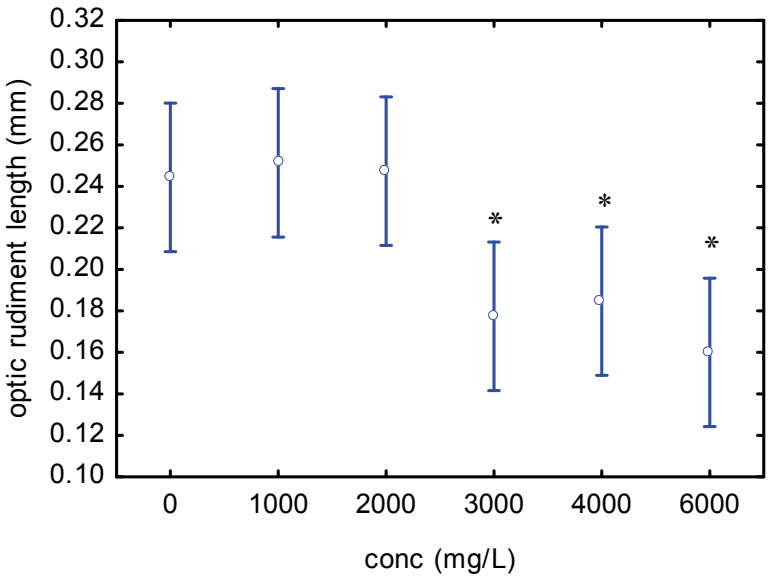

FIGURE 2.36 **Mean and 95% confidence intervals of optic rudiment length (mm) of *C. nilotica* embryos at 216 hours. Optic rudiments were significantly smaller at and above NaCl concentrations of 3000 mg/L than at 200 mg/L and below. The * indicates the concentrations where the optic rudiment length was significantly different from the control**

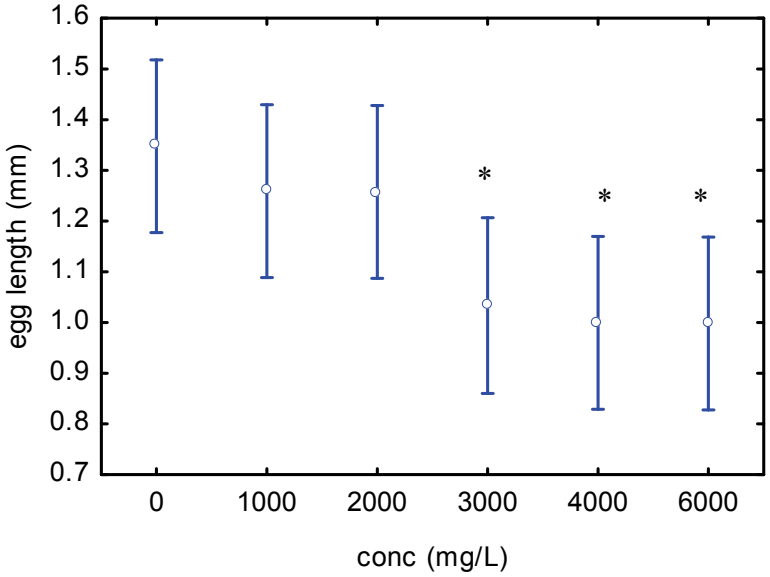

FIGURE 2.37 **Mean and 95% confidence intervals of egg length (mm) of *C. nilotica* embryos at 216 hours. Eggs were significantly smaller at and above NaCl concentrations of 3000 mg/L than at 200 mg/L and below. The * indicates the concentrations where the egg length was significantly different from the control**

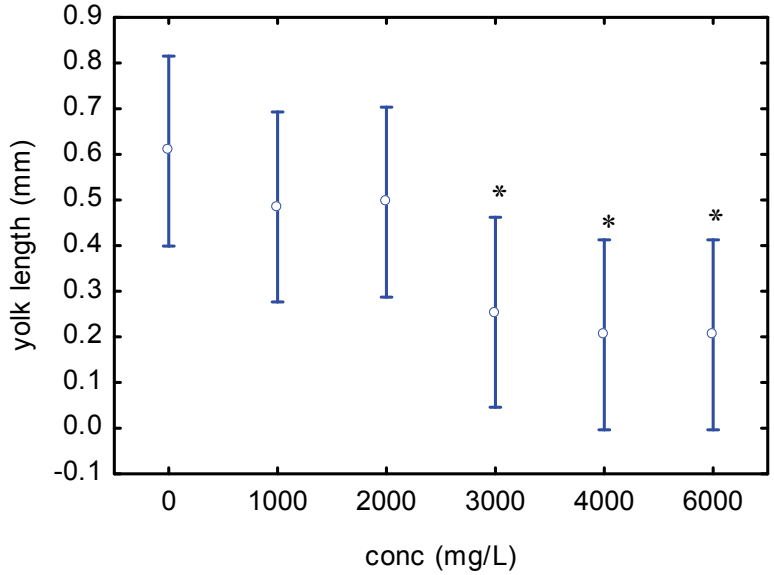

FIGURE 2.38 **Mean and 95% confidence intervals of yolk length (mm) of *C. nilotica* embryos at 288 hours. Yolks were significantly smaller at and above NaCl concentrations of 3000 mg/L than at 200 mg/L and below. The * indicates the concentrations where the yolk length was significantly different from the control**

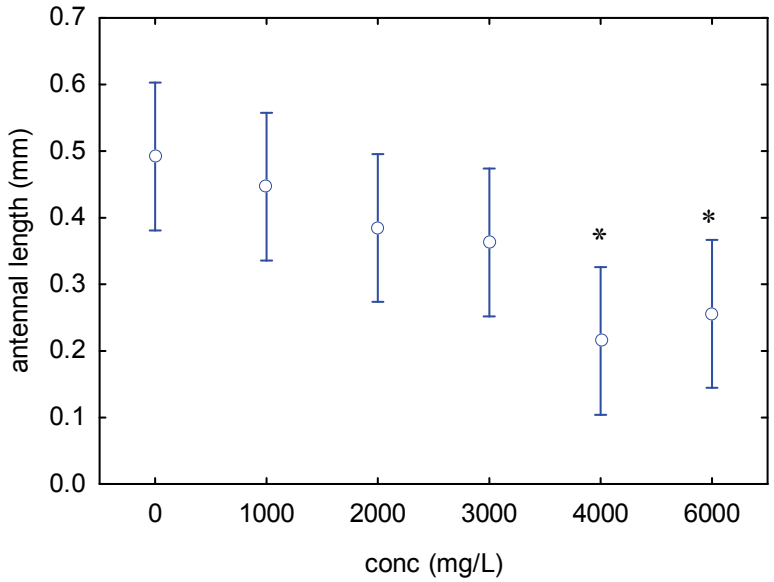

FIGURE 2.39 **Mean and 95% confidence intervals of antennal length (mm) of *C. nilotica* embryos at 288 hours. Antennaes were significantly smaller at and above NaCl concentrations of 4000 mg/L than at 300 mg/L and below. The * indicates the concentrations where the antennal length was significantly different from the control**

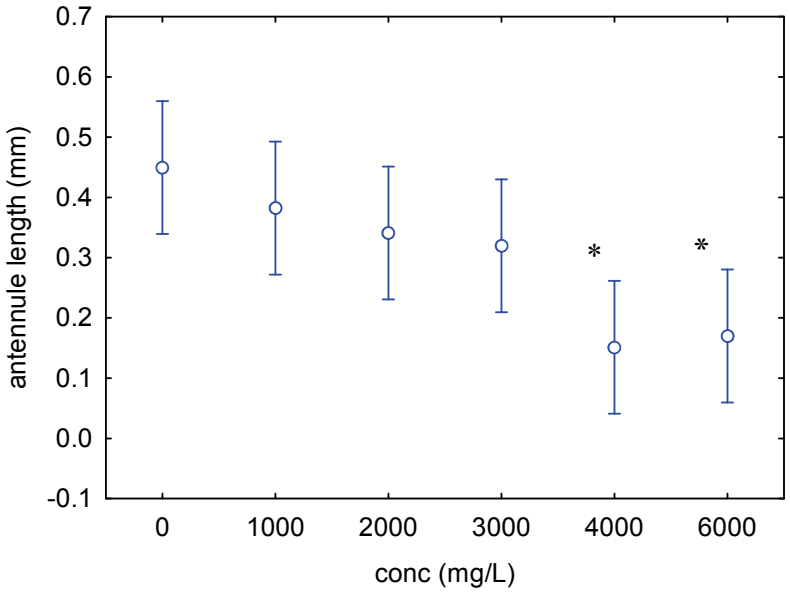

FIGURE 2.40 **Mean and 95% confidence intervals of antennule length (mm) of *C. nilotica* embryos at 288 hours. Antennules were significantly smaller at and above NaCl concentrations of 4000 mg/L than at 300 mg/L and below. The * indicates the concentrations where the antennule length was significantly different from the control**

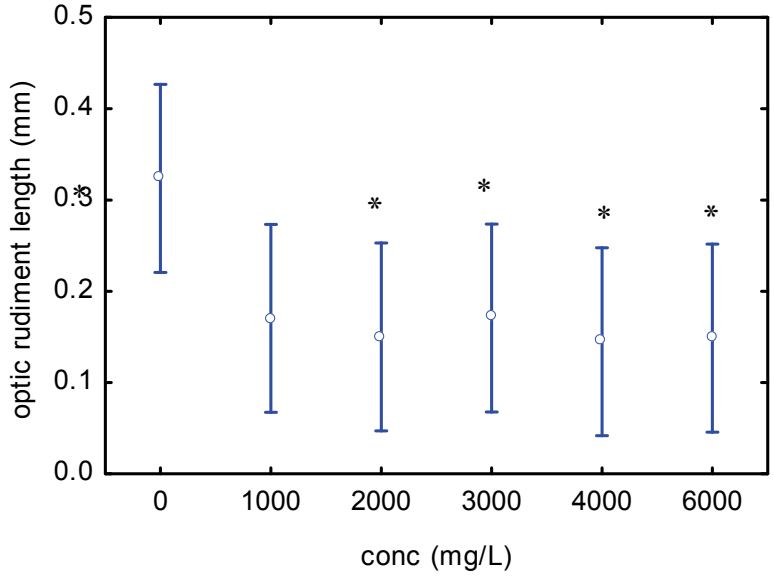

FIGURE 2.41 **Mean and 95% confidence intervals of optic rudiment length (mm) of *C. nilotica* embryos at 288 hours. Optic rudiments were significantly smaller at and above NaCl concentrations of 1000 mg/L. The * indicates the concentrations where the optic rudiment length was significantly different from the control**

Cadmium chloride

At 0.31 mg/L 13% of the eggs hatched while in the controls all the eggs hatched (Figure 2.42). At 24 hours mortality was observed in all the concentrations, and at 216 hours there was 100% mortality at 2.5 mg/L and 5 mg/L (Figure 2.43). At 288 hours there was 100% mortality at 1.25 mg/L and at 408 hours there was 100% mortality at 0.63 mg/L. This strongly suggests that the concentration range was too high.

After 24 hours exposure, a comparison of measured responses with controls showed a significant reduction in length, width and area of the features in all the concentrations. Over time until hatching the length, width and area of the features were smaller when compared to the control. The cadmium chloride proved to be highly toxic because most of the eggs did not reach the hatching stage. There was an insignificant mortality of 3% in the control, the allowed mortality in the control is 10% (Rand, 1995).

The LOEC was at 0.31 mg/L at 24 hours, which leads to an estimated NOEC of between 0 mg/L and 0.312 mg/L of $CdCl_2$ (Table 2.10).

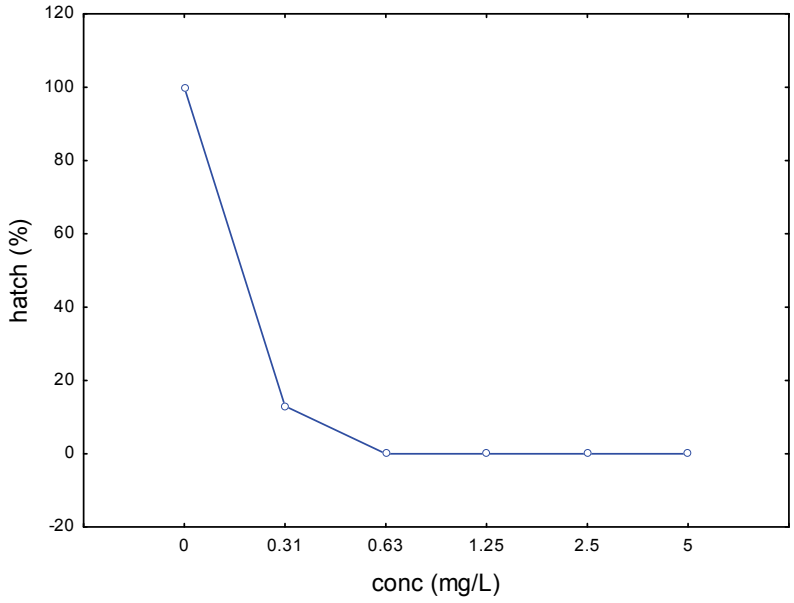

FIGURE 2.42 **Total hatching success (%) of *C. nilotica* embryos after 408 hours exposure to $CdCl_2$ at different concentrations**

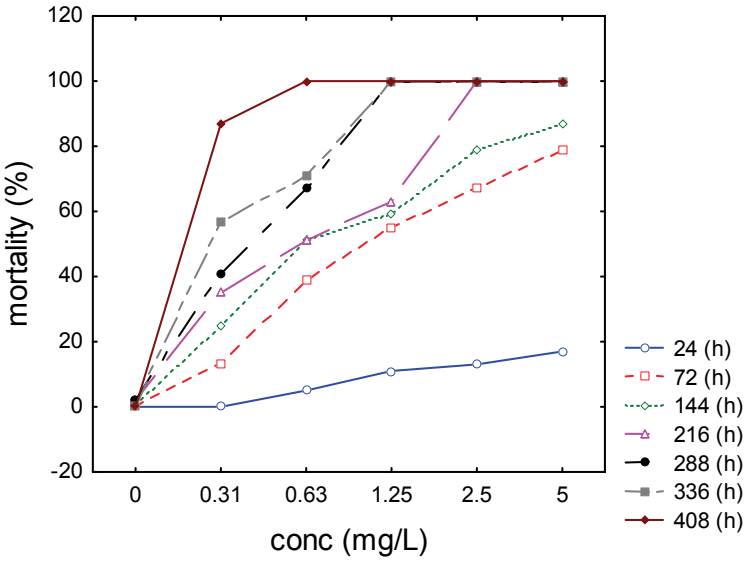

FIGURE 2.43 **Total mortality (%) of *C. nilotica* embryos exposed to increasing $CdCl_2$ concentrations. Each line represents a different exposure period. Data for the replicates were combined for each concentration**

TABLE 2.10 The estimated NOEC and LOEC values for NaCl, Na_2SO_4 and $CdCl_2$ over time, for the various developmental features of *C. nilotica*.

Toxicant	Time (hrs)	Feature	LOEC (mg/L)	NOEC(mg/L)
Na_2SO_4	144	egg	3000	2000
		yolk	2000	1000
		antennae	3000	2000
		antennule	3000	2000
		optic rudiment	2000	1000
	216	caudal papilla	3000	2000
	288	eye	4000	3000
	336	pleopods	3000	2000
NaCl	216	egg	3000	2000
		yolk	3000	2000
		antennae	3000	2000
		antennule	3000	2000
		optic rudiment	3000	2000
		caudal papilla	3000	2000
	288	eye	4000	3000
	336	pleopods	3000	2000
$CdCl_2$	24	egg	0.63	0.31
		yolk	0.31	< 0.31
	72	antennae	0.31	<0.31
		antennule	0.31	<0.31
		optic rudiment	0.31	<0.31
	144	caudal papilla	0.31	<0.31
	216	eye	0.31	<0.31
	288	pleopods		

In previous studies the hatching percentage of embryos from egg sacs has been the focus of embryo toxicity studies (Lee and Oshima, 1998, Lee et al., 1996). The toxicity tests undertaken in this study showed that most concentrations of Na_2SO_4, NaCl and $CdCl_2$ administered during embryonic development resulted in reduction of growth and increased mortality rate.

NaCl was the least lethal of the 3 toxicants (Table 2.10); the NOEC value for NaCl was the highest. Mortality started at 72 hours in 4000 mg/L (Figure 2.17), where 1% of the embryos died. NaCl is followed by Na_2SO_4 in lethality. At 24 hours there was 1% mortality at 5000 mg/L Na_2SO_4 (Figure 2.28) while $CdCl_2$ was most toxic, resulting in 5% mortality at 24 hours at 0.63 mg/L (Fig. 2.42). The pattern of Na_2SO_4 being more toxic than NaCl was evident in a study when Kefford et al. (2004) exposed *C. nilotica* to Na_2SO_4, in acute experiments Na_2SO_4 was more toxic than NaCl. Goetsch & Palmer (1997) investigated the salinity tolerance of macroinvertebrates in the Sabie River, and they discovered that the mayfly nymphs (*Tricorythus tinctus)* were more sensitive to Na_2SO_4 than to NaCl. The same trend is revealed in the UCEWQ database (Table 2.8). Table 2.11 shows the NOEC and LOEC values with the endpoints which were used to establish these values being the decrease in length, width, and area of the egg, yolk and optic rudiment of the developing embryo.

TABLE 2.11 The LOEC and NOEC values of the effects of Na_2SO_4, NaCl and $CdCl_2$ on the embryonic development of *C. nilotica.*

Toxicant	time (hrs)	Effect	Feature	Conc (mg/l)
Na_2 SO_4	144	NOEC		1000
		LOEC	yolk, optic rudiment	2000
NaCl	216	NOEC		2000
		LOEC	yolk, egg, antennae antennule and optic rudiment	3000
$CdCl_2$	24	NOEC		<0.31
		LOEC	Yolk	0.31

When Kefford et al. (2004) investigated the salinity tolerance of eggs and hatchlings of selected aquatic macroinvertebrates in south-east Australia and South Africa they came to the conclusion that the mean tolerance of the most sensitive younger life stage was barely half that of older life stages. Lee et al. (2000) suggested that this may be due to the fact that there is intense cellular activity and organ development over a relatively short period during embryonic development: it has been suggested that during cell division the more open chromosome is more susceptible to toxicant entrance. Thus, embryos exposed to toxicants for short periods may be more sensitive than longer exposure in later life stages.

Slaughter (2005) found the NOEC for Na_2SO_4 to be 1900 mg/L and the LOEC to be 2530 mg/L for growth and reproduction of *C. nilotica* over a period of 80 days. For NaCl the NOEC was 1900 mg/L and the LOEC was 2200 mg/L for growth, and for reproduction the NOEC was 1900 mg/L and LOEC was 2700 mg/L. Compared to the LOEC and NOEC values found in this study, the NOEC and LOEC values for NaCl found by Slaughter (2005) were lower. For Na_2SO_4 the NOEC and LOEC values found in this study were lower than the values found by Slaughter (2005).

The $CdCl_2$ concentrations were the most lethal to the embryos, as the mortality rate was higher even within the lowest concentrations. In other studies it has been determined that when older aquatic invertebrates were exposed to cadmium there was a decrease in growth and fecundity (Coetzee, 1989; De Nicola Guidici et al., 1987; Kogan et al., 2000; Lee et al., 1996; Muyssen and Janssen, 2004; Rodríguez and Medesani, 1994; Witeska et al., 1995). A full lifecycle test would allow the observation of the reproduction of the embryos that did hatch when they reach the mature stage. In a study undertaken by Muyssen and Janssen (2004), *Daphnia magna* were exposed to cadmium in acute and chronic tests. They found that *D. magna* in acute test became acclimated to the toxicant whereas in chronic tests there was a decrease in survival and reproduction, which shows the importance of a chronic toxicity method.

Further tests need to be done for NaCl, Na_2SO_4 and $CdCl_2$ and for other toxicants before embryonic development can be considered for regulatory testing. However, the reduction in length, width and area of the identified measured features show potential as an experimental endpoint with which to determine an effect on developing embryos of *Caridina nilotica*.

2.5 DISCUSSION

Laboratory methods for culturing and rearing *Caridina nilotica* have now been established. Using the method outlined in Section 2.2, test organisms can successfully be obtained from cultures maintained under known conditions for experimental purposes (acute and chronic toxicity tests, using different life-stages). Although methods require refining, mostly as more is known about the biology of the species (in particular its feeding preferences), method enhancement for improved rearing and culture maintenance can be introduced.

2.5.1 Growth of *C. nilotica* as a chronic test endpoint

The results seem to indicate that *C. nilotica* is fairly tolerant of elevated salinities. The NOEC values obtained for *C. nilotica* would probably not be protective of most other aquatic macroinvertebrates at a chronic exposure duration. Salinity tolerance information for this species are still important for setting salinity water quality guidelines in South Africa, as this is an indigenous species and new methods of setting guidelines, such as species sensitivity distributions, use all available tolerance data, and not just the most sensitive tolerance data (Warne, 1998).

C. nilotica may be more sensitive to toxicants other than the salts tested here. Sloof (1983), in an investigation on the comparative sensitivity of 22 freshwater species to 15 chemical compounds, found that crustaceans were among the most sensitive macroinvertebrates. The UCEWQ Ecotoxicology database contains the results of acute toxicity testing where this species was exposed to cadmium chloride. The LC_{50}s ranged from 0.05-0.09 mg/L. If compared to the LC_{50} values of approximately 0.15 mg/L for *Daphnia pulex* (a standard toxicity test species) on the database, *C. nilotica* appears to be sensitive for this chemical at least, although this needs to be confirmed for other toxicants.

C. nilotica is readily reared under laboratory conditions. The biology and life history of the species has also been researched (Hart, 1980; Hart, 1981; Hart, 1983). Equipment for rearing this species is relatively inexpensive. Food suitable for this species is also readily available and inexpensive. Egg to egg lifecycle takes approximately 3 months, which is long enough

for a comprehensive chronic toxicity test but short enough to not pose logistic problems. This species is also indigenous to South Africa. Individuals are fairly easy to handle at a mature age with full-grown animals reaching up to 2.5cm in length. Even young neonates are relatively easy to handle as they are visible with the naked eye and are readily transferable from one container to another using a pipette. One point of apparent concern is the high natural attrition rate of young neonates under 14 days old. However, using slightly older neonates as a starting point within a chronic toxicity test bypasses this problem. Using reproduction as an endpoint with this species in a toxicity test requires careful thought and planning. These shrimps are sexed according to the presence or absence of the appendix masculina on the excised 2nd pleopods, but reliable sex determination of living individuals is not possible (Hart, 1980). Therefore at the start of an experiment, it is impossible to control the ratio of sexes in the treatments. It is likely that all individuals in a treatment may be of the same sex. The sex of these shrimp may be related to size, with individuals changing from male to female when they reach a certain size (Okuthe et al., 2004). If individuals of the same age and size are used at the start of an experiment, and growth of all individuals are fairly standard, most individuals will be of the same sex at any particular time and reproduction will not be possible whereas a treatment where there is more variability in regards to the size of the individuals will probably contain greater heterogeneity in the sex of the individuals. It is also possible that larger individuals may be more tolerant to a certain stressor. In this case all smaller individuals in a treatment may perish effectively leaving survivors in larger size categories. All the survivors may then be female, which would exclude reproduction. A suitable reproductive endpoint that would bypass this problem would be mean number of eggs per female.

A further issue that should be considered when using *C. nilotica* as a toxicity test organism, is that individuals have a habit of jumping out of the experimental vessels. In this test, vessels were covered with cling wrap, which effectively prevented this. Unfortunately cling wrap tends to lose its effectiveness over time as corners of the covering start bunching up and small openings appear. The cling wrap coverings were replaced regularly in these experiments to prevent this. It is recommended that something more permanent be used to cover the vessels such as sheets of glass.

All points considered, *C. nilotica* is an excellent indigenous organism to use for chronic toxicity tests where growth as a biological response is measured. The potential use of this organism in short-term partial lifecycle chronic toxicity tests needs to be investigated.

2.5.2 Embryonic development of *C. nilotica* as a chronic test endpoint

The eggs are relatively easy to remove from the female and to handle. They are readily transferable from the females to the experimental brood chambers using a soft paint brush and a pipette, and can be removed easily from the experimental brood chambers to monitor embryo development.

The results of this study demonstrated that the easily identified developmental ages and morphological features of *C. nilotica* could be used in toxicity tests. Using a replicated experimental design and an ANOVA statistical analysis, statistically significant effects could be found, and ascribed to exposure to the toxicants. *C. nilotica* showed more tolerance to NaCl than to the other two chemicals tested. Similar studies by Goetsch & Palmer (1997) investigated the salinity tolerance of macroinvertebrates in the Sabie River to NaCl and Na_2SO_4. Mayfly nymphs (*Tricorythus tinctus*) were discovered to be more sensitive to

Na_2SO_4 than to NaCl. Data in the UCEWQ database (Table 2.8) also shows that NaCl is less toxic than Na_2SO_4.

The LOEC observed for NaCl was 3000 mg/L at 216 hours and the NOEC was 2000 mg/L, the morphological features which allowed the establishment of these values were the measured sizes of the egg, yolk, antenna, antennule and optic rudiment. The total percentage of the embryos that hatched after 408 hours was 33% and they hatched from concentration 1000 mg/L to 3000 mg/L. The Na_2SO_4 was shown to be more toxic than the NaCl. The LOEC of Na_2SO_4 was 2000 mg/L at 144 hours, and the estimated NOEC was 1000 mg/L, with the sizes of the yolk and the optic rudiment being the features which allowed the identification of the LOEC/NOEC values. The total percentage of the embryos that hatched after 408 hours was 18% and all the embryos were from the concentration of 1000 mg/L. The results presented for $CdCl_2$ indicate higher toxicity than the other two toxicants. The NOEC for $CdCl_2$ was found to be less than 0.31 mg/L and the LOEC was 0.31 mg/L at 24 hours, with the yolk being the morphological feature which allowed identification of the NOEC/LOEC values. A total of 2.6% of the eggs hatched after 408 hours and they were from 0.31 mg/L concentration. In order to establish a NOEC for $CdCl_2$, further experimental work must be undertaken.

Comparing the NOEC values of this study and the NOECs in the UCEWQ database and Slaughter (2005) for Na_2SO_4, it is possible to conclude that the early life stages of *C. nilotica* are more sensitive to chemicals than the older life stages for Na_2SO_4. The NOEC for Na_2SO_4 in my study was 1000 mg/L and in the UCEWQ database and in Slaughter (2005) it was 1900 mg/L. However, for NaCl the NOEC for this study was 2000 mg/L and Slaughter (2005) found an NOEC value of 1900 mg/L. Choosing concentrations between 1000 mg/L and 2000 mg/L might give an NOEC less than 1900 mg/L, and this warrants further investigation. The LC50s of $CdCl_2$ for older life stages of *C. nilotica* ranged from 0.05-0.09 mg/L from the UCEWQ database which, shows that lower concentrations need to be considered. However, based on the membrane of the *C. nilotica* eggs and the unknown toxicity of the $CdCl_2$ on the eggs a wide range of concentrations to test were chosen in this range finding study.

Data for the effects of NaCl and Na_2SO_4 on embryonic development of other freshwater shrimps could not be found. The combined effects of temperature and Cd on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos showed the LC_{50} at 26°C being 30.1 mg/L (Hallare et al., 2005). This value is very high, suggesting that the embryos of *C. nilotica* are more sensitive than the embryos of the zebrafish. Botton (2000) studied the toxicity of cadmium and mercury to horseshoe crab (*Limulus polyphemus*) embryos and larvae, and for embryos the LC_{50} value was 0.5 mg/L at 48 hours and 0.2 mg/L at 72 hours; no NOEC values could be established. However the LC_{50} values at 72 hours are lower than the LOEC values found in this study. Since horseshoe crabs are a marine species, it is not clear whether differences can be ascribed to the different environments.

2.5.3 General conclusions

The following general and salient points emerge from the research undertaken:

- Further biological information on *C. nilotica* is required if the test species is to be utilised as a test species for routine toxicity testing for regulatory purposes. Many assumptions have been made regarding optimising the laboratory conditions for maximising the production of a steady supply of test organisms. Although these

conditions have resulted in the establishment of a laboratory culture from which test organisms are routinely removed for experiments, there have been frequent culture crashes which suggest that conditions are not entirely optimum.

- Initial research work undertaken by Postdoctoral Research Fellow (Emily Okuthe, 2003/2004) on developmental aspects of *C. nilotica* yielded potentially interesting and promising results for a further chronic toxicity test. The species undergoes a sex change at approximately 3 months of age, changing from male to female (hence the need for a mixed size range in culture tanks). It was not established whether this was an age or size triggered switch, but suggested potential as a suitable test method specifically for those toxicants that are known endocrine disruptors. However, further biological investigations were not undertaken and a test method was never developed or refined due to time and funding constraints.
- The toxicants tested were limited to NaCl, Na_2SO_4 and $CdCl_2$. In order to adequately test the methods developed, a more diverse group of toxicants, with differing toxicological effects, will need to be tested.
- Salinity tolerance information for indigenous species is still important for setting salinity water quality guidelines in South Africa. The NOECs found in this study were compared with NOEC values of different test methods, to give an indication of the sensitivity of the test method developed in this study. Based on results from this study, *Caridina nilotica* is an excellent indigenous organism to use for chronic toxicity tests where growth of embryos as a biological response is measured although further testing of other chemicals is required to verify this. Whether teratogenic effects such as lesions, head malformation and spinal curvatures found in other studies (Rodríguez and Medesani, 1994, Witeska, 1995) can be found in *C. nilotica* still needs to be determined.
- The method developed to measure effects on growth and reproduction suggested good potential for further testing with other toxicants. Potentially, an effect on growth in juveniles can be measure within 20 days, which is significantly shorter than a full life cycle test, although effects of reproduction will then be missed.
- The results suggest that the embryonic development of *C. nilotica* can be a valuable indicator of contamination in freshwaters by aging the dead eggs and comparing their morphometry to the experimental values. Morphometric measurements of the developmental features could be an indication of an effect caused by chemicals. However, it would be necessary to extend this study to other pollutants to achieve more generalized conclusions useful for water quality monitoring. Embryonic development of *C. nilotica* could possibly be used in future studies in which the toxicity of complex effluents to the developing embryo is assessed. A preliminary experiment was undertaken during this study, exposing the eggs of *C. nilotica* to whole effluent collected from a textile industry. Results from this preliminary assessment suggest that the method developed in this study may be useful in the toxicity assessment of complex effluents.
- Comparisons between laboratory-reared and field-caught organisms will need to be undertaken, as differences in sensitivity between these two have been found in other species (*D. magna*; Muyssen et al., 2002) and this may have significant ramifications for environmental realism and using results to extrapolate from laboratory to field predictions and developing water quality guidelines. Out of necessity, due to culture crashes, the culture housed at the breeding facility at UCEWQ_IWR is a mixture from separate populations collected from 2 distinct river systems in South Africa (one from northern KwaZulu-Natal and another from the Eastern Cape). As the species reproduces sexually, genetic mixing has likely to have taken place and it is not known

whether this has rendered the population more or less sensitive than their wild-caught counterparts.

- The present results represent a stimulus for further studies of the effects of chemicals on developing embryos, including the establishment of teratogenic effects of aquatic pollutants on *C. nilotica* embryos. The results presented in this study will contribute to the development of chronic toxicity data for *C. nilotica* in the UCEWQ database, and contributes to national and international data available for the development of environmentally realistic water quality guidelines for aquatic ecosystems.

CHAPTER 3

DEVELOPING ORGANISM-LEVEL CHRONIC TOXICITY TEST METHODS FOR FLATWORM SPECIES

3.1 INTRODUCTION

Flatworms in general are easy to handle and inexpensive to maintain in the laboratory; available all year round and not subject to seasonal cycles; considered by some to be more susceptible to toxicants than some standard (Piontek 1999a, 1999b) or other species (Congiu et al., 1989); and their toxicological responses are considered by some to be comparable to vertebrates (Best and Morita, 1991). The latter facilitates analogies to be made between neurological development in the planaria with embryonic development in vertebrates, although with limitations. It is their regenerative abilities and behavioural responses that make these organisms well suited to assessing the toxicity of a wide variety of chemicals.

Dugesia are common representatives of the Class Turbellaria, found in freshwater habitats such as ponds, lakes, ditches, rivers and streams (Gilbertson, 1999). They are elongated, flattened and tapered at both ends with ocelli (eye spots) which detect the amount of light in the environment. These flatworms are photonegative and are usually found under rocks and debris during the day. Auricles (ear-like flaps) detect the amount of water current. Their colouration ranges from grey, brown and black with patterns of spots, to stripes or mottling. Flatworms are predators and scavengers. Their digestion tract consists of a central tubular pharynx, which is extended out of the body cavity for feeding. Digestive enzymes are excreted from the pharynx, and thus digestion begins outside of the body. Muscular pumping motions bring the liquefied food into a 3-branched intestine. Each branch consists of ceca, which deliver the nutrients to the body. Dugesia are blind-ended: food enters and excreta leave the body at one site (Gilbertson, 1999).

The planarians have been used for decades to detect the presence of water pollutants (Steinmann and Bresslau, 1913). Several authors have suggested *Dugesia* species in particular to be useful indicators of water quality pollution (Lewis and Suprenant, 1983; Kostelecky et al., 1989; Calevro et al., 1998 and 1999; Piontek, 1999a and b; Dasheiff and Dasheiff, 2002, Zhao and Zeng, 2004). The responses monitored include lethality, developmental neurotoxicity, teratogenesis, tumorigenesis and carcinogenesis (Best and Morita, 1991). Recently *Dugesia* species have been successfully used in microcosm tests in South Africa (Schulz et al., 2002). Although differences between species in their sensitivities to whole effluent, for example, have been found (Tamura et al., 1995), the choice of test species is dependent on how widely distributed they may be in unpolluted waters, and the availability of sources of populations in pristine conditions (for inclusion in site specific toxicity tests).

There were a number of reasons for selecting flatworms as possible test organisms for developing chronic toxicity test methods:

- Early studies revealed that flatworms can be kept for extended periods of time on a diet of raw liver (Wulzen, 1927) and it appeared that they would make good laboratory culture species.

- Use of flatworms in toxicity tests is scarce (Preza and Smith, 2001) and therefore the generation of toxicity test data for these species could be potentially invaluable, both nationally and internationally. Flatworms are an additional taxonomic grouping, which will be particularly useful for the development of water quality guidelines, as the key in the development of these is taxonomic diversity (ANZECC and ARMCANZZ, 2000).
- Preza and Smith (2001) reported low coefficient of variability between tests using a planarian species, suggesting that flatworms may be useful to develop as a standard test species, as test reproducibility appears acceptable.
- It is suggested that effects on fissioning (presumed for this study to include regrowth after cutting) and pharynx protrusion may be indicative of neurotoxicity (Best et al., 1981).

3.2 LABORATORY CULTURES OF FLATWORMS

The initial research to develop laboratory and culture methods for *Dugesia* spp. (Sluys, pers. comm.) was undertaken using organisms derived from two specimens collected from the Bell River, at the town of Rhodes (Eastern Cape; 30E47’48.96” S, 27E57’55.49” E), in 2000. However, subsequent to developing the methods present in this chapter, the culture "crashed" and a new culture was initiated with specimens collected from the Great Fish River, at Carlisle Bridge (Eastern Cape; 33E04'59.8"S, 26E13'31.8"E). The specimens collected were identified as a species of *Dugesia* (Sluys, pers. comm.).

A major part of establishing a chronic toxicity test method was to first develop culturing techniques which would allow the production of test organisms in sufficient numbers for routine use in toxicity tests. Although available literature suggested that keeping cultures of flatworms under laboratory conditions is relatively easy, developing methods to ensure consistent availability of test organisms has proven challenging and the method presented here is largely a result of trial and error in maintaining and increasing the numbers of individuals in the laboratory culture.

Further research is necessary, particularly with regards to the biology of the *Dugesia* species, to refine the method to ensure a constant production of test organisms for both acute and chronic toxicity tests.

3.2.1 EQUIPMENT, REAGENTS AND OTHER MATERIALS

The basic equipment and materials required for maintenance of laboratory cultures of *Dugesia* species are listed in Table 3.1. The laboratory breeding facility must be kept free of vapours, odours and dust that may be toxic to the flatworms, and ideally the test facility is kept separate from the culture facility.

Use analytical grade chemicals and dechlorinated tap water or equivalent for the preparation of solutions. Use deionised water for rinsing. All solutions can be stored for up to three months. All glassware, test vessels and non-disposal-non-glass containers used for breeding and test purposes is cleaned according to the following protocol:

- Rinse with tap water
- Soak in phosphate free soap (5% solution) for 15-30 minutes.
- Rinse three times with dechlorinated water

- Soak in 10% hydrochloric acid solution for 30 minutes
- Rinse three times with deionised water
- Rinse three times with dechlorinated water
- Air dry glassware
- All washed glassware should be stored in a dust free environment
- All glassware and equipment must be rinsed thoroughly with dilution water immediately before usage.

The necessary reagents are prepared as follows:

- Phosphate free soap, 5% solution: prepare by adding 1 L to 20 l dechlorinated tap water. The solution is stored in a polyethylene container between 15°C and 25°C and is stable for three months.
- Hydrochloric acid, 10% solution: prepare by adding 6.25 l, 32% HCl to 20 l dechlorinated tap water. The solution is stored in a polyethylene container between 15°C and 25°C and is stable for three months.
- Ethanol, 70% solution: prepare by adding 729.2 mL, 96% Ethanol to 1 l deionised tap water. The solution is stored in a polyethylene container between 15°C and 25°C and is stable for three months.

TABLE 3.1 Basic equipment necessary to establish a breeding culture of flatworm species.

Equipment	
Culture vessels: 10 l plastic basins	Polyethylene container with tap (25 l)
Glass beakers 50 mL, 100 mL and 500 mL	Pasteur pipettes or micropipette tips (1000µL), end cut off and fire-polished (opening approximately 5 mm), or disposable bulb pipettes (opening cut to approximately 5 mm) for handling and removal of dead flatworms from test containers
Air pumps	Plastic jugs (5 l)
Air tubing (5 mm)	Biolux and fluorescent light tubes
Tubing connectors, splitters and regulators	pH meter
Magnifying glass	Conductivity meter
Maximum/minimum thermometer	Dissolved oxygen (DO) meter
Paintbrushes	Toothbrushes
Glass cutting sheets	Single sided cutting blade
Reagents (all reagents must be analytical grade)	
Hydrochloric acid (HCl, 32%)	Ethanol (EtOH, 96%)
Phosphate free soap (Extran)	Deionised water
Food	
Bloodworm (chironomid larvae) – only use known brands	Boiled egg yolk from free range chickens

3.2.2 CULTURE MAINTENANCE AND BREEDING

Organisms are kept in culture vessels from where individuals of similar size are removed for use in toxicity tests. Dechlorinated tap water is used for both culture maintenance and breeding, and as negative control in experiments. Cultures are kept at room temperature,

although during winter it may be necessary to increase water temperature to ensure continued fissioning.

During the week, the organisms are exposed to a light regime of ambient laboratory illumination (artificial and/or day light), with a photoperiod of 12hrs light:12hrs dark. However, when no routine feeding or culture maintenance is taking place, the culture is kept covered (with black plastic) as flatworms are photonegative.

Culture and breeding tanks undergo routine daily, weekly and longer term maintenance, described in Table 3.2, and are set up as follows:

- Each culture vessel is given a unique label, noted on the data sheet
- Phosphate free soap is used to wash the vessels thoroughly
- Rinse thoroughly with dechlorinated tap water
- Fill with dechlorinated tap water to approximately 5cm from the top of the vessel
- Add an air pump and air tubing to each vessel
- Insert a cut pipette tip in the air tubing so sufficient bubbling occurs causing slight rippling of the water surface. Airflow must be controlled by a plastic control valve
- Allow the vessel to settle for one to two hours to reach room temperature before introducing the flatworms.

TABLE 3.2 Routine maintenance of flatworm culture.

Daily maintenance (Monday to Friday)	▪ Record temperatures (minimum, current and maximum). ▪ Ensure that aerators and air pumps are working. ▪ Compensate for evaporation by adding dechlorinated water to the vessels.
Weekly maintenance	▪ Feed flatworms twice weekly: 5 mm^3 per culture vessel of bloodworms once a week and 5 mm^3 per culture of boiled egg yolk. ▪ Transfer flatworms to a clean culture vessel one to three hours after feeding, to prevent accumulation of debris and waste. ▪ Gently squirt water onto the flatworm using a plastic pipette (pipette/micropipette tip with 5 mm diameter opening) to loosen the flatworm from the side of the dirty culture vessel. ▪ Suck the flatworm into the pipette (pipette/micropipette tip) and transfer to a new culture vessel, filled with dechlorinated tap water to approximately 5cm from the top of the vessel. ▪ Insert a new cut pipette tip in the air tubing and adjust airflow so sufficient bubbling occurs causing slight rippling of the water surface. ▪ Measure pH, conductivity and dissolved oxygen (DO): record ▪ Dilute with deionised water if conductivity >60 mS/m. ▪ pH should be between 6 and 8.5. ▪ DO should be >70% of saturation.

Flatworms can reproduce asexually by fission, in which each animal splits in half and then regenerates the lost body parts (Gilberston, 1999). Sexual reproduction between a pair of hermaphroditic flatworms results in a few eggs being laid in a stalked cocoon. This is rare and usually occurs under deteriorated conditions (Sluys, pers. comm.) and these cocoons have been noticed in laboratory cultures. Although it may seem tempting to increase the

numbers of individuals within cultures through cutting, particularly when fissioning appears to be happening slowly, this should not be done as it may result in complete culture failure.

High densities of flatworms within a culture vessel may suppress fissioning (Best et al., 1981). Although this has not been experienced in the UCEWQ-IWR laboratory cultures, it is suggested that not more than approximately 300 flatworms be maintained in each 10 l culture vessel. Depending on the demand for test organisms, this may require numerous culture vessels to ensure continuous fissioning of the test species in order to provide sufficient numbers for toxicity tests.

Maintenance and feeding the organisms remains relatively simple. Frozen commercially obtainable bloodworms and free-range (thereby ensuring no hormones and/or other chemicals, such as antibiotics) egg yolks are introduced to the cultures weekly. The organisms are allowed to feed for several hours, after which they are transferred to clean culture vessels.

3.2.3 OBTAINING TEST ORGANISMS FOR TOXICITY TESTS

Test specimens are acquired directly from the culture vessels. Using a pipette, the desired number of selected organisms are gently sucked into the pipette and transferred to a plastic tub from where they are randomly assigned to selected test vessels. Care is taken to ensure that individuals are taken from each of the culture vessels, to account for any possible differences between culture vessels. Individuals of approximately the same length (10 mm for this species) are used.

3.3 ACUTE TOXICITY TEST METHOD FOR FLATWORM SPECIES

Acute toxicity tests have been developed for the *Dugesia* species, independent of age of the organisms, exposed in both static and flow-through systems. All tests are undertaken in a temperature controlled facility, usually at 21ºC but not varying by more than 2°C during the test. Although ambient laboratory illumination is used (artificial light, with a photoperiod of 12hrs light:12hs dark, the test vessels are kept covered during the static test (this was not found possible for the flow-through system). Details of test conditions are provided in Table 3.3.

The same procedures can be followed for both single chemical toxicity tests as well as for complex mixtures (industrial effluents). The procedures for undertaking static toxicity tests for the *Dugesia* species are the same as those described in USEPA (1985) for fish 96 hour acute toxicity test. The test end point is mortality, or no movement of the body when the test individuals are prodded gently.

In flow-through and recirculating systems, it is important that a slow flow of test solution is maintained. The same protocol as described in DWAF (2000) is followed. Stones placed in the artificial streams provide habitat for the *Dugesia* species to escape from the ambient laboratory light (i.e. it is not necessary to cover and therefore darken the streams).

TABLE 3.3 Summary of test conditions for flatworm species in static toxicity tests.

Test parameter	Condition
Water temperature	21°C ± 2°C
Laboratory illumination	Biolux and Fluorescent
Photoperiod	12hrs dark: 12hrs light (although the test vessels are kept covered)
Oxygen concentration	As obtained by diluent
pH	As obtained by diluent
Feeding regime	None
Size of test container	600 mL
Volume of test sample	250 mL
Number of organisms per container	10
Number of containers	2
Total number of organisms per test	20
Control and diluent water	Dechlorinated tap water
Test duration	96hrs
Frequency of monitoring	12 hourly intervals until 96 hours

3.4 CHRONIC TOXICITY TEST METHOD FOR FLATWORM SPECIES

The chronic toxicity test for flatworm species described here follows the same basic procedures for *Dugesia* toxicity tests by Kostelecky (1988) and Kostelecky et al. (1989).

Most chronic toxicity tests involving flatworms use measures of regeneration and behavioural responses based on neurotoxic effects: *Dugesia* species have a well-organised nervous system with considerable behavioural responses which under toxic stress, can be quantified to toxicity end points (Best and Morita 1991, Dasheiff and Dasheiff 2002). The development of head abnormalities (either growth or resorption) and extrusion of the pharynx have been recognised as chronic responses to chemicals (Best et al., 1981). Regeneration tests were used by Piontek (1999a and b) over 10 days, who found *Dugesia tigrina* to be most sensitive during this period. Acute toxicity tests with both whole and cut individuals showed cut individuals to be 2 (to copper sulphate) to 140 (to cadmium chloride) times more sensitive than whole organisms, when tested against 18 metal salts; and significantly more sensitive with organic compounds. For two thirds of the metal salts, and more than half the organic compounds, *D. tigrina* was more sensitive than *Daphnia magna*.

Complications with observation interpretation may occur in regeneration toxicity tests. In looking for photonegative responses, Dasheiff and Dasheiff (2002) found younger heads counterintuitively lost this function initially (despite only needing to regenerate their tails), although regaining it over time. The development of heads from young planaria may serve as an ideal model for environmental toxins. However responses may be varied, depending on the toxin's physiological effects that may be complex, requiring nervous and locomotive system function and integration (Horvat et al., 2005). In testing the effects of a juvenile hormone insecticide on *Dugesia tigrina* at a chronic level, greater sensitivity to the toxicant was observed at the anterior compared to posterior regions (Mirjana et al., 2003). However, the posterior deformations remained, yet complete anterior recovery was seen after 10 days despite extreme histological and morphological damage.

Much of the regeneration research is based on observations at a cellular level at the point of cutting (Schürman and Peter, 1998). However, the preference for this project was to develop a test with quick and easy observations and scoring system. A number of responses have been used for scoring in toxicity tests (Calevro et al., 1998; Horvat et al., 2005). With so many responses to observe, the intention was not to conclude with mode(s) of toxic effects but rather satisfactory endpoints describing toxic effects. Numerous trial-and-error tests, with emphasis on salts, were initially completed before a final decision as to the final number of scored responses that could be observed, were included in this initial protocol (Table 3.4). Acephalic head development is regularly observed in toxicity regeneration tests (Villar et al., 1993) and thus formed a principal feature to score in this project. Recognition was given to the possibility of different toxins eliciting different responses. Thus, analyses of end points may differ from one toxicant to another. Further tests are considered necessary in developing the chronic toxicity test and endpoint described here for *Dugesia* before clarity on this can be made.

Calevro et al. (1998) recorded abnormal secretions of mucus in response to metal toxicity. However, UCEWQ found this difficult to observe and it was therefore not included in the observation sheets.

In addition to regeneration, a number of different behavioural responses by *Dugesia dorotocephala* on exposure phenol were described by Grebe and Schaeffer (1991). Although some of the identified behavioural responses were found not to be suitable for statistical analysis and therefore excluded from further consideration, there were others which were considered suitable for further analysis. Some of these were found to be independent of exposure duration or concentration (*i.e.* occurred immediately on exposure to any concentration of the chemical) such as restlessness, while others such as convulsing and pharynx protrusion occurred on at higher concentrations after long exposures. Responses such as slow movement are graded, becoming more prominent as the exposure duration increases.

Regeneration tests and behavioural responses therefore appeared obvious choices for chronic toxicity test endpoint measures for the *Dugesia* species cultured. In the developed test, *Dugesia* species are exposed to test samples for a period of 7 days in a static test. The process of head regeneration and other behavioural responses per organism is recorded and compared to those of the control organisms. It was decided to develop a numerical scoring system to record regeneration and behaviour. The final scoring system is shown in Table 3.5.

The zone of cutting is shown in Figure 3.1, with the regeneration under control conditions depicted in Figure 3.2. The protocol used to prepare the test organisms for the chronic experiment is described below (Section 3.4.1).

TABLE 3.4 Responses of flatworm species on exposure to toxicants (adapted from Grebe and Schaeffer, 1991).

Developmental Feature	Description
Bud development (blastema)	Bud development on cutting edge
Head developed (acephalia)	Head development beyond the blastema
Auricles normally formed	"Ear-flap" formation, may be 1 or 2 (see Fig. 3.1)
Eye spots seen	Concentration of black spots
Eye cavity developed	Apparent ornamentation around each eye spot
Fissioning observed	Anterior portion tears from the posterior region, each fragment regenerates
Behavioural Response	**Description**
Locomotive	
Normal gliding	Smooth gliding, flat on glide surface
Restlessness	Forward movement usually rapid, erratic, sharp; head or tail raised off glide surface; frequent position switches
Hyperkinesia	"Inch-worming" (slow); glide not smooth; almost always forward movement; few abrupt position switches
Morphological	
Body twisting/spiralling	Corkscrew-shaped movements; animal twists around cranio-caudal axis
Body curling	Horseshoe shape; head brought closer to tail either dorsally or ventrally
Body ripples	"Muscle" rippling
Protective	
Pharynx protrusion	Pharynx extends outside the body
Vomiting	Pharynx discharge
Lesions	Loss of structural integrity
Morbidity	
Death	Does not move when gently prodded or revived in dechlorinated tap water
Tail shrivelling	Noted at termination of test
Length at termination	Length of anesthetised *Dugesia*

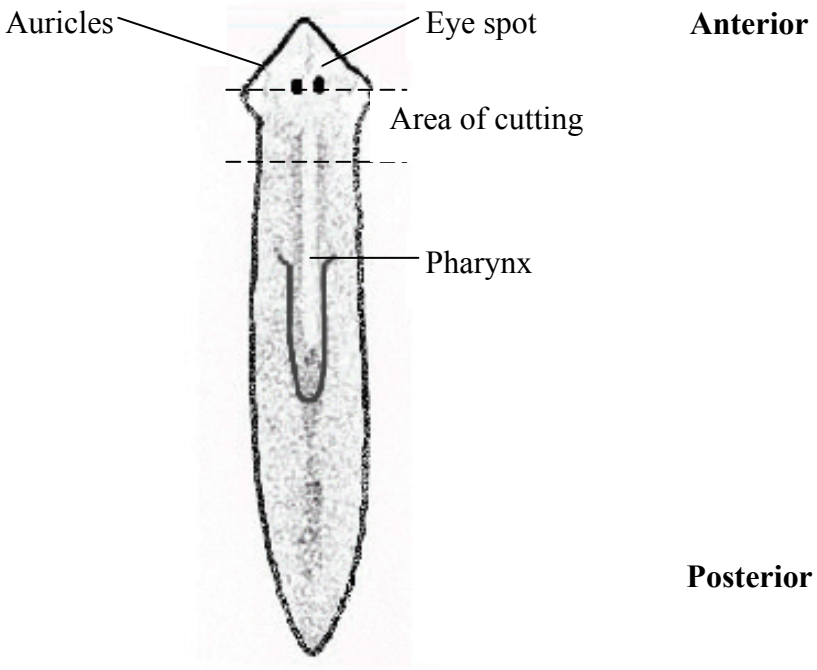

FIGURE 3.1 Schematic diagram of *Dugesia* sp. showing the proposed area of cutting.

TABLE 3.5 Suggested scoring system and data sheet for chronic toxicity tests for flatworm individuals.

TOXICANT:		**STARTING DATE:**												
CONCENTRATION:		**INDIVIDUAL NUMBER:**												
EXPOSURE DURATION	**DAY**													
	REAL TIME (HRS)													
FEATURE	**SCORE**													
Bud developed (blastema)	yes=1; no=0													
Head developed	yes=1; no=0													
Auricles normally formed	yes=1; none=0													
Eye spots seen	2=2; 1=1; 0=0													
Eye cavity developed	2=2; 1=1; 0=0													
Fissioning observed	yes=1; no=0													
Movement: normal gliding	yes=0; no=1													
Movement: restlessness	yes=1; no=0													
Movement: slow (hyperkinesia)	yes=1; no=0													
Body twisting/spiralling	yes=1; no=0													
Body curling	yes=1; no=0													
Body ripples	yes=1; no=0													
Pharynx protrusion	yes=1; no=0													
Vomiting	yes=1; no=0													
Lesions	yes=1; no=0													
Death	yes=1; no=0													
Tail shrivelling	yes=1; no=0													
Length at termination (mm)														

3.4.1 EXPERIMENTAL PROTOCOL FOR CHRONIC TOXICITY TEST FOR *DUGESIA*.

Remove flatworms from culture vessels. From the holding vessel, transfer individuals onto a glass sheet which is covered in a film of water, essential for the flatworms to glide on. Using a single sided blade, washed thoroughly with phosphate free soap and boiling water, cut flatworms squarely at a 90º angle with body once it is gliding in a straight line on the glass sheet. The incision is made dorsally midway between the head and the pharynx (Figure 3.1).

Remove the head from the glass sheet and discard. Place the cut posterior end of the *Dugesia* into the relevant test solution, ensuring that both blade and pipette (pipette/micropipette tip) are rinsed between each concentration to avoid cross-contamination.

A single cut posterior end of a *Dugesia* is placed in each test container, for individual observations, resulting in a total of 20 flatworms per test solution. As the organisms are photonegative, the test containers are placed in the controlled environment room (21°C ± 2°C) covered with plastic (to prevent evaporation) and shade netting (75%) for the duration of the test. Observations are made twice daily. Observe at 12hr intervals for the first 4 days and from day 5 up to and including day 7 observe 08:00 and 14:00 each day to ascertain if regeneration/fissioning had occurred. Observations are made using a light microscope (eyepiece x10, magnification x25) for the features and behavioural responses suggested in Tables 3.4 and 3.5. Record any dead *Dugesia* at 12hr intervals and the test is terminated after 7 days, when the control individuals have completely regenerated (Figure 3.2). Control mortality should remain less than 10% over the duration of the test. A summary of test conditions is provided in Table 3.6.

The test may be extended beyond 7 days. In this case, the test individuals will require feeding. The flatworms should be fed with bloodworm (chironomids) on day 7, left over night and placed into fresh test solutions in clean containers on day 8.

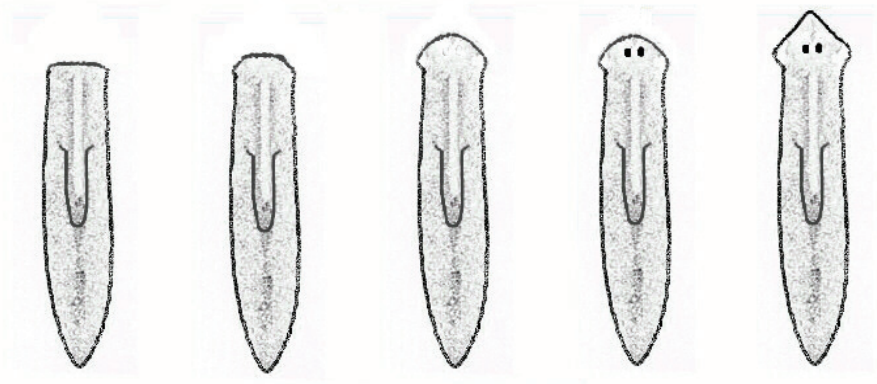

FIGURE 3.2 Diagrammatic representation of head regeneration as observed in control organisms.

TABLE 3.6 Summary of chronic test conditions for *Dugesia* chronic toxicity test

Test type	Static
Water temperature	21ºC (varying up to 2°C)
Laboratory illumination	Biolux and Fluorescent
Photoperiod	12hrs light
Oxygen concentration	As obtained
pH	As obtained
Feeding regime	None (for a 7 day test; longer test will require feeding)
Size of test container	30 mL
Volume of test sample	25 mL
Age of test organisms	N/A – size approximately the same (more or less 10 mm in length)
Number of organisms per container	1
Number of replicate containers	20
Total number of organisms per test	20
Control and diluent water	Dechlorinated tap water
Test duration	7 days
Test criteria	As described in section 8.6

3.4.2 NaCl CHRONIC TOXICITY TEST

Sodium chloride was chosen as the toxicant to develop and test the chronic toxicity test method for *Dugesia* sp. For a definitive experiment, the range of concentrations was based on the acute 96 hour LC_{50} of 5.6 mg/L, and test conditions are shown in Table 3.7. The duration endpoint selected was 7 days, to assess the suitability of the identified factors.

TABLE 3.7 Physico-chemical criteria measured at start of test.

NaCl concentration	pH	Conductivity ms/M
0 (Control)	7.27	33.6
1.6 mg/l	7.54	343.7
2.8 m/l	7.52	563.9
3.75 mg/l	7.35	747.4
5.0 mg/l	7.5	940.4

The glass beakers were found unsatisfactory for monitoring the individuals; many flatworms were found on the sides of the beakers at the time of monitoring, necessitating their forced displacement to the bottom of the beaker to allow for scoring. Under the higher concentrations, and therefore greater stress, this forced removal influenced their behaviour patterns to a degree, with some individuals in the highest concentration displaying obvious discomfort and added stress. It is suggested that test organisms are placed in glass or plastic (depending on the test substance) petri dishes to avoid this.

Head and eye observations represent positive patterns in the growth of the flatworms, the higher the score, the greater the development, the lower the toxic response. Movement represents stress levels, the higher the score, the greater the stress exhibited.

With few mortalities in any of the concentrations, no LC_{50} endpoint data could be calculated.

In considering the data scored and collated for *Dugesia* subjected to NaCl, the following data points were considered (in combination) for analysis purposes:

- head development: scores for bud development, head development, and the presence of auricles.
- eye development: scores for eye spots and cavities seen.
- head & eye: a combination of all head and eye data, therefore representing total head development.
- movement: movement defined by gliding, restlessness, hyperkinesias and body spiraling, twisting, curling or rippling.
- head, eye & movement: a combination of all data.

The scored features remaining (neurological and protective; Tables 3.4 and 3.5) had low or no scores with NaCl as the toxicant, and were therefore less likely to reveal statistical differences. These features could however be used for descriptive interpretation of toxicant effects of toxicants other than NaCl.

Data distributions for each feature, at each time of data capture, were drawn with analyses of linear fit, and correlation coefficients obtained (Table 3.8). Multiple range tests (Scheffé's and Dunnets tests) compared means between each concentration at each time interval, indicating significant differences ($p<0.01$) between the controls and the different concentrations. Dunnett's test comparing control means to toxicant means were also completed. These are shown in Table 3.8 as the suggested Lowest Observed Effect Concentrations (LOECs).

Head and eye data combined gave better linear fits to the data than only head development or only eye development (Table 3.8).

From 113hrs the head development at 1.6 mg/l and 2.8 mg/l are not distinguishable from the control, although with some individual variability at 2.8 mg/l and 145hrs. Head development at 3.75 mg/l suggests individual variability over all times recorded, but with a regression of development at 171hrs. Individuals within 5 mg/l did not reach full head development. At 94hours, the LOEC was 3.75 mg/l (Scheffé) or 2.8 mg/l (Dunnett); at 113hrs it remained at 5 mg/l sodium chloride suggesting a degree of recovery of head development.

Eye development, appearing after initial head development and therefore likely to show significant differences to the controls at later stages than head development, showed statistically significant differences to the controls from 113hrs, with a best linear fit at 119hrs. LOECs were at 2.8 mg/l (Dunnett) and 5 mg/l respectively. Combining the head and eye development, best fit for linear distribution was at 113hrs, with an LOEC of 2.8 mg/l.

TABLE 3.8 Results of data distribution analyses and multiple range comparisons of the chronic data. r^2 values in bold signify linear best fits for the data distribution of each feature. * most poorly fitting linear equations. LOEC: Lowest Observed Effect Concentration in mg/L.

Feature	Time hrs)	Correlation coefficient r^2	Scheffés test, LOEC (mg/L)	Dunnett's test LOEC (mg/L)
Head	94	**0.3993**	3.75	2.8
	113	**0.3414**	5	5
	119	0.2324	5	5
	138	0.3129	No differences	5
	145	0.0487*	No differences	No differences
	165	0.1151	No differences	5
	171	0.0761	No differences	No differences
Eye	94	0.1651*	No differences	5
	113	0.3756	5	2.8
	119	**0.4093**	5	5
	138	0.3214	5	5
	145	0.3743	5	5
	165	**0.4629**	5	5
	171	0.3792	5	5
HeadEye	94	0.4026	5	3.75
	113	**0.4423**	2.8	2.8
	119	**0.4175**	5	5
	138	0.3690	5	5
	145	0.3544	No differences	5
	165	0.3089	5	5
	171	0.2532*	5	5
Mvt	94	0.0674*	No differences	No differences
	113	0.0855	No differences	No differences
	119	0.2750	No differences	2.8
	138	0.3302	5	5
	145	0.3205	No differences	3.75
	165	**0.4033**	5	3.75
	171	**0.3977**	2.8	2.8

Movement scores gave no statistical differences from the controls until 119hrs (Dunnett) and 138hrs (Scheffé), increasing in stress levels to an LOEC of 2.8 mg/l at 171hrs.

Data distribution suggests a hyperbolic relationship: with longer exposure periods, higher concentrations are required for significant effects to be seen when examining anterior

development. However, the explanation of the developmental effects of NaCl are difficult to explain, because there is some recovery of head and eye development over time. Based on behavioural patterns, there were indications of increased stress over 165-171hrs (7 days) to reach a maximum toxic effect after 7 days.

For *Dugesia* sp. and using the protocol developed in this study, the LOEC for sodium chloride can [initially] be summarized as 2.8 mg/l after 119 hours (5 days) and 171hrs (7 days).

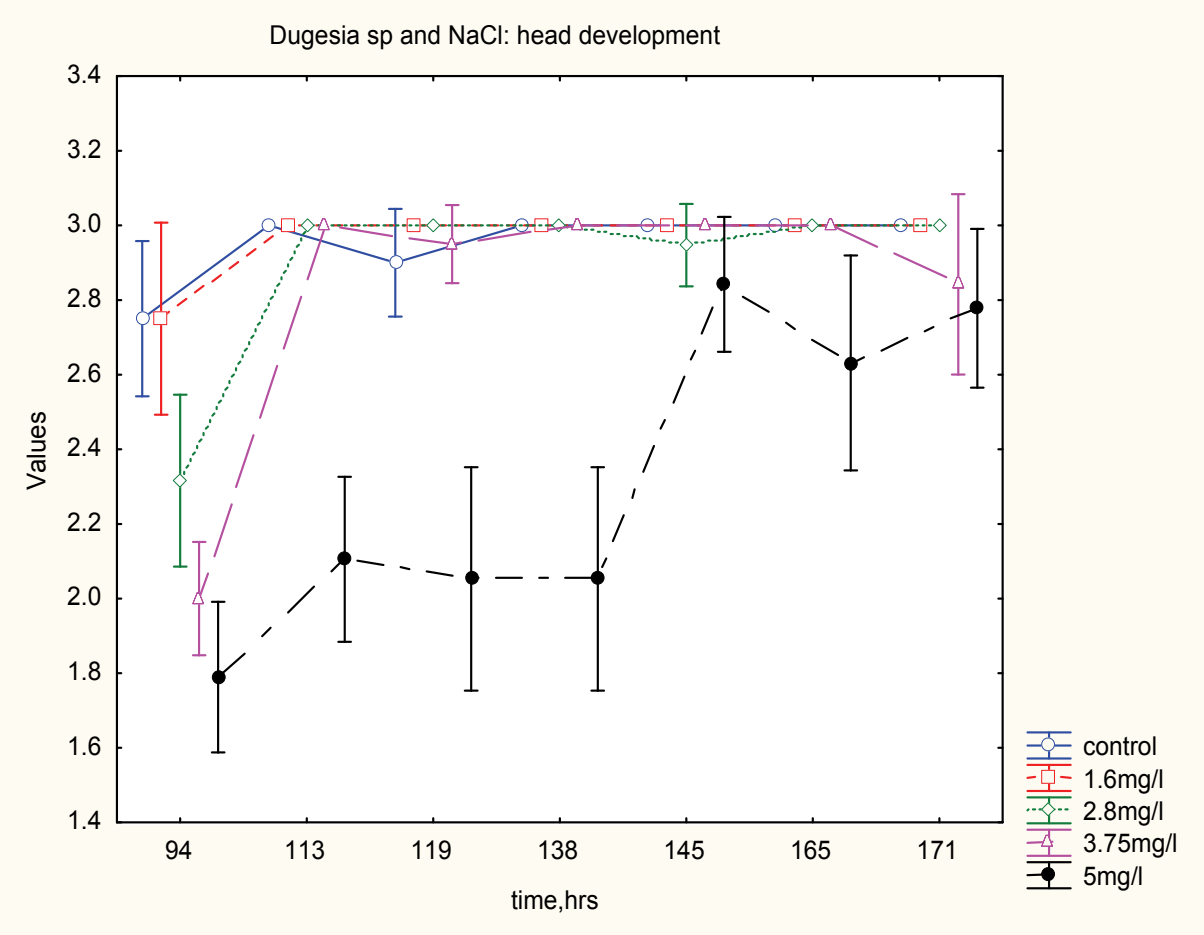

FIGURE 3.3 Plot of means and confidence intervals (95%) of Head development

3.4.3 Results and Discussion

A criticism of these initial trials is the lack of length data, taken with the assistance of relaxants. Individuals in 5 mg/l sodium chloride were, to the naked eye, significantly smaller than all other concentrations and the control. It is suggested in the protocol of chronic toxicity testing that the flatworms are measured for length at the end of the test.

Best et al. (1981) found that chlordane resulted in an increase in the incidence of fissioning at low concentrations in *Dugesia dorotocephala*. This suggests that *Dugesia* species may be used in studies investigating hormetic effects of chemicals.

Time to full head development was on average 113 hours in the controls. However, when asexual development occurs in the controls, this period needs to be extended to include the

regenerated individuals (Villar et al., 1993). Consequently a feed must be considered at 7 days with a change in toxicant solutions thereafter.

Further investigations into preferred or optimal dietary requirements is necessary, particularly calibrating the effects of protein type (presently a combination of egg yolk and liver are used). Not surprisingly, both protein composition and crowding have been shown to significantly affect rate of reproduction under sexual conditions (Kostelecky et al., 1989).

Dugesia species can be used to assess sublethal effects such as heat shock proteins (Hamana et al., 1995) and stress proteins (Schill et al., 2000; Guecheva et al., 2003) and osmoregulation shock (Steinbach 1962, Prusch 1976, Palladini et al., 1978, Hamana et al., 1995). Further research is warranted.

CHAPTER 4

CHRONIC TOXICITY TEST METHODS IN ENVIRONMENTAL WATER QUALITY MANAGEMENT: PROVIDING THE LINKS BETWEEN ENVIRONMENTAL WATER CHEMISTRY AND BIOMONITORING

4.1 Biochemical biomarkers as chronic toxicity test endpoints

Although the investigation on the usefulness of sub-lethal biomarkers in environmental water quality management in water resource management was commenced under this project, it was in fact superseded by WRC Project 1484/1/09 (Gordon et al., 2009 and references therein). Therefore a brief summary is provided as part of this report.

Water quality guidelines are an integral component of water resource management and are particularly important in the establishment of resource quality guidelines. It is therefore imperative that these water quality guidelines accurately reflect the true ecological sensitivity, or resilience, of indigenous species. Greater confidence is given to water quality guidelines that use data that are derived from chronic, or sub-lethal, toxicity tests due to their increased environmental realism. However, few chronic toxicity data are available due to the increased complexity, and associated human and other resource costs, of these toxicity tests and even fewer data are available for South African indigenous species. Toxicity test methods that provide reliable and good quality chronic toxicity data which can be used for the derivation and refinement of water quality guidelines are needed and it is thought that biochemical biomarkers will provide appropriate sublethal endpoints for toxicity tests.

Gordon et al. (2009) investigated the advantages and limitations of biochemical endpoints in the derivation of water quality guidelines. Although there has been criticism of the use of biochemical biomarkers in for regulatory purposes (including the derivation of water quality guidelines), there are many advantages that potentially outweigh the criticisms. Although there currently is no evidence that links biomarker endpoints from individual organisms to community and population level responses, biomarkers have been shown to be highly sensitive responders to environmental stressors and they can be found after short exposure durations. This suggests that these endpoints may result in increased environmental protection (although this could also be viewed negatively). In addition, the tests utilise fewer test organisms and are of shorter duration, thus reducing costs associated with lengthy and complex toxicity tests that provide other chronic data.

In the ensuing experiments undertaken by Gordon et al. (2009), it was demonstrated that the resultant lowest observed effect concentrations (LOEC), and subsequent no observed effect concentrations (NOEC), for the different biochemical biomarker methods selected varied widely. When these observed results were incorporated into the currently used water quality guideline derivation method for South Africa, the resultant guideline value which would ensure long-term protection of ecosystem was marginally less than similar guideline values for Australia and New Zealand as well as those obtained from complex mesocosm studies. The report concluded that biochemical biomarkers could be used as sensitive and accurate endpoints for the derivation of water quality guidelines but cautioned that quality assurance of these data are vital for their inclusion within an environmental water quality regulatory framework. A number of issues were identified for further investigation, including

establishing the relationship between individual organism biomarker result, health of the organism and the subsequent risk to ecosystem health and integrity. It was also suggested that a suite of biochemical markers, rather than only a single biomarker, would be more useful.

These results suggest that responses of species to complex effluents needs more research in order to sufficiently predict ecosystem responses and ensure adequate protection, as field population of species tend to be genetically more diverse than laboratory cultures (but they are also responding to a diversity of stressors, which then may make them more vulnerable – Duan et al., 2001). The importance of genetic variability in population responses to exposures to chemicals versus individual responses to chemicals may be significant in predicting ecosystem responses and therefore the inclusion of genetic variability (genetic structure of a population) as part of risk assessment procedures is strongly recommended (Barata et al., 2002).

It is therefore important to validate laboratory test results with those of field collected populations, which are genetically more diverse (or at least different from the laboratory cultures). For example, genotoxicity and genetic indicators for environmental monitoring: genetic distance analyses have been proposed as sensitive indicators of change in water quality which may indicate changes before any species are lost (Duan et al., 2001). However, neither of these approaches has been investigated in this study.

Barata et al. (2002) demonstrated that responses of organism to mixtures of metals was different to that of the same species exposed to single metal toxicants and that these responses are genetically mediated. Differences in sensitivity between genotypes have also been demonstrated in amphipods (Duan et al., 2001).

These results suggest that responses of species to complex effluents needs more research in order to sufficiently predict ecosystem responses and ensure adequate protection, as field population of species tend to be genetically more diverse than laboratory cultures (but they are also responding to a diversity of stressors, which then may make them more vulnerable – Duan et al., 2001). The importance of genetic variability in population responses to exposures to chemicals versus individual responses to chemicals may be significant in predicting ecosystem responses and therefore the inclusion of genetic variability (genetic structure of a population) as part of risk assessment procedures is strongly recommended (Barata et al., 2002).

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APPENDIX A:

TOXICITY TESTING USING THE STANDARD LABORATORY ORGANISM, *Daphnia pulex*

A.1 INTRODUCTION

The cladocerans, or daphnids, are recognised as useful species for acute toxicity tests (USEPA, 1993; Chapman, 1995; Slabbert, 2004). They are considered more sensitive to chemicals and effluents than many other animals (Persoone and Janssen, 1993), and have been chosen as a standard test organism because of their small size, short life cycle, ease of culture, high sensitivity to toxicants, and their clonal method of reproduction which lends itself to genetically homogeneous culture populations (Baird et al., 1989; Versteeg et al., 1997). Because of their short life span, acute and chronic toxicity tests are quick to complete, and endpoints such as immobility, mortality and reproductive output are easy to observe. The volumes of dilutions and thus toxicants used in toxicity tests are small, making the use of cladocerans practical and inexpensive. Two types of cladoceran toxicity tests are commonly used. Acute tests are over 48 or 96 hours, where end results are based on mortalities; and 21 day chronic toxicity tests involve reproductive effects and long-term survival.

Daphnia pulex (De Geer) has been chosen as a standard laboratory test organism in South African water quality testing because it is a cosmopolitan species present in surface waters in South Africa (Slabbert et al., 1998). It is principally a pond dweller, not usually found in lotic waters (USEPA, 1993a), and forms an important link in food chains (Gulatti, 1978). It is envisaged that *D. pulex* will provide the primary basis for general complex industrial water discharge management (DWAF, 2003), and is recommended for the general water quality monitoring countrywide by DWAF within the National Toxicity Monitoring Programme. Site-specific environmental water quality management will be based more on the use of an indigenous organism (DWAF, 2003). Consequently, for comparative purposes, each toxicity test completed with indigenous organisms at UCEWQ-IWR has at least one equivalent test completed with *D. pulex*. Some of the results are recorded here or given in Appendix B. These results can contribute to a national database for *D. pulex* acute and chronic toxicity tests (acute toxicity data for *D. pulex* are captured in the UCEWQ toxicity database and a database for capturing chronic toxicity data is currently under development).

As with all biological processes, a degree of variability is associated with each toxicity test undertaken. Genetic heterogeneity amongst the test organisms, which contributes to variability of response, is less of a concern with cladocerans because of their clonal reproduction with consequent genotypic homogeneity (although this does not preclude the possibility of mutations or sexual recombination occurring). Environmental heterogeneity, including under laboratory conditions, may also influence responses to toxins (Falconer, 1981). Maternal reserves are known to play a role in juvenile growth (Tessier et al., 1983) and response of their offspring (Baird et al., 1989) suggesting long term effects of toxicants can occur. Complete standardisation of culture methods and experimental conditions, both presently available for *D. pulex* (Slabbert, 2004), does alleviate part of the problem of environment influencing response (USEPA, 2000). However, variability within the UCEWQ-IWR laboratory culture of *D. pulex* has been periodically tested against reference toxicants (potassium dichloride and cadmium chloride).

A.2 CULTURE TECHNIQUES

D. pulex cultures have been maintained for a number of years by UCEWQ-IWR, principally following the Methods Manual produced by the South African Department of Water Affairs and Forestry (DWAF, 1998), although with differences, as indicated in Table A.1. The cultures of *D. pulex* at UCEWQ-IWR are fed daily on an artificial diet of trout pellets, dried yeast and dried alfalfa, prepared as a filtered solution with culture medium. This is also the food source for the chronic toxicity tests. The cultures are maintained from neonates produced parthenogenetically; if any ephippia (sexually produced eggs) are present, they are discarded to ensure the cultures are as genetically homogeneous as possible. The culture medium is also the diluent used in all toxicity tests.

Culture techniques (and conditions during chronic tests) have been considered generally problematic elsewhere (Stephenson and Watts, 1984) and are discussed further in Section A.5.

A.3 ACUTE TOXICITY TESTS

Through the years a number of definitive toxicity tests have been completed, according to the test method (as described in Slabbert, 2004). Those completed during the duration of the Chronic Toxicity Programme are listed in Appendix B. The tests are described as being static non-renewal with no aeration. Test replicates consist of 40 ml glass beakers, filled with 25 ml of test dilutions; four beakers per test solution and 5 neonates per beaker. LC_{50} values were calculated, when possible, using the Probit (Finney, 1971) and Trimmed Spearman-Kärber (Hamilton et al., 1977) methods.

The definitive acute toxicity tests have importantly provided the Programme with:

a) LC_{50} comparisons with indigenous species tested with the same toxicant,
b) ranges for *D. pulex* chronic tests, and
c) proficiency and individual variability test results with reference toxicants:
    i) Cadmium chloride results were generated through the three-monthly Proficiency Testing Scheme, co-ordinated by Rand Water and to which up to 8 laboratories around South Africa participate. Results are interpreted in terms of a Z-score used to check culture variability and also the performance of the laboratory. UCEWQ-IWR LC_{50} values consistently reflect a score close to zero (ideal) and always fall well within the ranges obtained from the 7 other laboratories.
    ii) Potassium dichromate was used as the reference toxicant in four assessments of inter-replicate and therefore individual variability, compared to the indigenous limpet *Burnupia stenochorias*, at the time being first considered for inclusion as a site-specific toxicity test organism (Davies-Coleman, 2002). Reaction to potassium dichromate proved to be non-monotonic over 48 hours, with LC_{50} values calculated using Trimmed Spearman-Kärber. Percentage trim was small (5%); LC_{50} values were within a three-fold range of each other; and the mean LC_{50} value was 1.41ppm potassium dichromate. Control mortalities did not occur during the potassium dichromate tests.

Results from i) and ii) suggest both laboratory techniques and good practise, and variability of the *D. pulex* cultures are well within accepted ranges for acute toxicity tests.

Table A.1 Comments relating to the culturing protocol for *Daphnia pulex*. Although the protocol given in 2004 is specific in its direction for all aspects of the culturing of *Daphnia pulex* (Slabbert, 2004), there are some differences in the methods used through the years at UCEWQ-IWR, and problems and practicalities that have arisen.

Culture condition specified in the protocol	Effects	Solution	Notes
Protocol stipulates ambient laboratory illumination: artificial and/or day light of 10 to 14hrs light.	Variations in ambient light intensities and prevailing day/night cycles in most laboratories do not seem to affect *Daphnia* growth and reproduction significantly (Slabbert, 2004).		UCEWQ-IWR cultures are kept with artificial light (a mixture of Biolux and Fluorescent light tubes) set at 16hrs light, 8hrs dark (as stipulated in USEPA, 1985).
Dust in the laboratory/test facility.	Despite the controlled environmental conditions dust accumulates on the surface of the test/culture beakers which tends to trap the *Daphnia*.	The culture/test beakers need to be covered at all times. Clear plastic sheeting works well.	Clear plastic is more practical than a glass covering.
Fungal slime on the surface of the culture beakers.	The problem with oxygen diffusion is associated with *Daphnia* mortalities. *Daphnia* also get trapped in the mycelia and die.	Aeration of the culture beakers.	The aeration possibly encourages algal growth on the glass surfaces.
Algal growth on the glass surfaces of the culture beakers.	*Daphnia* get trapped in the algae.	More frequent cleaning of the culture beakers than is stipulated in the culturing protocol. Each beaker is cleaned in a seven to ten day cycle.	UCEWQ algae were identified (C. Carelson, RQS, DWAF) as the non-toxic *Oscillatoria* sp. The reproduction rate of the *Daphnia* slows down in the presence of these algae.
Algal food, *Pseudokirchneriella subcapitata* (formerly named *Selenastrum capricornutum*) as food additive.	This is said to inhibit reproduction and encourages excessive algae growth in the culture beakers (pers. comm., laboratory managers at RQS, Ecosun & University of Johannesburg).	Feed only TYA (trout pellet, yeast and alfalfa) food.	Not, therefore, following the protocol.

Culture condition specified in the protocol	Effects	Solution	Notes
TYA (trout pellet, yeast and alfalfa) food.	Mass mortalities of *Daphnia* often seen, with excessive growth of algae and slime formation on the surface of the culture beakers; related to freshness of food.	A fresh batch of food is made every Friday for the following week's feeding regime.	
Washing of glassware not stipulated in the protocol (Slabbert 2004)	Inadequately cleaned glassware could cause mass mortalities.	UCEWQ applied the following method (according to Horning and Weber, 1985; IWQS method manual 1997) as a standard operating procedure to clean glassware. All glassware, test vessels and non-disposal-non-glass containers and equipment are washed as follows: 1) Rinsed with tap water. 2) Soaked in phosphate free soap solution for 15-30 minutes. 3) Rinsed three times with dechlorinated water. 4) Soaked in 10% hydrochloric acid solution for 30 minutes. 5) Rinsed three times with deionised water. 6) Rinsed three times with dechlorinated water. 7) Air dried. 8) Washed glassware stored in a dust free environment. 9) Glassware and equipment rinsed thoroughly with dilution water immediately before use.	

A.4 CHRONIC TOXICITY TESTS

Within chronic toxicity tests, reproduction is generally considered the most sensitive criterion in cladocerans, and an accurate and reliable endpoint for monitoring toxic effects (Canton and Adema, 1978). The most frequently used criterion is the total number of neonates produced over 21 days (APHA, 1992; Slabbert, 2004), although monitoring toxicity over 7 or 14 days has also been recommended (Gersich and Milazzo, 1990; USEPA, 1993b). Growth changes in cladocerans, measured as body length or caudal spine length, have also been used as sensitive toxicity parameters, although the ecological significance of such changes is not always clear (Van Leeuwen et al., 1985). In many other cases, survival of the parent population has been considered the most sensitive parameter to use in toxicity tests (Kühn et al., 1989).

The life span of *D. pulex*, from the release of eggs into the brood chamber until death of the adult, is variable and dependent on environmental conditions (Stephenson and Watts, 1984). The mean life cycle at 20°C is 50 days. Usually, clutches of 6-10 eggs are released into the brood chamber where they hatch after 2 days into juveniles or neonates. The time to reach sexual maturity and production of the first offspring is 6-10 days and depends on conditions and body size of the mother. *D. pulex* has 3 to 4 juvenile instars, moulting between instars. The adolescent stage is only one instar after which neonates are produced with each moult. The less favourable the conditions, the longer the instars and the fewer the number of neonates produced, although a decrease does occur in the number of neonates after approximately the tenth instar (Stephenson and Watts, 1984).

A number of 21 day chronic toxicity test assessing survival, growth [measured as caudal tail length measured using a Nikon bifocal microscope with a calibrated ocular graticule at magnification x40] and reproduction [number of neonates] were completed during the Chronic Toxicity programme, and are compared to results using potassium dichromate (Davies-Coleman, 2002). Tests used neonates <24 hours old, following the standard method presently described for South Africa (Slabbert, 2004), with 15 individuals in each concentration, each in a separate beaker. Feeding consisted of one drop (approximately 100 µl) per day of the artificial food used in the culturing of *D. pulex*, which was considered not limiting. The beakers were covered with transparent plastic to prevent evaporation. Monitoring of water quality (pH, dissolved oxygen and conductivity) was performed twice per week after solution renewal. Those neonates that died before 21 days were excluded from the calculations of reproductive output.

Results for experiments in which *D. pulex* were exposed to sodium salts, cadmium chloride and textile whole effluent are presented.

A.4.1 RESULTS FOR SODIUM CHLORIDE EXPOSURE EXPERIMENTS

Experiment September 2004

Concentrations tested were 0, 50, 100, 200, 300, 400 and 500 mg/L NaCl. Table A.2 provides a summary of the results obtained, and survival and reproduction results are presented in Figures A.1 and A.2.

Toxicity endpoints in terms of mortalities:

- LC20 (21 days) not estimated from drawn equation.

- LC50 (21 days) did not fit Probit model. TSK % trim too high.
- Correlation coefficients for linear fit to mortality data:
- R^2 (lethality) = 0.15 with intercept = 26.7, std error = 9.7, p = 0.07.
- *Toxicity endpoints in terms of growth:*
- Size (based on caudal length) at 21 days: Students 't' test (two-sample assuming equal variances) gave no significant differences ($p>0.05$).
- *Toxicity endpoints in terms of reproduction (total number neonates per individual):*
- EC values unable to define in terms of Probit or TSK analyses. Inhibition not monotonic, and with some increases in numbers of neonates (at 50 and 400 mg/l) compared to the controls.
- EC values unable to estimate from drawn equation based on change from the controls (as opposed to % inhibition), as significantly poor fit to data (see graph).
- Correlation coefficients for linear fit to mortality data:
    - R^2 (neonates / individual) = 0.02, intercept = 11.44, std error = 3.14, p=0.015.

Summary of experiment results:

- It was not possible to estimate toxicity endpoints in terms of mortalities.
- Growth, based on caudal tail length, was not a useful criterion to estimate the NOEC over 21 days.
- Unable to estimate EC values in reproductive terms.
- The overall NOEC = <50 mg/l NaCl over 21 days but with a highly significant degree of uncertainty because of poor data distribution.

Table A.2 **Data analysis of *Daphnia pulex* 21-day exposure experiment to NaCl with mortality and reproduction as experimental endpoints. Significant differences to controls are indicated in italics. The toxicity endpoint of >20% change is based on recommendations by Slabbert (2004).**

Criterion	Statistic	Concentration NaCl mg/L							Significantly different to control
		0	50	100	200	300	400	500	
Mortalities	%	20	*46.7*	*33.3*	*0*	*6.7*	*6.7*	*13.3*	*>20% = toxicity:* 50, 100 mg/l
Number neonates /breeding animal	Average	10.4	*16.4*	*8.0*	*9.7*	*7.6*	*13.1*	10.1	*50,100,200, 300,400 mg/l
	CL (95%)	6.0	11.5	3.2	3.6	4.5	4.9	3.4	
	Std error	2.7	4.84	1.34	1.67	2.05	2.24	1.58	
	% Change	0	*+5.7*	*-22.2*	*-6.7*	*-27.3*	*+25.5*	*-3.2*	*>20% = toxicity.*
Number neonates / brood	Average	2.8	*3.9*	2.7	*3.1*	*2.3*	*3.4*	2.7	*50, 200, 300, 400 mg/l
	Std error	0.45	0.74	0.32	0.36	0.32	0.42	0.31	
	% Change	0	*+39.2*	*-3.3*	*+10.9*	*-16.0*	*+21.0*	*-5.0*	*>20% = toxicity.*
Time to first brood (days)	Average	13.2	*9.9*	13.7	*11.2*	12.5	*11.4*	*11.4*	50, 200, 400, 500 mg/l
	Std error	1.07	0.51	1.13	0.62	1.05	1.01	1.03	
	% Change	0	*-24.8*	+3.7	*-14.9*	*-4.9*	*-13.6*	*-13.6*	

* Students t-Test, two-sample assuming equal variances

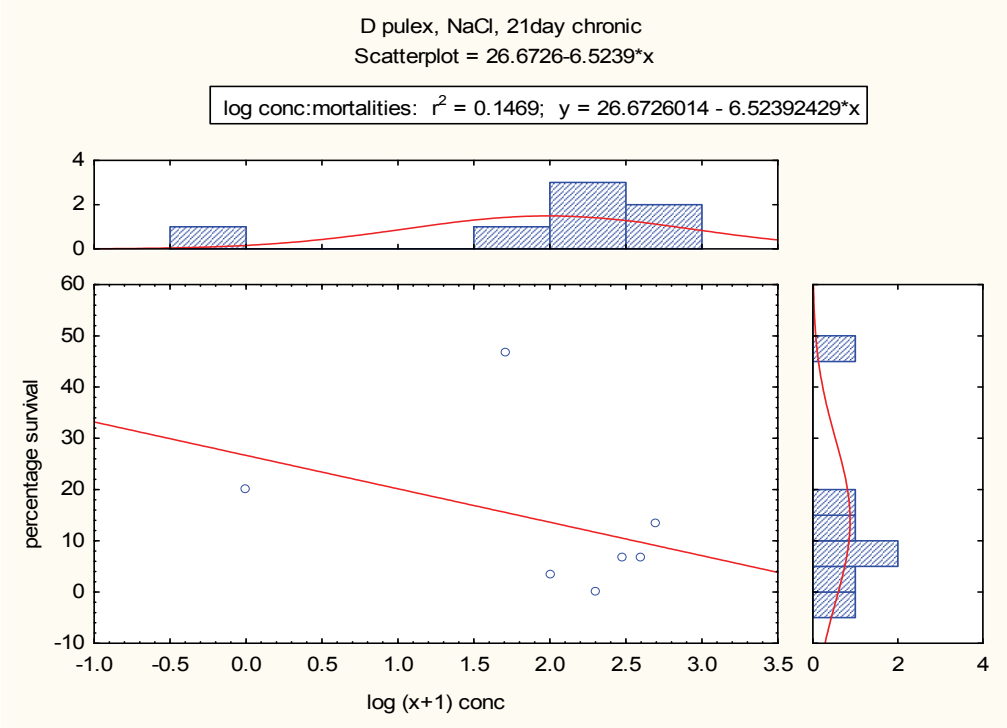


Figure A.1 ***Daphnia pulex*** **survival over 21 days exposure to NaCl.**

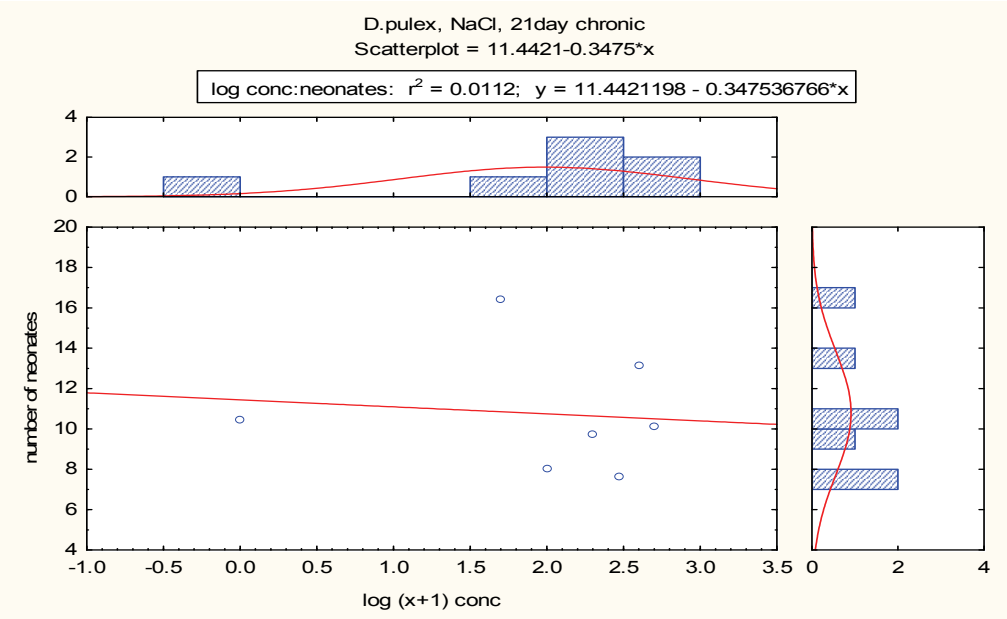


Figure A.2 **Comparison of number of** ***Daphnia pulex*** **neonates produced over 21 days exposure to NaCl, expressed as a linear fit.**

Experiment June 2005

The concentrations used were 0, 100, 200, 300, 400 and 500 mg/L NaCl. The test was terminated after a significant proportion of neonates died in all concentrations, including the controls after 6 days (Figure A.3). The salt exacerbated the mortality problems.

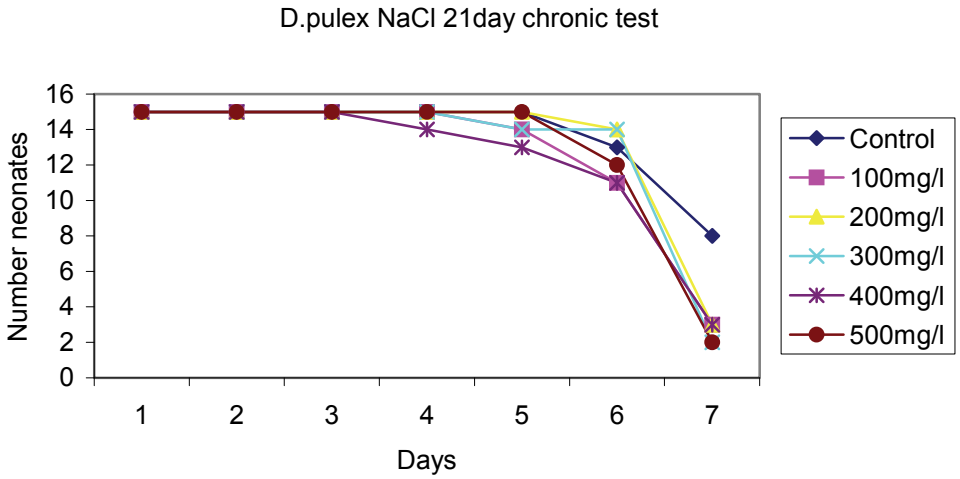


Figure A.3 *Daphnia pulex* survival over 7 days exposure to NaCl.

*Toxicity endpoints in terms of mortalities:*none
*Toxicity endpoints in terms of growth:*none
*Toxicity endpoints in terms of reproduction:*none

Summary of experiment results:

- It was not possible to estimate toxicity endpoints and a NOEC could not be estimated.

Experiment July 2005

Concentrations included were 0, 100, 200, 300, 400 and 500 mg/L NaCl. The test was terminated after a significant proportion of neonates died in all concentrations, including the controls, after 5 days. The salt only initially appeared to exacerbate the mortality problems; control mortalities suggested problems with the daphnids chosen from the culture (Figure A.4).

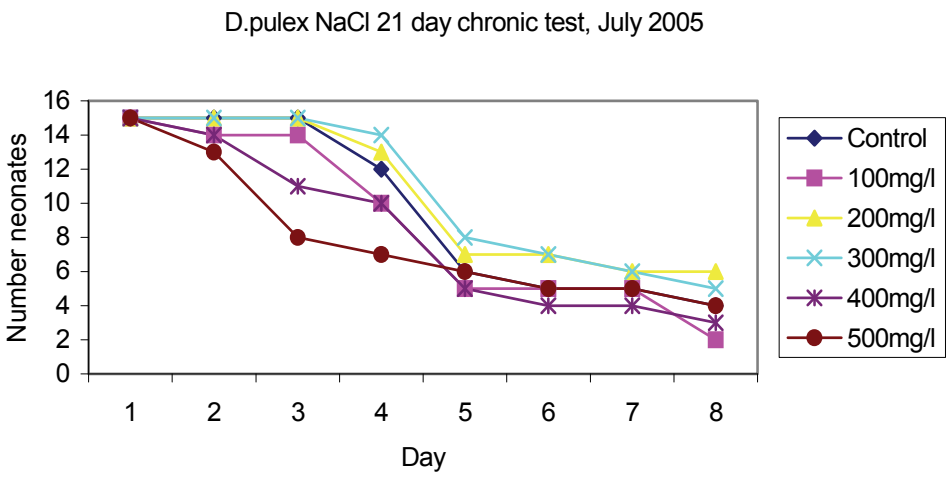


Figure A.4 *Daphnia pulex* survival over 8 days exposure to NaCl.

A.4.2 Results for Sodium Sulphate Exposure Experiments

Experiment April 2004

Concentrations tested were 0, 50, 100, 200, 300, 400 and 500 mg/L Na_2SO_4. The test was terminated after a significant proportion of neonates died in all concentrations including the control after 4 days.

Experiment April 2005

Concentrations tested were 0, 100, 200, 300, 400 and 500 mg/L Na_2SO_4. The test was terminated after a significant proportion of neonates died in most concentrations after 4 days, with fewer mortalities in the controls and 500 mg/l but sufficient to warrant terminating the test. Results are presented graphically in Figure A.5.

*Estimated toxicity endpoints in terms of mortalities:*none
*Estimated toxicity endpoints in terms of growth:*none
*Estimated toxicity endpoints in terms of reproduction:*none

Summary of experiment results:
As it was not possible to estimate any toxicity endpoints, a NOEC could not be established.

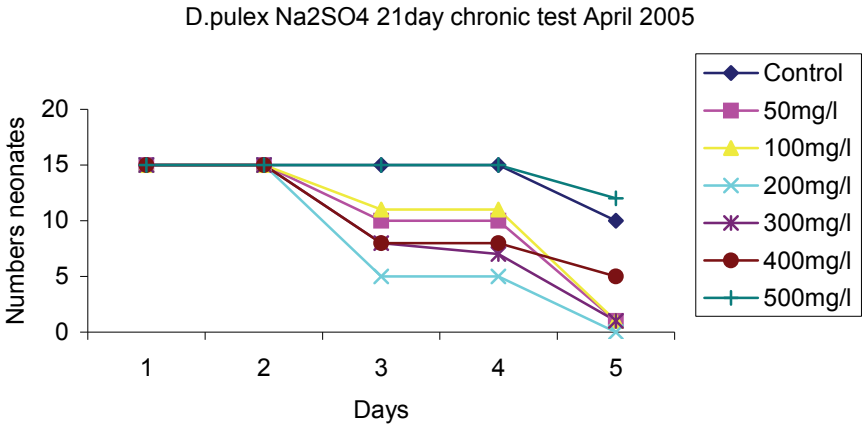


Figure A.5 *Daphnia pulex* survival over 5 days exposure to Na_2SO_4.

A.4.3 Results for Cadmium chloride exposure experiments

The same methods were followed as for the sodium salts (Sections A.4.1 and A.4.2). Concentrations tested were 0, 0.006, 0.013, 0.025, 0.05 and 0.1 mg/L $CdCl_2$ (based on acute toxicity data for *D. pulex*). 100% mortality occurred in 0.05 and 0.1 mg/L $CdCl_2$ and therefore these variables were not included in the assessment of growth (results are presented in Table A.3 and Figures A.6 to A.8).

Toxicity endpoints in terms of mortalities:

- LC15 (21 days) Probit = 0.010 mg/l (Confidence limits 0.001-0.02 mg/l).
- LC20 (21 days) estimated from drawn equation = 0.001 mg/l based on curvilinear fit.
- LC50 (10 days) Probit = 0.074 mg/l (Confidence limits 0.04-0.449 mg/l) (first death of control at 11 days).
- LC50 (21 days) TSK with trim 11.5% = 0.03 mg/l (Confidence limits 0.02-0.03 mg/l)
- Correlation coefficients for linear fit to mortality data:
  R^2 (lethality) = 0.79 with intercept = 3.28, std error = 1.66, p = 0.11.

Toxicity endpoints in terms of growth:

- Size (based on caudal length) at 21 days: Students 't' test (two-sample assuming equal variances) gave no significant differences ($p>0.05$).

Toxicity endpoints in terms of reproduction (total number neonates per individual):

- EC15 (21 days) Probit = 0.001 mg/l (confidence limits 0.000-0.004 mg/l).
- EC20 (21 days) estimated from drawn equation = <0.001 mg/l based on exponential curve.
- EC50 (21 days) Probit = 0.004 mg/l (confidence limits 0.000-0.008 mg/l but chi squared for heterogeneity 12.3).
- EC50 (21 days) TSK with trim not able to calculate.
- Correlation coefficients for linear fit to reproductive data:
  R^2 (neonates per individual) = 0.40 with intercept = 10.29, std error = 4.12, p = 0.07.

Table A.3 **Data analysis of *Daphnia pulex* 21-day exposure experiment to $CdCl_2$ with mortality and reproduction as experimental endpoints. Significant differences to controls are indicated in italics. The toxicity endpoint of >20% change is based on recommendations by Slabbert (2004).**

Criterion	Statistic	Concentration mg/l						Signif diff to control
		0 (control)	0.006	0.013	0.025	0.05	0.1	
Mortalities	%	13.3	*26.7*	*20*	*53.3*	*100*	*100*	*>20% = toxicity* All
Number neonates /breeding animal	Average	21.7	*6.5*	*5.7*	*0.6*	*0.3*	*0*	*All
	CL (95%)	3.9	2.9	1.66	0.81	0.57	N/a	
	Std error	1.85	1.65	1.33	0.37	0.27	N/a	
	% Inhibition	0	*70.2*	*75.2*	*97.2*	*98.7*	*100*	*>20% = toxicity* *All
Number neonates / brood	Average	4.3	*2.5*	*1.6*	*0.6*	*[4]* [A]	*0*	*All
	Std error	0.34	0.32	0.21	0.25	[0.26] [A]	N/a	
	% Inhibition	0	*42.9*	*62.7*	*84.6*	*[7.0]* [A]	*100*	*>20% = toxicity* *All
Time to first brood (days)	Average	10.5	12.1	*13.3*	*17*	*11*	*N/a*	*0.013, 0.025, 0.05, 0.1 ($p<0.001$)
	Std error	0.43	0.95	0.78	1.5	N/a [A]	N/a	
	% Delay	0	15.2	*26.7*	*61.9*	N/a [A]	N/a	*>20% = toxicity* *All

* Students t-Test, two-sample assuming equal variances

[A] Only one animal survived to 21 days and therefore statistics skewed. Results taken as significant difference to control.

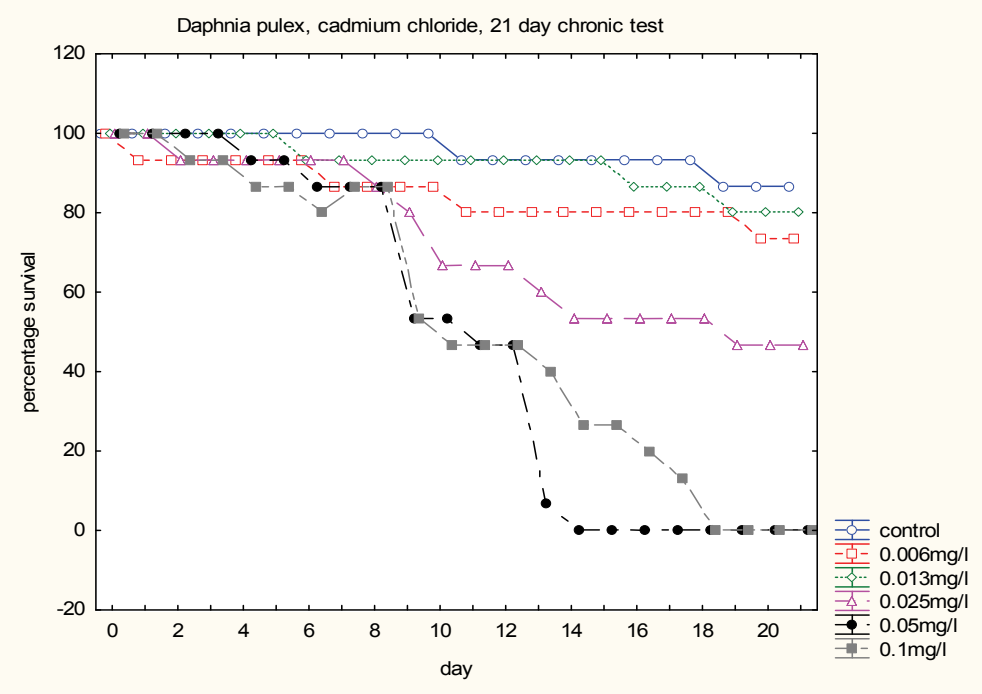


Figure A.6 *Daphnia pulex* survival over 21 days' exposure to $CdCl_2$.

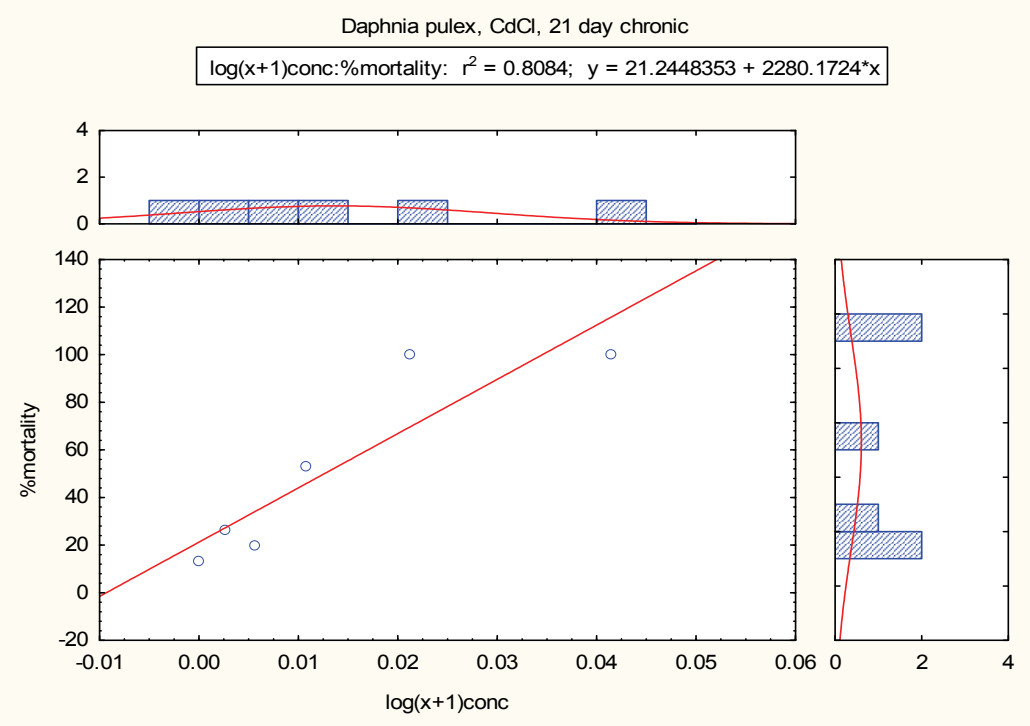


Figure A.7 Linear fit log (x+1) concentration of $CdCl_2$ against percentage mortalities of *Daphnia pulex* after 21 days.

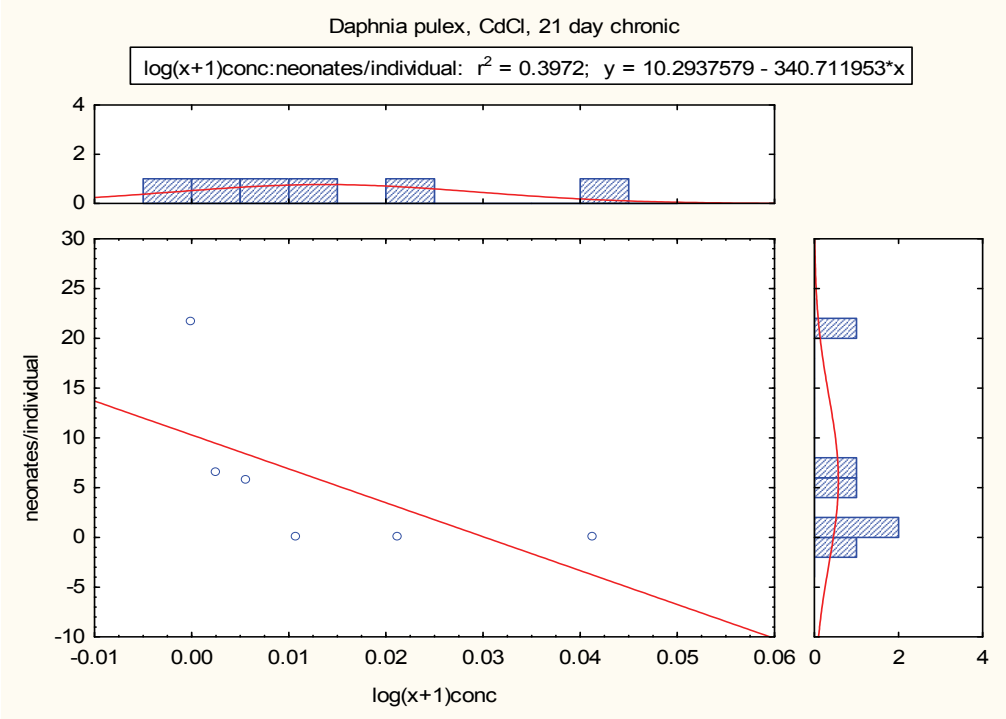


Figure A.8 Linear fit of log (x+1) concentration of $CdCl_2$ against number of *Daphnia pulex* neonates per individual after 21 days.

Test Validity:
For a test to be valid there are a number of criteria that have to be fulfilled (Slabbert, 2004). The principal criterion is based on a comparison of historic geometric means of 21 day EC_{50} values. As this is the first UCEWQ-IWR chronic toxicity test using the reference toxicant $CdCl_2$, this comparison could not be undertaken.

This toxicity test was considered valid based on the test duration (reached 21 days), and adult lethality and numbers of living males were <20% after 21 days. However, it failed validity in the following ways (Slabbert, 2004):

i) mean number of control offspring was 21.7 (not >40).
ii) R^2 for linear fit to mortality data is 0.39 (< 0.9).

Summary of experiment results:

- Survival data proved useful in estimations of the No Observed Effect Concentration (NOEC).
- Growth, based on caudal tail length, was not a useful criterion to estimate the NOEC over 21 days.
- Reproductive data showed wide variability and poor data distribution with exponential fit, but calculations of the LOEC were still possible.
- The overall NOEC = <0.006 mg/l CdCl over 21 days.

A.4.4 RESULTS FOR TEXTILE WHOLE EFFLUENT EXPOSURE EXPERIMENTS

For the protection of the ecological Reserve in South Africa, the proposed introduction of compulsory toxicity testing in the licensing of effluent discharges necessitates the development of whole effluent toxicity testing. The elucidation of the effects of effluent on the local indigenous populations of organisms is essential before hazard and risk assessment can be undertaken. Acute and chronic textile whole effluent toxicity tests were completed to compare the responses of *D. pulex* (representing a standard laboratory organism) to those of *Burnupia stenochorias* an indigenous limpet under consideration as a toxicity test organism (Palmer et al*.,* 2004). In a previous report to the Water Research Commission, the results of Davies-Coleman (2002) are given, principally referring to the textile whole effluent chronic toxicity tests using the freshwater limpet *Burnupia stenochorias* (Pulmonata, Ancylidae) as the test organism. The entire thesis is available as a PDF on the Internet: http://www.ru.ac.az/library/theses/collection.html#Zoology. The *D. pulex* chronic toxicity results are summarised in this report.

It was hypothesised that *D. pulex* would provide less ambiguous whole effluent toxicity test results than *B. stenochorias.* Two 21 day chronic tests were completed (1998 and 1999). The same methods described above were used. The effluent was end-of-pipe collected from an Eastern Cape textile factory, flowing into the Mlakalaka Stream and soon after, the Buffalo River, north of East London. Sufficient quantities were collected for both tests and sub-samples defrosted when required. The effluent consisted of dye particles that slowly adhered to the sides of the beakers but was renewed every two or three days in an attempt to maintain the constituents of the effluent as constantly as possible over the entire test period. As per the present protocol (Slabbert, 2004), at the time of effluent renewal, each glass beaker was replaced with a clean beaker. Oxygen levels, pH and Total Dissolved Salts of each effluent dilution and the culture medium (controls) were monitored with each renewal. Metal and inorganic analyses were made through the Institute for Water Quality Services (now called Resource Quality Services), Department of Water Affairs.

In analysing for differences in survival between concentrations of effluent, actual data were compared for statistical differences between diluents and concentrations using the Fisher's Exact Test (Finney, 1948) following the method stipulated by the USEPA (1993a). To determine differences between concentrations in time to first death, Student's two sample 't' tests assuming equal and unequal variances were used. The time to first brood production data were $\log_{10}$ transformed to normalize data and analysed for differences between 1998 and 1999 using Analysis of Variance (ANOVA) (Statistica, 1999). Growth (change in caudal tail size) was compared using the Tukey's Honest Significance Test (ANOVA, $p < 0.05$).

Inorganic and metal ion analyses of the textile effluent are as per samples used in the chronic toxicity tests with *B. stenochorias* (Table A.4). When comparing total dissolved salts (TDS) and pH values of the 1998 and 1999 100% effluent samples (Table A.5), significant differences were found (Student's two sample 't' test for means, $t = -6.98$ [TDS] and -4.79 [pH], $p < 0.01$). The 1998 and 1999 responses of *D. pulex* should therefore be compared with caution. No significant differences occurred between the TDS and pH control values.

Toxicity endpoints in terms of survival:

- Because each individual *D. pulex* was in a separate beaker, no within treatment testing of variability was possible. Survival of neonates over 21 days was generally greater in 1999 than 1998 (Figure A.9), with survival in 100% textile effluent equal to or greater

than survival in the controls for each year. The times to first death were not significantly different between 1998 and 1999 (Student's 't' test, t = 0.312, p = 0.38). No LC_{50} data over the 21 days were obtainable as 50% mortality did not occur. Toxicity of the defrosted textile effluent could be loosely defined as follows:
100% = 12.5% < control = 6.25% < 50% = 25% [1998]
100% = control < 1.1% < 10% < 20% = 3% [1999].

- However, differences in survival in each effluent dilution compared to the controls were not significant (Fisher's Exact Test, p>0.05), with the consequent allocation of 12.5% textile whole effluent as the No Observed Effective Concentration (NOEC) at 21 days. Because the range of tested effluent concentrations was not at a factor of 0.5 or more (USEPA, 1993a), and the dilution below 100% was only 20% effluent, it seemed appropriate to allocate the NOEC value between 10% and 20% textile whole effluent (Hoekstra and Van Ewijk, 1993).

Toxicity endpoints in terms of growth:

- Use of the caudal spine as a measure of growth was not satisfactory, as many of the caudal spines had broken off.
- However, comparison of the tails of the remaining cladocerans gave a significant difference in size (longer) between cladocerans in 100% effluent and the controls (Tukey's Honest Significance Test, ANOVA, p < 0.05; Figure A.11).
- Variability in length between the cladocerans within each dilution was always within a range of two standard deviations at each dilution (USEPA, 2000).

Toxicity endpoints in terms of reproduction:

- Because *Daphnia* neonates are usually produced every second day (Persoone and Janssen, 1993), those produced on two consecutive days are often counted as one brood (Stephenson and Watts, 1984). However, the *D. pulex* studied here regularly produced broods on a daily basis for a number of days. Consequently, each day that *D. pulex* neonates were produced was considered an independent brood, irrespective of the pattern of brood production within each solution tested.
- No statistical differences between 1998 and 1999 occurred in the mean time to first production within each dilution, including between the controls (ANOVA, p>0.05).
- In 1998 all concentrations significantly decreased the time to first reproduction compared to the controls, by approximately 2 days (Student's 't' test, p<0.01), where the controls had a mean time of 11.07 days (Table A.6). In 1999 only 20% effluent significantly decreased the time to first production from a mean control time of 11.23 days (Student's 't' test, p<0.05; Table A.6).
- A significantly greater number of total neonates were produced per test solution in 1999, both in the control and effluent solutions (Figure A.10).
- The number of neonates per cladoceran day and the size of the first three broods generally increased with increasing effluent concentration (Table A.6).

Table A.4 **Major inorganic determinands and trace metals in carbon filtered tap water (diluent) and defrosted textile effluent. The 'Factory' source refers to the analyses of the post-irrigated effluent [the same source as sampled for chronic testing] completed by the textile factory chemistry department from 1996 to 1998. Number of samples (tap water and effluent) = 26. Cobalt, beryllium, nickel, molybdenum, cadmium, mercury and lead were not detectable. TWQR = Target Water Quality Range; CEV = Chronic Effect Value; AEV = Acute Effect Value (DWAF, 1996). [a] refers to the USA recommended sodium content of drinking water (Bunce, 1994). [b c d] refer to typical figures for upland stream, lowland stream and large lowland river (Dallas and Day, 1993). * indicates value measured within effluent above the CEV.**

Determinand	Source	Range mg/l	Mean mg/l	TWQR mg/l	CEV mg/l	AEV mg/l
* Ammonium as N	Tap water	0.05-4.20	1.40	0.007	0.02	0.10
	Effluent	0.21-7.13	1.01			
	Factory	12.00-29.00	18.00			
* Nitrate and Nitrite as N	Tap water	0.04-0.23	0.12	0.007	0.02	0.10
	Effluent	0.04-0.17	0.08			
	Factory	2.00-16.00	4.00			
* Kjeldahl Nitrogen as N	Tap water	0.23-0.29	0.26	0.007	0.02	0.10
	Effluent	8.28-11.28	10.12			
* Fluoride as F	Tap water	0.6-0.1	0.2	□0.8	1.5	2.5
	Effluent	0.3-0.4	0.4			
* Chloride as Cl	Tap water	10.0-15.5	13.9	□0.0002	0.0035	0.0050
	Effluent	104.0-318.0	192			
Sulphate as SO4	Tap water	30-35	32			
	Effluent	177-1515	307			
Alkalinity as Calcium Carbonate	Tap water	23-42	39			
	Effluent	498-1196	820	[very hard]		
Total Phosphate	Tap water	0.09-0.11	0.10			
	Effluent	2.67-4.02	3.52	[Hypertrophic]		
	Factory	1.93-16.09	4.95			
Ortho Phosphate as P	Tap water	0.006-0.051	0.019			
	Effluent	1.901-5.600	2.211			
* Sodium as Na	Tap water	30-84	74	[<100 ppm][a]		
	Effluent	403-844	648			

Determinand	Source	Range mg/l	Mean mg/l	TWQR mg/l	CEV mg/l	AEV mg/l
	Factory	326-840	555			
Calcium as Ca	Tap water	18.0-23.0	20.0			
	Effluent	3.8-55.0	19.2			
	Factory	4.4-20.9	10.1			
Magnesium as Mg	Tap water	15.0-20.0	15.5			
	Effluent	17.0-32.0	25.7			
	Factory	1.2-40.7	6.2			
Potassium as K	Tap water	2.0-2.8	2.2			
	Effluent	15.7-26.2	20.3			
Silicon as Si	Tap water	0.4-2	1.3			
	Effluent	8.6-19.2	11.6			
Electrical Conductivity (mS/m)	Tap water	46.8-67.3	63.7			
	Effluent	203.0-372.0	276.5			
* Aluminium, acid soluble	Tap water	0.06-0.52	0.14	□0.01	0.01	0.1
	Effluent	0.06-0.13	0.08			
Titanium, acid soluble	Tap water	0.007	0.007			
	Effluent	0.007-0.300	0.024			
Chromium, acid soluble	Tap water	<0.007	<0.007	□0.007	0.01	0.2
	Effluent	<0.007-0.032	0.024			
* Manganese, dissolved	Tap water	<0.003	0.008	□0.18	0.37	1.3
	Effluent	0.201-0.400	0.279			
Iron, acid soluble	Tap water	<0.02-0.09	0.03			
	Effluent	0.30-0.82	0.60			
* Copper, acid soluble	Tap water	<0.011	<0.011	□0.0014	0.0028	0.012
	Effluent	<0.011-0.073	0.025			
* Zinc, acid soluble	Tap water	<0.01-0.06	0.02	□0.002	0.04	0.36
	Effluent	0.03-0.16	0.07			
Barium	Tap water	0.01-0.02	0.02			
	Effluent	<0.01-0.06	0.04			
Dissolved Organic Carbon as C	Tap water	3.5-4.2	4.0			
	Effluent	51.7-75.3	62.3			

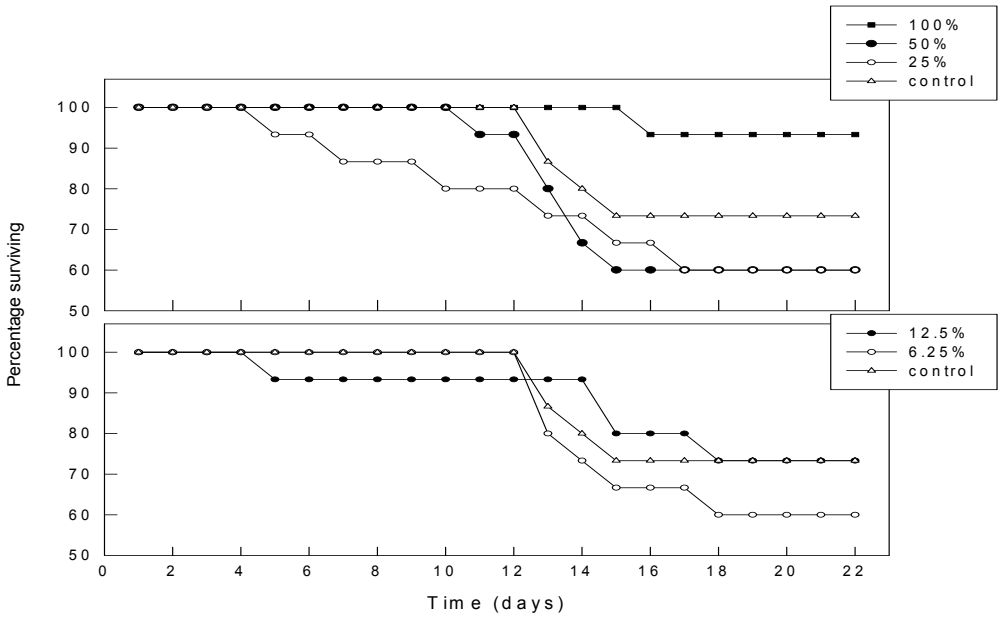
Determinand	Source	Range mg/l	Mean mg/l	TWQR mg/l	CEV mg/l	AEV mg/l
* Biological Oxygen Demand	Effluent	75.0-135.0	102.0	[0.5-2.0][b]	[2.0-5.0][c]	[3.0-7.0][d]
	Factory	129.0-341.0	197.0			
Chemical Oxygen Demand	Effluent	610-734	629			
	Factory	698-2427	1125			

Table A.5 Total dissolved salts (TDS) and pH readings for the two chronic textile effluent *Daphnia pulex* toxicity tests. Std.dev. = standard deviation; C.V. = percentage coefficient of variation.

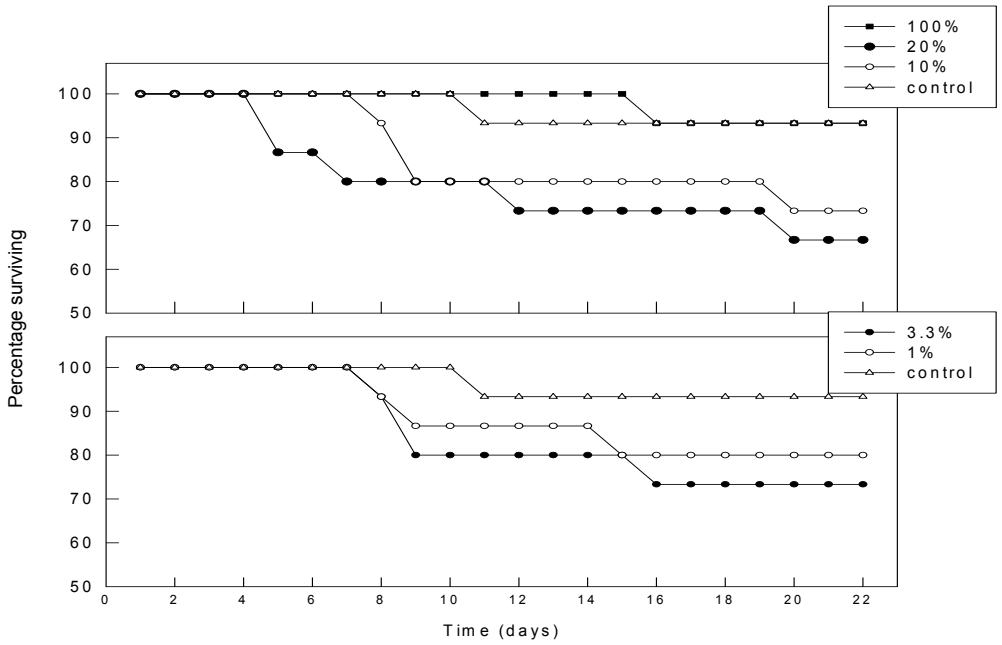
Year	Effluent conc	TDS mean	± Std. dev.	C.V.	pH mean	± Std. dev.	C.V. %
1998	100.0%	1480.0	158.12	10.7	8.44	0.25	3.14
	50.0%	848.5	83.11	0.98	8.61	0.19	2.22
	25.0%	587.4	81.12	13.81	8.59	0.16	1.98
	12.5%	388.3	70.61	18.19	8.62	0.17	2.15
	6.25%	297.3	19.81	6.67	8.67	0.19	2.21
	[control]	209.9	29.73	14.17	8.67	0.23	2.76
1999	100.0%	1709.0	66.6	38.96	9.21	0.24	2.61
	20.0%	618.2	4.91	7.91	9.18	0.20	2.22
	10.0%	425.3	3.25	7.70	9.09	0.19	2.16
	3.33%	305.0	6.16	19.81	8.89	0.34	3.82
	1.0%	272.2	4.27	15.54	8.72	0.25	2.54
	[control]	247.9	4.86	17.60	8.70	0.23	2.95

Toxicity endpoints in terms of F1 generation:

- Acute mortalities in the F1 generation of *D. pulex* subjected to textile effluent were not significantly different to those in the parent acute toxicity tests, with only a 5% mortality occurring in 100%, 20% and 1% effluent, and 10% in 1% effluent, with no control deaths. The effluent was therefore not acutely toxic to the F1 generation, although an increase in mortality did occur, compared to the parent generation.



a) 1998



b) 1999

Figure A.9 Percentage survival of *Daphnia pulex* per concentration of effluent compared to the controls over 21 days. Each year's control is duplicated for ease of comparison.

Table A.6. **Survival and reproduction rates of *Daphnia pulex*, after 21 days' exposure to textile effluent. Ephippia were not produced in any of the beakers. * numbers in brackets refer to actual number of neonates dead; ** = total percentage increase relative to the controls; Conc. = concentration; no. = number of; St. dev. = standard deviation. Daily mortality rate was calculated as the total number of neonates dead ÷ the total number of observation days (21). Production rate / cladoceran day calculated from the total number of cladocerans produced per day per concentration ÷ the total number of days limpets survived. [a] significantly different ($p<0.01$) to 1998 control; [b] significantly different ($p<0.05$) to 1999 control.**

Year	% conc. effluent	Total % dead *	Time at first death (days)	Daily mortality rate	Mean time first reprodn (days)	Mean no. neonates / adult	St. dev. / adult	Prodn rate / cladoceran day	Total % increase in no. neonates **	Mean size first 3 broods
1998	100	26.7 (4)	7	0.191	9.43[a]	27.13	16.46	1.519	72.5	5.62
	50	40.0 (6)	12	0.286	8.87[a]	27.07	11.79	1.498	72.1	4.67
	25	40.0 (6)	7	0.286	8.92[a]	24.40	15.28	1.469	55.2	6.08
	12.5	26.7 (4)	7	0.191	9.71[a]	32.86	15.76	1.723	108.9	6.07
	6.25	33.3 (5)	12	0.238	9.13[a]	26.93	17.22	1.474	71.2	3.83
	Control	33.3 (5)	12	0.238	11.07	15.73	13.09	0.834	-	3.50
1999	100	6.7 (1)	16	0.047	10.66	48.33	13.45	2.201	104.8	8.02
	20	33.3 (5)	5	0.238	9.31[b]	44.46	28.35	2.595	88.4	8.72
	10	26.7 (4)	6	0.143	10.08	43.0	24.19	2.363	82.2	7.97
	3.33	33.3 (5)	5	0.238	10.41	32.40	19.44	1.800	37.3	5.72
	1.1	20.0 (3)	9	0.143	10.92	30.67	21.61	1.614	29.9	4.69
	Control	6.7 (1)	12	0.047	11.23	23.60	16.62	1.161	-	4.36

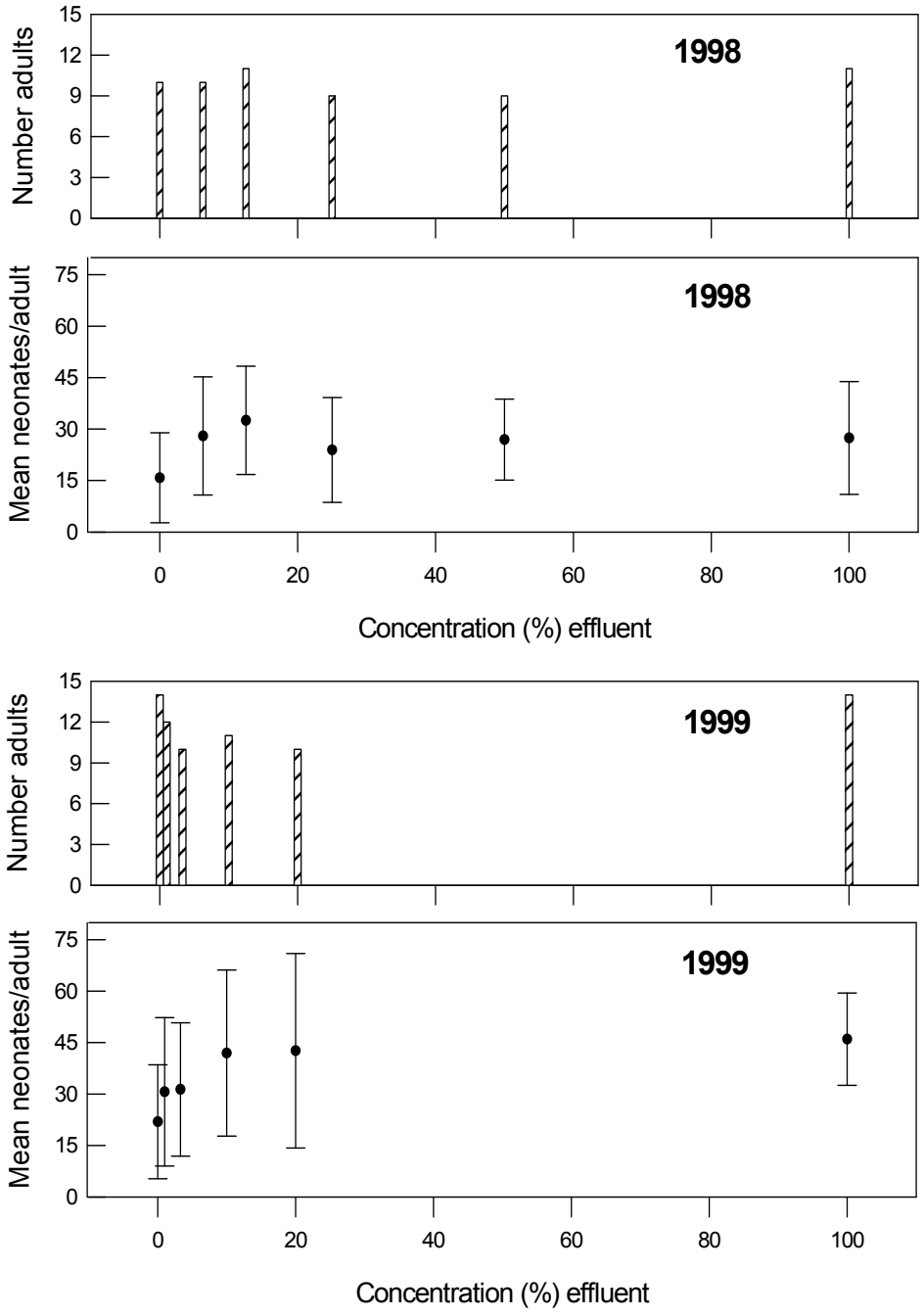


Figure A.10 **Total number of adults within each concentration of effluent after 21 days. Differences between 1998 and 1999 in the mean aggregate of neonates per adult at each concentration of textile effluent are also shown. Concentration (%) effluent 0 = control of culture medium**.

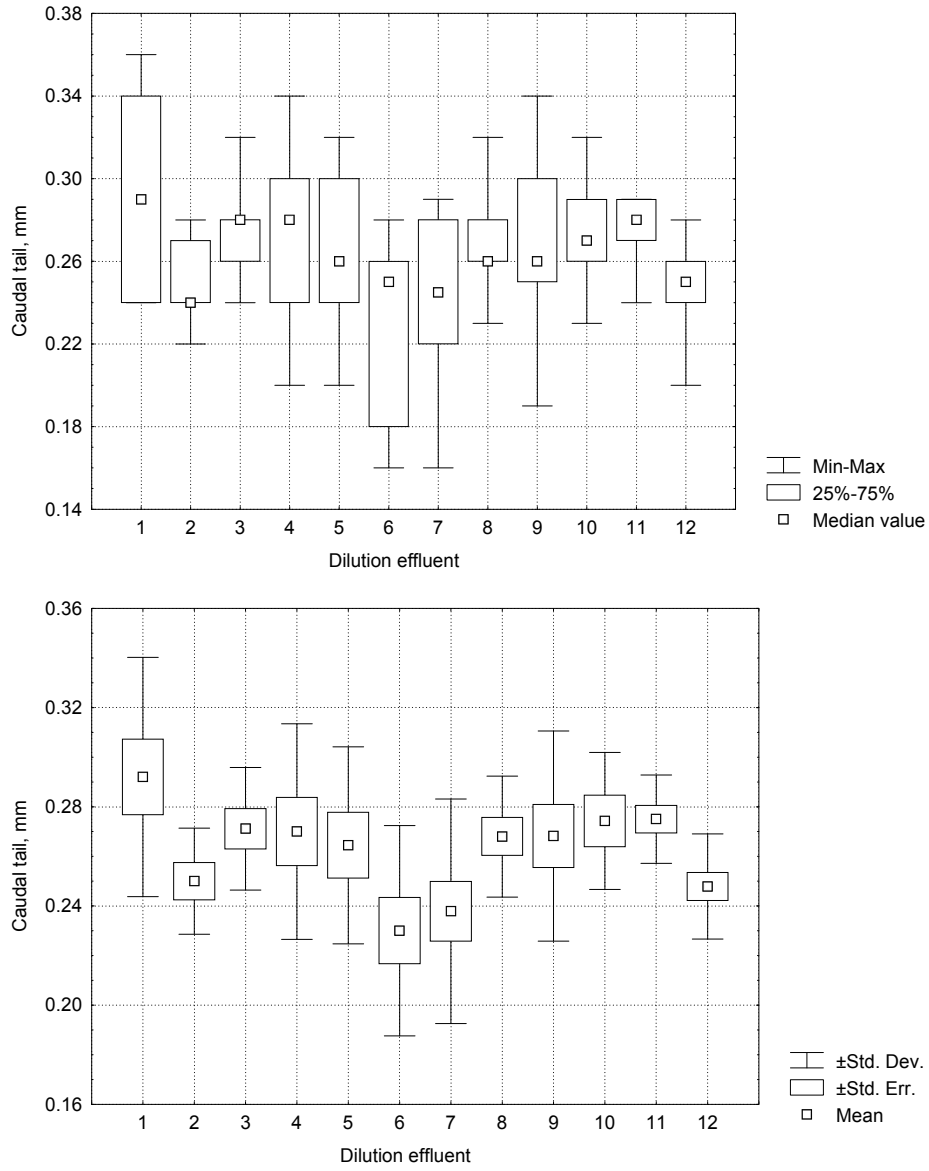


Figure A.11 Respective lengths of *Daphnia pulex* caudal tails at each dilution after 21 days. The dilution effluent figures on the x axis represent the following dilutions: 1998 concentrations: 1 = 100%, 2 = 50%, 3 = 25%, 4 = 12.5%, 5 = 6.25%, 6 = control; 1999 concentrations: 7 = 100%, 8 = 20%, 9 = 10%, 10 = 3.33%, 11 = 1%, 12 = control.

Summary of test results:

- No LC_{50} toxicity of the textile effluent to *D. pulex.*
- Growth differences only recorded at 100% effluent.
- Stimulation of neonate production <100% effluent, but significant differences found between numbers of neonates produced between the 2 years, despite the same techniques and food source.
- F1 generation demonstrated increased mortalities in effluent, but not significantly different to controls.
- Overall NOEC for toxicity = <20% effluent.

A.5 DISCUSSION

The assessment of toxicity, the control of effluent discharges, or the registration of a new chemical requires the collection of consistent and comparable toxicity response data (APHA, 1992; Rand, 1995; USEPA, 2000). Depending on the reference toxicant and test species used in acute toxicity tests, coefficients of variation have previously been calculated between 6% and 124% (Nebeker, 1982; Cabridene, 1986; Parkhurst et al., 1992). Variability of acute responses of UCEWQ-IWR *D. pulex* to both cadmium chloride and potassium dichromate reference toxicants has been within an approximate three fold range (equivalent to approximately 30% coefficient of variation), and LC_{50} values fall comfortably within the South African Proficiency Test ranges.

However, a disappointing mortality rate occurred after 5 to 7 days in some of the chronic toxicity tests within the last few years at UCEWQ-IWR, as described. Reasons for this are likely to be interrelated; some possibilities are listed:

1) *Differences in culture techniques*: (see Table A.1) There are conflicting viewpoints within South Africa as to the best feeding regime and the value of adding *Selenastrum* species, possibly affecting the validity criterion of 40 neonates per individual required for chronic toxicity endpoints. Both the quantity (Baird et al., 1989) and quality (Belanger et al., 1989) of food potentially affect the maternal production of neonates.

2) *Moulting*: Chronic toxicity test ranges are selected based on acute endpoints. Moulting is thought to influence LC_{50} values obtained in acute toxicity tests. It is the opinion of Lee and Buikema (1979) that ideally, acute toxicity tests should last long enough for all neonates used in the tests to moult at least once; over the 48hours recommended for South Africa, moulting does not occur. Present selection of endpoints may not reveal the wider responses of *D. pulex* to the toxicant under chronic conditions. [This however does not explain why control mortalities are regularly experienced.]

3) *Salt toxicity*: The site(s) of toxicity with sodium chloride and sodium sulphate are likely to be complex and are not understood. The present WRC project "Osmoregulation in freshwater invertebrates in response to exposure to salt pollution" [project number K1585] may provide some answers. However, chemical complexities confuse this further [Table A.7; sodium sulphate and food interactions]. The difficulty UCEWQ has in obtaining *D. pulex* chronic toxicity results with the sodium salts and with the reference toxicant cadmium chloride suggests a precautionary note with DWAF's DEEEP recommendations (DWAF, 2003).

Further comments on toxicity results and data interpretation:

- *Risk assessment based on toxicity data*

Most risk assessments tend to estimate intrinsic rate of population increase. South Africa's presently recommended 21 day chronic *D. pulex* toxicity test does not include this overall calculation. The significant increases in reproduction of *D. pulex* when subjected to the textile whole effluent suggested complications for data interpretation. Such stimulation could be an indication of overproduction leading eventually to reduction in reproductive effort. However, stimulation of reproduction in the cladocerans does occur (Naudin et al., 1995),

linked to the maximum genetically determined rate of reproduction and growth (scope for reproduction or growth) (Van Leeuwen et al., 1985).

The extrapolation of LC_{50} data has been found to be an inaccurate predictor of populations with many invertebrates, including *Daphnia pulex* (Stark, 2005). Present recommendations are based on EC values estimated from linear graphs [ill-fitting in our examples] drawn from the toxicity data. Similarly interpretation of toxicity data is often based on the calculation and interpretation of Toxicity Units, also based on LC_{50} values.

Necrosis is a strong possibility with whole effluents, not considered with acute or chronic tests and consequent risk assessment.

Further consideration and collation of data is needed in South Africa with consideration being given to form of data being collected as toxicity databases are developed (Hobbs et al*.,* 2005).

- *The necessity for alternative test species*

D. pulex is an initial step in the toxicity test tiers to be recommended by DWAF for toxicity testing of whole effluents (DWAF, 2003). During any textile toxicity tests for example, dye particles tend to adhere to the test containers. The interpretation of *D. pulex* (free swimming) toxicity endpoints may bear little resemblance to those for invertebrates such as the indigenous limpet *Burnupia stenochorias* (attached to substrate and therefore immersed amongst the dyes). This possibly explains to a degree the significantly less toxicity shown by *D. pulex* to the effluent in both acute and chronic tests than *B. stenochorias* (Section A.6; Davies-Coleman, 2002). Zokufa's toxicity results with the same textile effluent (2001) underline the need for other, indigenous test species.

Where appropriate, further analyses such as dye particle removal by centrifugation and separate toxicity testing could be considered a necessity for risk assessment (Timofeeva, 1991).

Table A.7 Comments on problems and practicalities relating to the chronic toxicity protocol (Slabbert, 2004).

CHRONIC TOXICITY TESTS (REPRODUCTION TESTS)		
Culture condition	**POINT OF CONCERN**	**Comments and notes**
Protocol Section 7.4 – test environment.	Use ambient laboratory illumination (artificial and/or day light:10 to 14hrs light).	Conditions used by UCEWQ are the same ambient illumination as used for the cultures (16hrs light).
Protocol Section 7.5.3 – daphnia food.	Algal suspension (2.5×10^7 cells/mℓ), included into the food mixture.	UCEWQ does not use algal suspension for feeding *Daphnia* (as per the cultures).
Protocol Section 7.8.1 – Serial dilutions on wastewater for a definitive test.	Prepare the same dilutions for each renewal (three times per week).	For the testing of chemical solutions, dilutions are made up twice weekly. The protocol refers rather to wastewater that is likely to contain pathogens and bacteria or spores. These may alter the treatments/concentrations, interfere with effects, and thus affect interpretation of end results.
Protocol Section 7.8.2 – add daphnia food to samples.	Before distributing samples into test containers, add TYA food and algal suspension to sample and mix well.	UCEWQ feeds small aliquot portions of food to each of the test beakers daily and not at time of toxicant exchange (twice weekly).
Protocol Section 7.9 – data analyses and expression of results.	If any parent dies before the end of the test or is identified as a male, exclude the replicate from the data analysis for reproduction.	Are males ever present in a culture and how are they identified? Should this be added to the protocol?

Protocol Section 7.11 – test validity.	The mean number of offspring per parent in the controls is >40.	UCEWQ has only ever had a maximum of 30 neonates in the control. Despite small differences in the techniques, we suggest this number may need refining or alternatively confidence limits given in the protocol, particularly as inter-laboratory differences in number of offspring may occur.
Other test condition issues	**Effect**	**Comments and notes**
During the testing of salts, in particular Na_2SO_4, the TYA food appears to combine with the salts, forming a slimy substance on the glass surface.	*Daphnia* get trapped in the slime layer and die if not removed. Chronic test duration significantly impaired.	1) Remove *Daphnia* from slime at least once a day. 2) Solutions can be changed more often but is too labour intensive. 3) Feeding less frequently. There are further implications in putting this into practice as it diverges from the protocol procedure.

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APPENDIX B

RESULTS OF ACUTE AND CHRONIC TOXICITY TESTS COMPLETED WITHIN THE CHRONIC TOXICOLOGY PROJECT OR RELEVANT TO THE PROGRAMME

TABLE B.1 ***Caridina nilotica*** **acute toxicity test results for NaCl and Na_2SO_4. All *Caridina nilotica* laboratory cultured. Expt ID refers to the UCEWQ database entry number. LCL: lower confidence level, UCL: upper confidence level; Diluent;1 = dechlorinated tap water.**

Species	Toxicant	Date	Expt ID	Chi Square Calculated	Chi Square Tabulated	Duration hrs	LC_{50}	Conc	LCL	UCL	Diluent	Unit
Caridina nilotica	NaCl	2002-07-09	233	0.56	7.815	48	50	5978.707	4823.105	7058.461	1	mg/L
Caridina nilotica	NaCl	2002-09-03	246	7.168	15.507	48	50	5954.507	5099.727	6751.841	1	mg/L
Caridina nilotica	NaCl	2002-10-15	253	13.95	16.919	48	50	4450.134	3708.684	5195.741	1	mg/L
Caridina nilotica	NaCl	2003-12-04	315	6.597	14.067	48	50	5486.76	4528.17	6446.06	1	mg/L
Caridina nilotica	Na_2SO_4	2002-05-29	239	3.145	12.592	48	50	5734.084	5083.878	6614.3	1	mg/L
Caridina nilotica	Na_2SO_4	2002-06-14	236	8.295	14.067	48	50	5989.305	4873.772	7180.567	1	mg/L
Caridina nilotica	Na_2SO_4	2002-07-21	238	16.659	12.592	48	50	7001.675	4710.227	9024.049	1	mg/L
Caridina nilotica	Na_2SO_4	2002-08-06	258	1.738	24.996	48	50	5445	4806	5976	1	mg/L
Caridina nilotica	Na_2SO_4	2005-01-02	364	1.625	11.07	48	50	1785.025	1040.866	2598.983	1	mg/L

TABLE B.2 ***Caridina nilotica*** **chronic toxicity test results for NaCl and Na_2SO_4. All *Caridina nilotica* laboratory cultured. Expt ID refers to the UCEWQ database entry number. EC: Effective concentration; LCL: lower confidence level, UCL: upper confidence level; Diluent: 1 = dechlorinated tap water.**

Species	Toxicant	Date	Expt ID	Source	Chi Square Calculated	Chi Square Tabulated	Duration hrs	EC	Conc	LCL	UCL	Diluent	Unit
Caridina nilotica	NaCl	2002-09-22	254	Laboratory culture	2.979	14.067	96	50	8568.334	7545.879	9483.086	1	mg/L
Caridina nilotica	NaCl	2002-09-22	254	Laboratory culture	3.071	14.067	96	50	1572.16	1399.93	1726.431	1	mS/m
Caridina nilotica	NaCl	2003-12-02	338	Laboratory culture	3.855	9.488	96	50	879.137	796.48	965.955	1	mg/L
Caridina nilotica	NaCl	2003-12-02	338	Laboratory culture	2.202	9.488	96	50	1514.991	1389.953	1647.721	1	mS/m
Caridina nilotica	NaCl	2003-12-02	338	Laboratory culture	0.044	9.488	240	50	466.164	422.65	511.813	1	mg/L
Caridina nilotica	NaCl	2003-12-02	338	Laboratory culture	0.323	9.488	240	50	868.376	791.542	943.7	1	mS/m
Caridina nilotica	Na_2SO_4	2000-06-24	25	Mpisini stream	11.115	23.685	96	50	4955.347	4553.501	5405.65	1	mg/L
Caridina nilotica	Na_2SO_4	2000-06-24	25	Mpisini stream	13.519	19.675	240	50	1751.558	1521.666	2006.389	1	mg/L
Caridina nilotica	Na_2SO_4	2001-05-21	203	Laboratory culture	21.066	24.996	96	50	5615.491	5013.497	6310.238	1	mg/L
Caridina nilotica	Na_2SO_4	2001-05-21	203	Laboratory culture	18.711	24.996	240	50	3149.265	2755.012	3510.686	1	mg/L
Caridina nilotica	Na_2SO_4	2001-05-21	203	Laboratory culture	22.776	24.996	96	50	744.893	681.81	815.167	1	mS/m
Caridina nilotica	Na_2SO_4	2001-05-21	203	Laboratory culture	19.843	24.996	240	50	473.453	425.565	516.24	1	mS/m
Caridina nilotica	Na_2SO_4	2002-07-04	226	Laboratory culture	18.631	24.996	96	50	6582.666	6198.092	6950.156	1	mg/L
Caridina nilotica	Na_2SO_4	2002-07-04	226	Laboratory culture	30.573	24.996	168	50	3994.754	3214.359	4507.259	1	mg/L
Caridina nilotica	Na_2SO_4	2002-07-04	226	Laboratory culture	12.163	24.996	240	50	3248.947	2348.85	3684.828	1	mg/L
Caridina nilotica	Na_2SO_4	2002-07-04	226	Laboratory culture	20.888	24.996	96	50	4848.74	4609.45	5075.219	1	mg/L-TDS
Caridina nilotica	Na_2SO_4	2002-07-04	226	Laboratory culture	45.044	24.996	168	50	3214.085	2525.091	3600.024	1	mg/L-TDS
Caridina nilotica	Na_2SO_4	2002-07-04	226	Laboratory culture	15.301	24.996	240	50	2701.686	1966.564	3021.977	1	mg/L-TDS
Caridina nilotica	Na_2SO_4	2002-07-04	226	Laboratory culture	20.888	24.996	96	50	734.659	698.403	768.973	1	mS/m
Caridina nilotica	Na_2SO_4	2002-07-04	226	Laboratory culture	35.731	24.996	168	50	483.646	391.669	539.287	1	mS/m
Caridina nilotica	Na_2SO_4	2002-07-04	226	Laboratory culture	15.309	24.996	240	50	409.282	297.953	457.824	1	mS/m

Species	Toxicant	Date	Expt ID	Source	Chi Square Calculated	Chi Square Tabulated	Duration hrs	EC	Conc	LCL	UCL	Diluent	Unit
Caridina nilotica	Na_2SO_4	2002-07-22	255	Laboratory culture	5.027	14.067	96	50	6820.183	5614.508	7885.71	1	mg/L
Caridina nilotica	Na_2SO_4	2002-07-22	255	Laboratory culture	4.953	14.067	96	50	909.1	774.853	1024.834	1	mS/m

TABLE B.3 ***Caridina nilotica*** **acute toxicity test results for $CdCl_2$. All *Caridina nilotica* laboratory cultured. Expt ID refers to the UCEWQ database entry number. EC: Effective concentration; LCL: lower confidence level, UCL: upper confidence level; Diluent: 1 = dechlorinated tap water.**

Species	Date	Expt ID	Chi Square Calculated	Chi Square Tabulated	Duration hrs	EC	Conc	LCL	UCL	Diluent	Unit
Caridina nilotica	2001-04-03	193	4.735	14.067	48	50	0.054	0.023	0.078	1	mg/L
Caridina nilotica	2001-04-11	194	12.927	16.919	48	50	0.07	0.043	0.098	1	mg/L
Caridina nilotica	2001-04-24	195	11.241	16.919	48	50	0.09	0.043	0.144	1	mg/L

TABLE B.4 ***Caridina nilotica*** **chronic toxicity test results for Deltamethrin and Linear Alkylbenzene Sulfonate (LAS). All *Caridina nilotica* laboratory cultured. Expt ID refers to the UCEWQ database entry number. EC: Effective concentration; LCL: lower confidence level, UCL: upper confidence level; Diluent: 1 = dechlorinated tap water.**

Species	Toxicant	Date	Expt ID	Chi Square Calculated	Chi Square Tabulated	Duration hrs	EC	Conc	LCL	ULL	Diluent	Unit
Caridina nilotica	Deltamethrin	2005-05-30	381	2.783	9.488	96	50	0.000005	0	0	1	mg/L
Caridina nilotica	Deltamethrin	2005-07-05	382	3.512	9.488	96	50	0.000025	0.000013	0.00004	1	mg/L
Caridina nilotica	Deltamethrin	2005-08-01	383	N/a	N/a	96	100% mortalities	N/a	N/a	N/a	1	mg/L
Caridina nilotica	Deltamethrin	2005-08-08	384	1.963	9.488	96	50	0.000003	0.000001	0.000004	1	mg/L
Caridina nilotica	Deltamethrin	2005-08-29	385	3.84	9.488	96	50	0.000001	0.000001	0.000002	1	mg/L
Caridina nilotica	Deltamethrin	2005-09-05	386	N/a	N/a	96	100% mortalities	N/a	N/a	N/a	1	mg/L
Caridina nilotica	Deltamethrin	2005-09-11	387	N/a	N/a	96	50	0.00001	0.00001	0.00002	1	mg/L

Species	Toxicant	Date	Expt ID	Chi Square Calculated	Chi Square Tabulated	Duration hrs	EC	Conc	LCL	ULL	Diluent	Unit
Caridina nilotica	LAS	2005-06-25	376	8.676	9.488	96	50	9.121	5.907	12.529	1	mg/L
Caridina nilotica	LAS	2005-06-25	376	11.259	9.488	96	50	10.351	0	0	1	mg/L

TABLE B.5 ***Dugesia*** **sp. NaCl and Na_2SO_4 acute toxicity test results. Source indicates the origin of the test organisms (i.e. laboratory reared or collected from known reference site). Expt ID refers to the UCEWQ database entry number. EC: Effective concentration; LCL: lower confidence level, UCL: upper confidence level; Diluent: 1 = dechlorinated tap water.**

Species	Toxicant	Date	Expt ID	Source	Chi Square Calculated	Chi Square Tabulated	Duration hrs	EC	Conc	LCL	UCL	Stream Type	Diluent	Unit
Dugesia sp.	NaCl	2002-10-18	260	Kat River			240	50	5297.86	4534.95	6189.1	Aquarium	1	mg/L
Dugesia sp.	NaCl	2004-02-23	343	Laboratory culture	0.863	16.919	96	50	5640.44	5359.5	5939.12	Beaker	1	mg/L
Dugesia sp.	NaCl	2004-02-23	343	Laboratory culture	0.689	16.919	96	50	934.244	894.254	977.109	Beaker	1	mS/m
Dugesia sp.	Na_2SO_4	2003-11-13	314	Kat River	5.173	12.592	96	50	10132.227	7659.608	22324.787	Beaker	1	mg/L
Dugesia sp.	Na_2SO_4	2003-11-13	314	Kat River	7.792	12.592	240	50	9177.18	6820.79	20187.84	Beaker	1	mg/L
Dugesia sp.	Na_2SO_4	2003-11-13	314	Kat River	5.458	12.592	96	50	1106.775	876.236	2151.477	Beaker	1	mS/m
Dugesia sp.	Na_2SO_4	2003-11-13	314	Kat River	7.864	12.592	240	50	1050.768	818.025	2047.126	Beaker	1	mS/m
Dugesia sp.	Na_2SO_4	2004-04-20	344	Laboratory culture	4.539	16.919	96	50	8153.258	7221.594	10447.823	Beaker	1	mg/L
Dugesia sp.	Na_2SO_4	2004-04-20	344	Laboratory culture	1.351	16.919	96	50	944.393	870.869	1150.189	Beaker	1	mS/m
Dugesia sp.	Na_2SO_4	2004-10-01	345	Laboratory culture	5.609	15.507	96	50	5236.635	4836.341	5624.988	Beaker	1	mg/L
Dugesia sp.	Na_2SO_4	2004-10-01	345	Laboratory culture	5.34	14.067	96	50	662.488	617.953	705.117	Beaker	1	mS/m

TABLE B.6 ***Dugesia*** **sp. NaCl chronic toxicity test results. All test organisms were laboratory reared. LOEC: Lowest Observed Effect Concentration.**

Species	Date	Duration	Concentration range	Test completed	LOEC	Unit
Dugesia sp.	2005-07-01	7 days	0, 5.3	Yes	Not calculated; development of criteria	mg/L
Dugesia sp.	2005-07-07	7 days	0, 5.3	Yes	Not calculated; development of criteria	mg/L
Dugesia sp.	2005-07-21	7 days	0, 1.6, 2.8, 3.75, 5	Yes	2.8 mg/l	mg/L

TABLE B.7 ***Dugesia*** **sp. Deltamethrin acute toxicity test results. All test organisms laboratory cultured. Expt ID refers to the UCEWQ database entry number. EC: Effective concentration; LCL: lower confidence level, UCL: upper confidence level; Diluent: 1 = dechlorinated tap water.**

Species	Date	Expt ID	Source	Chi Square Calculated	Chi Square Tabulated	Duration hrs	EC	Conc	LCL	UCL	Diluent	Unit
Dugesia sp.	2005-06-20	380	Laboratory culture	N/a	N/a	96	No mortalities	N/a	N/a	N/a	1	mg/L

TABLE B.8 ***Burnupia stenochorias*** **acute toxicity test results for NaCl and Na_2SO_4. Expt ID refers to the UCEWQ database entry number. Source indicates the origin of the test organisms. EC: Effective concentration; LCL: lower confidence level, UCL: upper confidence level; Diluent: 1 = dechlorinated tap water.**

Species	Toxicant	Date	Expt ID	Source	Chi Square Calculated	Chi Square Tabulated	Duration hrs	EC	Conc	LCL	UCL	Stream Type	Diluent	Unit
Burnupia stenochorias	NaCl	2003-11-13	320	Kat River	9.271	12.592	96	50	3899.41	3572.82	4298.699	Channel	1	mg/L
Burnupia stenochorias	NaCl	2003-11-13	320	Kat River	10.306	12.592	96	50	4577.86	4218.37	5017.48	Channel	1	mg/L-TDS
Burnupia stenochorias	NaCl	2003-11-13	320	Kat River	9.316	12.592	96	50	660.058	613.135	717.918	Channel	1	mS/m
Burnupia stenochorias	Na_2SO_4	2003-11-13	321	Kat River	7.124	11.07	96	50	5272.404	4836.43	5800.604	Channel	1	mg/L
Burnupia stenochorias	Na_2SO_4	2003-11-13	321	Kat River	7.012	11.07	96	50	6111.698	5630.71	6699.364	Channel	1	mg/L-TDS
Burnupia stenochorias	Na_2SO_4	2003-11-13	321	Kat River	6.649	11.07	96	50	610.801	570.363	658.413	Channel	1	mS/m

TABLE B.9 ***Burnupia stenochorias*** **acute $K_2Cr_2O_7$ toxicity test results. Expt ID refers to the UCEWQ database entry number. Source indicates the origin of the test organisms. EC: Effective concentration; LCL: lower confidence level, UCL: upper confidence level; Diluent: 1 = dechlorinated tap water.**

Species	Date	Expt ID	Source	Duration Hrs	EC	Conc	%Trim	LCL	UCL	Stream Type	Diluent	Unit
Burnupia stenochorias	1998-09-14	52	Botha River	96	50	4.17	40	0.6	28.94	Bucket	1	mg/L
Burnupia stenochorias	1998-09-14	52	Botha River	96	50	8.71	30	3.49	21.76	Bucket	1	mg/L
Burnupia stenochorias	1998-09-14	52	Botha River	96	50	8.82	20	5.98	13.01	Bucket	1	mg/L
Burnupia stenochorias	1998-09-23	53	Kubusi River	96	50	14.35	33.3	5.8	35.5	Bucket	1	mg/L
Burnupia stenochorias	1998-09-23	53	Kubusi River	96	50	8.31	22.2	4.31	16.01	Bucket	1	mg/L
Burnupia stenochorias	1998-09-23	53	Kubusi River	96	50	7.45	22.2	3.68	15.1	Bucket	1	mg/L
Burnupia stenochorias	1999-02-16	50	Botha River	96	50	9.78	10	6.25	15.3	Bucket	1	mg/L
Burnupia stenochorias	1999-02-16	50	Botha River	96	50	9.87	0	6.25	15.3	Bucket	1	mg/L
Burnupia stenochorias	1999-02-16	50	Botha River	96	50	13.83	22.2	7.89	24.25	Bucket	1	mg/L

Species	Date	Expt ID	Source	Duration Hrs	EC	Conc	%Trim	LCL	UCL	Stream Type	Diluent	Unit
Burnupia stenochorias	1999-08-17	49	Botha River	96	50	17.01	5	11.89	24.33	Bucket	1	mg/L
Burnupia stenochorias	1999-08-17	49	Botha River	96	50	17.04	10	10.99	26.4	Bucket	1	mg/L
Burnupia stenochorias	1999-08-17	49	Botha River	96	50	15.38	20	8.6	27.52	Bucket	1	mg/L

TABLE B.10 ***Burnupia stenochorias*** **acute aquarium salts toxicity test results. Expt ID refers to the UCEWQ database entry number. Source indicates the origin of the test organisms. EC: Effective concentration; LCL: lower confidence level, UCL: upper confidence level; Diluent: 1 = dechlorinated tap water.**

Species	Date	Expt ID	Source	Duration hrs	EC	Conc	%Trim	LCL	UCL	Stream Type	Diluent	Unit
Burnupia stenochorias	2002-07-30	229	Botha River	96	50	1145.1	5.19	1023.3	1281.3	Channel	1	mS/m
Burnupia stenochorias	2002-07-30	229	Botha River	96	50	6967.8	5.19	6127.8	7923.1	Channel	1	mg/L
Burnupia stenochorias	2002-07-30	244	Botha River	24	50	7610.4	0	7254.7	7983.7	Aquarium	1	mg/L
Burnupia stenochorias	2002-07-30	244	Botha River	96	50	1228.5	0			Aquarium	1	mS/m
Burnupia stenochorias	2002-07-30	244	Botha River	96	50	7746	0			Aquarium	1	mg/L

TABLE B.11 ***Daphnia pulex*** **acute toxicity test results for NaCl and Na_2SO_4. Expt ID refers to the UCEWQ database entry number. EC: Effective concentration; LCL: lower confidence level, UCL: upper confidence level; Diluent: 1 = reconstituted laboratory tap water.**

Species	Toxicant	Date	Expt ID	Chi Square Calculated	Chi Square Tabulated	Duration hrs	EC	Conc	LCL	UCL	Diluent	Unit
Daphnia pulex	NaCl	2001-01-24	166	5.455	12.592	48	50	2925.942	2868.401	2989.283	1	mg/L
Daphnia pulex	NaCl	2001-01-24	167	1.089	9.488	24	50	3827.209	3669.686	3979.08	1	mg/L
Daphnia pulex	NaCl	2001-10-31	202	0.709	7.815	24	50	4072.337	3935.797	4203.329	1	mg/L
Daphnia pulex	Na_2SO_4	2002-04-03	207	10.243	9.488	48	50	609.578	56.341	10359.548	1	mg/L

Species	Toxicant	Date	Expt ID	Chi Square Calculated	Chi Square Tabulated	Duration hrs	EC	Conc	LCL	UCL	Diluent	Unit
Daphnia pulex	Na_2SO_4	2002-04-05	208	12.834	11.07	48	50	3445.655	2670.915	4427.773	1	mg/L
Daphnia pulex	Na_2SO_4	2002-04-07	209	29.339	16.919	48	50	2565.354	1217.701	15873.881	1	mg/L
Daphnia pulex	Na_2SO_4	2002-07-09	237	15.137	14.067	48	50	3268.724	2801.361	3748.716	1	mg/L
Daphnia pulex	Na_2SO_4	2005-02-02	363	7.135	9.488	48	50	2413.989	2235.227	2571.633	1	mg/L
Daphnia pulex	Na_2SO_4	2005-02-02	363	3.997	9.488	48	50	295.542	282.272	308.214	1	mS/m
Daphnia pulex	Na_2SO_4	2005-02-09	361	1.051	11.07	48	50	2083.141	1938.473	2217.25	1	mg/L
Daphnia pulex	Na_2SO_4	2005-04-11	390	13.102	7.815	48	50	1757.498	0	0	1	mg/L
Daphnia pulex	Na_2SO_4	2005-04-18	389	31.234	16.919	48	50	0.048	0	0	1	mg/L
Daphnia pulex	Na_2SO_4	2005-08-16	391	13.544	9.488	48	50	1616.569	598.825	23851.061	1	mg/L

TABLE B.12 *Daphnia pulex* $CdCl_2$ acute toxicity test results. Expt ID refers to the UCEWQ database entry number. EC: Effective concentration; LCL: lower confidence level, UCL: upper confidence level; Diluent: 1 = reconstituted laboratory tap water.

Species	Date	Expt ID	Chi Square Calculated	Chi Square Tabulated	Duration hrs	EC	Conc	LCL	UCL	Diluent	Unit
Daphnia pulex	2001-04-10	273	11.623	9.488	48	50	0.086	0.007	0.133	1	mg/L
Daphnia pulex	2001-05-29	274	2.32	9.488	48	50	0.149	0.137	0.161	1	mg/L
Daphnia pulex	2001-06-28	275	9.133	9.488	48	50	0.131	0.117	0.145	1	mg/L
Daphnia pulex	2001-08-08	276	3.115	9.488	48	50	0.104	0.089	0.116	1	mg/L
Daphnia pulex	2001-11-13	277	0.42	9.488	48	50	0.134	0.122	0.147	1	mg/L
Daphnia pulex	2002-02-13	278	3.165	9.488	48	50	0.14	0.127	0.153	1	mg/L
Daphnia pulex	2002-05-15	279	0.93	9.488	48	50	0.146	0.134	0.159	1	mg/L
Daphnia pulex	2002-08-20	240	107.947	7.815	48	50	0.162	0	0	1	mg/L
Daphnia pulex	2002-08-20	241	2.124	9.488	48	50	0.148	0.136	0.162	1	mg/L
Daphnia pulex	2002-11-13	280	2.536	9.488	48	50	0.131	0.117	0.144	1	mg/L

Species	Date	Expt ID	Chi Square Calculated	Chi Square Tabulated	Duration hrs	EC	Conc	LCL	UCL	Diluent	Unit
Daphnia pulex	2003-02-20	281	0.658	9.488	48	50	0.133	0.117	0.147	1	mg/L
Daphnia pulex	2003-05-07	282	1.406	9.488	48	50	0.127	0.114	0.138	1	mg/L
Daphnia pulex	2003-08-11	302	1.408	9.488	48	50	0.149	0.135	0.165	1	mg/L
Daphnia pulex	2003-11-03	360	2.392	9.488	48	50	0.129	0.118	0.139	1	mg/L
Daphnia pulex	2004-02-14	340	1.109	9.488	48	50	0.085	0.072	0.097	1	mg/L
Daphnia pulex	2005-02-07	362	33.545	12.592	48	50	0.132	0.041	0.176	1	mg/L
Daphnia pulex	2005-05-16	374	87.955	12.592	48	50	0.182	0	0	1	mg/L
Daphnia pulex	2005-08-09	377	3.837	9.488	48	50	0.125	0.118	0.132	1	mg/L

TABLE B.13 *Daphnia pulex* effluents acute toxicity test results. Expt ID refers to the UCEWQ database entry number. EC: Effective concentration; LCL: lower confidence level, UCL: upper confidence level; Diluent: 1 = reconstituted laboratory tap water; 2 = river water.

Species	Toxicant	Date	Expt ID	Chi Square Calculated	Chi Square Tabulated	Duration hrs	EC	Conc	LCL	UCL	Diluent	Unit
Daphnia pulex	General Textile Effluent	1998-11-17	137	4.964	12.592	48	50	8.288	6.69	10.263	1	Percent
Daphnia pulex	Artificial Metal Plating Industry Effluent	1999-05-06	151	0.355	14.067	48	50	0.412	0.301	0.522	1	Percent
Daphnia pulex	Artificial Metal Plating Industry Effluent	1999-05-13	153	0.6	12.592	48	50	0.208	0.151	0.289	1	Percent
Daphnia pulex	Artificial Metal Plating Industry Effluent	1999-05-13	154	214.9	12.592	48	50	0.83	0	0	2	Percent
Daphnia pulex	Artificial Metal Plating Industry Effluent	1999-05-16	156	17.32	14.067	48	50	1.239	0.706	2.748	2	Percent
Daphnia pulex	Detergent	2000-07-07	44	0.573	11.07	48	50	42.264	31.79	57.07	1	mg/L
Daphnia pulex	Citrus Agriculture Effluent 2	2003-04-02	310	3.778	7.815	48	50	1.286	0.713	2.281	1	Percent

Species	Toxicant	Date	Expt ID	Chi Square Calculated	Chi Square Tabulated	Duration hrs	EC	Conc	LCL	UCL	Diluent	Unit
Daphnia pulex	Citrus Agriculture Effluent 1	2003-04-09	309	6.405	7.815	48	50	0.175	0.139	0.22	1	Percent
Daphnia pulex	Citrus Agriculture Effluent 2	2003-04-09	311	0.035	7.815	48	50	0.815	0.677	1	1	Percent
Daphnia pulex	Citrus Agriculture Effluent 3	2003-04-09	313	0.894	5.991	48	50	0.008	0	0.018	1	Percent
Daphnia pulex	Tannery Soaking Effluent	2003-05-06	284	3.72	9.488	48	50	0.078	0.029	0.157	1	Percent
Daphnia pulex	Tannery Liming Effluent	2003-05-08	285	0.009	9.488	48	50	0.218	0.131	0.379	1	Percent
Daphnia pulex	Tannery Final 1 Effluent	2003-06-16	290	11.195	9.488	48	50	17.658	0	0	1	Percent
Daphnia pulex	Tannery Final 2 Effluent	2003-06-18	291	N/a	N/a	48	50	17.78	11.39	27.77	1	Percent
Daphnia pulex	Tannery Bleaching Effluent	2003-06-19	286	N/a	N/a	48	50	0.06	0.04	0.09	1	Percent
Daphnia pulex	Tannery Degreasing Effluent	2003-06-20	287	0.097	9.488	48	50	0.028	0.016	0.051	1	Percent
Daphnia pulex	Tannery Tanning Effluent	2003-06-24	288	N/a	N/a	48	50	0.5	0.33	0.76	1	Percent
Daphnia pulex	Tannery Colouring Effluent	2003-06-25	289	N/a	N/a	48	50	2.82	2.24	3.54	1	Percent
Daphnia pulex	Tannery Soaking Effluent	2003-07-08	292	285.788	11.07	48	50	0.45	0	0	1	Percent
Daphnia pulex	Tannery Liming Effluent	2003-07-10	293	0.044	11.07	48	50	0.224	0.16	0.313	1	Percent
Daphnia pulex	Tannery Bleaching Effluent	2003-08-16	294	12.548	11.07	48	50	0.045	0.01	0.099	1	Percent
Daphnia pulex	Tannery Degreasing Effluent	2003-08-18	295	0	11.07	48	50	0.008	0.006	0.011	1	Percent
Daphnia pulex	Tannery Tanning Effluent	2003-08-19	296	0.719	11.07	48	50	0.377	0.31	0.473	1	Percent
Daphnia pulex	Tannery Colouring Effluent	2003-08-20	297	6.073	11.07	48	50	2.167	1.815	2.691	1	Percent
Daphnia pulex	Tannery Final 1 Effluent	2003-08-21	298	0.22	11.07	48	50	8.777	4.127	11.553	1	Percent
Daphnia pulex	Tannery Final 2 Effluent	2003-08-22	300	13.812	11.07	48	50	1.804	0.029	4.925	1	Percent
Daphnia pulex	Tannery Final 2 Effluent	2003-09-10	301	0	9.488	48	50	12.513	11.35	13.786	1	Percent
Daphnia pulex	Tannery Final 1 Effluent	2003-09-12	299	3.767	9.488	48	50	16.951	14.1	20.109	1	Percent
Daphnia pulex	Sewage	2004-04-26	346	0.795	7.815	48	50	23.864	19.99	28.483	1	Percent
Daphnia pulex	Sewage	2004-05-16	347	0.369	5.991	48	50	14.18	6.946	19.187	1	Percent
Daphnia pulex	Sewage	2004-06-09	348	19.63	7.815	48	50	31.245	0	0	1	Percent

Species	Toxicant	Date	Expt ID	Chi Square Calculated	Chi Square Tabulated	Duration hrs	EC	Conc	LCL	UCL	Diluent	Unit
Daphnia pulex	Sewage	2004-06-17	349	63779	5.991	48	50	37.784	0	0	1	Percent
Daphnia pulex	Sewage	2004-08-31	353	8.563	7.815	48	50	42.202	0	0	1	Percent
Daphnia pulex	Sewage	2004-09-01	351	6.684	7.815	48	50	30.024	23.87	37.906	1	Percent
Daphnia pulex	Sewage	2004-09-06	352	3.877	5.991	48	50	0	0	0	1	Percent
Daphnia pulex	Sewage	2004-09-06	354	1.124	7.815	48	50	30.953	0	0	1	Percent
Daphnia pulex	General Textile Effluent	2005-09-15	378	N/a	N/a	48	No mortalities	N/a	N/a	N/a	1	Percent
Daphnia pulex	General Textile Effluent	2005-09-21	379	N/a	N/a	48	No mortalities	N/a	N/a	N/a	1	Percent

TABLE B.14 *Daphnia pulex* NaCl chronic toxicity test results. LOEC: Lowest Observed Effect Concentration.

Species	Date	Duration	Conc range	Test completed	NOEC	Unit
Daphnia pulex	2004-09-20	21 days	0, 50, 100, 200, 300, 400, 500	Yes	< 50 (21 days) with high degree of uncertainty	mg/L
Daphnia pulex	2005-07-06	9 days	0, 100, 200, 300, 400, 500	No		mg/L
Daphnia pulex	2005-06-28	6 days	0, 50, 100, 200, 300, 400, 500	No		mg/L

TABLE B.15*Daphnia pulex* Na_2SO_4 chronic toxicity test results. LOEC: Lowest Observed Effect Concentration.

Species	Date	Duration	Conc range	Test completed	NOEC	Unit
Daphnia pulex	2005-08-16	12 days	0, 46.875, 93.75, 187.5, 375, 750, 1500, 3000	No		mg/L
Daphnia pulex	2005-04-04	4 days	0, 50, 100, 200, 300, 400, 500	No		mg/L
Daphnia pulex	2005-04-11	4 days	0, 50, 100, 200, 300, 400, 500	No		mg/L

TABLE B.16 *Daphnia pulex* $CdCl_2$ chronic toxicity test results. LOEC: Lowest Observed Effect Concentration.

Species	Date	Duration	Conc range	Test completed	NOEC	Unit
Daphnia pulex	2005-07-26	21 days	0, 0.006, 0.013, 0.025, 0.05, 0.1	Yes	< 0.006 (21 days)	mg/L

Figures for results of experiments of *Caridina nilotica* exposure to Na_2SO_4, NaCl and $CdCl_2$

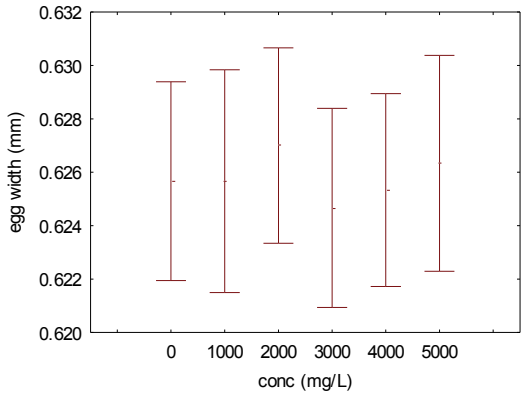

FIGURE A **Mean egg width (mm) for the embryos of *C. nilotica* at 72 hours, shows there is no difference between the concentrations of Na_2SO_4 and the control. Lines show the standard deviations of the egg length in the different concentrations.**

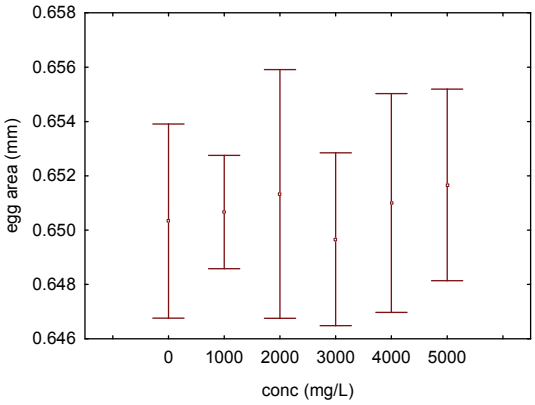

FIGURE B **Mean egg area (mm) for the embryos of *C. nilotica* at 72 hours, shows there is no difference between the concentrations of Na_2SO_4 and the control. Lines show the standard deviations of the egg length in the different concentrations.**

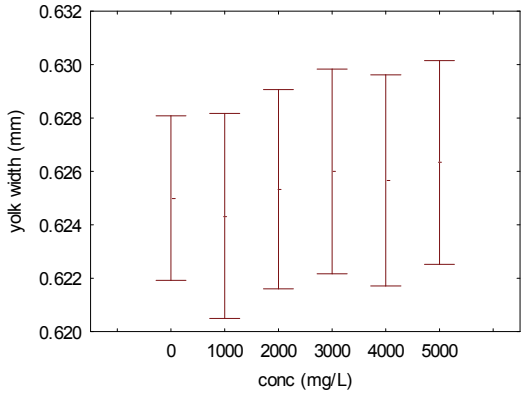

FIGURE C **Mean yolk width (mm) for the embryos of *C. nilotica* at 72 hours, shows there is no difference between the concentrations of Na_2SO_4 and the control. Lines show the standard deviations of the egg length in the different concentrations**.

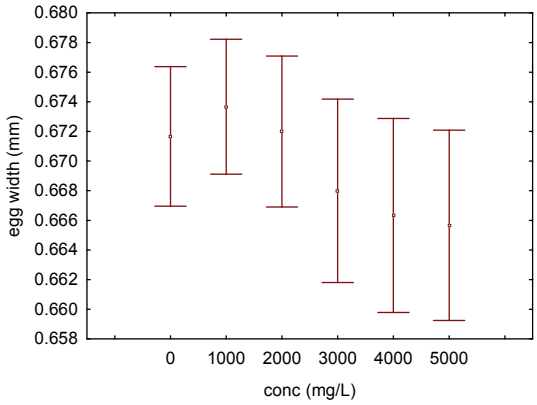

FIGURE D **Mean egg width (mm) for the embryos of *C. nilotica* at 144 hours, shows a decrease in egg width at 3000 mg/L to 6000 mg/L of Na_2SO_4. Lines show the standard deviations of the egg width in the different concentrations**

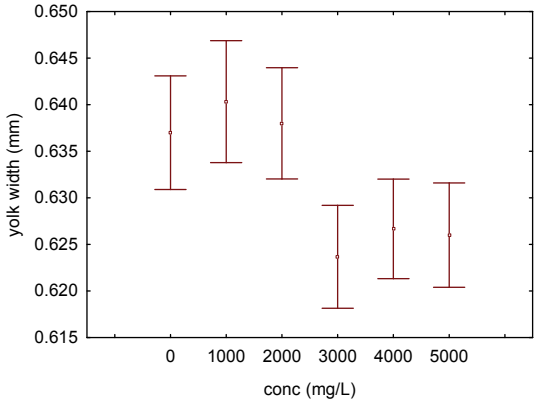

FIGURE E **Mean yolk width (mm) for the embryos of *C. nilotica* at 144 hours, shows a decrease in egg width at 3000 mg/L to 6000 mg/L of Na_2SO_4. Lines show the standard deviations of the yolk width in the different concentrations**

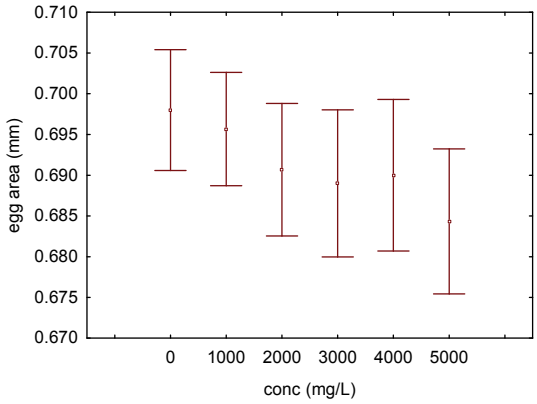

FIGURE F **Mean egg area (mm) for the embryos of *C. nilotica* at 144 hours, shows a decrease in egg width at 3000 mg/L to 6000 mg/L of Na_2SO_4. Lines show the standard deviations of the egg area in the different concentrations**

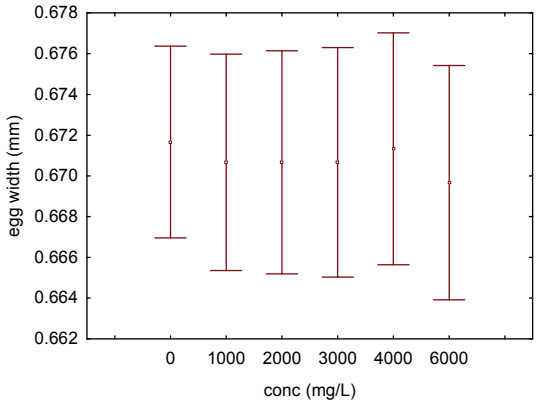

FIGURE G **Mean egg width (mm) of embryos of *C. nilotica* at 144 hours, shows there is no difference in egg width between concentrations of NaCl and control. Lines show the standard deviations of the egg width in the different concentrations**

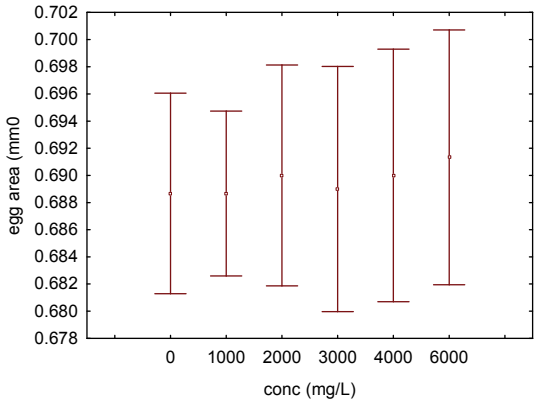

FIGURE H **Mean egg area (mm) of embryos of *C. nilotica* at 144 hours, shows there is no difference in egg area between concentrations of NaCl and control. Lines show the standard deviations of the egg area in the different concentrations**

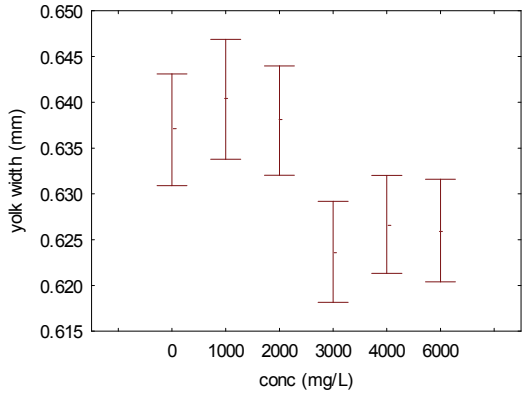

FIGURE I **Mean yolk width (mm) of embryos of *C. nilotica* at 216 hours, shows a decrease in yolk width at 3000 mg/L to 6000 mg/L of NaCl. Lines show the standard deviations of the yolk width in the different concentrations**

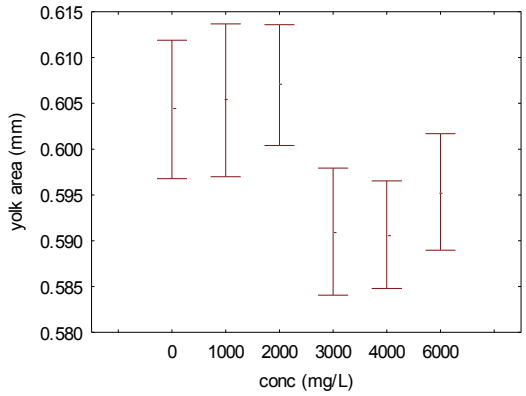

FIGURE J **Mean yolk area (mm) of embryos of *C. nilotica* at 216 hours, shows a decrease in yolk area at 3000 mg/L to 6000 mg/L of NaCl. Lines show the standard deviations of the yolk area in the different concentrations**

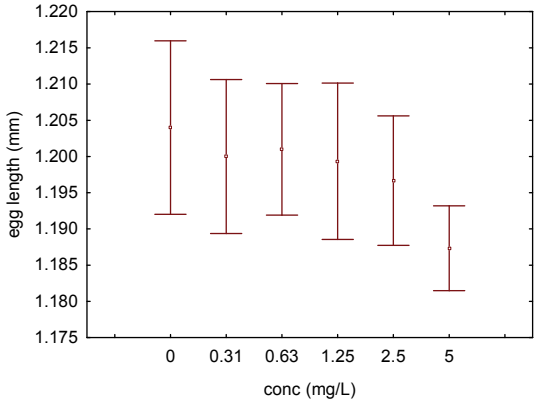

FIGURE K **Mean egg length (mm) of embryos of *C. nilotica* at 24 hours, shows a decrease in egg length at 5 mg/L of $CdCl_2$. Lines show the standard deviations of the egg length in the different concentrations**

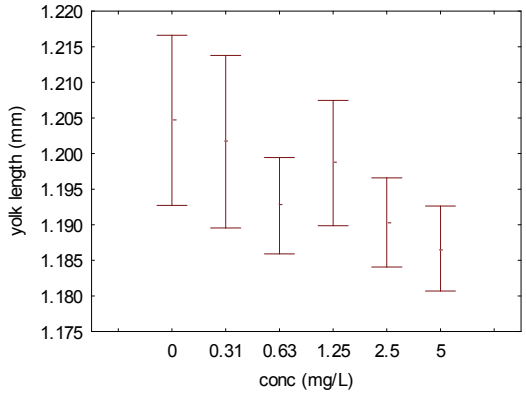

FIGURE L **Mean yolk length (mm) of embryos of *C. nilotica* at 24 hours, shows a decrease in yolk length at 0.63 mg/L to 5 mg/L of $CdCl_2$. Lines show the standard deviations of the yolk length in the different concentrations**

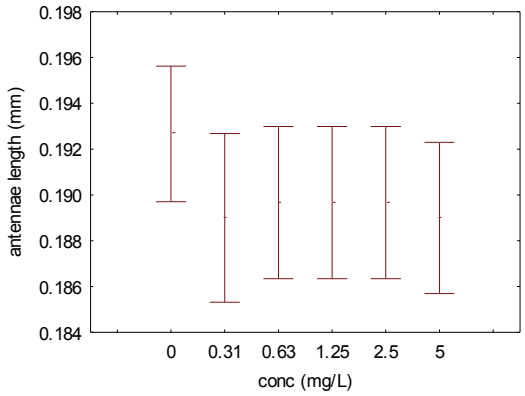

FIGURE M **Mean antennae length (mm) of embryos of *C. nilotica* at 72 hours, shows a decrease in antennae length in all concentrations of $CdCl_2$. Lines show the standard deviations of the antennae length in the different concentrations**

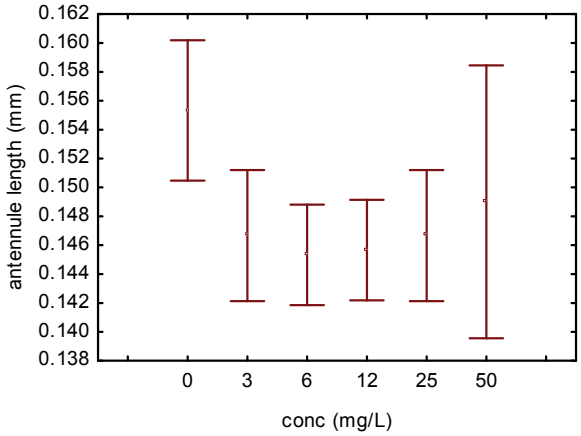

FIGURE N **Mean antennule length (mm) of embryos of *C. nilotica* at 144 hours, shows a decrease in antennule length in all concentrations of $CdCl_2$. Lines show the standard deviations of the antennule length in the different concentrations**