GENERATION OF NEW ECOTOXICITY DATA FOR SALTS USING INDIGENOUS SOUTH AFRICAN FRESHWATER MACROINVERTEBRATE: UPDATING THE NATIONAL SALTS TOXICITY DATABASE

Report to the

WATER RESEARCH COMMISSION

by

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The publication of this report emanates from a project entitled *Generation of new ecotoxicity data for salts using indigenous South African freshwater invertebrate: Updating the national salts toxicity database* (WRC Project No. K8/1075)

EXECUTIVE SUMMARY

BACKGROUND

Scherman and Palmer (2013) reviewed the historical and current trends of Environmental Water Quality (EWQ) in South Africa. Based on the review, they identified research gaps for which they proposed a co-ordinated set of projects that need to be commissioned and executed to fill these gaps. These co-ordinated set of projects include update of TEACHA (Tool for Ecological Aquatic Chemical Habitat Assessment); update of the national salt toxicity database; integration of Resource Directed Measures (RDM) components (i.e. Ecological Reserve, Resource Quality Objectives (RQOs) and Classification); integrating water quality and quantity; evaluation of the RDM participatory process based on research and current understandings of complex social-ecological systems and ecosystem services; and integrating RDM and SDC (Source Direct Control) to ensure coherent link between the two. The overarching aim of the above set of projects is to support implementation of the National Water Resource Strategy 2 (NWRS2). Therefore, the main objective of this project to contribute to addressing the second research gap listed above (i.e. updating the national salt toxicity database). Noting that the database contains only data on single salts with nothing on salt mixtures, the project also generated binary salt mixtures data for the database, in addition to generating data for single salts.

RATIONALE

Salinisation is an important problem facing freshwater resource managers in South Africa. Data on macroinvertebrate responses to salts strongly informed water quality management strategies but the national salts toxicity database has been not updated for over a decade. Additionally, upsurge of complex chemical mixtures in the environment in recent years meant that the call to update the database was very important for freshwater protection.

OBJECTIVES AND AIMS

1. Generation of short-term and long-term toxicity data for single salts.
2. Generation of short-term and long-term toxicity data for binary salt mixture.
3. Development of a procedure for salt mixtures exposure experiment.
4. Update the national salt toxicity database by incorporating the new dataset.

METHODOLOGY

Key toxicological importance major salts (TIMS) including magnesium sulphate ($MgSO_4$), magnesium chloride ($MgCl_2$), and sodium sulphate (Na_2SO_4), as well as binary mixtures of NaCl+Na_2SO_4, $MgCl_2$+$MgSO_4$, NaCl+$MgSO_4$ and $MgCl_2$+Na_2SO_4 were exposed to juvenile and adult stages of the indigenous South African freshwater shrimp *Caridina nilotica*. Short-term lethal tests (96 h) and long-term lethal tests (240 h) static experimental methods were used to determine the lethal concentration values of the test salts for juvenile and adult shrimps. Based on the principles, theories and outcome of the binary mixture experiments, a procedure for conducting salt mixture experiments was developed. The mortality data for both 96 and 240 h exposure tests were used to estimate LC50 values for the various salts and salt mixtures.

RESULTS AND DISCUSSION

For single salt data, juvenile *C. nilotica* 96 h LC50 values for $MgSO_4$, $MgCl_2$ and Na_2SO_4 were 10.80 (8.06-14.59), 1.67 (1.34 -2.09) and 1.76 (1.39-2.24) g/L, respectively; while adult *C. nilotica* 96 h LC50 values for $MgSO_4$, $MgCl_2$ and Na_2SO_4 were 11.57 (5.43-50.93), 6.48 (5.31-7.91) and 2.06 (1.73-2.45) g/L, respectively. Similarly, single salt data for juvenile *C. nilotica* 240 h LC50 values for $MgSO_4$, $MgCl_2$ and Na_2SO_4 were 4.85 (3.61 -6.17), 0.99 (0.47-1.99) and 0.77 (0.35-1.63) g/L, respectively; while adult *C. nilotica* 240 h LC50 values for $MgSO_4$, $MgCl_2$ and Na_2SO_4 were 3.60 (1.19-13.28), 4.61 (3.79-5.59) and 0.82 (0.30-2.10) g/L, respectively.

For binary salt mixture data, juvenile *C. nilotica* 96 h LC50 values for $MgCl_2$+$MgSO_4$, NaCl+Na_2SO_4, $MgCl_2$+Na_2SO_4 and NaCl+$MgSO_4$ were 1.76 (1.39-2.24), 2.56 (2.18-3.02), 7.34 (2.48-39.84) and 7.06 (5.73-8.73) g/L, respectively; while adult *C. nilotica* 96 h LC50 values for $MgCl_2$+$MgSO_4$, NaCl+Na_2SO_4, $MgCl_2$+Na_2SO_4 and NaCl+$MgSO_4$ were 7.26 (1.95-54.16), 3.67 (0.00-0.00), 8.39 (4.50-17.20) and 7.94 (5.04-12.82), respectively. Similarly, binary salt mixture data for juvenile *C. nilotica* 240 h LC50 values for $MgCl_2$+$MgSO_4$, NaCl+Na_2SO_4, $MgCl_2$+Na_2SO_4 and NaCl+$MgSO_4$ were 0.72 (0.31-1.58), 1.98 (1.46-2.67), 2.66 (0.00-0.00) and 3.95 (3.31-4.72) g/L, respectively; while adult *C. nilotica* 240 h LC50 values for $MgCl_2$+$MgSO_4$, NaCl+Na_2SO_4, $MgCl_2$+Na_2SO_4 and NaCl+$MgSO_4$ were 0.80 (0.21-1.49), 1.90 (0.00-0.00), 2.26 (0.79-5.46) and 2.58 (2.25-2.96), respectively.

In summary, conducting a binary salt mixture experiments may be done according to the following procedure:

1. Determining what type of binary mixture experiment to do base on similar or dissimilar cations of the single salts involved.
2. Determining the concentrations of binary salt mixtures by determination of LC50s separately for single salts in a binary salt mixture.
3. Determination of the relative toxic unit (RTU) of the mixture using the LC50s of the two salts by calculating and adding the relative toxic fractions (RTFs).
4. Estimation of concentration range and proportion of individual single salts in the salt mixture.
5. Apply standard exposure methods such as 96 h static non-renewal for short-term and 240 h static renewal for long-term.

CONCLUSIONS

Toxicity data for both single and binary salt mixture were generated and attached as appendices to this report. Data generated are attached as appendices to this report and ready to be added to the national salt toxicity database host by the Unilever Centre for Environmental Water Quality, Institute for Water Research, Rhodes University.

RECOMMENDATIONS FOR FUTURE RESEARCH

It is recommended that there should be further research to include more single salts and salt mixtures (binary, ternary and quaternary) data in the database. This must involve other freshwater macroinvertebrates and other taxonomic groupings. The possibility of making the national salt toxicity database more accessible to local and international communities should be considered. The application of these data in RDM and SDC processes should also be considered in other related WRC projects.

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LIST OF ABBREVIATIONS

AMD:	Acid mine drainage
DWA:	Department of Water Affairs
EWQ:	Environmental Water Quality
IUA:	Integrated Units of Analysis
IWR:	Institute for Water Research
LC:	Lethal concentration
LC50:	Midian lethal concentration
MOA:	Mechanism of action
MoA:	Mode of action
NOAEL:	No Observable Adverse Effect Level
NWRS2:	National Water Resource Strategy 2
OECD:	Organisation for Economic Cooperation and Development
RDM:	Resource Directed Measure
RGR:	Relative growth rate
RQO:	Resource Quality Objective
RQS:	Resource Quality Services
RTF:	Relative toxic fractions
RTFs:	Relative toxic fractions
RTU:	Relative toxic unit
SDC:	Source Direct Control
SSD:	Species sensitivity distributions
TEACHA:	Tool for Ecological Aquatic Chemical Habitat Assessment
TIMS:	Toxicological importance major salts
TNP:	Towards a New Paradigm
UCEWQ:	Unilever Centre for Environmental Water Quality
USEPA:	United States Environmental Protection Agency
WRC:	Water Research Commission

1 INTRODUCTION

1.1 Background

Scherman and Palmer (2013) reviewed the historical and current trends of Environmental Water Quality (EWQ) in South Africa. Based on the review, they proposed a co-ordinated set of projects as enumerated below, as gaps to be filled:

A. *TEACHA functionality update*

TEACHA (Tool for Ecological Aquatic Chemical Habitat Assessment) programme should be revised so that it is usable on a generally accessible platform with Department of Water Affairs (DWA) water quality data. At present it requires MATLAB which is expensive, technically demanding, and therefore not generally accessible. This is a necessary first step to update the water quality methods within an ecological Reserve determination. This will require collaboration between DWA's division of Resource Quality Services (RQS) and EWQ researchers.

B. Salt toxicity update

That since TEACHA is premised on ion and salt toxicity, there is a need to update the salt toxicity data used in the programme, and to assess the need for additional ecotoxicity experiments using local species. This would enable an understanding of the ecotoxicity of particular ions that are currently thought to be under- or over-estimated by TEACHA (for example magnesium, potassium and sulphate). This project would then feed into the update of TEACHA (Project 1). Project 1 and 2 will result in a validated use of TEACHA.

C. Integration of RDM components

The application of the methods and procedures for Resource Directed Measures (RDM) components have evolved at different times and attention has not been paid to integrating their premises, or the implications for the resulting practice. Issues include, for example, the up-scaling from resource units, which have Resource Quality Objectives (RQOs), to the Integrated Units of Analysis (IUA), which have management classes and an unclear way of amalgamating, or prioritising the RQO's within and IUA. There are no guidelines for such prioritisation which would need to take into account a consideration of the role of refugia, and the possibility decisions not to rehabilitate. This project should include a set of national workshops to canvass practice-based experience, and must include the active participation of the RDM Chief Directorate.

D. Integrating Water quality and quantity

There has been a long standing call for the integration of water quality and flow in RDM (and SDC) processes. Currently there is research on the development of user-friendly water quality/quantity models. These need to be fast-tracked into RDM processes and into robust, transparent, meaningful stakeholder participation in during the RDM processes.

E. RDM participatory processes

Participatory processes are important and challenging. There needs to be research-based evaluation of current participatory process. This can draw on the WRC initiative for social science in water research, and will result in more theoretically supported participatory processes that are integrated, transparent and robust. Current understandings of complex social-ecological systems and ecosystem services will be included in this. The concept of ecosystem services and benefits can act to mediate social and ecological values.

F. Integrating RDM and SDC

To date there is little connection in ensuring coherent links between EWQ RDM and SDC measures. A Unilever Centre for Environmental Water Quality (UCEWQ) MSc student recently completed the first empirical study to link waste water treatment works with licence requirements and green drop performance with in-stream river health – a useful starting point. This task needs to be tackled at a range of levels through policy, legislation, governance, and practice – supported by research. The project will include all work to date on the Waste Discharge Charge System.

Justification

The overarching aim of the above set of projects is to support implementation of the National Water Resource Strategy 2 (NWRS2). Projects A and B will specifically support the implementation of NWRS2 by fulfilling principle 1 of the NWRS2, which is protection of the resources through classification of the resource with the Reserve as a priority (DWA, 2013). This principle recognises using the gazetted classification process to classify all major rivers, wetlands and aquifers as critical resource protection activity that needs to be undertaken in the next five years. Resource classification draws on knowledge about ecological and societal water needs that is quantified and described through Reserve determinations and setting RQOs. Projects A and B will develop a sound scientific basis for all the RDM components to work together in an integrative manner, as determination of water quality components of the ecological Reserve and RQOs depend on these Projects, which support Project C).

Thus, the first three projects clearly support and give justification to EWQ RQO's necessary for transparent stakeholder participation, which is Principle 2 of the NWRS2. It is important to observe that successes of Projects D-F clearly depend on successful completion of Projects A-C.

Projects A-F should contribute to integration into the Towards a New Paradigm (TNP) for IWRM process. If these projects are undertaken with and understanding of complex social ecological systems in an integrated way, taking a systems approach – this will support and feed into and support the TNP project (WRC proposal 1003122).

Since Scherman and Palmer (2013) proposal for the update of TEACHA, recent discussions with the main author of TEACHA, Dr Sebastian Jooste of the Water Quality Services division of the Department of Water Affairs, suggested that updating of the tool on the basis of the current form is probably not a good idea. This is because the basic principles upon which TEACHA was built were not given much thought at the time it was authored. Therefore, it would not seem appropriate to build on it. Based on this information, it was important to seek new ways of studying salts and ions toxicity. The salt mixture procedure developed in this study aims to contribute to this effect.

1.2 Rationale

Salinisation is an important problem facing freshwater resource managers in South African. Data on macroinvertebrate responses to salts strongly informed water quality management strategies. The development of a salinity ecotoxicity database (Palmer et al., 2004) focussed on using NaCl as a model for agriculturally-related salinisation because of the dominance of Na+ and Cl^- ions; and on using Na_2SO_4 as model for mining related salinisation because of elevated $SO4^{2-}$ ions. The database also includes results of exposure to $MgSO_4$, CaSO4, salt mixtures and saline effluents. Such an ecotoxicity database is valuable resource for the derivation of salt-specific species sensitivity distributions (SSDs), a very important water resource management tool. However, not many salts are included in the database (Palmer et al., 2004), and it has not seen any update since 2004 when it was first set up. The review by Scherman and Palmer (2013) proposed generation of new toxicity data for salts and subsequent update of the national salt toxicity database as key research projects.

More importantly, there is no existing data on the binary effect of salt mixtures (e.g. the combined effect of NaCl and Na_2SO_4) on any indigenous species. This is probably because there is no locally based methodology written for that purpose. This proposal seeks to address these gaps.

1.3 Aims and objectives

The aim of the study is to generate new ecotoxicity data for selected salts using indigenous South African species so as to use the data to update the national salt toxicity database. Specific objectives include:

- Expose of the test organisms to the test salts and use lethality data to calculate LC50 values.
- Compare the LC50 values obtained from acute and chronic tests.
- Obtain first ever set of data for a salt mixture exposure tests using South African indigenous species.
- Write a procedure for salt mixtures exposure tests.
- Update the national salt toxicity database by incorporating the new dataset.

2 SALTS AS SOURCE OF FRESHWATER POLLUTION

2.1 Salinisation of South African freshwater resources

South Africa is a semi-arid, water-stressed country so management of water pollution is of paramount concern. The climate varies from desert and semi-desert in the west, to sub-humid along the eastern coastal area. The average annual rainfall is 450 mm, which is below the world's average of 860 mm per annum, while evaporation is comparatively high (DWAF, 2004). The association of arid and semi-arid areas with high rates of salinisation is a common phenomenon, especially where these regions are associated with shallow, saline water tables (Jorenush and Sepaskhah, 2003; Shanyengana and Sanderson, 2004). Therefore, it is likely for different areas of South Africa to experience various degrees of salinisation since the country is typically characterised by large semi-arid areas.

Factor that affect the rate of salinisation include various environmental components such as annual rainfall, ratio of precipitation to evaporation, groundwater hydrology and surface run-off. Seasonal and inter-annual variations in climate are also major drivers of solute concentrations in rivers (Interlandi and Crockett, 2003). South Africa's climate and landscape exacerbate the process of salinisation due to high evaporation-precipation ratios and low run-off-rainfall ratios. The country's runoff coefficient is only 10 %, which means that 90 % of rainfall is lost through evapotranspiration. Some of South Africa's rivers, large dams, canals and farm dams experience salinisation due to evaporative losses from surface waters.

The process of salinisation can also be driven by the nature of geological formations of a particular area. The mass and type of mineral dissolved in solution depends on geochemical characteristics of soil and surrounding environment. Limestone bedrock and other calcareous sedimentary deposits can contribute to increasing solutes (Interlandi and Crockett, 2003). Decomposed shales release high concentrations of sodium, chloride and sulphate ions, while decomposed dolomites contribute principally calcium, magnesium and carbonate ions (Loewenthal, 1995). The mixing of leachates with formation water varies, depending on hydrological conditions (Farber et al., 2004). In South Africa, many water bodies are naturally high in dissolved salts, especially where rivers flow over old marine sediments such as the Karoo series (O'Keeffe et al., 1992). Dominance of specific ions is correlated with geographical patterns. For example, ground waters of much of the country's coastal belt and all of the Karoo were categorised as 'highly mineralized chloride-sulphate waters with TDS values greater than 1000mg/L (Day and King, 1995).

Although salinisation can be a result of natural processes, it can also be driven or exacerbated by anthropogenic activity, which will lead to high levels of salinity in the natural environment. In South Africa, urbanisation, industrialisation and irrigation have increased salinisation, which greatly threaten the usefulness of the country's freshwater resources (O'Keeffe et al., 1992). The mining sector within South Africa is diverse and water usage patterns and impacts of increased salinisation vary significantly throughout the country. Salinisation due mineral salts derived from irrigation seepages, mining and industrial effluents, and storm runoff from mining areas has been documented as creating serious problems from as early as the 1970's in the Commission of Enquiry into Water Matters (DWAF, 1986), contributing significantly to the country's salinity problems. Among the problems associated with mining effluent is acid mine drainage (AMD) and sulphate pollution. In the process of coal mining in South Africa, coal deposits, which contain pyretic formations, under certain conditions are oxidised to sulphuric acid and iron sulphate. Resultant AMD from these by-products are extremely acidic and can be treated with hydrated lime ($Ca(OH)_2$) before discharge into the environment. The resultant effluent is saline (gypsiferous) water, mainly due to Ca^{2+} and SO_4^{2-} in solution (Aube, 2005).

Another potential contributor to sulphate-enriched effluent is in the process of heavy mineral extraction from dune sand. The chemical impacts relating to smelting processes are of environmental concern. Effluent resulting from the smelter complex is most likely to cause raised salinity levels in the receiving aquatic ecosystems, particularly due to the contribution of SO_4^{2-} ions (Aube, 2005).

The Olifants and upper Vaal River catchments are among South Africa catchments that have been subjected to intense pressure from mining activities. The Olifants River catchment formed the basis of one of the first comprehensive ecological Reserve determinations carried out in the country. The assessment revealed that various segments of the river are highly impacted by numerous coal mining and power generation activities and discharges from slime dams (DWAF, 2000).

Agricultural activity can contribute to salinisation on a large geographical scale with irrigation being the main contributing factor to salt loading, especially when saline groundwater is the significant or sole source of water (Oren et al., 2004). This may lead to the recycling of salts (mainly Cl^-, SO_4^{2-}, Na^+ and Ca^{2+}) dissolved in irrigation water. When water containing salts in solution is lost by evaporation and transportation, salts precipitate out; this causes salt concentrations to increase. Major salts are not taken up substantially by plants and return to rivers and groundwater from runoff, and by percolating through soil.

The problem intensifies as repeated irrigation results in increasing salt accumulation. High rates of evapotranspiration and the lack of flushing by rain of near-surface and soil root zones in arid and semi-arid areas only increases salt concentrations. Furthermore, infiltrating water from agricultural fields can cause water table levels to rise, increasing chances of evaporation.

Various factors have resulted in agriculturally-induced salinisation becoming a major problem in freshwater ecosystems worldwide. These include reduced annual flows, over-irrigation and insufficient drainage systems, over-use of salt-generating agrochemicals, the dumping of diverted saline springs or wastewater into freshwater systems, the intrusion of seawater into freshwater systems, and the accumulation of surface runoffs in low-lying areas (Kotb et al., 2000). Most of these factors are linked either to insufficient planning (in the case of poorly designed irrigated systems) or inadequate catchment management (in the case of poor land-use practices). Various countries have experienced problems in managing saline water bodies either caused by anthropogenic-related activities or due to rising saline groundwater tables and expanding saline lakes.

There are increased concentrations of major ions or anions in various ecosystems (e.g. Mg, Ca, Na, SO4 and Cl) due to natural and various anthropogenic processes (Zalizniak et al., 2007; Kunz et al. 2013). This has led to a substantial interest in the effects of salinity on aquatic ecosystems. Often times, the concentrations of these ions are mostly a direct reflection of all activities that occur in catchment areas and have a significant impact on the ecological integrity of freshwater ecosystems (Ollis et al., 2006). Salts pose grave concerns in various South African freshwater ecosystems (Palmer et al., 2002; Scherman et al., 2003; Slaughter, 2005). Studies that allow good predictions of the impacts of increased salinity on aquatic ecosystems are insufficient (Kunz et al., 2013). Salinity is the consequence of naturally occurring, essential elements, altered by agricultural and industrial activity and, if salt concentrations are high enough, the result is mortality (Kefford et al., 2002).

Natural salinity levels occurring in freshwater bodies depend on the geographical location although anthropogenic threats may not necessarily be different from one location to another (Slaughter, 2005). Freshwater salinisation has long been regarded as the single greatest threat facing the environment in some countries such as Australia (Hart et al., 1991; Palmer et al., 2004; Marshall and Bailey, 2004; Dunlop et al., 2008; Horrigan et al., 2007). In Southern Africa, the geographical patterns of ionic dominance that occur in the rivers have classified inland water systems based on major ion chemistry (Slaughter, 2005).

This has partly led to a number of studies to predominantly investigate salt effects on various taxa in the formation of water quality guidelines using acute toxicity test methods, rather than the preferred chronic tests due to lack of such data which provide far strong reliability and confidence limits (Zokufa et al., 2001: Scherman et al., 2003; Slaughter, 2005; Holland et al., 2011).

Some aquatic macroinvertebrates respond adversely to various salt exposures although the effects on individual species are poorly understood. Early evidence indicated that salts with magnesium ions are more toxic to freshwater macroinvertebrates (Jooste and Rossouw, 2002). This information is embedded in the Ecological Reserve methodology of the South African Department of Water Affairs and Sanitation (DWAS). There is emerging evidence that the toxicity of magnesium salts is inconsistent in the Ecological Reserve boundaries because it either overestimates or underestimates these boundaries (Scherman, 2009; Scherman, 2010; Holland et al., 2011). For instance, Holland et al., (2011) reported that magnesium sulphate ($MgSO_4$) salt boundary guidelines were inconsistent with electrical conductivity and biotic response data; and this has led to the uncertainty of important water resource management processes like resource classification and setting resource quality objectives (RQOs) in South Africa. The challenge currently is that there is limited ecotoxicity data on a wide variety of salts with different ion combinations and comparisons on what age group of organisms are sensitive in order to review the methodology of concern critically.

Furthermore, the South African National Water Act (No. 36 of 1998) requires the sustainable management of water resources through resource protection and use. Thus, the understanding of different salt toxicity effects for freshwater macroinvertebrates protection is ecologically imperative in the development of water quality guidelines. Therefore, this study was undertaken to compare the ecotoxicity between magnesium sulphate and magnesium chloride salts in short-term and long-term lethal experiments by using the shrimp *Caridina nilotica* as test organisms. Magnesium sulphate was chosen because it is considered as the most toxicological important salt among those that are used in the Present Ecological State assessments, and also makes it a core water quality variable for ecological water quality Reserve assessments (Holland et al., 2011). Insufficient understanding of the effects of magnesium chloride ($MgCl_2$) on freshwater macroinvertebrates (Dallas and Day, 2004) and for comparison with magnesium sulphate necessitated its inclusion in this study. The freshwater shrimp *Caridina nilotica* has been used as a model indigenous crustacean species in ecotoxicological studies.

Caridina nilotica is often used as a toxicity test organism within the Unilever Centre for Environmental Water Quality (UCEWQ), Rhodes University, South Africa, for testing salts and other pollutants such as pesticides and herbicides. It is a prevalent organism in South Africa and easy to collect (Scherman et al., 2003; Slaughter et al., 2010; Holland et al., 2011; Mensah et al. 2012; Mensah et al. 2013). As a water-stressed country, any threat to the limited freshwater resources needs to be tackled with the deserved attention. Thus, the current study sought to evaluate the ecotoxicity of magnesium sulphate and magnesium chloride on juvenile and adult *C. nilotica* under laboratory conditions.

2.2 Toxicology of chemical mixtures

A chemical mixture is any set of multiple chemicals regardless of source that may or may not be identifiable and may contribute to joint toxicity in a target population. Whenever humans are exposed to chemicals, whether simultaneous or sequential, they are not exposed to just one chemical at a time but to chemical mixtures since a large number of chemicals pervade our environment (Mumtaz et al., 2010). Although almost all applied and basic science underpinning current regulations test one chemical at a time, several environmental laws acknowledge the significance of potential exposure to, and the health effects of, chemical mixtures. This is the origin of and motivation for the study of chemical mixtures, and subsequently making cognitive transition and logical progression from single to multiple chemical risk evaluation (Mumtaz et al., 2010).

Although the toxicity of the single toxicant might be well known, organisms can be exposed to a mixture of different toxicants in the environment and the simultaneous presence of these toxicants might induce non-overlapping toxic effects. This makes the study of interactions among toxicants to be importance in toxicological sciences (Goldoni and Johansson, 2007). In studying chemical mixtures, the term ‘‘additivity’’ is used when two or more toxicants act without any interaction among them and the total effect does not differ from what can be expected from the dose-effect relations of the individual agents. However, when there is an interaction among toxicants such that the total effect is lower than expected, it is termed “antagonistic”, whereas it is termed synergistic when the effect is than expected (Groten et al., 2001; Goldoni and Johansson, 2007).

In molecular toxicology, the toxicity of a toxicant depends on its affinity to target sites at cellular level, inter alia. This toxicity might be decreased or increased by the presence of other (toxic) substances that biologically modify cellular conformation and expression, sometimes affecting the cellular defence system and detoxification capability. For most toxicants, there are numerous potential target sites and even less is known about possible interactions. Only in a few cases are the exact toxicological mechanisms of a compound perfectly known and represented by a definite binding site (Goldoni and Johansson, 2007).

Many studies have reported on the effects of individual stressors such as salinity, metals, and pesticides on aquatic ecosystems, but not many studies have given sufficient consideration to the interactions and coexistence of these stressors in aquatic ecosystems. Thus, although there have been many studies investigating the effects of different salts on aquatic organisms in South Africa, there is paucity of information about the ecotoxicological evaluations of their combination in the aquatic environment.

The notion of environmental realism dictates that interactions do not only occur between salts but also between salts and other elements such as metals. Leblebici et al., (2011) studied the effects of salinity on the growth, the content of the photosynthetic pigments (chlorophyll a, b, and carotenoid), and heavy metal uptake by the aquatic macrophytes *Spirodela polyrrhiza*. They reported that at a high levels of salinity (100 and 200 mM NaCl), the relative growth rate (RGR) of the plant decreased, and the content of photosynthetic pigments negatively correlated with the salt level. They also found that high levels of salinity caused a decrease in the accumulation cadmium (Cd) and nickel (Ni) by *S. polyrrhiza*. Leblebici et al., (2011) suggested that salinity is affect metal accumulation, physical and biochemical properties, and other properties of freshwater organisms. For instance, metal toxicity in seaweed has been found to increase with decrease salinity. In separate experiments with seaweed (*Fucus vesiculosus*), copper toxicity was found to increase under reduced salinity (Connan and Stengel, 2011), while zinc accumulation capacity decreased at higher salinity (Munda and Hudnik, 1988).

Methods of chemical mixture exposures often involve simple assumptions of additivity, which are usually based on determinations of toxicological similarity or dissimilarity among the mixture components (Teuschler, 2007). Such methods can further be developed and refined through research to provide guidance on their appropriate applications. Similarly, in-depth research into the emergence of new methods in response to complexities of chemical mixture exposures is necessary to ascertain their usefulness and application (Teuschler, 2007).

Thus, to develop an appropriate method for chemical mixture exposures require meeting certain criteria, which may include the following, among others: (i) appropriate use of generalised approaches for chemical mixtures (e.g. approaches that generalise by similar modes of action or dissimilar modes of action), (ii) applying the appropriate decision criteria to show that several chemicals share a similar toxic mode of action (MoA) or have similarly shaped dose-response curves, (iii) the use of appropriate statistical, chemical or toxicological evidence to ascertain that two complex chemical mixtures are sufficiently similar in nature such that known toxicity data on one mixture is useful for estimating the toxicity of the other, (iv) finding the appropriate means to incorporate information on toxicological interactions into a risk assessment, (v) finding the appropriate exposure levels and mixing ratios at which a simple additivity model can be applied to the data, (vi) finding appropriate methods that can be used to evaluate a complex mixture containing a large fraction of unidentified chemicals.

2.2.1 Response addition and dose addition models in relation to chemical mixture toxicology

Response addition (also called independence) and dose addition (also called non-independence) models are both ‘‘non-interaction’’ models, in that they assume chemicals are simply additive, and neither synergistic nor antagonistic, when combined in mixtures (Borgert et al., 2004). The combined action (i.e. the toxicity produced when chemicals are combined in mixtures) of the response addition model assumes that the toxicity of a mixture is the sum of the toxic effects of each constituent. For instance, it predicts that a mixture of chemicals will not exert an adverse effect when individual chemicals in that mixture are present below their individual No Observable Adverse Effect Level (NOAEL). Response addition model has be suggested to be used for mixtures of chemicals that produce the same toxic effect in the same target organ, but which do so via dissimilar mechanisms of action (U.S.EPA, 2000a; ATSDR, 2001a, 2001b). In comparison, the combined action of dose addition model assumes that non-interacting chemicals in a mixture behave as dilutions of one another and, therefore, may be related by potency factors. For example, the model predicts that a mixture of three chemicals, each present at a concentration one-half its toxic threshold, would produce a measurable toxic effect (Borgert et al., 2004). Dose addition has been suggested to be used for chemicals that produce the same toxic effect in the same target organ via the same mechanism of action (U.S.EPA, 2000a; ATSDR 2001a, 2001b).

2.2.2 Mode of action and mechanism of action in relation to chemical mixture toxicology

‘‘Mode of action’’ and ‘‘mechanism of action’’ are two different biological concepts which have been used to determine the extent to which chemicals exhibit similar mechanistic features, and therefore, to select the model of combined action for those chemicals in a mixture. Although the terms ‘‘mode’’ and ‘‘mechanism’’ are well defined, the toxicological literature on mixtures and regulatory guidance documents for mixture assessments often fail to make clear distinctions between these terms (Borgert et al., 2004). Notwithstanding, the distinction between ‘‘mode’’ and ‘‘mechanism,’’ is critical to conducting a mixtures risk assessment. This is because choice of a model to predict the effects of chemical mixtures (i.e. a dose addition model versus a response addition model) can turn on whether mechanistic data for the chemical components of the mixture are described in terms of the mode or mechanism of action (Borgert et al., 2004). Because of the importance of these concepts for choosing between dose addition and response addition models, it is important to understand the differences between these concepts and how common practice has blurred the distinction.

A mode of action (MoA) describes a functional or anatomical change, at the cellular level, resulting from the exposure of a living organism to a chemical substance. It refers to the type of response produced in an exposed organism or to only the critical steps or features of the mechanism required for production of the particular biological response. Thus, mode of action is defined by a common set of physiological and behavioural signs that characterise a type of adverse biological response, or a common set of mechanisms that shares general features critical to the production of toxicity. In general, the mode of action classification should consider some aspect of the critical biochemical pathway as well as the resultant physiological and behavioural changes produced by alterations in that pathway by the toxicant. A mode of action is important in classifying chemicals as it represents an intermediate level of complexity in between molecular mechanisms and physiological outcomes, especially when the exact molecular target has not yet been elucidated or is subject to debate.

Conversely, a mechanism of action (MOA) describes such changes at the molecular level. It denotes the molecular sequence of events leading from the absorption of an effective concentration of a toxicant to the production of a specific biological response in the target organ. Thus, understanding the mechanism of action of a toxicant involves understanding of the causal and temporal relationships between the steps leading to a particular effect, as well as the steps that lead to an effective concentration of the toxicant at the relevant biological target(s) of action (Borgert et al., 2004). In comparison, a mechanism of action of a chemical could be "binding to DNA" while its broader mode of action would be "transcriptional regulation". Table 1 present differences between these two concepts based on mechanistic data.

Table 1: Differences between mode of action and mechanism of action based on mechanistic data

Terminology	**Use of mechanistic data**	**Definition or criteria**	**References**
Mode	Decide relevance of animal data; identify sensitive subpopulations; high to low dose extrapolation and predict threshold.	Mode of action is composed of key events and processes starting with interaction of an agent with a cell, through operational and anatomical changes, resulting in cancer formation. Mechanism of action implies a more detailed, molecular description of events than mode of action. To demonstrate mode, an understanding of the complete sequence of events at the molecular level (mechanism) is not expected; instead, use empirical observations at different levels of biological organization: biochemical, cellular, physiological, tissue, organ, system, and determine causal relationship between the events.	U.S.EPA, 2001. Pages 1-15.
Mode	Reduce uncertainty in carcinogen risk assessment; improve extrapolation of animal data to humans; predict thresholds.	Emphasizes the importance of understanding how environmental agents are changed through metabolism, the dose at the affected organ system, how an agent produces its adverse effect at high and low doses. ‘‘It	Dellarco and Wiltse, 1998. Mutation Research, 405, 273-277.

		should be noted that the term mode of action is deliberately chosen in these new guidelines in lieu of mechanism to indicate using knowledge that is sufficient to draw a reasonable working conclusion without having to know the processes in detail at the molecular level, as the term mechanism might imply.''	
Mode	To support the cancer risk assessment of 2,3,7,8-TCDD and related compounds.	One aid to the use of more information in risk assessment has been the definition of mode versus mechanism of action. Mechanism of action is defined as the detailed molecular description of a key event in the induction of cancer or other health endpoints.	U.S.EPA, 2000b. Page 41.
Mechanism	To identify chemicals that will be modeled by dose additivity based on common action.	Common mechanism means the same, or essentially the same, sequence of major biochemical events such that the underlying basis of the toxicity is the same, or essentially the same.	U.S.EPA, 1999. Page 4.
Mechanism	To identify chemicals that will be modeled by dose additivity based on common action.	''Common mechanism is described as the major steps leading to an adverse health effect following interaction of a pesticide with biological targets. An understanding of	Mileson, B. E.; Chambers, J. E.; Chen, W. L.; et al., 1998. Toxicol Sci., 41(1), 8-20.

		all steps leading to an effect is not necessary, but identification of the crucial events following chemical interaction is required to describe a mechanism of toxicity.'' Common mechanisms means (a) cause the same critical effect, (b) act on same molecular and tissue target, (c) act by same biochemical mechanism and possibly share a common toxic intermediate.	
Mechanism	To choose a model of joint toxic action.	Should include information on events occurring at the molecular or receptor site level and at higher levels of biochemical, physiological, or pathogenic activities, such as toxicological response in the whole animal. Dose additivity means that chemicals behave as dilutions of one another, differing only in potency, and DRCs are parallel.	ATSDR, 2001a. Page 8; ATSDR, 2001b. Pages 26-39.
Mode or mechanism	To choose between dose additivity and response additivity models.	Chemicals are dose additive if 'chemical B is a functional clone of chemical A'. Dose additive chemicals have 'similar uptake, metabolism, distribution, elimination, and toxicological properties', and there is a 'constant proportionality between	U.S.EPA, 2000a. Pages 20-22, 28, 75-76.

		effectiveness' such that their DRCs are 'congruently shaped', that is, 'parallel'.	

2.3 Macroinvertebrates for water quality studies

The indigenous South African freshwater shrimp *Caridina nilotica* was used as the principal model organism for this study. However, other freshwater macroinvertebrates including mayfly nymphs (Ephemeroptera) were also considered. These indigenous species, which are established species for toxicity testing in South Africa (Scherman et al., 2003), were either laboratory cultured in the UCEWQ or collected from unimpacted rivers in Eastern Cape, South Africa. The present report, however, focused on salt exposure to *C. nilotica*.

Caridean shrimps are true-freshwater crustaceans that belong to the Class Malacostraca, Sub-Class Eumalacostraca, Super-order Eucarida, Order Decapoda, Sub-Order Macrura and Family Atyidae, and are widely distributed in African inland waters (Day, 2001). Crustacea may be classified as a Sub-phylum of the Phylum Arthropoda of the Kingdom Animalia. Their bodies are bilaterally symmetrical and metamerically segmented, and have jointed limbs on all or some of the segments. The entire body architecture is covered with a calcium-containing exoskeleton, which is shed during ecdysis to allow for growth (Hart et al., 2001). There are over 40,000 species of crustaceans the world over. The majority of these live in marine and estuarine environments, with only a few freshwater species existing today (Hart et al., 2001). Crustaceans have unique biological characteristics, which make them suitable candidates for toxicity testing. These features include their morphology, physiology, behaviour, adaptability, life history and reproductive patterns (Rinderhagen et al., 2000).

Caridina nilotica is the most common of four indigenous freshwater caridean species found in the Southern Africa sub-region. The others are *C. typus*, *C. africana* and *C. indistinct* (Hart et al., 2001). *Caridina nilotica* inhabits both lentic and lotic waters of Mozambique, and the greater part of eastern and northern South Africa, from as far south as the Gamtoos River, extending westwards to the lower Orange River (Hart et al., 2001). They thrive in temperatures between 10 to 30° C but their oxygen tolerances are not well known (Hart et al., 2001). They are considered important role players in the freshwater ecosystems as they form part of most food webs.

Caridina nilotica is an omnivorous-detritivorous surface scrapers that feed on periphyton scraped from hydrophytes and on plant detritus. They also scavenge on remains of animals such as fish, insects and shrimps. This mode of feeding is useful in clearing debris and epiphytic microflora from leaves of submerged macrophytes, thereby enhancing macrophyte photosynthesis and recycling organic matter (Hart, 1981, Hart et al., 2001). *C. nilotica*, an important member of the communities of submerged macrophyte beds and the profundal benthos, provides food for other members of the community as it is preyed upon by predators such as herons, lake-terns and the Nile perch, *Lates nilotica*. *C. nilotica* is reportedly eaten by humans as a delicacy and therefore has economic value (Budeba, 1999).

Caridina nilotica has been suggested as a good model for developing partial life-cycle, full life-cycle, or multigenerational toxicity testing protocols that can be used to assess ecologically relevant effects of chemicals on growth and reproduction (Okuthe et al., 2004). *C. nilotica* toxicity tests have been developed for acute toxicity tests for neonate, juvenile and adult life history stages (Scherman and Palmer, 2000). Chronic test methods for embryotoxicity and partial life-cycle tests have also been conducted (Slaughter, 2005; Ketse, 2006). The present report focused on study, hypothesised that each life history stage of *C. nilotica* can potentially be used in routine and regulatory testing for glyphosate-based herbicides. In the present study, *C. nilotica* was used as a model freshwater organism to investigate the separate effects of single salts and binary salt mixtures on this aquatic shrimp.

3 GENERATION OF DATA FOR SINGLE KEY TOXICOLOGICAL IMPORTANT MAJOR SALTS

3.1 Methodology

3.1.1 Test organism and test salts

The UCEWQ maintains a laboratory culture of *C. nilotica* used for ecotoxicological studies. They are maintained in aquaria 30-L glass tanks h in a controlled environment of temperature 24° C ± 1 and 12:12 h light:dark regime. Shrimps were fed TetraMin fish flakes (morning and late afternoon) as well as algae which grow naturally in the stocking tanks. Gravid shrimps were collected from all stocking tanks on same day and placed in breeding tanks as they became available to obtain a representative age group of the offspring. Once a gravid female releases its eggs it was removed from the breeding tanks to avoid cannibalising its own eggs. Most eggs hatched within 2-3 weeks. Hatched shrimps remained in the breeding tanks until they were removed and kept in separate tanks for acclimation 24 h before an exposure tests began. After acclimation, shrimps were individually transferred into experimental vessels using a modified hand-net. In the present study, juvenile (>7<20 days post hatch (dph)) and young adult (>20 dph) of *C. nilotica* were exposed to increasing concentrations of key toxicological importance salts (TIMS) including magnesium sulphate ($MgSO_4$), magnesium chloride ($MgCl_2$) and sodium sulphate (Na_2SO_4) were used as single salts.

3.1.2 Test design and procedure

This study employed a static experimental method to determine the lethal concentration values of the test salts for juvenile and adult shrimps. The test methods used were short-term lethal tests (96 h) and long-term lethal tests (240 h) using 600-mL grade A beakers as experimental vessels, which were pre-acid washed by following the Acid Glass Wash Procedure used at UCEWQ. The test medium was dechlorinated water, same used during culture of the shrimps. For each single salt or binary salt mixture, different fresh concentrations (more than 5) were prepared for juvenile and adult exposure tests. Each concentration contained 10 shrimps and replicated three times. Dead shrimps were recorded twice daily and removed from experimental vessels. The cumulative number of mortality were recorded at the end of 48, 96 and 240 h. Data obtained after 48-96 h of exposure was considered short-term, while that obtained 240 h after exposure was considered long-term.

Test solutions were changed every fourth day to minimise build-up of algae and nutrients within the test vessels for the long-term tests. Shrimps were not fed during the experimental period. Swimming behaviour of shrimps due to the exposure to salts were observed and recorded.

Water quality parameters including temperature, electrical conductivity (EC), hydrogen ion concentration (pH), dissolved oxygen (DO) and temperature were recorded daily. The test endpoint was mortality or immobilisation, which was assessed by prodding the organism and checking for movement. Acceptable control mortality was restricted to 10% for the short-term exposure tests.

3.1.3 Data analysis

Probit statistical software version 1.5 (USEPA, 1990) was used to estimate the lethal concentration (LC) values and their 95% confidence limits, using mortality data obtained from the various ecotoxicity tests with salts and *C. nilotica*. One-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison post hoc tests were used to compare mean mortality values between control and exposed groups. Statistics were performed using Statistica Version 12 and all statistical decisions were made at alpha = 0.05 a priori.

3.2 Results

3.2.1 Juvenile and adult *C. nilotica* exposure to $MgSO_4$

For juvenile shrimps, at 48, 96 and 240 h after exposure, one way analysis of variance (ANOVA) revealed a significant difference ($p < 0.001$) between control group and $MgSO_4$ exposed groups. At 48 h after exposure, a post hoc analysis with Newman-Keuls multiple comparison tests showed that shrimp mortality in concentrations 0.25, 0.5, 1 and 2 g/L were not statistically different from control group, but significantly lower than the other treatment groups ($p < 0.05$). Mortality in 4 g/L and 8 g/L were not statistically different from each other but were significantly lower that mortality in 16 g/L and 32 g/L. Mortality in 16 g/L was significantly lower than mortality in 32 g/L (Figure 1). At 96 h after exposure, Newman-Keuls multiple comparison test showed that adult shrimp mortality in concentrations 0.25, 0.5, 1 and 2 g/L were not statistically different from control group, but significantly lower than the other treatment groups ($p < 0.05$). Mortality in 4 g/L was significantly lower than mortality in 8, 16 and 32 g/L. However, mortality in 32 g/L was significantly higher than mortality in 8 g/L and 16 g/L, which were not statistically different from other (Figure 2).

At 240 h after exposure, Newman-Keuls multiple comparison test showed that adult shrimp mortality in concentrations 0.25, 0.5, 1 and 2 g/L were not statistically different from control group, but significantly lower ($p < 0.05$) than the other treatment groups (i.e. 4, 8, 16 and 32 g/L). Mortality in 4, 8, 16 and 32 g/L were statistically different from each other ($p < 0.05$), increasing monotonically (Figure 3).

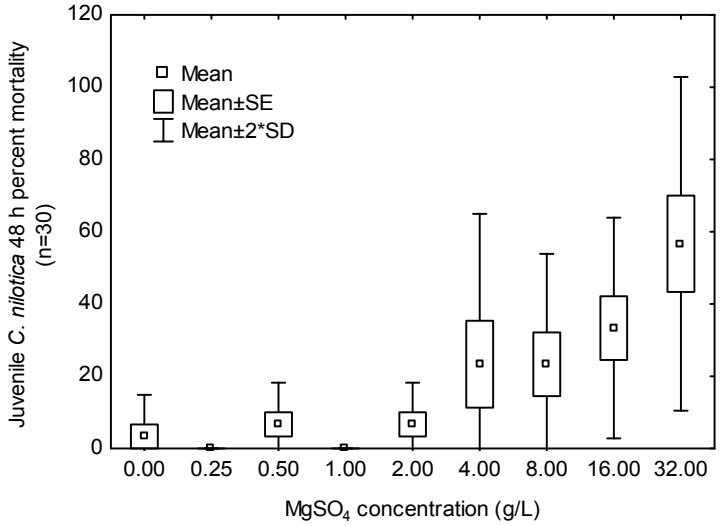

Figure 1: Juvenile *C. nilotica* mean mortality after 48 h exposure to $MgSO_4$

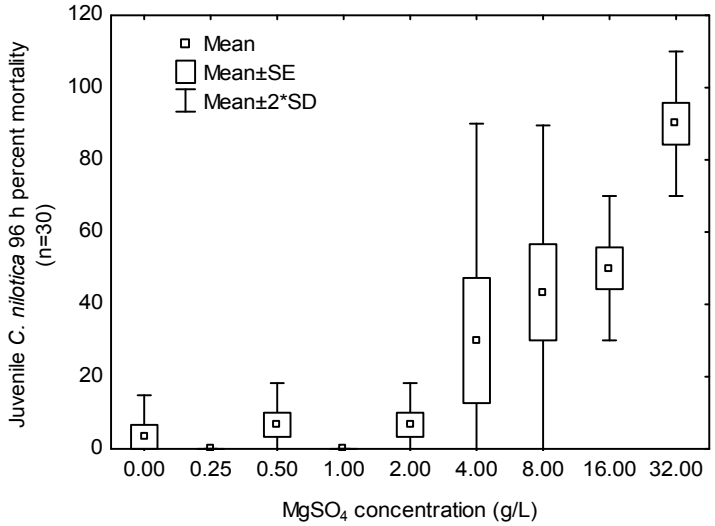

Figure 2: Juvenile *C. nilotica* mean mortality after 96 h exposure to $MgSO_4$

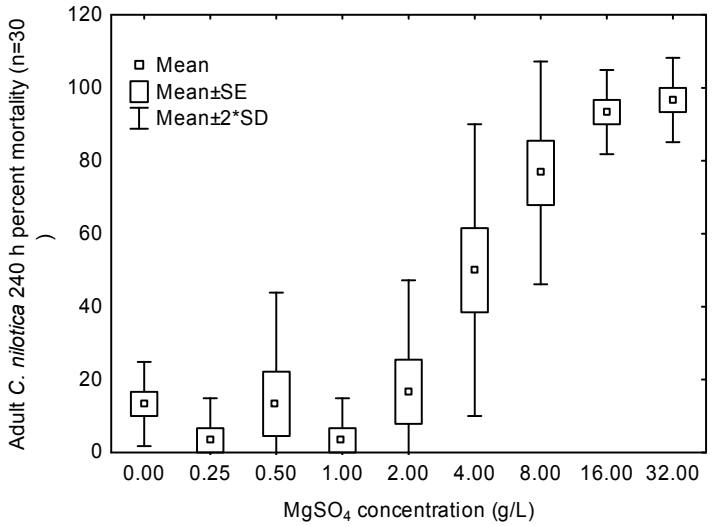

Figure 3: Juvenile *C. nilotica* mean mortality after 240 h exposure to $MgSO_4$

For adult shrimps, at 48, 96 and 240 h after exposure, one way analysis of variance (ANOVA) revealed a significant difference ($p < 0.001$) between control and $MgSO_4$ exposed groups. At 48 h after exposure, a post hoc analysis with Newman-Keuls multiple comparison test showed that shrimp mortality in concentrations 0.06, 0.125, 0.25, 0.5, 1 and 2 g/L were not statistically different from control group, but significantly different lower than the other treatment group. Mortality in 4, 8, 16 and 32 g/L were significantly different from each other, with mortality increasing monotonically (Figure 4). At 96 h after exposure, Newman-Keuls multiple comparison test showed that adult shrimp mortality in concentrations 0.06, 0.125, 0.25, 0.5 and 1 g/L were not significantly different from control group, but significantly different from the other treatment groups. Mortality in 2 g/L was significantly different from mortality in 4, 8, 16 and 32 g/L are significantly different from each other, with mortality increasing monotonically, but mortality in 4, 8, 16 and 32 g/L were not significantly different from each other (Figure 5). At 240 h after exposure, Newman-Keuls multiple comparison test showed that adult shrimp mortality in concentrations 0.06, 0.125, 0.25 and 0.5 g/L were not significantly different from control group, but significantly lower than the other treatment groups. Mortality in 1, 2, 4, 8, 16 and 32 g/L were significantly different from each other as well as the lower concentrations (Figure 6).

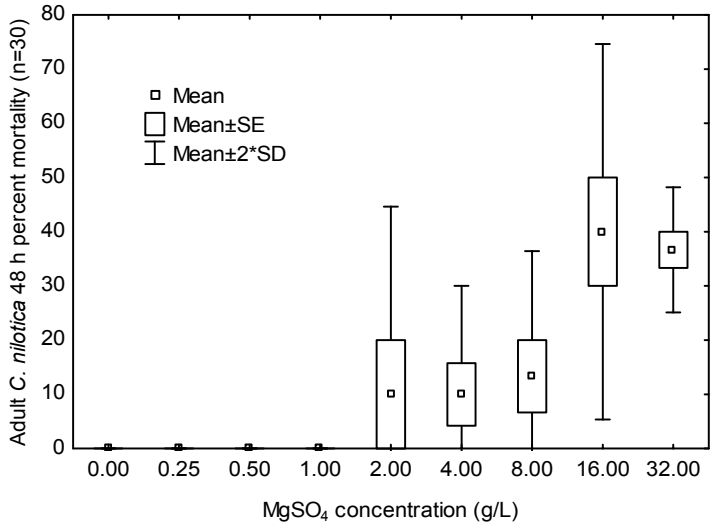

Figure 4: Adult *C. nilotica* mean mortality after 48 h exposure to $MgSO_4$

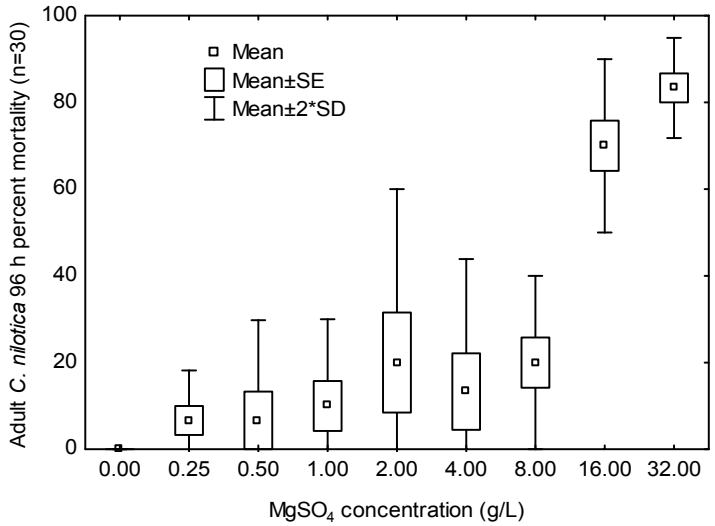

Figure 5: Adult *C. nilotica* mean mortality after 96 h exposure to $MgSO_4$

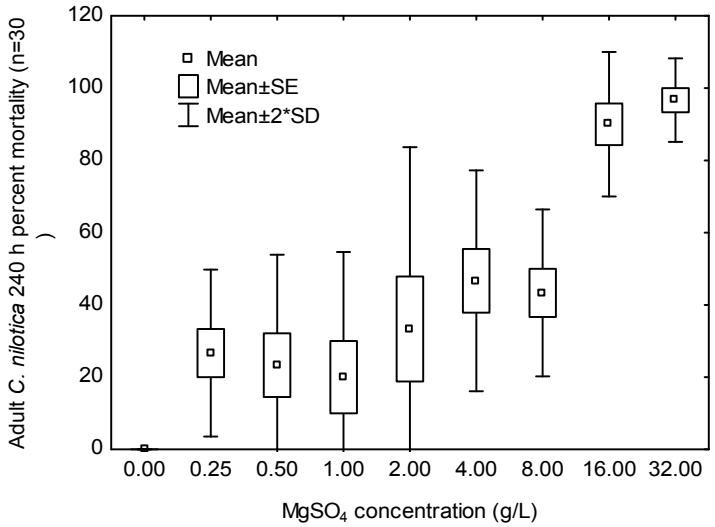

Figure 6: Adult *C. nilotica* mean mortality after 240 h exposure to $MgSO_4$

3.2.2 Juvenile and adult *C. nilotica* exposure to $MgCl_2$

For juvenile shrimps, at 48 h after exposure, Newman-Keuls multiple comparison tests showed that mortality in concentrations 0.06, 0.125, 0.25, 0.5 and 1 g/L were not significantly different from control group, but significantly lower than the other treatment groups. Mortality in 2 and 4 g/L were not significantly different but significantly lower than in 8, 16 and 32 g/L. However, mortality in 16 and 32 g/L were not significantly different but significantly higher than in 8 g/L. Similar observations were at 96 h and 240 h after exposure (Figures 7-9).

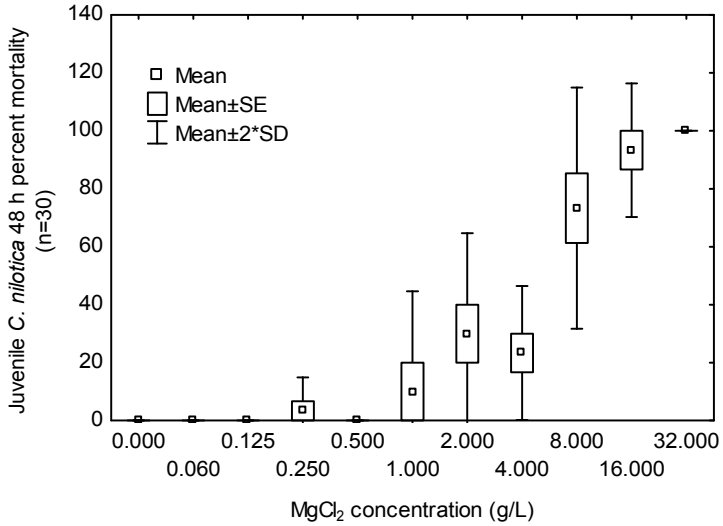

Figure 7: Juvenile *C. nilotica* mean mortality after 48 h exposure to $MgCl_2$

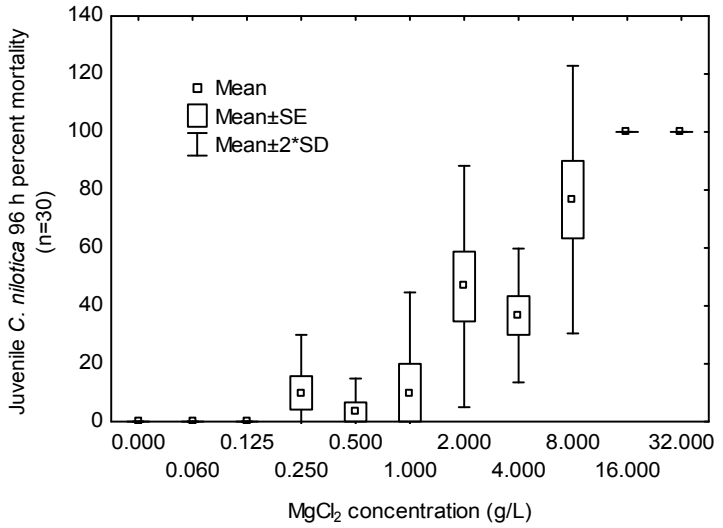

Figure 8: Juvenile *C. nilotica* mean mortality after 96 h exposure to $MgCl_2$

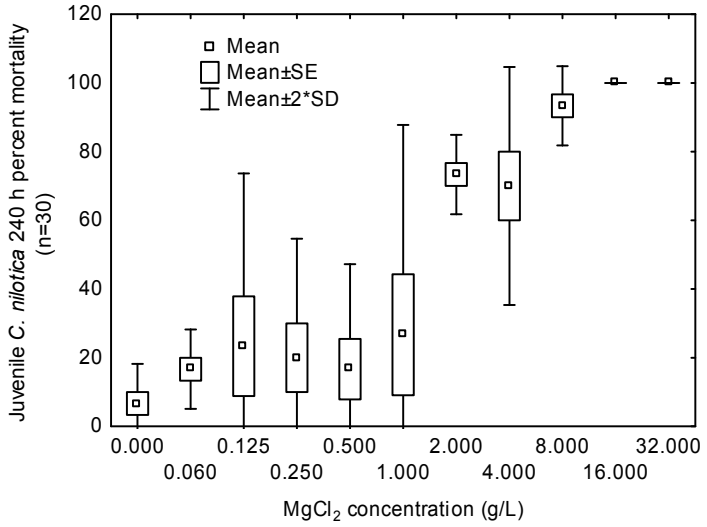

Figure 9: Juvenile *C. nilotica* mean mortality after 240 h exposure to $MgCl_2$

For adult shrimps, at 48, 96 and 240 h after exposure, one way analysis of variance (ANOVA) of mortality revealed a significant difference ($p < 0.001$) between control and $MgCl_2$ exposed groups. At 48 h after exposure, Newman-Keuls multiple comparison test showed that adult shrimp mortality in concentrations 0.06, 0.125, 0.25, 0.5, 1 and 2 g/L were not significantly different from control group, but significantly lower than the other treatment groups. Mortality in 4 g/L and 8 g/L were not significantly different from each other just as mortality in 16 and 32 g/L were not significantly different. Nevertheless, mortality in 16 and 32 g/L were significantly higher than in 4 g/L and 8 g/L (Figure 10). At 96 h after exposure, Newman-Keuls multiple comparison test of mortality revealed similarities to mortalities at 48 h after exposure (Figure 11). At 240 h after exposure, Newman-Keuls multiple comparison test showed that adult shrimp mortality in concentrations 0.06, 0.125, 0.25, 0.5 and 1 g/L were not significantly different from control group, but significantly lower than the other treatment groups. Mortality in 2, 4 and 8 g/L were significantly different from each, increasing monotonically. However, there were no significant differences in mortality between 16 and 32 g/L, which recorded the highest mortality (Figure 12).

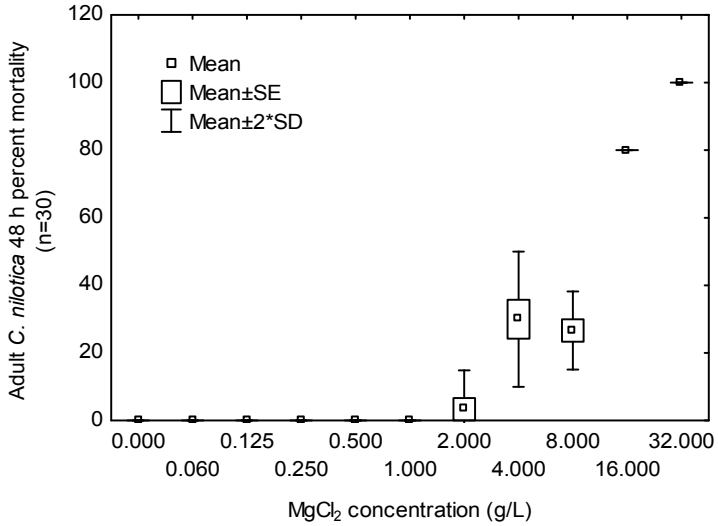

Figure 10: Adult *C. nilotica* mean mortality after 48 h exposure to $MgCl_2$

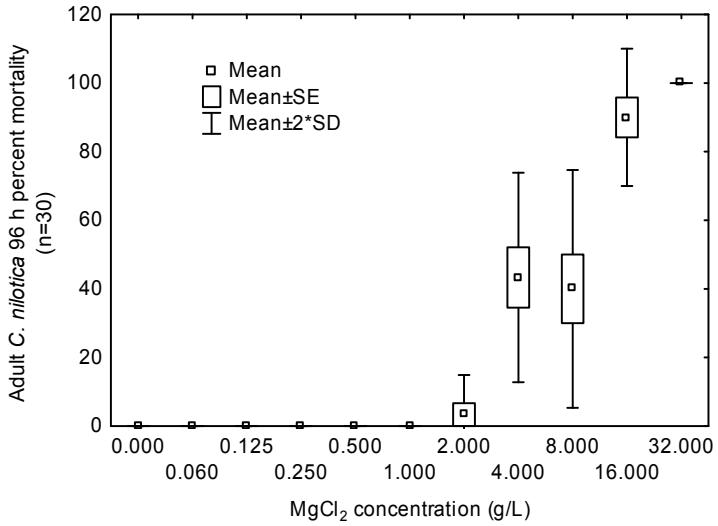

Figure 11: Adult *C. nilotica* mean mortality after 96 h exposure to $MgCl_2$

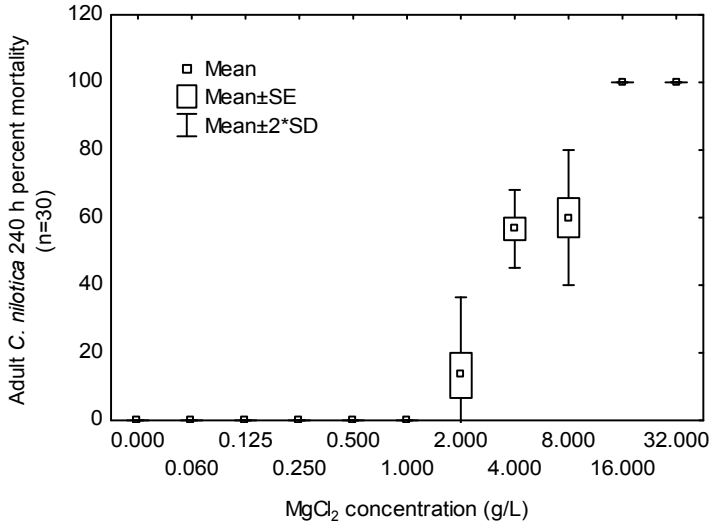

Figure 12: Adult *C. nilotica* mean mortality after 240 h exposure to $MgCl_2$

3.2.3 Juvenile and adult *C. nilotica* exposure to Na_2SO_4

For juvenile shrimps, at 48, 96 and 240 h after exposure, one way analysis of variance (ANOVA) revealed a significant difference ($p < 0.001$) between control and Na_2SO_4 exposed groups. At 48 h after exposure, Newman-Keuls post hoc multiple comparison tests showed that shrimp mortality in concentrations 0.06, 0.125, 0.25, 0.5, 1 and 2 g/L were not significantly different from control group, but significantly lower than the other treatment groups. Mortality in 4 g/L was significantly lower than in 8, 16 and 32 g/L, but mortality in 8, 16 and 32 g/L were not significantly different (Figure 13). At 96 h after exposure, Newman-Keuls multiple comparison test showed that juvenile shrimp mortality in concentrations 0.06, 0.125 and 0.25 g/L were not significantly different from control groups, but significantly different from the other treatment groups. Mortality in 0.5 and 1 g/L were not significantly different, but statistically different from mortality in 2, 4, 8, 16 and 32 g/L. mortality in 2 and 4 g/L were not significantly different, but significantly different from 8, 16 and 32. Although mortality in these last three concentrations were not statistically different, mortality in these concentrations were significantly higher than the lower concentrations (Figure 14). At 240 h after exposure, Newman-Keuls multiple comparison test showed that juvenile shrimp mortality in concentrations 0.06, 0.125 and 0.25 g/L were not significantly different from control groups, but significantly different from the other treatment groups. Mortality in 0.5 and 1 g/L were not significantly different, just as mortality in 2, 4, 8, 16 and 32 g/L were not statistically different. That notwithstanding, mortality generally increased monotonically (Figure 15).

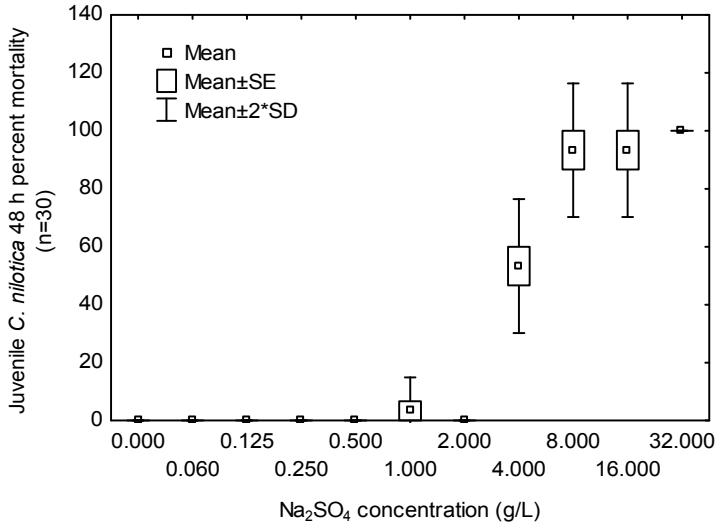

Figure 13: Juvenile *C. nilotica* mean mortality after 48 h exposure to Na_2SO_4

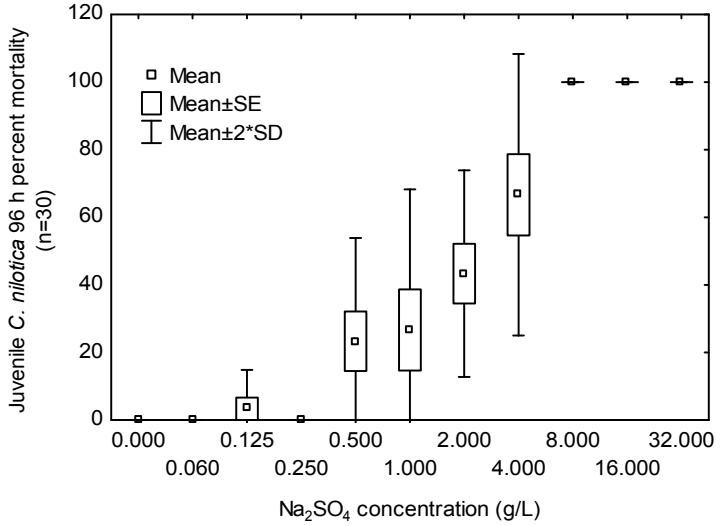

Figure 14: Juvenile *C. nilotica* mean mortality after 96 h exposure to Na_2SO_4

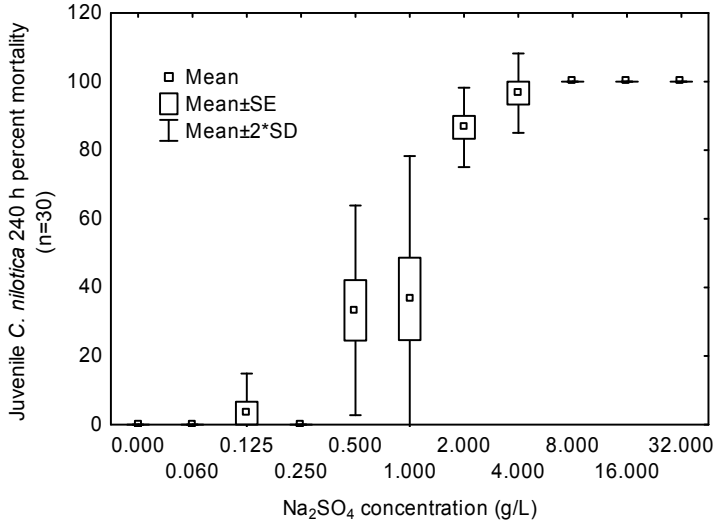

Figure 15: Juvenile *C. nilotica* mean mortality after 240 h exposure to Na_2SO_4

For adult shrimps, at 48, 96 and 240 h after exposure, one way analysis of variance (ANOVA) revealed a significant difference ($p < 0.001$) between control and Na_2SO_4 exposed groups. At 48 h after exposure, Newman-Keuls post hoc multiple comparison tests showed that mortality in concentrations 0.06, 0.125, 0.25, 0.5 and 1 g/L were not significantly different from control group. Similarly, mortality in 2 and 4 g/L were statistically not different but significantly lower than mortality in 8, 16 and 32 g/L. However, mortality in 8, 16 and 32 g/L were statistically not different but higher than all other treatment groups (Figure 16). At 96 h after exposure, Newman-Keuls multiple comparison test showed that adult shrimp mortality in concentrations 0.06, 0.125, 0.25, 0.5 and 1 g/L were not significantly different from control group, but significantly different from the other treatment groups. Mortality in 2 g/L was significantly lower than mortality in 4, 8, 16 and 32 g/L. Mortality in the last four concentrations (i.e. 4, 8, 16 and 32 g/L) were statistically not different but higher than the lower concentrations (Figure 17). At 240 h after exposure, Newman-Keuls multiple comparison test showed that adult shrimp mortality in concentrations 0.06, 0.125, 0.25 and 0.5 g/L were not statistically different from control group, but significantly lower the other treatment groups. Mortality in 1 g/L was significantly lower than in 2, 4, 8, 16 and 32 g/L. Mortality in the last five concentrations (i.e. 2, 4, 8, 16 and 32 g/L) were statistically not different but higher than the lower concentrations (Figure 18).

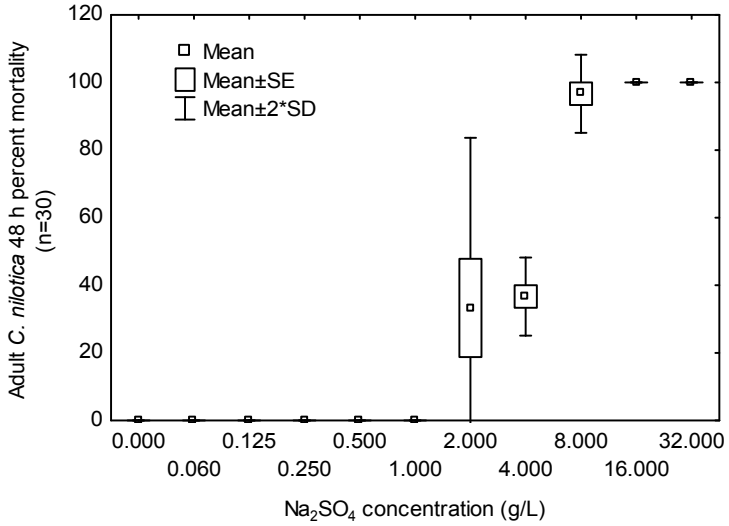

Figure 16: Adult *C. nilotica* mean mortality after 48 h exposure to Na_2SO_4

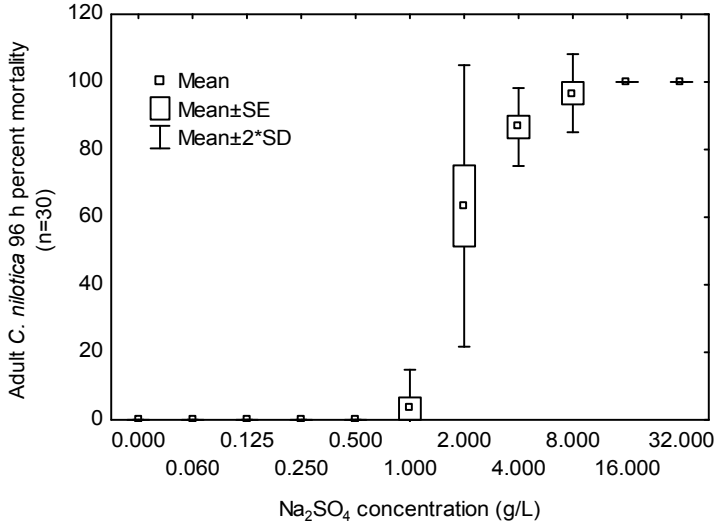

Figure 17: Adult *C. nilotica* mean mortality after 96 h exposure to Na_2SO_4

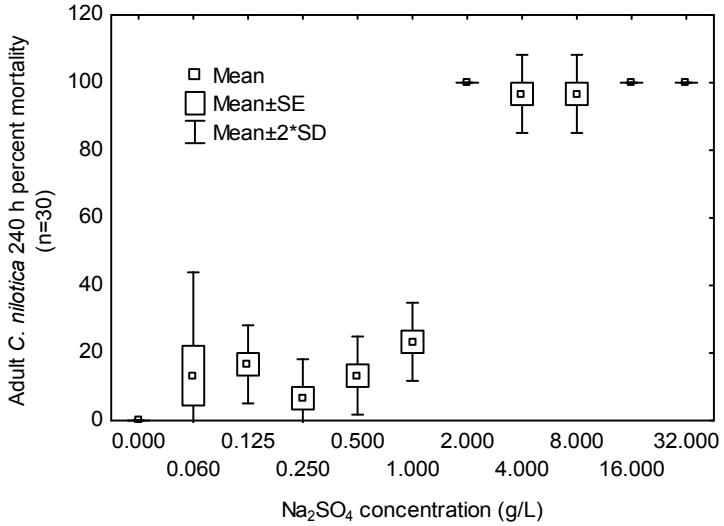

Figure 18: Adult *C. nilotica* mean mortality after 240 h exposure to Na_2SO_4

3.2.4 Lethal concentrations of the tests salts to *C. nilotica*

The LC1, LC10 and LC50 values estimated using PROBIT regression based on responses of adult and juvenile *C. nilotica* exposure tests with single salts are presented in Table 2. A fuller version of the estimated LC values is attached as Appendix 1. It should be noted that the smaller the LC value, the more sensitive is the organism to the test substance. In other words, the salt with the least LC value is the most sensitive. Juveniles were found to be more sensitive than adults in most cases, but adult were also found to be sensitive than juveniles in some cases.

For short-term (48 h) exposure tests and at the level of LC50, the most toxic salt was Na_2SO_4 with an LC50 of 3.51 g/L for adult *C. nilotica*, while $MgSO_4$ was the least toxic with an LC50 of 42.66 g/L for adult *C. nilotica* (Table 3). Similarly, for short-term (96 h) exposure tests and at the level of LC50, $MgCl_2$ was the most toxic with an LC50 of 1.67 g/L for juvenile *C. nilotica*, while $MgSO_4$ was the least toxic with an LC50 of 11.57 g/L for adult *C. nilotica* (Table 4). For long-term (240 h) exposure tests and at the level of LC50, Na_2SO_4 was the most toxic salt with an LC50 of 0.77 g/L for juvenile *C. nilotica*, while $MgSO_4$ was the least toxic with an LC50 of 4.85 g/L for adult *C. nilotica* (Table 5).

Table 2**:** Estimated lethal concentration (LC) values for *C. nilotica* juvenile and adult exposed to single salts

Single salts	**Life stage**	**Test duration**	**Lethal concentration (in g/L) (Lower limit-Upper limit)**		
			LC1	**LC10**	**LC50**
$MgSO_4$	Juvenile	48h	0.52 (0.03-1.54)	3.10 (0.79-5.59)	27.61 (17.34-66.62)
	Adult	48 h	0.58 (0.12-1.31)	4.01 (2.05-6.23)	42.66 (24.36-119.82)
	juvenile	96h	0.79 (0.22-1.54)	2.55 (1.22-3.88)	10.80 (8.06-14.59)
	Adult	96 h	0.16 (0.01-0.66)	1.11 (0.11-2.61)	11.57 (5.43-50.93)
	juvenile	240h	0.69 (0.25-1.20)	1.65 (0.86 -2.41)	4.85 (3.61-6.17)
	Adult	240	0.03 (0.00-0.20)	0.25 (0.00-0.84)	3.60 (1.19-13.28)
$MgCl_2$	Juvenile	48h	0.37 (0.09-0.77)	1.13 (0.46 1.85)	4.36 (2.86-6.78)
	Adult	48 h	1.37 (0.74-2.02)	3.10 (2.12-4.11)	8.42 (6.89-10.37)
	juvenile	96h	0.17 (0.09-0.27)	0.48 (0.32-0.64)	1.67 (1.34 -2.09)
	Adult	96 h	1.12 (0.62-1.63)	2.46 (1.70-3.17)	6.48 (5.31-7.91)
	juvenile	240h	0.01 (0.00-0.06)	0.10 (0.02-0.24)	0.99 (0.47-1.99)
	Adult	240	0.86 (0.49-1.23)	1.82 (1.27-2.33)	4.61 (3.79-5.59)
Na_2SO_4	Juvenile	48h	1.01 (0.59-1.40)	1.92 (1.37-2.41)	4.26 (3.55-5.10)
	Adult	48 h	0.83 (0.49-1.16)	1.588 (1.13-1.99)	3.51 (2.93-4.20)
	juvenile	96h	0.14 (0.07-0.22)	0.43 (0.28-0.59)	1.76 (1.39-2.24)
	Adult	96 h	0.56	1.00	2.06

			(0.33-0.76)	(0.72-1.24)	(1.73-2.45)
	juvenile	240 h	0.05 (0.00-0.14)	0.16 (0.02-0.35)	0.77 (0.35-1.63)
	Adult	240 h	0.04 (0.00-0.15)	0.16 (0.01-0.39)	0.82 (0.30-2.10)

Table 3**:** *C. nilotica* tolerances to the tested single salts at LC50 48 h after exposure

Salt	**Life stage**	**Test duration**	**LC50 (g/L)**
Na_2SO_4	Adult	48 h	3.51
Na_2SO_4	Juvenile	48h	4.26
$MgCl_2$	Juvenile	48h	4.36
$MgCl_2$	Adult	48 h	8.42
$MgSO_4$	Juvenile	48h	27.61
$MgSO_4$	Adult	48 h	42.66

Table 4**:** *C. nilotica* tolerances to the tested single salts at LC50 96 h after exposure

Salt	**Life stage**	**Test duration**	**LC50 (g/L)**
$MgCl_2$	juvenile	96h	1.67
Na_2SO_4	juvenile	96h	1.76
Na_2SO_4	Adult	96 h	2.06
$MgCl_2$	Adult	96 h	6.48
$MgSO_4$	juvenile	96h	10.80
$MgSO_4$	Adult	96 h	11.57

Table 5**:** *C. nilotica* tolerances to the tested single salts at LC50 240 h after exposure

Salt	**Life stage**	**Test duration**	**LC50 (g/L)**
Na_2SO_4	juvenile	240h	0.77
Na_2SO_4	Adult	240h	0.82
$MgCl_2$	juvenile	240h	0.99
$MgSO_4$	Adult	240h	3.60
$MgCl_2$	Adult	240h	4.61
$MgSO_4$	juvenile	240h	4.85

4 DEVELOPMENT OF A PROCEDURE FOR MIXTURE ECOTOXICITY TESTING AND GENERATION OF DATA FOR BINARY SALT MIXTURES

The procedure used in section 3 to generate data for the single salts followed a general experimental procedure well documented in the field of ecotoxicology. These include Guidelines for the Testing of Chemicals by Organisation for Economic Cooperation and Development (OECD); Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms by the United States Environmental Protection Agency (USEPA), as well as A Protocol for acute toxicity testing using selected riverine invertebrates in artificial stream systems by Scherman and Palmer (2000). These documents provide a sound scientific base for conducting ecotoxicity tests and the procedures they describe were modified in most cases to suit experimental needs. Unfortunately, these documents do not provide a procedure for mixing two or more salts. Furthermore, there is no specific procedure for salts or mixture experiments even though many have propounded theories and models to describe characteristics of such mixtures. Thus, in this section, the development of a procedure for salt mixtures is first described. This procedure is then used to generate data for binary salt mixtures.

4.1 Description of a procedure for binary salt mixture experiments

4.1.1 Relative toxic unit and relative toxic fractions of chemical substances in a mixture

An organism exposed to a mixture of chemical substances will experienced the combined toxic effect (whether synergistic or antagonistic) of the mixture. The combined toxicity of the components may be called relative toxic unit (RTU). In a mixture of chemical substances, each of the components comes with its own toxicity relative to the other components. That is, each component has its own relative toxic fractions (RTF). Thus, the RTU of a binary salt mixture is made up of individual relative toxic fractions (RTFs) of the 2 salts; the RTU of a ternary salt mixture is made up of individual RTFs of the 3 salts; and the RTU of a quaternary mixture is made up of individual RTFs of the 4 salts. Examples of RTU for binary, ternary and quaternary salt mixtures are shown in Table 6. The RTU is the sum of all individual RTFs in a mixture; hence it has a total value of 1.

Table 6**:** Exemplar calculations of relative toxic units of different salt mixture types

Salt mixture type	**Salt mixture type example**	**Calculation of RTU**
Binary	$NaCl+MgCl_2$ mixture	$RTU = {}_{RTF}NaCl + {}_{RTF}MgCl_2$
Ternary	$NaCl+MgCl_2+CuSO_4$ mixture	$RTU = {}_{RTF}NaCl + {}_{RTF}MgCl_2 + {}_{RTF}CuSO_4$
Quaternary	$NaCl+MgCl_2+CuSO_4+ZnCl_2$ mixture	$RTU = {}_{RTF}NaCl + {}_{RTF}MgCl_2 + {}_{RTF}CuSO_4 + {}_{RTF}ZnCl_2$

The lethal concentration (LC) of a chemical substance represents its toxicity to a certain percentage of population exposed to that chemical substance, although this also depends on the duration of exposure, which is often included in the definition. The medial lethal concentration, which is the concentration at which 50 % of the test population died, is generally used as an indicator of a chemical substance's acute (i.e. short-term) toxicity. A lower LC50 of a chemical substance to a particular organism indicates higher toxicity, and vice versa. In this study, the 96 h LC50 values for the individual salts in the different salt mixture types were used to represent the relative toxic fractions (RTFs) in the mixture.

4.1.2 Mixing of salts based on similar or dissimilar cations for binary salt mixtures experiment

One of the most important questions to ask in salt mixture experiments is what salts to mix. Salts are formed as a result of neutralization reaction between acid and base (alkali). The toxicity of salts depends on the ions they form in solution. There are indications that some of the alkali cations (such as Na^+ and K^+) and alkali earth cations (such as Ca^{2+} and Mg^{2+}) are physiologically important, having reported as being toxic to living organisms. Furthermore, studies show that the toxicity of salts such as $CuSO_4$, $ZnSO_4$, $CuCl_2$, $ZnCl_2$ $CdCl_2$ and NaCN formed from transition metals can be attributed to the cations (such as Cd 2+) or an anion (such as CN^-). However, for common salts such as NaCl, Na_2SO_4, $MgCl_2$ and $MgSO_4$ formed from alkali and alkali earth metals, it is considerably more difficult to attribute the toxic effect of such salts to either their cationic or anionic component. Therefore, the approach used in this study is based on the measured toxicity of salts formed from alkali and alkali earth metals. Salts where mixed depending on the cations components, i.e. either as similar cations or dissimilar cations as shown in Table 7 below:

Table 7**:** Mixing of salts based on similar or dissimilar cations

Similar cations	**Dissimilar cations**
MgCl_2+**Mg**SO_4	**Mg**Cl_2+**Na$_2$**SO_4
NaCl+**Na$_2$**SO_4	**Na**Cl+**Mg**SO_4

4.1.3 Determining the concentrations of binary salt mixtures

Another relevant factor in salt mixture experiments is the determination of the different concentrations that have to be used. In this study, the concentrations of the mixtures were determined according to the following steps:

1. Determination of LC50s separately for single salts in a binary salt mixture

For each binary salt mixture (i.e. $MgCl_2$+$MgSO_4$, NaCl+Na_2SO_4, $MgCl_2$+Na_2SO_4 and NaCl+$MgSO_4$), separate experiments were conducted to determine the LC50s for the individual salts (i.e. NaCl, $MgCl_2$, Na_2SO_4 and $MgSO_4$) as presented in Table 8.

Table 8**:** Different salts 96 h LC values for *C. nilotica*

Salts	**96 h LC50 (g/L)**
NaCl	10.53
$MgCl_2$	1.67
Na_2SO_4	1.76
$MgSO_4$	10.80

2. Determination of the relative toxic unit (RTU) of the mixture

Using the LC50s of the two salts, the RTU is calculated by adding the relative toxic fractions (RTFs) of each individual salt (Table 9). Note that the sum of the RTFs should be equal to 1 (i.e. equations (1) + (2) = 1).

$$\mathrm{RTFMgCl2} = \frac{\mathrm{LC50\ MgCl2}}{\mathrm{LC50\ MgCl2\ +\ LC50\ MgSO4}} = \frac{1.67}{1.67\ +\ 10.80} = 0.134 \qquad (1)$$

$$\mathrm{RTFMgSO4} = \frac{\mathrm{LC50\ MgSO4}}{\mathrm{LC50\ MgCl2\ +\ LC50\ MgSO4}} = \frac{10.80}{1.67\ +\ 10.80} = 0.866 \qquad (2)$$

Table 9**:** Examples of binary salt mixture and calculations of RTUs

Binary salt mixture	**Calculation of RTU**
$MgCl_2$+$MgSO_4$	RTU = $_{RTF}$ $MgCl_2$ + $_{RTF}$$MgSO_4$ = 0.134 + 0.866 = 1
NaCl+$MgSO_4$	RTU = $_{RTF}$NaCl + $_{RTF}$$MgSO_4$ = 0.494 + 0.506 = 1
$MgCl_2$+Na_2SO_4	RTU = $_{RTF}$$MgCl_2$ + $_{RTF}$$Na_2SO_4$ = 0.487 + 0.513 = 1
NaCl+Na_2SO_4	RTU = $_{RTF}$NaCl + $_{RTF}$$Na_2SO_4$ = 0.857 + 0.143 = 1

3. Estimation of concentration range and proportion of individual salts in the salt mixture

Use the single salt experiments as basis to estimate the concentrations to use in the salt mixture experiment. Studies have shown that the same concentrations range used in the single salt experiments can be reconciled and used in the binary mixture experiments. For instance, in the present experiment, the concentrations range used for the single salt experiments was between 0 (control) and 32 mg/L. Hence, concentrations ranges used for the binary mixture experiments were also between 0 and 32 mg/L.

For each concentration, determine the proportion of each individual salt in the mixture by multiplying the concentration with the RTF of that particular salt. In this study, the concentrations used for the binary salt mixture exposure tests were 0 (control), 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 mg/L. Each concentration was multiplied by the respective RTF to obtain the proportions of salt needed in that each binary mixture.

4.2 Exposure experiments of organisms to binary salt mixtures

4.2.1 Test design and procedure

In order to test the described procedure for conducting binary salts experiments, juvenile *Caridina nilotica* were exposed to binary mixture of $MgCl_2$+$MgSO_4$, NaCl+Na_2SO_4 (similar cations), $MgCl_2$+Na_2SO_4, NaCl+$MgSO_4$ (dissimilar cations) in separate experiments. The aim was to find out whether the individual salts in the mixture interact and thereby possibly increase the toxic effect from what would be expected on the basis of single-salt data.

This study employed a static experimental method to determine the lethal concentration values of the test binary salt mixtures for juvenile *Caridina nilotica*, an indigenous freshwater shrimp. The test methods used were short-term lethal tests (96 h) and long-term lethal tests (240 h) using 600-mL grade A beakers as experimental vessels, which were pre-acid washed. The test medium was dechlorinated tap water, same used during culture of the shrimps. For each binary salt mixture, different fresh concentration range between 0 (control) and 32 mg/L were prepared for juvenile and adult exposure tests. Tables 10-13 give the concentration range as well as proportions of single salts used for $MgCl_2$+$MgSO_4$, NaCl+Na_2SO_4, $MgCl_2$+Na_2SO_4, NaCl+$MgSO_4$ binary salt mixtures. Each concentration contained 10 shrimps and replicated three times. Dead shrimps were recorded twice daily and removed from experimental vessels. The cumulative number of mortality were recorded at the end of 48, 96 and 240 h. Data obtained after 48-96 h of exposure were considered short-term, while that obtained 240 h after exposure were considered long-term. Test solutions were changed every fourth day to minimise build-up of algae and nutrients within the test vessels for the long-term tests. Shrimps were not fed during the short-term experimental periods but were fed after 96 h of exposure. Swimming behaviour of shrimps due to the exposure to salts were observed and recorded. Water quality parameters including temperature, electrical conductivity (EC), hydrogen ion concentration (pH), dissolved oxygen (DO) and temperature were recorded daily. The test endpoint was mortality or immobilisation, which was assessed by prodding the organism and checking for movement. Acceptable control mortality was restricted to 10% for the short-term exposure tests.

Table 10: Proportions of single salts required to form the different concentrations of $MgCl_2$+$MgSO_4$ binary mixture

Concentration of $MgCl_2$+$MgSO_4$ (mg/L)	**Proportion of $MgSO_4$ (mg/L) ($_{RTF}MgSO_4$ = 0.866)**	**Proportion of $MgCl_2$ (mg/L) ($_{RTF}MgCl_2$ = 0.134)**
0.000	0.000	0.000
0.063	0.055	0.008
0.125	0.108	0.017
0.25	0.217	0.034
0.5	0.433	0.067
1	0.866	0.134
2	1.732	0.268
4	3.464	0.536
8	6.928	1.072
16	13.856	2.144
32	27.712	4.288

Table 11: Proportions of single salts required to form the different concentrations of NaCl+Na_2SO_4 binary mixture

Concentration of NaCl+Na_2SO_4 (mg/L)	**Proportion of NaCl (mg/L) ($_{RTF}$ NaCl = 0.857)**	**Proportion of Na_2SO_4 (mg/L) ($_{RTF}Na_2SO_4$ = 0.143)**
0.000	0.000	0.000
0.063	0.054	0.009
0.125	0.107	0.018
0.25	0.214	0.036
0.5	0.429	0.072
1	0.857	0.143
2	1.714	0.286
4	3.428	0.572
8	6.856	1.144
16	13.712	2.288
32	27.424	4.576

Table 12: Proportions of single salts required to form the different concentrations of $MgCl_2$+$MgSO_4$ binary mixture

Concentration of $MgCl_2$+Na_2SO_4 (mg/L)	**Proportion of $MgCl_2$ (mg/L) ($_{RTF}MgCl_2$ = 0.487)**	**Proportion of Na_2SO_4 (mg/L) ($_{RTF}Na_2SO_4$ = 0.513)**
0.000	0.000	0.000
0.063	0.031	0.032
0.125	0.061	0.064
0.25	0.122	0.128
0.5	0.244	0.257
1	0.487	0.513
2	0.974	1.026
4	1.948	2.052
8	3.896	4.104
16	7.792	8.208
32	15.584	16.416

Table 13: Proportions of single salts required to form the different concentrations of NaCl+$MgSO_4$ binary mixture

Concentration of NaCl+$MgSO_4$ (mg/L)	**Proportion of NaCl (mg/L) ($_{RTF}$NaCl = 0.494)**	**Proportion of $MgSO_4$ (mg/L) ($_{RTF}MgSO_4$ = 0.506)**
0.000	0.000	0.000
0.063	0.031	0.032
0.125	0.062	0.063
0.25	0.124	0.127
0.5	0.247	0.253
1	0.494	0.506
2	0.988	1.012
4	1.976	2.024
8	3.952	4.048
16	7.904	8.096
32	15.808	16.192

4.2.2 Data analysis

Probit statistical software version 1.5 (USEPA, 1990) was used to estimate the lethal concentration (LC) values and their 95% confidence limits, using mortality data obtained from the various ecotoxicity tests with binary salt mixtures and *C. nilotica*. One-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison post hoc tests were used to compare mean mortality values between control and exposed groups. Statistics were performed using Statistica Version 12 or R statistical software and all statistical decisions were made at alpha = 0.05 a priori.

4.3 Results of binary salt mixtures exposure tests

4.3.1 Juvenile and adult *C. nilotica* exposure to $MgCl_2$+$MgSO_4$

For juvenile *C. nilotica* exposed to $MgCl_2$+$MgSO_4$, at 48, 96 and 240 h after exposure, one way analysis of variance (ANOVA) of mortality revealed a significant difference ($p < 0.001$) between control and treatment groups. At 48 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in concentrations 0.06, 0.125, 0.25, 0.5, 1 and 2 g/L were not significantly different from control group, but significantly lower than the other treatment groups. Mortality in 8, 16 and 32 g/L were statistically not different, but significantly higher than mortality in 4 g/L (Figure 19). At 96 h after exposure, Newman-Keuls multiple comparison test showed that mortality in 0.06, 0.125 and 0.25 g/L were not significantly different from control group, but statistically different from the other treatment groups ($p < 0.05$). Mortality in 0.5 and 1 g/L were not significantly, but lower than mortality in 2, 4, 8, 16 and 32 g/L. However, mortality in 8, 16 and 32 g/L were statistically not different but higher than in the lower concentrations (Figure 20). At 240 h after exposure, Newman-Keuls multiple comparison test showed that juvenile shrimp mortality in concentrations 0.06, 0.125 and 0.25 g/L were not statistically different from control groups, but significantly different from the other treatment groups. Mortality in 0.5 and 1 g/L were not significantly different, just as mortality in 2, 4, 8, 16 and 32 g/L were not statistically different. However, mortality generally increased monotonically (Figure 21).

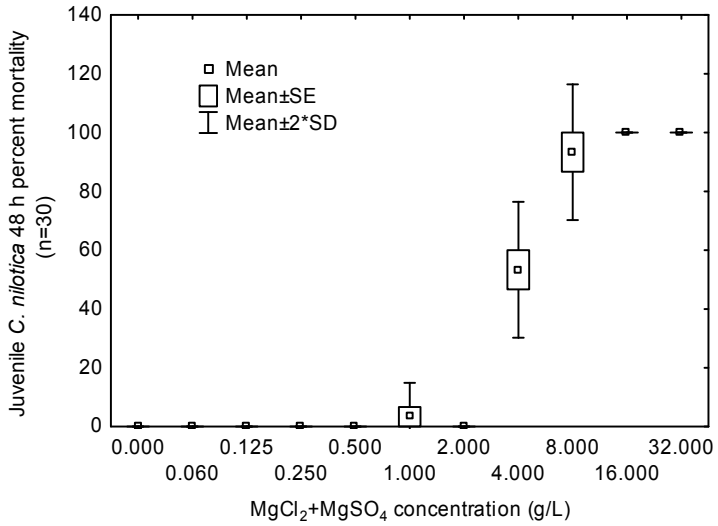

Figure 19: Juvenile *C. nilotica* mean mortality after 48 h exposure to $MgCl_2$+$MgSO_4$

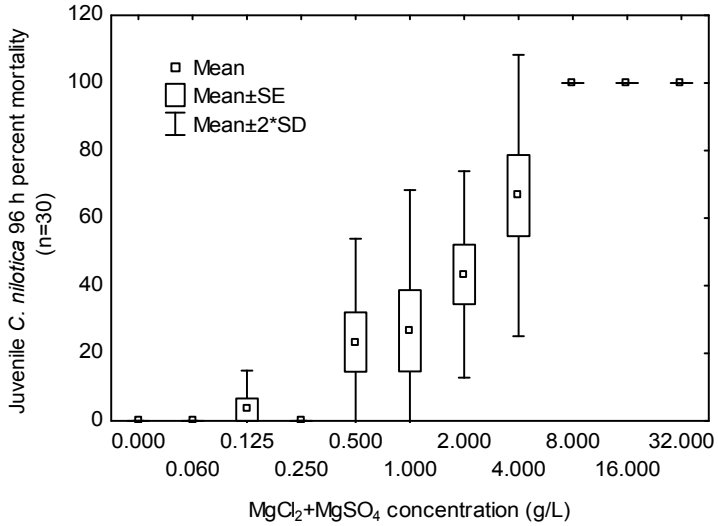

Figure 20: Juvenile *C. nilotica* mean mortality after 96 h exposure to $MgCl_2$+$MgSO_4$

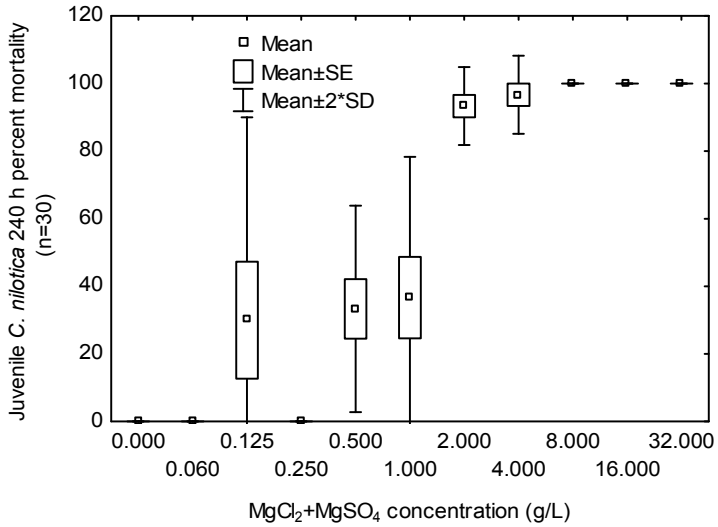

Figure 21: Juvenile *C. nilotica* mean mortality after 240 h exposure to $MgCl_2$+$MgSO_4$

For adult *C. nilotica* exposed to $MgCl_2$+$MgSO_4$, at 48, 96 and 240 h after exposure, one way analysis of variance (ANOVA) of mortality revealed a significant difference ($p < 0.001$) between control and treatment groups. At 48 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in 0.06, 0.125, 0.25, 0.5 and 1 g/L were not significantly different from control group, but statistically different from the other treatment groups ($p < 0.05$). Conversely, mortality in 2, 4, 8, 16 and 32 g/L were not significantly different, but higher than in the lower concentrations (Figure 22). However, at 96 h after exposure, Newman-Keuls multiple comparison test showed that mortality in 0.06, 0.125, 0.25, 0.5 and 1 g/L were significantly different from control group, but mortality in the upper treatment groups remain statistically no significant from each other (Figure 23). At 240 h after exposure, Newman-Keuls multiple comparison test showed that the recorded shrimp mortality was similar to the observations made at 96 h after exposure, except that mortality increased with time (Figure 24).

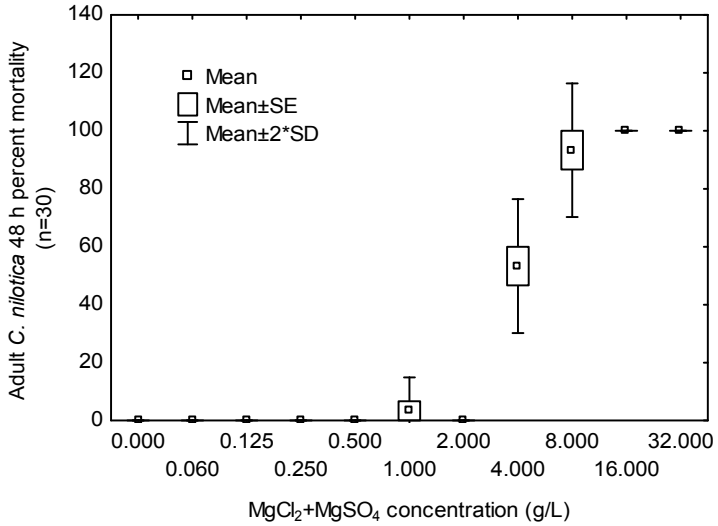

Figure 22: Adult *C. nilotica* mean mortality after 48 h exposure to $MgCl_2$+$MgSO_4$

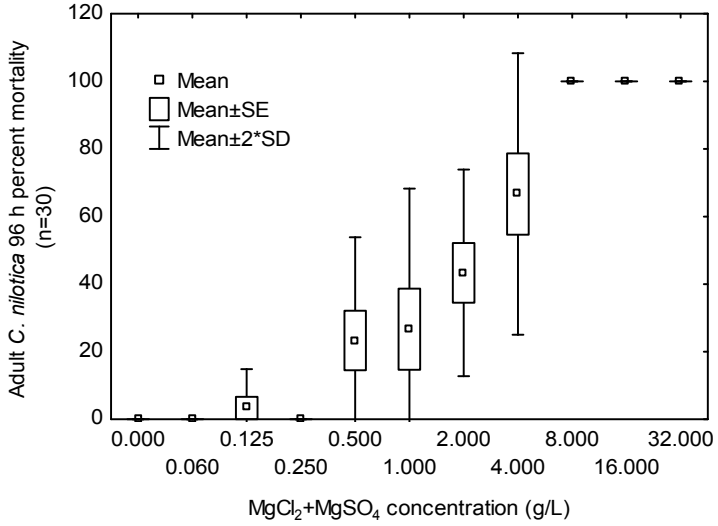

Figure 23: Adult *C. nilotica* mean mortality after 96 h exposure to $MgCl_2$+$MgSO_4$

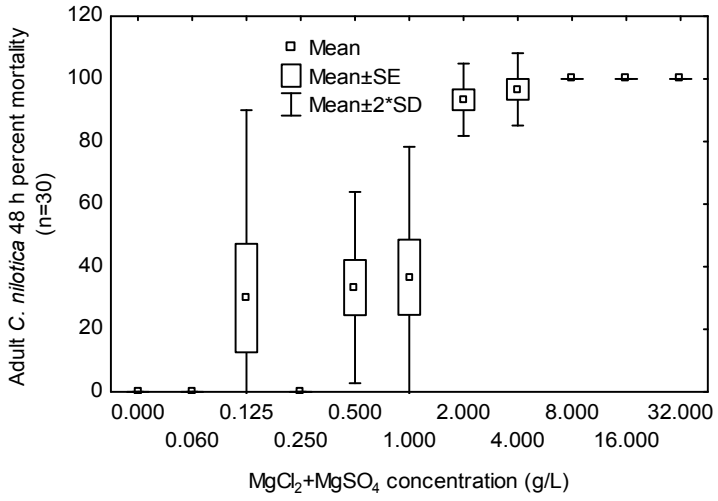

Figure 24: Adult *C. nilotica* mean mortality after 240 h exposure to $MgCl_2$+$MgSO_4$

4.3.2 Juvenile and adult *C. nilotica* exposure to $NaCl+Na_2SO_4$

For juvenile *C. nilotica* exposed to $NaCl+Na_2SO_4$, at 48, 96 and 240 h after exposure, one way analysis of variance (ANOVA) of mortality revealed a significant difference ($p < 0.001$) between control and treatment groups. At 48 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in concentrations 0.06, 0.125, 0.25, 0.5 and 1 g/L were not significantly different from control group, but significantly lower than the other treatment groups. Mortality in 2, 4 and 8 g/L were significantly different, increasing monotonically. However, mortality in 16 and 32 g/L showed no statistically significant difference, but mortality were significantly higher in these two $NaCl+Na_2SO_4$ exposed groups than in the other treatment groups (Figure 25). At 96 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in concentrations 0.06, 0.125, 0.25, 0.5 and 1 g/L were not statistically different from control group, but significantly different from the other treatment groups. Mortality in 2 g/L was significantly lower than mortality in 4, 8, 16 and 32 g/L. Mortality in the last four concentrations (i.e. 4, 8, 16 and 32 g/L) were statistically not different but higher than the lower concentrations (Figure 26). At 240 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in concentrations 0.06, 0.125, 0.25 and 0.5 1 g/L were not statistically different from control group, but significantly lower than the other treatment groups. Mortality in 1 g/L was significantly lower than in 2 g/L, but mortality in both exposed groups (i.e. 1 and 2 g/L) were significantly lower than mortality in 4, 8, 16 and 32 g/L. Mortality in the last four concentrations (i.e. 4, 8, 16 and 32 g/L) were statistically not different but higher than the preceding concentrations (Figure 27).

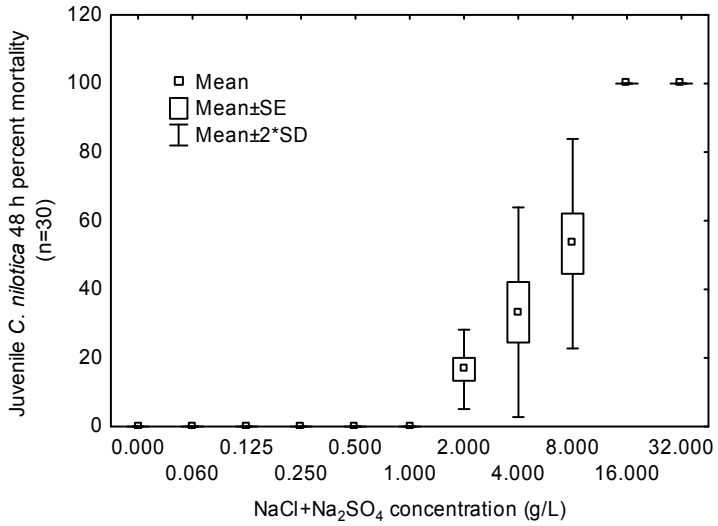

Figure 25: Juvenile *C. nilotica* mean mortality after 48 h exposure to $NaCl+Na_2SO_4$

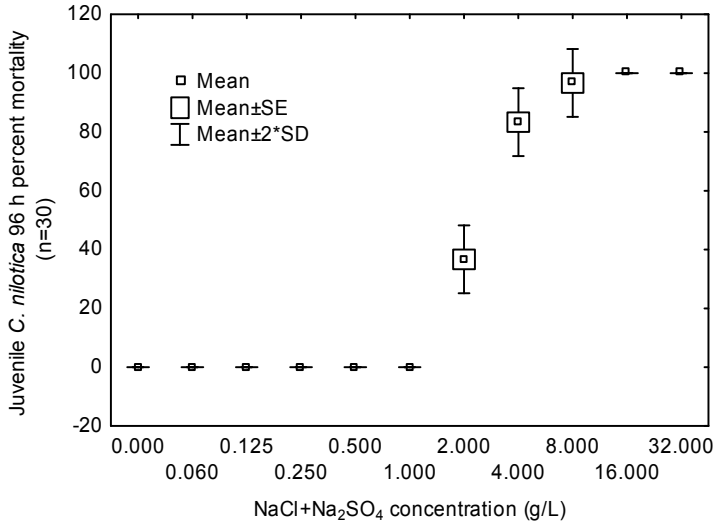

Figure 26: Juvenile *C. nilotica* mean mortality after 96 h exposure to $NaCl+Na_2SO_4$

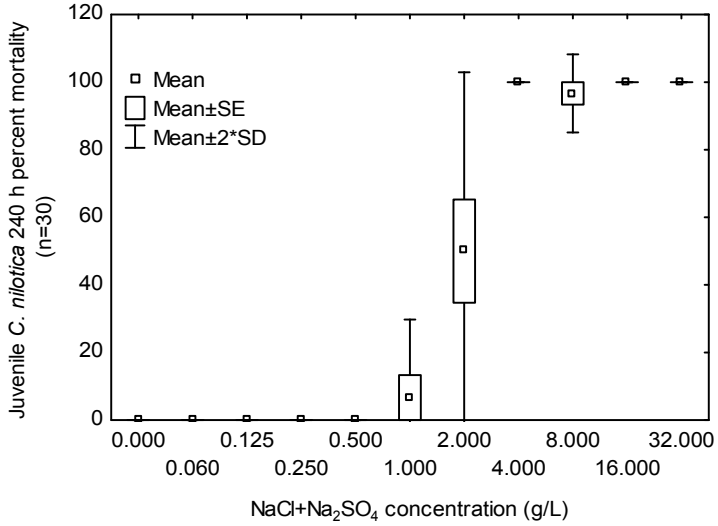

Figure 27: Juvenile *C. nilotica* mean mortality after 240 h exposure to $NaCl+Na_2SO_4$

For adult *C. nilotica* exposed NaCl+Na_2SO_4, at 48, 96 and 240 h after exposure, one way analysis of variance (ANOVA) of mortality revealed a significant difference ($p < 0.001$) between control and treatment groups. At 48 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in 0.06, 0.125, 0.25, 0.5, 1 and 2 g/L were not significantly different from control group, but statistically different from the other treatment groups ($p < 0.05$). Nonetheless, mortality 16 and 32 g/L were not significantly different, but higher than in the lower concentrations, while adult shrimp mortality was significantly lower in 4 g/L than in 8 g/L (Figure 28). Similar mortality observations were made at 96 h after exposure but at this time, Newman-Keuls multiple comparison test showed that adult shrimp mortality in concentrations 8, 16 and 32 g/L were not significantly different (Figure 29). At 240 h after exposure, Newman-Keuls multiple comparison test showed that adult shrimp mortality in concentrations 4, 8, 16 and 32 g/L were not statistically different from each other but was significantly higher than mortality in the lower treatment groups (Figure 30).

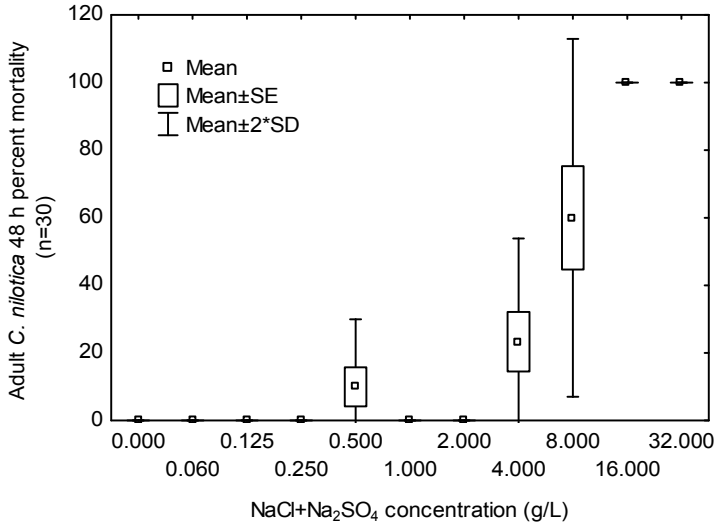

Figure 28: Adult *C. nilotica* mean mortality after 48 h exposure to $NaCl+Na_2SO_4$

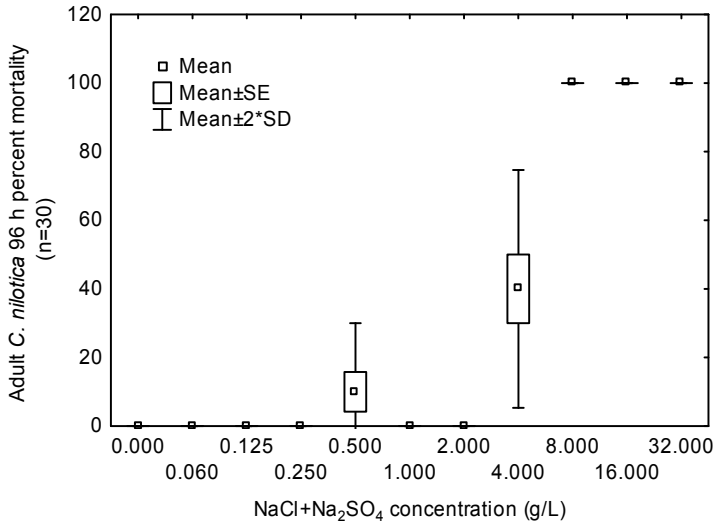

Figure 29: Adult *C. nilotica* mean mortality after 96 h exposure to $NaCl+Na_2SO_4$

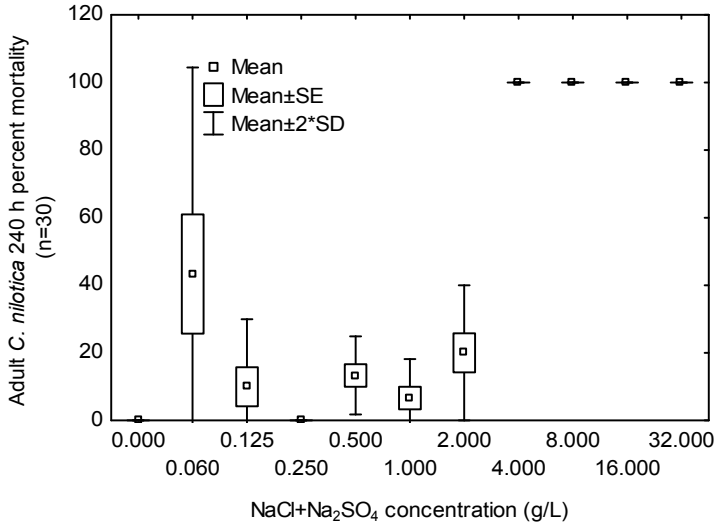

Figure 30: Adult *C. nilotica* mean mortality after 240 h exposure to $NaCl+Na_2SO_4$

4.3.3 Juvenile and adult *C. nilotica* exposure to $MgCl_2$+Na_2SO_4

One way analysis of variance (ANOVA) of juvenile *C. nilotica* mortality after 48, 96 and 240 h revealed a significant difference ($p < 0.001$) between control and $MgCl_2$+Na_2SO_4 exposed groups. At 48 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in concentrations 0.06, 0.125, 0.25, 0.5, 1 and 2 g/L were not significantly different from control group, but significantly lower than the other treatment groups. Mortality in 4, 8 and 16 g/L were significantly different, increasing monotonically. Mortality in 32 g/L was statistically higher than in all other treatment groups (Figure 31). At 96 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in concentrations 0.06, 0.125, 0.25, 0.5, 1 and 2 g/L were not statistically different from control group, but significantly different from the other treatment groups. Mortality in 4 and 8 g/L was not significantly different but significantly lower than mortality in 16 g/L, while shrimps exposed to 32 g/L experienced the highest significant mortality than in all other treatment groups (Figure 32). At 240 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in concentrations 0.06, 0.125, 0.25, 0.5, 1 and 2 g/L were not statistically different from control group, but significantly different from the other treatment groups. Similar observations were made at 48 and 96 h after exposure. Mortality in 8, 16 and 32 g/L were significantly not different but higher than the preceding concentration of 4 g/L (Figure 33).

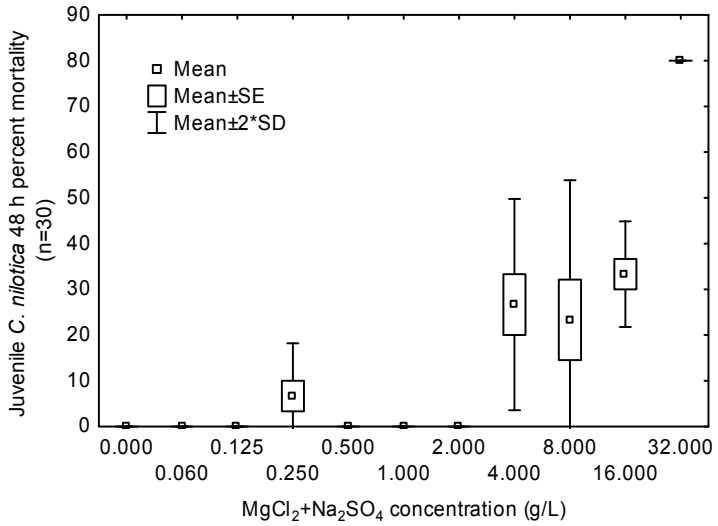

Figure 31: Juvenile *C. nilotica* mean mortality after 48 h exposure to $MgCl_2$+Na_2SO_4

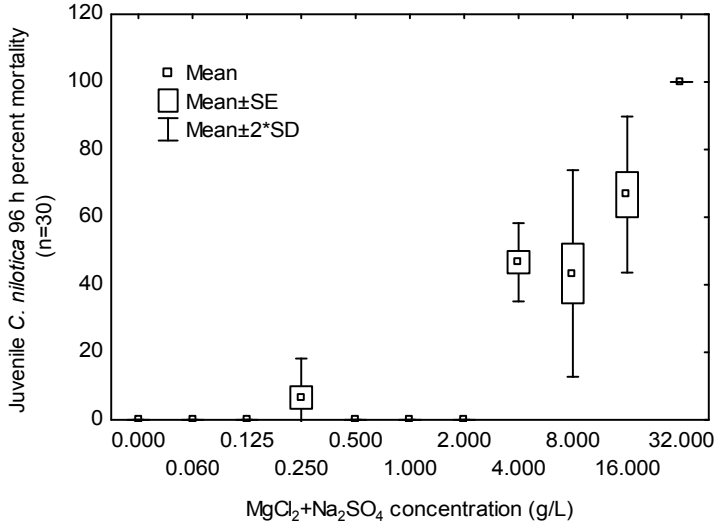

Figure 32: Juvenile *C. nilotica* mean mortality after 96 h exposure to $MgCl_2$+Na_2SO_4

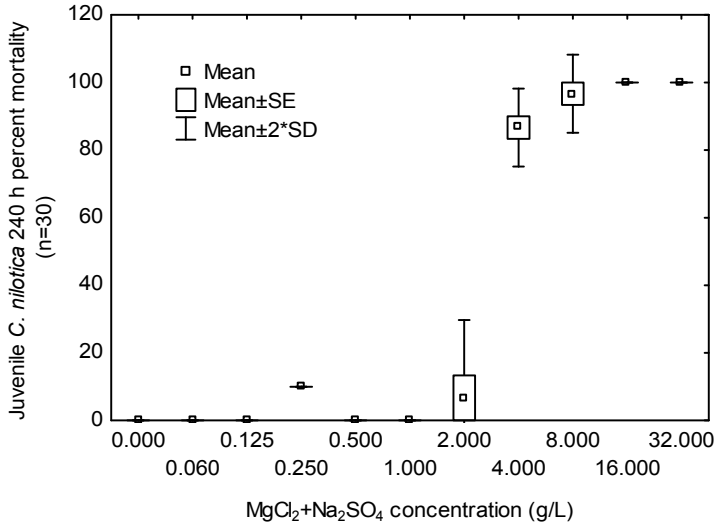

Figure 33: Juvenile *C. nilotica* mean mortality after 240 h exposure to $MgCl_2$+Na_2SO_4

For adult *C. nilotica* exposed to $MgCl_2$+Na_2SO_4, one way analysis of variance (ANOVA) of mortality after 48, 96 and 240 h revealed a significant difference ($p < 0.001$) between control and treatment groups. At 48 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in concentrations 0.5, 1 and 2 g/L were not significantly different from control group, but significantly lower than the other treatment groups. Similarly, mortality in 4, 8 and 16 g/L were significantly lower than mortality in 16 g/L. However, the highest mortality at 48 h after exposure was recorded in 32 g/L (Figure 34). At 96 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in concentrations 0.5, 1 and 2 g/L were not statistically different from control group, but significantly lower than mortality in the other treatment groups. Adult shrimp mortality in 4 and 8 g/L were statistically not different from each other, but significantly lower than mortality in 16, which also recorded lower mortality than that of 32 g/L (Figure 35). At 240 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in concentrations 0.5, 1 and 2 were significantly lower than control mortality but not statistically different from each other. Similarly, mortality in 4 g/L was significantly lower than mortality of shrimps in 8, 16 and 32 g/L, but mortality in these three concentrations were statistically not different from each other (Figure 36).

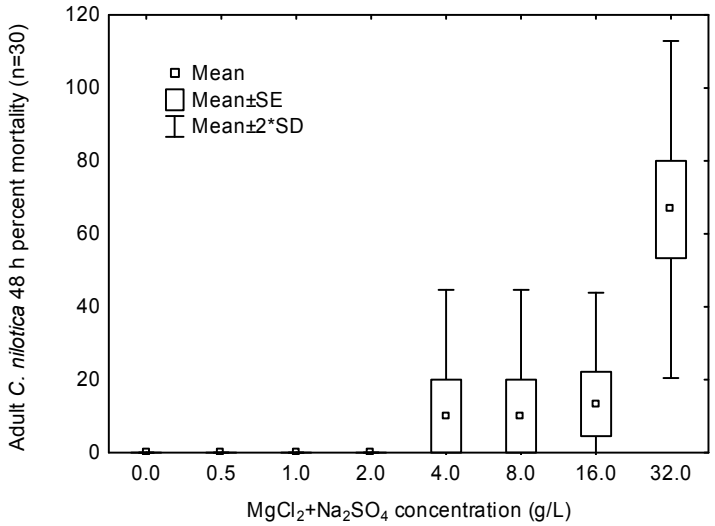

Figure 34: Adult *C. nilotica* mean mortality after 48 h exposure to $MgCl_2$+Na_2SO_4

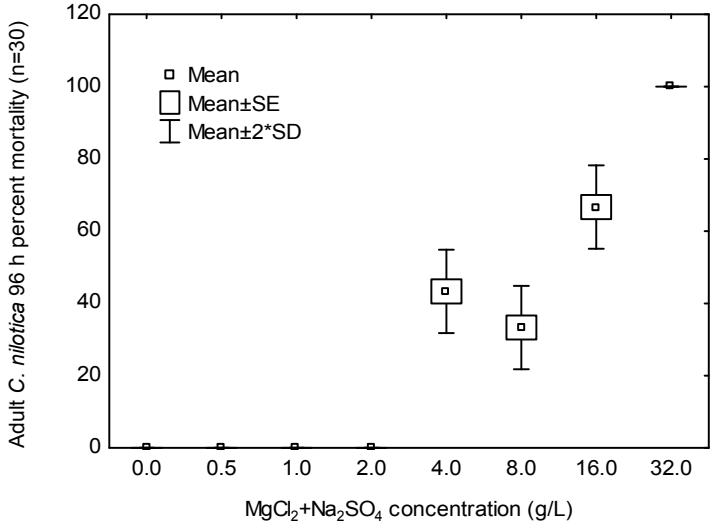

Figure 35: Adult *C. nilotica* mean mortality after 96 h exposure to $MgCl_2$+Na_2SO_4

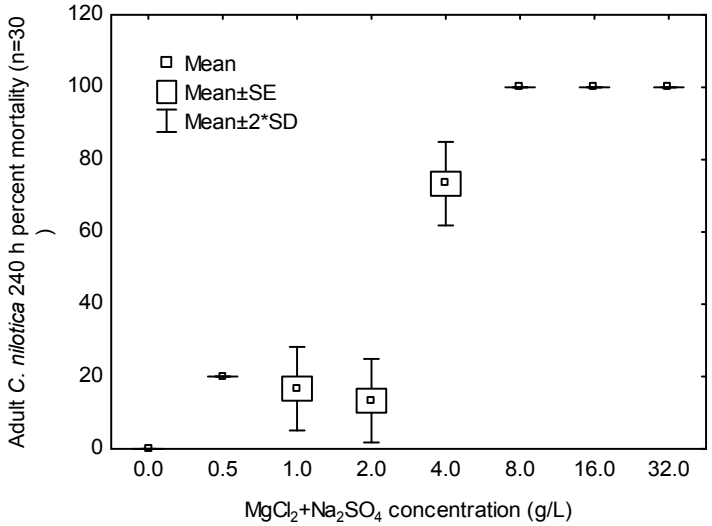

Figure 36: Adult *C. nilotica* mean mortality after 240 h exposure to $MgCl_2$+Na_2SO_4

4.3.4 Juvenile and adult *C. nilotica* exposure to NaCl+$MgSO_4$

One way analysis of variance (ANOVA) of juvenile *C. nilotica* mortality after 48, 96 and 240 h revealed a significant difference ($p < 0.001$) between control and NaCl+$MgSO_4$ exposed groups. At 48 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in in concentrations 0.06, 0.125, 0.25, 0.5, 1 and 2 g/L were not significantly different from control group, but significantly lower than the other treatment groups. Mortality in 4, 8 and 16 g/L were not significantly different from each other, but significantly lower in 32 g/L, which recorded highest significant mortality (Figure 37). At 96 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in concentrations 0.06, 0.125, 0.25, 0.5, 1 and 2 g/L were not significantly different from control group, but significantly lower than the other treatment groups. Similarly, mortality in 4 and 8 g/L were not significantly difference but significantly lower than mortality in 16 and 32 g/L. Shrimp mortality in 16 g/L was significantly higher than in 32 g/L, which recorded the highest statistically significant mortality (Figure 38). At 240 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in concentrations 0.06, 0.125, 0.25, 0.5, 1 and 2 g/L were not statistically different from control group, but significantly different from the other treatment groups. Similar observations were made at 48 and 96 h after exposure. Mortality in 4 and 8 g/L were statistically not significant, but were statistically lower than mortality in 16 and 32 g/L. However, mortality in the last two highest concentrations, i.e. 16 and 32 g/L, which was higher than in all other treatment groups, were not significantly difference (Figure 39).

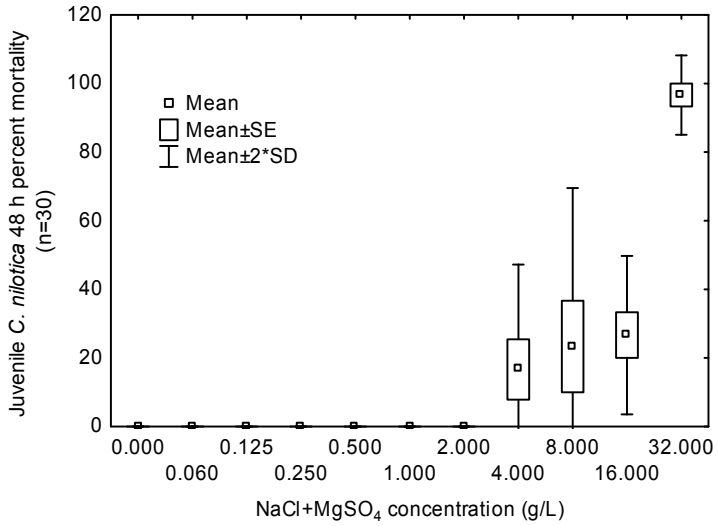

Figure 37: Juvenile *C. nilotica* mean mortality after 48 h exposure to $NaCl+MgSO_4$

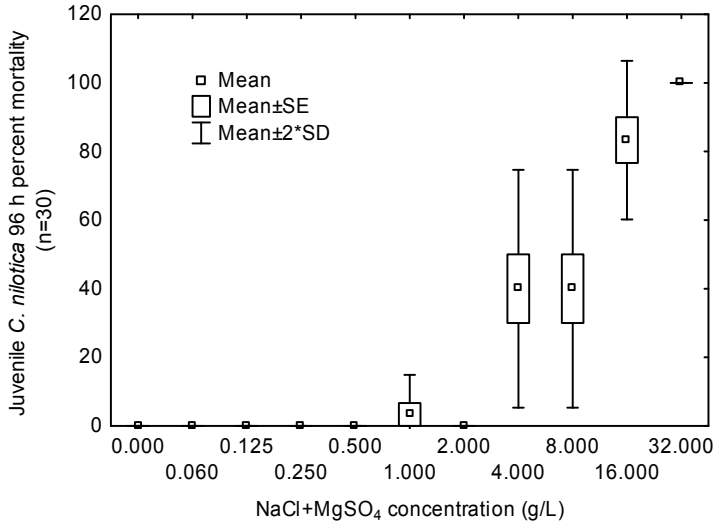

Figure 38: Juvenile *C. nilotica* mean mortality after 96 h exposure to $NaCl+MgSO_4$

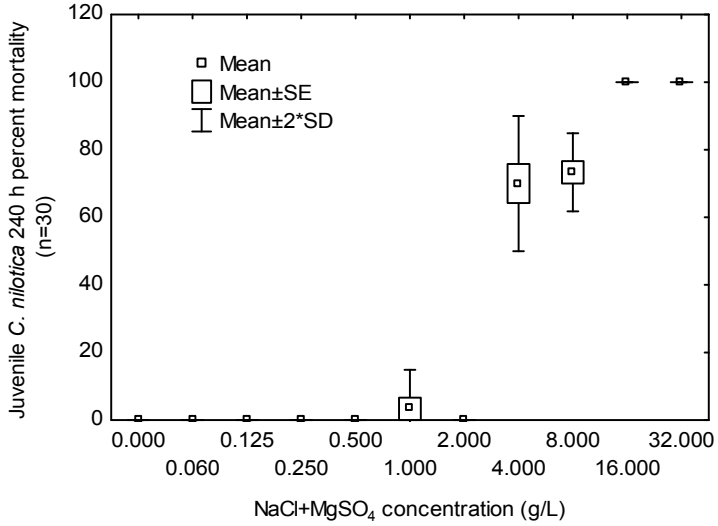

Figure 39: Juvenile *C. nilotica* mean mortality after 240 h exposure to $NaCl+MgSO_4$

For adult *C. nilotica* exposed to NaCl+$MgSO_4$, one way analysis of variance (ANOVA) of mortality after 48, 96 and 240 h revealed a significant difference ($p < 0.001$) between control and treatment groups. At 48 h after exposure, Newman-Keuls multiple comparison tests showed that shrimp mortality in in concentrations 0.5, 1, 2 and 4 g/L were not significantly different from control group, but significantly lower than the other treatment groups. Mortality in 8, 16 and 32 g/L were significantly different from each other, increasing monotonically (Figure 40). At 96 h after exposure, mortality in concentrations 0.5, 1 and 2 g/L were not significantly different from control group, while mortality in 4 and 8 g/L were statistically not different from each other but lower than mortality in the lower concentrations. There were statistically significant differences in mortality in concentrations 16 and 32 g/L, with highest mortality being recorded in 32 g/L (Figure 41). At 240 h after exposure, mortality in concentrations 0.5 and 1 g/L were not statistically different from mortality in control group but significantly lower than that of other treatment groups. Mortality in 2 g/L was significantly lower than mortality in 4 g/L, which in turn recorded lower mortality than that recorded in concentrations 8, 16 and 32 g/L. Mortality recorded in these last three concentrations where not significantly different from each other (Figure 42).

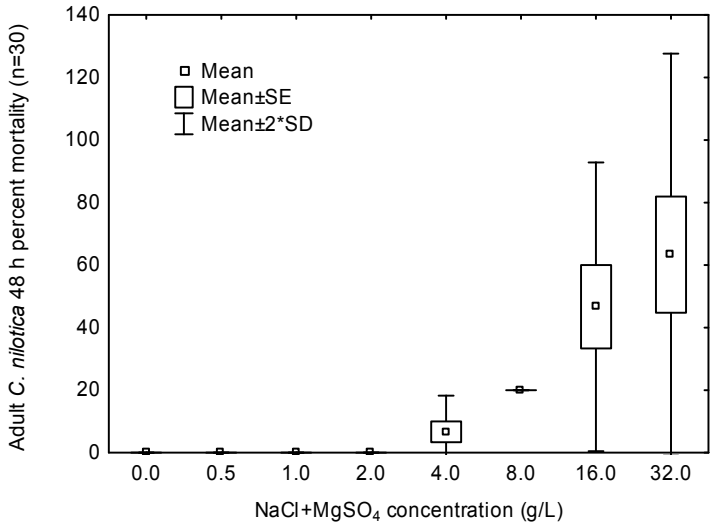

Figure 40: Adult *C. nilotica* mean mortality after 48 h exposure to $NaCl+MgSO_4$

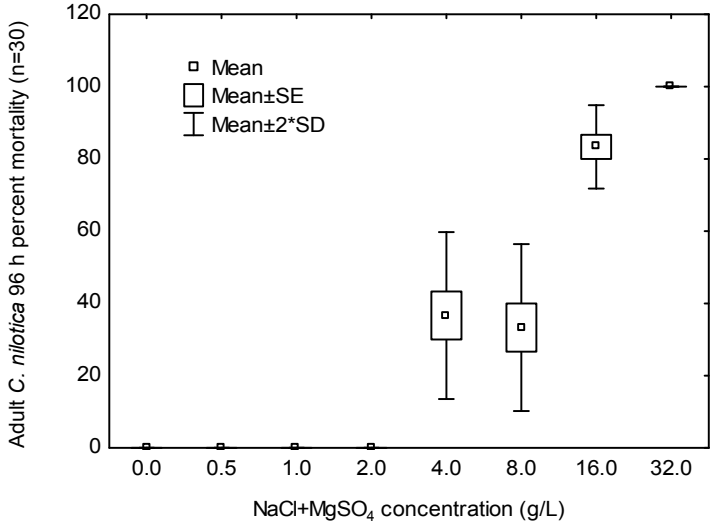

Figure 41: Adult *C. nilotica* mean mortality after 96 h exposure to $NaCl+MgSO_4$

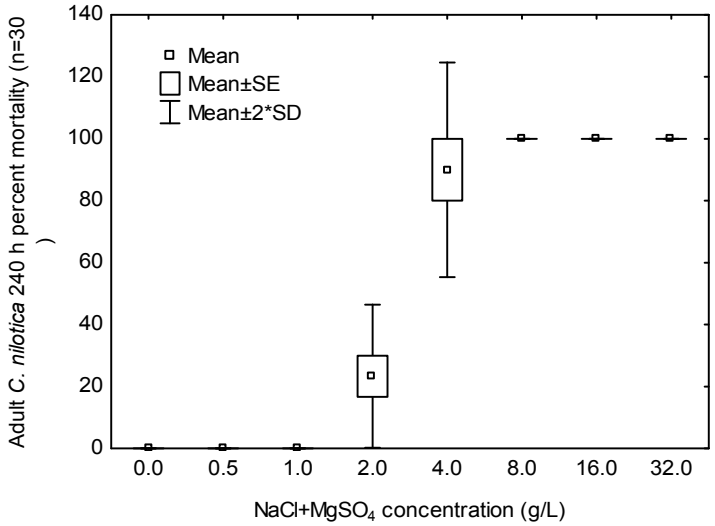

Figure 42: Adult *C. nilotica* mean mortality after 240 h exposure to $NaCl+MgSO_4$

4.3.5 Lethal concentrations of the tests binary salt mixture to *Caridina nilotica*

The LC1, LC10 and LC50 values estimated using PROBIT regression based on responses of adult and juvenile *C. nilotica* exposure tests with binary salt mixtures are presented in Table 14. A fuller version of the estimated LC values is attached as Appendix 2. It should be noted that the smaller the LC value, the more sensitive is the organism to the test substance. In other words, the salt with the least LC value is the most sensitive. Juveniles were found to be more sensitive than adults in most cases, but adult were also found to be sensitive than juveniles in some cases.

For short-term (48 h) exposure tests and at the level of LC50, the most toxic salt was $MgCl_2$+$MgSO_4$ with an LC50 of 3.97 g/L for juvenile *C. nilotica*, while $MgSO_4$ was the least toxic binary mixture salt was $MgCl_2$+Na_2SO_4 with an LC50 of 28.06 g/L for adult *C. nilotica* (Table 15). Similarly, for short-term (96 h) exposure tests and at the level of LC50, $MgCl_2$+$MgSO_4$ was the most toxic with an LC50 of 1.76 g/L for juvenile *C. nilotica*, while $MgCl_2$+Na_2SO_4 was the least toxic with an LC50 of 8.93 g/L for adult *C. nilotica* (Table 16). For long-term (240 h) exposure tests and at the level of LC50, $MgCl_2$+$MgSO_4$ was the most toxic salt with an LC50 of 0.72 g/L for juvenile *C. nilotica*, while NaCl+$MgSO_4$ was the least toxic with an LC50 of 3.95 g/L for juvenile *C. nilotica* (Table 17).

Table 14**:** Estimated lethal concentration (LC) values for *C. nilotica* juvenile and adult exposed to single salt and binary salt mixtures

Binary salt mixtures	**Life stage**	**Test duration**	**Lethal concentration (in g/L) (Lower limit-Upper limit)**		
			LC1	**LC10**	**LC50**
$NaCl+Na_2SO_4$	Juvenile	48 h	0.96 (0.23-1.81)	2.22 (0.92-3.43)	6.16 (4.14-9.27)
	Adult	48 h	0.72 (0.00-2.35)	1.81 (0.00-4.45)	5.60 (0.51-102.86)
	Juvenile	96 h	0.80 (0.49-1.07)	1.35 (0.98-1.65)	2.56 (2.18-3.02)
	Adult	96 h	0.68	1.45	3.67
	Juvenile	240 h	0.63 (0.21-0.98)	1.06 (0.53-1.43)	1.98 (1.46-2.67)
	Adult	240 h	0.07 (0.00-0.49)	0.31 (0.00-1.34)	1.90 (0.00-0.00)
$MgCl_2+MgSO_4$	Juvenile	48 h	1.27 (0.78-1.70)	2.12 (1.56-2.58)	3.97 (3.37-4.66)
	Adult	48 h	2.84	7.04	21.41
	Juvenile	96 h	0.14 (0.07-0.22)	0.43 (0.28-0.59)	1.76 (1.39-2.24)
	Adult	96 h	0.19 (0.00-1.04)	0.962 (0.00-2.89)	7.26 (1.95-54.16)
	Juvenile	240 h	0.05 (0.00-0.15)	0.16 (0.02-0.36)	0.72 (0.31-1.58)
	Adult	240 h	0.01 (0.00-0.04)	0.049 (0.00-0.20)	0.80 (0.21-1.49)
$MgCl_2+Na_2SO_4$	Juvenile	48 h	0.42 (0.00-1.700)	2.29 (0.06-5.78)	18.44 (7.42-415.10)
	Adult	48 h	2.32 (0.79-3.98)	7.10 (4.21-9.73)	28.06 (20.52-46.99)
	Juvenile	96 h	0.45 (0.00-1.65)	1.58 (0.02-3.94)	7.34 (2.48-39.84)
	Adult	96 h	1.07 (0.05-2.52)	2.70 (0.46-4.91)	8.39 (4.50-17.20)
	Juvenile	240 h	0.40	0.94	2.66

	Adult	240 h	0.25 (0.00-0.73)	0.67 (0.02-1.44)	2.26 (0.79-5.46)
$NaCl+MgSO_4$	Juvenile	48 h	1.72 (0.760-2.76)	4.68 (2.96-6.29)	16.01 (12.61-21.50)
	Adult	48 h	1.82 (0.73-3.02)	5.32 (3.27-7.23)	19.84 (15.23-28.49)
	Juvenile	96 h	1.00 (0.54-1.51)	2.41 (1.63-3.16)	7.06 (5.73-8.73)
	Adult	96 h	1.34 (0.21-2.62)	2.97 (0.99-4.76)	7.94 (5.04-12.82)
	Juvenile	240 h	1.00 (0.59-1.39)	1.86 (1.33-2.31)	3.95 (3.31-4.72)
	Adult	240 h	1.18 (0.77-1.49)	1.68 (1.28-1.97)	2.58 (2.25-2.96)

Table 15**:** *C. nilotica* tolerances to the tested binary salt mixtures at LC50 48 h after exposure

Binary salt mixture	**Life stage**	**Exposure period**	**LC50 (g/L)**
$MgCl_2+MgSO_4$	Juvenile	48 h	3.97
$NaCl+Na_2SO_4$	Adult	48 h	5.60
$NaCl+Na_2SO_4$	Juvenile	48 h	6.16
$NaCl+MgSO_4$	Juvenile	48 h	16.01
$MgCl_2+Na_2SO_4$	Juvenile	48 h	18.44
$NaCl+MgSO_4$	Adult	48 h	19.84
$MgCl_2+MgSO_4$	Adult	48 h	21.41
$MgCl_2+Na_2SO_4$	Adult	48 h	28.06

Table 16**:** *C. nilotica* tolerances to the tested binary salt mixtures at LC50 96 h after exposure

Binary salt mixture	Life stage	Exposure period	LC50 (g/L)
$MgCl_2$+$MgSO_4$	Juvenile	96 h	1.76
NaCl+Na_2SO_4	Juvenile	96 h	2.56
NaCl+Na_2SO_4	Adult	96 h	3.67
NaCl+$MgSO_4$	Juvenile	96 h	7.06
$MgCl_2$+$MgSO_4$	Adult	96 h	7.26
$MgCl_2$+Na_2SO_4	Juvenile	96 h	7.34
NaCl+$MgSO_4$	Adult	96 h	7.94
$MgCl_2$+Na_2SO_4	Adult	96 h	8.39

Table 17**:** *C. nilotica* tolerances to the tested binary salt mixtures at LC50 240 h after exposure

Binary salt mixture	Life stage	Exposure period	LC50 (g/L)
$MgCl_2$+$MgSO_4$	Juvenile	240 h	0.72
$MgCl_2$+$MgSO_4$	Adult	240 h	0.80
NaCl+Na_2SO_4	Adult	240 h	1.90
NaCl+Na_2SO_4	Juvenile	240 h	1.98
$MgCl_2$+Na_2SO_4	Adult	240 h	2.26
NaCl+$MgSO_4$	Adult	240 h	2.58
$MgCl_2$+Na_2SO_4	Juvenile	240 h	2.66
NaCl+$MgSO_4$	Juvenile	240 h	3.95

5 DISCUSSION

5.1 Single and binary salt mixtures toxicity

It is known that magnesium ions are commonly found in nature and so do the chloride and sulphate ions, although very little is known about its effects on aquatic organisms (Dallas and Day 2004). Most South African rivers are at risk of salinity, which has been recognised as problematic with known natural water chemistry variations in various catchments. Areas such as the Olifants catchment system in Mpumalanga and Breed River in the Western Cape are characterised by sulphates and chloride salinisation due to predominant agricultural, industrial and mining activities in the catchment areas (Scherman et al. 2003; Holland et al. 2011). The concern is that the excessive contents of a certain component of total dissolved solids may harm life activities of individual species, which potentially limit their distribution pattern, growth and reproduction in an ecosystem (Berezina 2003; Nielsen et al. 2003; Miranda et al. 2010). Therefore, this study has highlighted the significance of conducting ecotoxicity tests for various salts in order to protect aquatic ecosystems, which host various organisms including macroinvertebrates, as mandated by the South African National Water Act (No. 38 of 1998).

This study has shown that both juvenile and adult *C. nilotica* are adversely affected when exposed to magnesium sulphate or magnesium chloride based on mortalities observed. Under similar laboratory conditions, small quantities of $MgCl_2$ have proved to be lethal to adult *C. nilotica* at 96 h as compared to $MgSO_4$, which required four times more to be effective on the same age group and time interval. This trend was found in all age groups whereby double or triple the amount of $MgSO_4$ salt were needed to yield the same effect under same conditions. Magnesium chloride was found to be more lethal than magnesium sulphate in both juvenile and adult shrimps. When the organisms were treated with $MgSO_4$ and $MgCl_2$ in each of the experiments, mortalities started to occur first in experimental vessels that were treated with $MgCl_2$.

The findings of this current study also revealed that juvenile *C. nilotica* is more sensitive to $MgCl_2$ than its adult stage. However, a study by Mensah et al. (2011) revealed that neonate *Caridina nilotica* treated with Roundup herbicide are most sensitive to the herbicide compared to juveniles and adults. This designates that further studies for a particular salt or contaminant on a specific species should primarily be conducted on all life stages to determine their sensitivities. It is thus imperative to ensure that water quality guidelines developed for protection of freshwater species should be inclusive of the life stage of

organisms as age was found to be a factor according in the species under this present study.

Furthermore, the current study findings can also be used to define and refine the existing ecological reserve boundaries for magnesium sulphate which has been reported to be inconsistent. However, further research on other taxa would have to be conducted to present a representative and clear boundaries for the salt. This study has shown that both $MgCl_2$ and Na_2SO_4 are more toxic than $MgSO_4$ although this may be species dependent and other factors. It also clearly shows that the salt is more lethal on young individuals than adults with increasing concentrations and time intervals.

In this study, analysis of binary mixtures was to find out whether the individual salts in the mixture interact and thereby possibly increase the toxic effect from what would be expected on the basis of single-salt data. The analysis is therefore aimed at describing the no interaction or additivity relationship among individual salts in the salt mixtures. It appears that interactions between salts increase the intensity of salts toxicity as demonstrated by the tested binary salt mixtures. Toxicity of the tested binary salt mixtures showed that $MgCl_2$+$MgSO_4$ is most toxic at 48 h, 96 h and 240 h. This implies that irrespective of the exposure time, that is, whether short-term or long-term, $MgCl_2$+$MgSO_4$ poses potential threat to *C. nilotica*. It was also observed that similar cations salts tend to be more toxic than dissimilar cations. In fact, at the end of 48, 96 and 240 h of exposure, all the first three most toxic binary salt mixtures involved similar cations salts (Tables 15-17) (i.e. **Mg**Cl_2+**Mg**SO_4, **Na**Cl+**Na$_2$**SO_4 and **Na**Cl+**Na$_2$**SO_4 for 48-96 h; **Mg**Cl_2+$MgSO_4$, **Mg**Cl_2+**Mg**SO_4 and **Na**Cl+**Na$_2$**SO_4 for 240 h). These observations may be interpreted to mean that similar cations exert synergistic effect, which is not the case of dissimilar cations. Furthermore, it appears that interactions between salts increase the intensity of salts toxicity as demonstrated by the tested binary salt mixtures. Toxicity of the tested binary salt mixtures showed that $MgCl_2$+$MgSO_4$ is more toxic than NaSO4+NaCl, although the later binary salt mixture is more toxic than some of the tested single salts.

5.2 Development of procedure for binary salt mixtures

In discussing salt mixture exposures, the focus may either be on dose responsiveness whereby both the amount of mixture (i.e. level of exposure concentration) and the mixing proportions may impact response, or on only the mixing proportions and concentration does not impact response. The former will be applicable to mixture exposures of salts formed by alkali and alkali earth metals, while the later will be applicable to mixture exposures of salts formed by transition metals. In this study, the focus is on dose responsiveness whereby the amount of mixture and the mixing proportions are both associated with response.

One way of describing interaction among individual salts in salt mixtures is by using the concept of “change in slope”. This is based on the fact that the slope (i.e. steepness) of a dose-response curve of a chemical changes in the presence of one or more other components in a mixture. Thus, if the slope of the dose-response curve of a chemical is not changed in the presence of another chemical, then the chemicals are said to exhibit no interaction or they are said to combine additively (i.e. no-interaction). The no-interaction or additivity concept is not only simple but has also been described as “general solution” and “mechanism-free” because it is based on empirical information (Gennings, 2010).

In a chemical mixture, c, let E_i represent the concentration/dose of the ith component alone that yields a fixed response, and let x_i represent the concentration/dose of the ith component in mixture with the c agents that yields the same response. According to this definition of additivity, if the substances combine with zero interaction, then

$$\sum_{i=1}^{c} \frac{x_i}{E_i} = 1.$$

If the left-hand side of the equation is less than 1, then a greater than additive response (i.e. synergism) can be claimed at the combination of interest. If the left-hand side of the equation is greater than 1, then a less than additive response (i.e. antagonism) can be claimed at the combination (Gennings, 2010). This definition of additivity implies that under additivity contours of constant response are planar as the equation is that of a plane in c dimensions. Furthermore, it is important to note that:

- This general definition of additivity as expressed in the equation places no constraint on the single-chemical slopes.
- The chemicals in the mixture do not need to have similar shaped dose-response curves.
- The mixture may include active and inactive compounds (Gennings, 2010).

As it has been shown with the example exposure tests, binary mixtures toxicology is necessary to determine the dose-response effects as oppose that of single-salt toxicity. As was observed, the interactions between salts may increase the intensity of toxicity, suggesting additivity relationship among individual salts in the salt mixtures.

6 CONCLUSION

The present study reports data generated for single salts and binary salts mixtures in both short-term lethal and long-term lethal exposure tests. The results have provided data for some of the toxicological important salts found in South African freshwater systems using an indigenous freshwater species *C. nilotica*. Data has also been obtained, for the first time, on binary salt mixtures, which will form the basis for future salt mixture research in the country. Further tests need be carried out on other salts and freshwater macroinvertebrates to generate more lethal concentrations (LCs) data. The LC values that this study has generated, including LC1, LC10 and LC50 values, in conjunction with other salt data from the current national salt toxicity database, can be subjected to a Species Sensitivity Distribution (SSD) determination. The SSDs can then be used to calculate species protection boundary values according to Warne et al., 2004. Such boundary values can then be compared to the benchmark boundary values currently in use for Reserve assessments in South Africa so as to protect the Ecological Reserve.

7 RECOMMENDATIONS

It is recommended that there should be further research to include more single salts and salt mixtures (binary, ternary and quaternary) data in the database. This must involve other freshwater macroinvertebrates and other taxonomic groupings. The possibility of making the national salt toxicity database more accessible to local and international communities should be considered. The application of these data in RDM and SDC processes should also be considered in other related WRC projects.

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APPENDIX 1

Probit estimates of lethal concentration values for single salts

$MgSO_4$ 48 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.523	0.031	1.539
LC5	1.671	0.260	3.525
LC10	3.104	0.793	5.593
LC15	4.716	1.651	7.789
LC50	27.608	17.338	66.616
LC85	161.624	66.877	1551.623
LC90	245.516	90.000	3341.891
LC95	456.152	139.104	10463.401
LC99	1457.803	312.211	89726.242

$MgSO_4$ 96 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% c confidence limit
LC 1	0.785	0.224	1.535
LC5	1.691	0.678	2.800
LC10	2.547	1.216	3.883
LC15	3.358	1.795	4.867
LC50	10.800	8.059	14.591
LC85	34.730	23.661	66.892
LC90	45.785	29.617	98.850
LC95	68.952	41.027	177.511
LC99	148.620	74.729	538.397

$MgSO_4$ 240 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.685	0.252	1.196
LC5	1.215	0.564	1.883
LC10	1.650	0.864	2.408
LC15	2.028	1.149	2.850
LC50	4.850	3.614	6.174
LC85	11.602	8.922	17.031
LC90	14.260	10.687	22.388
LC95	19.359	13.818	33.923
LC99	34.347	21.980	75.287

$MgSO_4$ 48 h LC values for adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.584	0.117	1.306
LC5	2.052	0.780	3.506
LC10	4.011	2.049	6.226
LC15	6.305	3.744	9.631
LC50	42.660	24.355	119.824
LC85	288.616	106.723	2212.804
LC90	453.690	149.966	4452.852
LC95	886.776	247.596	12581.804
LC99	3116.815	630.834	88730.203

$MgSO_4$ 96 h LC values for adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.163	0.002	0.661
LC5	0.569	0.025	1.579
LC10	1.106	0.108	2.607
LC15	1.733	0.278	3.785
LC50	11.570	5.427	50.929
LC85	77.243	24.530	2958.748
LC90	121.041	33.503	8092.576
LC95	235.481	52.644	36296.402
LC99	820.441	120.682	616815.688

$MgSO_4$ 240 h LC values for adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.027	0.000	0.197
LC5	0.114	0.000	0.501
LC10	0.245	0.002	0.844
LC15	0.410	0.009	1.224
LC50	3.595	1.192	13.283
LC85	31.559	9.736	2317.455
LC90	52.762	13.962	9007.710
LC95	112.987	23.297	68831.711
LC99	471.298	58.727	3234062.750

$MgCl_2$ 48 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.374	0.087	0.774
LC5	0.767	0.260	1.357
LC10	1.126	0.462	1.850
LC15	1.459	0.675	2.299
LC50	4.361	2.855	6.777
LC85	13.032	8.130	29.652
LC90	16.884	10.070	43.480
LC95	24.782	13.692	43.480
LC99	50.901	23.923	232.703

$MgCl_2$ 96 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.173	0.092	0.267
LC5	0.336	0.208	0.471
LC10	0.479	0.320	0.642
LC15	0.608	0.427	0.794
LC50	1.672	1.336	2.091
LC85	4.595	3.519	6.552
LC90	5.837	4.352	8.730
LC95	8.320	5.928	13.429
LC99	16.177	10.478	30.431

$MgCl_2$ 240 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.014	0.001	0.056
LC5	0.049	0.005	0.142
LC10	0.095	0.015	0.236
LC15	0.149	0.030	0.337
LC50	0.988	0.466	1.992
LC85	6.572	3.033	28.079
LC90	10.290	4.364	56.848
LC95	19.994	7.333	164.952
LC99	69.498	18.752	1259.029

$MgCl_2$ 48 h LC values for adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	1.369	0.742	2.022
LC5	2.331	1.475	3.152
LC10	3.096	2.116	4.106
LC15	3.749	2.690	4.747
LC50	8.423	6.885	10.365
LC85	18.926	14.782	26.982
LC90	22.921	17.443	34.356
LC95	30.442	22.187	49.371
LC99	51.834	34.543	98.302

$MgCl_2$ 96 h LC values for adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	1.119	0.623	1.631
LC5	1.871	1.205	2.510
LC10	2.461	1.703	3.174
LC15	2.961	2.144	3.732
LC50	6.475	5.312	7.908
LC85	14.157	11.191	19.704
LC90	17.035	13.150	24.823
LC95	22.409	16.621	35.118
LC99	37.479	25.566	67.917

$MgCl_2$ 240 h LC values for adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.855	0.486	1.231
LC5	1.400	0.914	1.862
LC10	1.821	1.273	2.333
LC15	2.175	1.588	2.725
LC50	4.605	3.793	5.593
LC85	9.750	7.775	13.374
LC90	11.643	9.081	16.680
LC95	15.14	11.375	23.247
LC99	24.79	17.202	43.715

Na_2SO_4 48 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	1.006	0.591	1.403
LC5	1.535	1.027	1.992
LC10	1.923	1.373	2.412
LC15	2.239	1.665	2.753
LC50	4.256	3.553	5.099
LC85	8.093	6.580	10.884
LC90	9.421	7.509	13.201
LC95	11.801	9.093	17.649
LC99	18.006	12.912	30.682

Na_2SO_4 96 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.138	0.071	0.220
LC5	0.291	0.176	0.419
LC10	0.434	0.284	0.593
LC15	0.567	0.390	0.754
LC50	1.764	1.392	2.235
LC85	5.485	4.121	7.990
LC90	7.174	5.234	10.993
LC95	10.679	7.415	17.741
LC99	22.517	14.098	44.009

Na_2SO_4 240 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.045	0.002	0.139
LC5	0.104	0.010	0.253
LC10	0.162	0.023	0.354
LC15	0.218	0.041	0.450
LC50	0.768	0.349	1.630
LC85	2.710	1.335	13.144
LC90	3.652	1.703	23.184
LC95	5.680	2.394	54.856
LC99	13.009	4.372	286.052

Na_2SO_4 48 h LC values for adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.832	0.489	1.159
LC5	1.268	0.849	1.645
LC10	1.588	1.134	1.991
LC15	1.848	1.375	2.272
LC50	3.508	2.929	4.201
LC85	6.659	5.416	8.951
LC90	7.750	6.180	10.852
LC95	9.702	7.480	14.499
LC99	14.788	10.612	25.175

Na_2SO_4 96 h LC values for adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.555	0.331	0.759
LC5	0.814	0.552	1.042
LC10	0.999	0.722	1.238
LC15	1.147	0.862	1.396
LC50	2.057	1.731	2.445
LC85	3.689	3.032	4.909
LC90	4.235	3.417	5.865
LC95	5.197	4.062	7.668
LC99	7.630	5.574	12.776

Na_2SO_4 240 h LC values for adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.040	0.000	0.147
LC5	0.097	0.004	0.274
LC10	0.155	0.010	0.389
LC15	0.214	0.021	0.501
LC50	0.820	0.300	2.102
LC85	3.151	1.372	27.289
LC90	4.332	1.777	55.348
LC95	6.943	2.540	161.974
LC99	16.820	4.748	1269.607

APPENDIX 2

Probit estimates of lethal concentration values for binary salt mixtures

$MgCl_2$+$MgSO_4$ 48 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	1.274	0.779	1.699
LC5	1.776	1.226	2.228
LC10	2.121	1.556	2.584
LC15	2.391	1.822	2.864
LC50	3.965	3.373	4.660
LC85	6.575	5.488	8.625
LC90	7.411	6.082	10.101
LC95	8.849	7.055	12.816
LC99	12.340	9.251	20.177

$MgCl_2$+$MgSO_4$ 96 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.138	0.071	0.220
LC5	0.291	0.176	0.419
LC10	0.434	0.284	0.593
LC15	0.567	0.390	0.754
LC50	1.764	1.392	2.235
LC85	5.485	4.121	7.990
LC90	7.174	5.234	10.993
LC95	10.679	7.415	17.741
LC99	22.517	14.098	44.009

$MgCl_2$+$MgSO_4$ 240 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.048	0.001	0.147
LC5	0.106	0.008	0.259
LC10	0.161	0.019	0.356
LC15	0.215	0.035	0.447
LC50	0.717	0.312	1.580
LC85	2.393	1.166	13.355
LC90	3.183	1.472	23.943
LC95	4.858	2.034	58.098
LC99	10.735	3.593	318.098

$MgCl_2$+$MgSO_4$ 48 h LC values for adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	2.847	-	-
LC5	5.141	-	-
LC10	7.044	-	-
LC15	8.713	-	-
LC50	21.405	-	-
LC85	52.582	-	-
LC90	65.041	-	-
LC95	89.127	-	-
LC99	160.930	-	-

$MgCl_2$+$MgSO_4$ 96 h LC values for adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.185	0.000	1.043
LC5	0.542	0.000	1.994
LC10	0.962	0.000	2.893
LC15	1.416	0.001	3.806
LC50	7.262	1.946	54.156
LC85	37.248	12.658	272256.344
LC90	54.839	16.368	2462915.250
LC95	97.273	23.423	65816684.000
LC99	284.985	44.283	%32352917504.000

$MgCl_2$+$MgSO_4$ 240 h LC values for adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.005	0.000	0.039
LC5	0.022	0.000	0.111
LC10	0.049	0.002	0.195
LC15	0.084	0.004	0.284
LC50	0.797	0.205	1.490
LC85	7.600	4.685	17.065
LC90	12.958	7.420	40.216
LC95	28.567	13.713	153.156
LC99	125.836	40.452	2017.549

$NaCl+Na_2SO_4$ 48 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.963	0.227	1.808
LC5	1.659	0.569	2.727
LC10	2.216	0.918	3.430
LC15	2.695	1.260	4.033
LC50	6.160	4.135	9.265
LC85	14.081	9.347	30.914
LC90	17.123	10.972	42.476
LC95	22.879	13.779	68.671
LC99	39.400	20.758	172.070

$NaCl+Na_2SO_4$ 96 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.797	0.485	1.068
LC5	1.122	0.771	1.413
LC10	1.346	0.984	1.646
LC15	1.523	1.157	1.830
LC50	2.563	2.176	3.019
LC85	4.314	3.590	5.676
LC90	4.880	3.991	6.674
LC95	5.857	4.651	8.516
LC99	8.247	6.151	13.555

$NaCl+Na_2SO_4$ 240 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.634	0.205	0.976
LC5	0.885	0.383	1.248
LC10	1.057	0.530	1.434
LC15	1.191	0.657	1.582
LC50	1.976	1.464	2.667
LC85	3.278	2.469	5.946
LC90	3.696	2.724	7.371
LC95	4.413	3.128	10.210
LC99	6.156	4.000	19.069

$NaCl+Na_2SO_4$ 48 h LC values for Adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.717	0.000	2.346
LC5	1.310	0.000	3.481
LC10	1.805	0.000	4.454
LC15	2.242	0.000	5.427
LC50	5.602	0.513	102.861
LC85	13.999	5.772	-
LC90	17.386	6.963	-
LC95	23.967	8.843	-
LC99	43.762	13.042	-

$NaCl+Na_2SO_4$ 96 h LC values for Adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.683	_	_
LC5	1.118	_	_
LC10	1.454	_	_
LC15	1.736	_	_
LC50	3.674	_	_
LC85	7.776	_	_
LC90	9.285	_	_
LC95	12.076	_	_
LC99	19.770	_	_

$NaCl+Na_2SO_4$ 240 h LC values for Adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.070	0.000	0.488
LC5	0.183	0.000	0.908
LC10	0.307	0.000	1.343
LC15	0.436	0.000	1.846
LC50	1.901	0.000	-
LC85	8.297	1.954	-
LC90	11.758	2.645	-
LC95	19.708	3.868	-
LC99	51.925	7.137	-

$MgCl_2$+Na_2SO_4 48 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.418	0.000	1.700
LC5	1.267	0.007	3.605
LC10	2.289	0.059	5.775
LC15	3.411	0.229	8.544
LC50	18.436	7.418	415.104
LC85	99.637	26.610	182446
LC90	148.519	34.394	805851
LC95	268.312	49.785	7355019
LC99	813.582	97.734	474316576

$MgCl_2$+Na_2SO_4 96 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.450	0.00	1.646
LC5	1.020	0.003	2.844
LC10	1.577	0.018	3.944
LC15	2.117	0.056	5.066
LC50	7.342	2.484	39.837
LC85	25.462	9.845	3532.56
LC90	34.171	12.216	11395.193
LC95	52.842	16.457	66026.727
LC99	119.694	27.740	1848459.5

$MgCl_2$+Na_2SO_4 240 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.401	_	_
LC5	0.698	_	_
LC10	0.938	_	_
LC15	1.145	_	_
LC50	2.658	_	_
LC85	6.173	_	_
LC90	7.535	_	_
LC95	10.124	_	_
LC99	17.618	_	_

$MgCl_2$+Na_2SO_4 48 h LC values for Adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	2.319	0.785	3.980
LC5	4.813	2.378	7.042
LC10	7.104	4.211	9.734
LC15	9.240	6.086	12.326
LC50	28.055	20.522	46.987
LC85	85.184	49.931	248.250
LC90	110.784	61.004	371.791
LC95	163.516	81.863	678.171
LC99	339.384	141.407	2104.437

$MgCl_2$+Na_2SO_4 96 h LC values for Adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	1.069	0.051	2.516
LC5	1.955	0.217	3.845
LC10	2.697	0.463	4.906
LC15	3.351	0.761	5.858
LC50	8.392	4.501	17.196
LC85	21.013	11.667	115.104
LC90	26.110	13.805	191.073
LC95	36.020	17.468	410.604
LC99	65.862	26.499	1767.063

$MgCl_2$+Na_2SO_4 240 h LC values for Adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	0.246	0.001	0.732
LC5	0.471	0.008	1.127
LC10	0.666	0.023	1.444
LC15	0.841	0.049	1.730
LC50	2.258	0.794	5.461
LC85	6.062	3.027	73.070
LC90	7.658	3.669	152.796
LC95	10.825	4.751	468.219
LC99	20.721	7.381	3995.980

$NaCl+MgSO_4$ 48 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	1.717	0.7602.	2.759
LC5	3.301	1.857	4.693
LC10	4.679	2.961	6.288
LC15	5.920	4.028	7.716
LC50	16.010	12.614	21.496
LC85	43.293	30.185	78.356
LC90	54.781	36.589	107.911
LC95	77.638	48.480	174.020
LC99	149.322	81.624	429.336

$NaCl+MgSO_4$ 96 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	1.004	0.538	1.506
LC5	1.778	1.114	2.433
LC10	2.410	1.633	3.160
LC15	2.960	2.107	3.783
LC50	7.056	5.728	8.734
LC85	16.818	13.024	24.116
LC90	20.655	15.571	31.151
LC95	28.006	20.191	45.739
LC99	49.576	32.576	94.855

$NaCl+MgSO_4$ 240 h LC values for juvenile *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	1.003	0.594	1.385
LC5	1.499	1.010	1.930
LC10	1.857	1.334	2.314
LC15	2.146	1.605	2.624
LC50	3.953	3.314	4.715
LC85	7.283	5.956	9.739
LC90	8.415	6.752	11.717
LC95	10.426	8.095	15.477
LC99	15.580	11.284	26.298

$NaCl+MgSO_4$ 48 h LC values for Adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	1.819	0.728	3.024
LC5	3.662	1.953	5.297
LC10	5.319	3.265	7.232
LC15	6.842	4.572	9.015
LC50	19.835	15.232	28.490
LC85	57.503	37.535	121.730
LC90	73.970	45.880	173.818
LC95	107.423	61.574	295.605
LC99	216.282	106.267	805.180

NaCl+$MgSO_4$ 96 h LC values for Adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	1.335	0.213	2.616
LC5	2.251	0.584	3.834
LC10	2.974	0.989	4.756
LC15	3.589	1.400	5.546
LC50	7.942	5.036	12.816
LC85	17.574	11.241	47.733
LC90	21.208	13.072	67.751
LC95	28.017	16.170	115.074
LC99	47.23	23.631	316.905

NaCl+$MgSO_4$ 240 h LC values for Adult *C. nilotica*

Point	Concentration (g/L)	Lower 95% confidence limit	Upper 95% confidence limit
LC 1	1.183	0.769	1.485
LC5	1.486	1.075	1.779
LC10	1.679	1.282	1.965
LC15	1.822	1.440	2.107
LC50	2.579	2.250	2.956
LC85	3.649	3.154	4.624
LC90	3.961	3.381	5.194
LC95	4.473	3.736	6.191
LC99	5.620	4.476	8.659