OSMOREGULATION IN FRESHWATER INVERTEBRATES IN RESPONSE TO EXPOSURE TO SALT POLLUTION

Report to the

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by

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EXECUTIVE SUMMARY

Introduction

Apart from natural causes, agricultural practices and industrial activities have been identified as major contributors to increasing salinisation and deterioration of resource water quality both in South Africa and worldwide (Walmsley et al., 1999; Kefford et al., 2004). Pressure to develop infrastructure and provide food security has resulted in a rapid expansion of the industrial and agricultural sectors (Goetsch and Palmer, 1997). This expansion has increased pressure on the country's water resources and has resulted in elevated levels of inorganic salt pollution in rivers by increasing salinisation (Goetsch and Palmer, 1997; Kefford et al., 2004).

The South African National Water Act (No. 36 of 1998) provides for an ecological Reserve which is intended to protect fresh water ecosystems and resources from degradation as a result of misuse, and to maintain vital ecological functions within these systems (Palmer et al., 2004). Water quality guidelines are an important tool in the management of these water resources, aiming to adequately balance protection of aquatic ecological systems with sustainable human use needs. Jooste and Rossouw (2002) proposed guidelines or boundary values for inorganic salts to be included in the ecological Reserve. These boundary values for inorganic salts were derived as follows, acute lethality data (LC_{50}s) from the ECOTOX database maintained by the USEPA were projected to 336 h and the 5^{th} percentile determined as a lethality benchmark, analogous to the Fair/Poor boundary. Similarly, the 5^{th} percentile of available sublethal data was determined as the sublethality benchmark and analogous with the Natural/Good boundary value. The Good/Fair boundary was the mean value between Natural/Good and Fair/Poor values. It has been suggested however, that these guidelines might not be entirely appropriate as they were derived without including tolerances of South African biota. Furthermore, the accuracy for some salt boundary values have been questioned (Scherman, 2009; Scherman, 2010).

In order to address these issues, there is a need to increase understanding of the physiological responses of organisms to salinity and for the generation of toxicity response data from indigenous species which might improve the accuracy of the guidelines.

In general, it is understood that biota react adversely to increases in salinisation, although the effects on individual species are poorly understood (Hart et al., 1991). In particular it may affect the osmoregulation of both invertebrates and vertebrate species while negatively affecting oxygen uptake (Schmidt-Nielsen, 1998). Consequently, it was decided to investigate the oxygen consumption of two fish species and the haemolymph osmolality of a fresh water crustacean. Furthermore, an alternative approach to deriving magnesium sulphate ($MgSO_4$) guideline boundary values using indigenous mayfly lethality data was investigated.

Indigenous mayfly responses to $MgSO_4$ exposure

The objective of this experiment was to compare sensitivities of three different mayfly species to $MgSO_4$ and generate 96 h lethality data. These data, together with other lethality data from organisms exposed to $MgSO_4$ in international studies, were used to calculate guideline values for $MgSO_4$ using a species sensitivity distribution (SSD) approach.

Nymphs of three different mayfly (Ephemeroptera) genera: *Afronurus barnardi* (Heptageniidae); *Tricorythus discolor* (Tricorythidae); and *Euthraulus elegans* (Leptophlebiidae) were collected from the Kat River, Eastern Cape, South Africa, and exposed to increasing concentrations of $MgSO_4$ in recirculating channel systems on three different occasions. Toxicity tests were conducted over a 10 day (240 h) period with LC_{50} values determined after 96 h considered acute endpoints, and LC_{50} values determined after 240 h considered chronic endpoints.

The geometric means of LC_{50}s over the three experiments were 3.16 g/L for *E. elegans*, 5.96 g/L for *T. discolor* and for 7.55 g/L for *A. barnardi*. An evaluation of the current Reserve boundary values was undertaken by combining these indigenous mayfly 96 h LC_{50} data (see Chapter 2) with international acute lethality data from the ECOTOX database (USEPA, 2004) and deriving protective concentration values (PCVs) according to methods outlined in Warne et al. (2005). A comparison of the current Reserve boundary values and the PCVs determined in this study show the PCVs to be more conservative at the Natural/Good boundary, but less conservative at the Good/Fair boundary and considerably so at the Fair/Poor boundary (Table 5.4).

In recent assessments of the water quality component of the ecological Reserve (Scherman, 2009; Scherman, 2010), the $MgSO_4$ boundary value guidelines have been shown to be inconsistent with EC and biotic response data assessed concurrently. This suggests that the salt is either being overestimated by the analytical tool TEACHA (Tool for Ecological Aquatic Chemical Habitat Assessment) which is used to determine the inorganic salt concentrations from the available salt ions found in solution, or that the guideline boundary values may be over-protective. This situation has particularly problematic implications when only desktop analyses of water quality data for water use licenses are undertaken, as biotic response data are generally not available for comparative assessment purposes. Consequently, the PCV derivation approach should be investigated further in order to determine if it may provide more realistic boundary values for $MgSO_4$. Although it is possible to use only acute lethality data in deriving guidelines and then apply an acute to chronic ratio (ARC), further research should investigate the use of chronic/sublethal data

Fish responses to NaCl and Na_2SO_4 exposure

The objective of this experiment was to determine whether a change in dissolved oxygen (DO) could be used as a measure of the physiological response in guppies, *Poecilia reticulata* and zebra fish, *Danio rerio* when exposed to increasing concentrations of sodium chloride (NaCl) and sodium sulphate (Na_2SO_4). By using fish species in toxicity tests a more comprehensive approach to toxicity testing is provided through incorporating another trophic level in addition to that of invertebrates.

The two freshwater fish species used for this experiment were the guppy, *P. reticulata* and the zebra danio, *D. rerio*. Both species are exotic to South Africa, however are used globally in toxicity tests (Boisen et al., 2003). These two species were exposed to increasing concentrations of the inorganic salts Na_2SO_4 and NaCl in separate experiments.

A NOEC (no observed effect concentration) for NaCl of 0.5 g/L was determined for both *D. rerio* and *P. reticulata*. For Na_2SO_4, only a LOEC of 0.375 g/L for both species could be determined and a MATC (maximum allowable toxicant concentration) of 0.188 g/L was calculated by dividing the LOEC by two. These data indicate little difference in the sensitivity of the two species to either salt.

As sublethal data were used in the derivation of the Natural/Good Reserve boundary values, physiological response data such as the oxygen consumption data measured in *D. rerio* and *P. reticulata* could be used to evaluate this boundary value. For NaCl, a NOEC of 0.5 g/L was determined for both species. When compared with the sublethal toxicity data used by Jooste and Rossouw (2002) to derive the Reserve boundary values for NaCl (Table 5.5) it is evident that the physiological response of oxygen consumption has the potential to contribute as a sensitive endpoint in the determination of a realistic but protective guideline. The types of sublethal endpoints used in the derivation of the Reserve boundary values (e.g. growth, reproduction etc) are not detailed in Jooste and Rossouw (2002) and thus it is difficult to interpret the significance of the difference in NOEC value obtained for *D. rerio* in the current study as compared to the NOEC listed in Table 5.5.

A NOEC could not be obtained for oxygen consumption as a physiological response in Na_2SO_4 exposed *D. rerio* and *P. reticulata*, although a LOEC could, allowing the calculation of a MATC of

0.188 g/L. The MATC (calculated by dividing the LOEC by half) is sometimes, in the absence of a NOEC, used as a sublethal endpoint in guideline derivation. When comparing this endpoint to the NOECs used by Jooste and Rossouw (2002) to derive the Reserve boundary values for Na_2SO_4 (Table 5.5), it is again evident that oxygen consumption can contribute as a sensitive endpoint in the determination of suitable guidelines.

Indigenous crustacean response to NaCl and Na_2SO_4 exposure

Osmoregulatory capacity (OC) is the difference between the osmolality of haemolymph and that of the external medium (Charmantier et al., 1989) and has been suggested by Lignot et al. (2000) as a tool for monitoring physiological stress in crustaceans. The freshwater shrimp *Caridina nilotica* has been used as a model indigenous crustacean species in acute and chronic toxicity testing in South Africa (Slaughter et al., 2008). Therefore the physiological endpoint of osmoregulatory capacity (OC) was determined in *C. nilotica* exposed to increasing concentrations of sodium chloride (NaCl) and sodium sulphate (Na_2SO_4). *Caridina nilotica* used within this study were collected from the Bushmans River in Alicedale, South Africa.

Results generated (Chapter 4) indicate no evidence of osmotic stress in *C. nilotica* with haemolymph osmolality levels remaining steady with increasing exposure to the selected inorganic salts. At 96 h, shrimp exposed to the highest concentration of Na_2SO_4 died, but there was no evidence at 72 h that osmoregulatory capacity in these organisms was failing. Hence osmoregulatory capacity (OC) could not be applied as an indicator for osmotic stress in *C. nilotica* exposed to the inorganic salts NaCl and Na_2SO_4.

Consequently, due to the hyper-hypo-regulatory mechanism employed by freshwater shrimp exposed in this project (Chapter 4), a negative impact on the osmoregulatory mechanism of these animals could not be determined for either salt and consequently NOECs could not be calculated. To successfully evaluate current Reserve boundary values using osmoregulation as endpoint, test organisms whose mechanisms of osmoregulation are measurably impacted by increasing concentrations of inorganic salts should be utilised. As internal haemolymph osmolality levels may vary between taxa, the use of multiple species is also recommended in order to increase confidence in derived guidelines.

Conclusions and future research

The lack of confidence in the $MgSO_4$ Reserve boundary value guidelines has recently led to a review of the guideline and a revision of derivation methods for salts being included as sub-tasks in a Water Research Commission (WRC) / Department of Water Affairs (DWA) proposal for further development of the water quality methods of the ecological Reserve, submitted in August 2010. Results from the current study, particularly the demonstration of the PCV derivation approach, could make a contribution to this project and should be further investigated.

Usually there are very few sublethality data available to derive the Natural/Good Reserve boundary value using the method described by Jooste and Rossouw (2002), leading to lower confidence in the resultant guideline. Although the most reliable PCVs are also derived using sublethality data, it is still possible to utilise acute lethality data in deriving PCVs and apply a default or, preferably, experimentally determined acute-to-chronic ratio. Ultimately, however, sublethal endpoints generated using indigenous aquatic organisms are necessary in order to derive realistic protective guidelines and the generation of these data should be prioritised.

Problematic issues encountered in producing and utilising sublethality endpoints at sub-organism levels in water quality management, such as osmoregulatory capacity, are well documented (Clark et al. 1999; Tannenbaum 2005; Forbes et al. 2006). Issues raised are: the inherent variability of the endpoints measured (mainly related to the assay protocol and the differences in tolerances at low

levels of organisation among exposed individuals); complicated time- or dose-dependent responses are frequently measured, but are difficult to explain and to derive endpoints such as NOECs or EC_{50}s from; confounding nonchemical influences such as temperature, nutritional state, reproductive state and lifecycle stage often impact results and; there are unclear or undetermined links between sub-organism endpoints and the fitness of the individual, and especially, fitness of the population and community. These issues need to be considered when undertaking sublethal toxicity tests, and applying these data to guideline derivation.

Lastly, the EWQ management approach to salinity should reconsider the use of electrical conductivity as an additional tool, particularly in combination with biological response data. The process to determine individual salt concentrations (TEACHA) is complex, not well understood and requires salt ion data that is often not available. In addition, the accuracy of the Reserve boundary values for some salts have been questioned (Scherman, 2009; Scherman, 2010). Electrical conductivity, however, is easy to measure and the data are readily available in most cases. Further research should be conducted to determine advantages and limitations of using electrical conductivity data, either alone or in combination with biological data, in EWQ management practices.

Capacity Building

This project was utilised as an opportunity to develop scientific thinking, experimentation and writing skills in a number of students and early career water scientists based within the Institute for Water Research at Rhodes University. Much of the experimental work was undertaken by undergraduate students, supported by the incumbent IWR research intern, and overseen by the project manager Dr Muller.

A 3rd year undergraduate project was completed by Mr Guy Williams, who generated data for Chapter 2 of this report. Mr Greg Tutt completed his Honours project whilst generating data which contributed substantially to Chapter 3 of this report. In addition, three research interns worked in turn on this project whilst undertaking their MSc/PhDs. This project offered them training in research and scientific writing and broadened their aquatic scientific expertise.

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ABBREVIATIONS

ACR	acute-chronic ratio
ANOVA	analysis of variance
BOD	biological oxygen demand
C_{50}	lethal concentration at which 50% of the tested population dies
COD	chemical oxygen demand
Conc	Concentration
CV	coefficient of variation
DEEEP	Direct Estimation of Ecological Effects Potential
DO	dissolved oxygen
DWA	Department of Water Affairs
DWAF	Department of Water Affairs and Forestry
EC	electrical conductivity
EC_{50}	effective concentration at which 50% of the tested population shown an effect
ECOTOX	USEPA database of ecotoxicology data
EWQ	environmental water quality
IWR	Institute for Water Research
LOEC	lowest observed effect concentration
MATC	maximum allowable toxicant concentration
$MgSO_4$	magnesium sulphide
NaCl	sodium chloride
Na_2SO_4	sodium sulphide
NOEC	no observed effect concentration
NWA	National Water Act
OC	osmoregulatory capacity
PC	protective concentration
PCV	protective concentration value
RDM	Resource Directed Measures
RUESC	Rhodes University Ethical Standards Committee
SDC	Source Directed Controls
SSD	species sensitivity distribution
Std. Dev.	Standard deviation
T	temperature
TEACHA	Tool for Ecological Aquatic Chemical Habitat Assessment
TSK	Trimmed Spearman-Kärber
UCEWQ	Unilever Centre for Environmental Water Quality
USEPA	United States Environmental Protection Agency
WRC	Water Research Commission
WQG	Water Quality Guideline

1 GENERAL INTRODUCTION

Conservation physiology was defined by Wikelski and Cooke (2006) as “the study of physiological responses of organisms to human alteration of the environment that might cause or contribute to population declines”. Wide arrays of disciplines, including environmental toxicology, are able to make contributions towards sustainable environmental management and conservation (Wikelski and Cooke, 2006). This research field may, therefore, be useful in contributing towards achieving a balance between water resource protection (and conservation of biodiversity) and resource use, a requirement in achieving long-term sustainability as required by the South African National Water Act (National Water Act, 1998). Wikelski and Cooke (2006) consider that physiological characteristics (of key species) may prove useful in predicting and anticipating environmental problems, thus ensuring that corrective management interventions can be instituted to achieve desired conservation measures.

This study was initiated as a developmental process towards establishing a possible role for physiological responses such as osmoregulation in water resource management tools. In this project a strong emphasis was placed on building capacity through the participation of students and early career scientists.

1.1 Managing environmental water quality

Apart from natural causes, agricultural practices and industrial activities have been identified as major contributors to increasing salinisation and deterioration of resource water quality both in South Africa and worldwide (Walmsley et al., 1999; Kefford et al., 2004). In order to achieve a balance of resource protection while ensuring long-term and optimal resource use (National Water Act, 1998) the Environmental Water Quality (EWQ) approach has been proposed for application in managing environmental water quality in both Resource Directed Measures (RDM) and Source Directed Controls (SDC) (Scherman et al., 2003). EWQ is an approach which recognises the value of using aquatic organisms for resource protection and monitoring, combining biomonitoring and ecotoxicology with the traditionally used physico-chemical measurements when defining ecologically acceptable water quality parameters (Palmer et al., 2004). In general it is understood that biota react adversely to increases in salinisation, although the effects on individual species is poorly understood (Hart et al., 1991). Urgent attention therefore needs to be paid to assessing the effects of salts on biota in water resources in order to optimise resource protection and resource utilization.

1.2 Endpoints in toxicology assessments in water resource management:

1.2.1 Developing water quality guidelines

The South African National Water Act (No. 36 of 1998) provides for an ecological Reserve which is intended to protect fresh water ecosystems and resources from degradation as a result of misuse, and to maintain vital ecological functions within these systems (Palmer et al., 2004). In order to create ecological Reserves that adequately balance protection of aquatic ecological systems and sustainable human use needs, accurate and if possible site-specific water quality guidelines (WQGs) need to be created through an integrated understanding of physico-chemical, biomonitoring, and ecotoxicological data (Palmer et al., 2005).

Ecotoxicology provides valuable and highly reliable data for the creation of boundary values used to establish WQGs (Warne et al., 2005) as it relates biological responses of test organisms to physico-chemical values in a concentration-response relationship (Scherman et al., 2003). Although acute toxicity test data can be found in abundance internationally, it is widely recognized that site-specific data using indigenous species and long term chronic tests are ultimately required to provide precise

local ecospecs (Scherman et al., 2003). Indeed, the accuracy or reliability of using acute toxicity data has been much debated (Roux et al., 1996). Warne (1998) argued that acute data could not be used to show long term exposure effects and that it was important to incorporate sub-lethal chronic toxicity data in the process of deriving these guidelines.

Site specific WQGs for inorganic salts, proposed by Jooste and Rossouw (2002), were derived without including tolerances of South African biota (Slaughter et al., 2004). The need for widespread site- and indigenous species-specific water chemistry and toxicology testing is a reoccurring theme (Scherman and Palmer, 2000; Palmer et al., 2004; Warne et al., 2005; Browne, 2005; Palmer et al., 2005) and a major reason for the initiation of this study.

1.2.2 Toxicity assessment approaches

The current methodology for water quality assessments includes the determination of boundary values for specific salts, based on biological effects data. Aquatic ecotoxicology is central to the EWQ approach, although currently much of the data are based on acute (lethal) toxicity tests due to the paucity of chronic (sub-lethal) toxicity data. To add to uncertainty, even fewer data based on indigenous species are available. Extrapolation methods from acute to chronic endpoints have been shown statistically possible in deriving accurate chronic endpoints by exposing the freshwater shrimp *Caridina nilotica* to inorganic salts (Slaughter, 2005).

Riverine macroinvertebrates are excellent indicators of water-borne pollutants (they are in constant contact with pollutants in the water column), and are suitable laboratory-test organisms. They hold a key position in aquatic food chains but little information is available on their tolerances to increased salinities. Methods have been developed by UCEWQ for generating chronic toxicity test data for selected indigenous species, at both organism and sub-organism levels (Gordon et al., 2009). However, accurate interpretation of toxicity test results remains elusive as very little is known of the biology, and physiology, of these indigenous toxicity test species. Physiological functions, including endocrine control mechanisms, mediate the relationship of the organism to its environment (Ricklefs and Wikelski, 2002). Thus it has been argued that comparative physiology does have an important role to play in informing a variety of assumptions made in macro-ecology, including tolerances to pollutants (Chown et al., 2002).

1.3 Selected physiological responses to environmental water quality stress

In general freshwater animals are termed hyperosmotic, meaning they have a higher concentration of solute (or salts) than the water surrounding them. As a result freshwater animals constantly have to excrete water in order to maintain equilibrium, and in so doing lose some solutes (Schmidt-Nielsen, 1998). Freshwater animals therefore continually need to take up ions to replace those lost through diffusion to the environment (Boisen et al., 2003). Although some animals are able to tolerate and adapt to a wide range of salinities (euryhaline), most are stenohaline (have a narrow range of tolerance) (Schmidt-Nielsen, 1998). Therefore changes in salinity, for example through addition of inorganic salts from industrial effluents and agricultural runoff, are likely to affect the ability of organisms to effectively osmoregulate. This in turn may affect such factors as endocrine balance, and oxygen consumption following chronic exposures, with subsequent changes in physiological processes. Elevated energy expenditures may occur until a threshold of intolerance is reached. Thresholds may in turn differ between species even of the same genus (Rowe, 2002).

1.3.1 Oxygen consumption as a measure of physiological stress

An indirect indicator of metabolic rate in fish is the rate of oxygen consumption usually expressed in mg oxygen per gram dry weight of the test species per hour (Chech, 1990). Oxygen consumption has been used to assess the energetic cost of osmoregulation in several fish species when exposed to increasing salinities (Altinok and Grizzle, 2003; Zheng et al., 2000). Differences in oxygen consumption found in a range of species tested seem to be partly based on the developmental stage of the species (Moser and Hettler, 1989; Aristizabal-Abud, 1992) and their degree of euryhalinity or stenohalinity. As fish metabolic expenditures rise, ventilation-related osmotic and ionic activities will increase (Rao, 1968). In this study oxygen consumption could not accurately be measured and therefore changes in DO were used as a surrogate measure.

1.3.2 Osmoregulation as a measure of physiological stress

Main sites for osmoregulation in both fish and invertebrates are the gills, which are also responsible for active uptake of lost solutes. The sodium pump (Na^++K^+-ATPase) is the main mechanism for moving ions across the gills in aquatic animals (Lucu and Towle, 2003). Freshwater fish primarily use their kidneys for maintaining water balance and excreting harmful substances. The mechanism of osmoregulation used is dependent on the developmental status of the animal, for example pre-larval fish osmoregulate largely through the skin, whereas larval stages regulate through the gills (Varsamos and Charmantier, 2005). Insects, in addition, possess a network of Malpighian tubules lined with secretory cells extending throughout much of the body cavity and attached to the alimentary canal between the midgut and hindgut, where ions get reabsorbed before waste is excreted (Dettner and Peters, 1999).

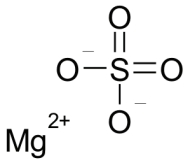
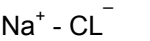
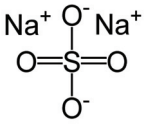
Osmoregulation can be monitored by measuring osmolarity or osmolality, depending on the mechanism used to determine endpoints. Osmolarity is the concentration of osmotically active particles in solution, which may be quantitatively expressed in osmoles of solute per litre of solution, whereas osmolality is expressed in osmoles of solute per kilogram of solvent (Schmidt-Nielsen, 1998). Osmolality of the haemolymph (in the case of macroinvertebrates) will give an indication of the osmotic concentration of the transport fluid when the animal is exposed to higher concentrations of inorganic salts.

1.4 Selection of environmental water quality stressors: Inorganic salts

South Africa is largely a semi-arid country with an average rainfall of 450 mm per annum, almost half the global average (DWAF, 2004), making it a water-scarce country. Much emphasis is placed on the conservation and management of the water resource. In addition to meeting ecological needs (The Ecological Reserve) this resource also needs to meet human needs (Basic Human Needs Reserve) (NWA, 1998). Pressure to develop infrastructure and provide food security has resulted in the industrial and agricultural sectors expanding rapidly over the last few years (Goetsch and Palmer, 1997). This expansion has increased pressure on the country's water resources and has resulted in elevated levels of inorganic salt pollution in rivers by increasing salinisation (Goetsch and Palmer, 1997; Kefford et al., 2004). Three main causes for increased salinisation have been cited by Goetsch and Palmer (1997): the geology of the area, agricultural practices and industrial activities. This increase in salinisation can have severe impacts on the biota in these river systems. In particular it may affect the osmoregulation of both invertebrates and vertebrate species while negatively affecting the uptake of oxygen of these biota (Schmidt-Nielsen, 1998). Sodium sulphate (Na_2SO_4) and sodium chloride (NaCl) have been identified as suitable indicators of salinisation as most agricultural salts are dominated by NaCl while industrial salts are dominated by SO_4^{2-} (Dallas and Day, 1993). According to

Palmer et al. (2005) $MgSO_4$ is more toxic than Na_2SO_4 and NaCl making it the most toxic of the six common inorganic salts listed in the Reserve process (Jooste and Rossouw, 2002). It was specifically suggested by Browne (2005) that $MgSO_4$ be tested on indigenous South African organisms because no such tests have yet been undertaken. Thus, Na_2SO_4, NaCl, and $MgSO_4$ were selected as the three salts to be tested for this study (Table 1.1).

Table 1.1 Choice of three inorganic salts used for this study

Salt (abbreviation)	Chemical structure	Reasons for choice
Magnesium sulphate ($MgSO_4$)		- considered the most toxicologically important salt of those used in Present Ecological State assessments - therefore a core water quality variable for ecological water quality Reserve assessments. - consistently responsible for Poor to Fair water quality class classification (Jooste and Rossouw, 2002).
Sodium chloride (NaCl)		- dominant naturally-occurring salt of inland and south western parts of South African waters (Day, 1993). - dominates agricultural salts - necessary core water quality variable for ecological water quality Reserve assessments.
Sodium sulphate (Na_2SO_4)		- dominates industrial effluent - core water quality variable for ecological water quality Reserve assessments.

1.5 Selection of test organisms

Test organisms selected to investigate osmoregulatory responses to inorganic salt exposure are listed in Table 1.2.

Mayflies (Ephemeroptera) were selected as indigenous insect representatives as they are abundant in South African rivers, widespread, easy to collect and are established as suitable toxicity test organisms (Palmer et al., 2004). Mayflies have also been exposed to salts in previous toxicity tests (Goetsch and Palmer, 1997). Organisms were collected in the field and identified in our laboratories prior to conducting toxicity tests. Representatives from three different genera were collected and identified: Heptageniidae (*Afronurus barnardi*), Tricorythidae (*Tricorythus discolor)*, and Leptophlebiidae (*Euthraulus elegans)* as used previously in Palmer et al. (2004).

The shrimp, *Caridina nilotica,* was chosen as indigenous crustacean representative. This species is frequently used as a toxicity test organism within UCEWQ for testing salts and other pollutants like pesticides and herbicides. The freshwater shrimp is widespread in South Africa and easy to collect. Organisms were field collected from a known relatively unimpacted reference site in the Eastern Cape, South Africa.

Two species of fish were chosen as representation of aquatic vertebrates: the guppy (*Poecilia reticulata*) and the Zebra fish (*Danio rerio*). Both species are commonly used in toxicity testing internationally and are not indigenous to South Africa. At the time of this study however, tests with

both species were warranted as there were no test protocols available for indigenous species at the time. Since then, Rall et al. (2010) have described breeding and toxicity test methods for indigenous fish such as *Barbus trimaculatus,* which could be considered for use in future testing. Zebrafish (*Danio rerio*) are easily bred and kept in captivity, and are commonly used as a test standard in toxicology studies. However little is known about their physiology when coping with osmoregulation (Boisen et al., 2003). The guppy (*Poecilia reticulata*) is a standard species for toxicology tests due to their ease of breeding in captivity and relatively short life cycle. Guppies and Zebra fish were obtained from a local breeder.

Table 1.2 Choice of test species to investigate osmoregulatory responses to inorganic salt exposure

Test animal	Common name	Reasons for choice
Afronurus barnardi, *Tricorythus discolor*, *Euthraulus elegans*	Mayflies	- abundant - indigenous - widespread in South Africa - easy to collect - toxicity test protocol exists (Goetsch and Palmer, 1997)
Caridina nilotica	Freshwater Shrimp	- indigenous - widespread in South Africa - easy to collect - used for lethal and sublethal toxicity testing, at UCEWQ-IWR laboratories (WRC project number K5/1313)
Poecilia reticulata	Guppy	- available in sufficient numbers from a local breeder - recommended for short term fish toxicity testing in the National Direct Estimation of Ecological Effect Potential (DEEEP) (DWAF, 2004).
Danio rerio	Zebra fish	- available in sufficient numbers from a local breeder - recommended for long term chronic fish development toxicity testing in the National Toxicity Monitoring Programme (DWAF, 2005).

2 FRESHWATER MACROINVERTEBRATE RESPONSES TO SELECTED INORGANIC SALTS

2.1 Introduction

The objective of this experiment was to compare sensitivities of three different mayfly species to magnesium sulphate ($MgSO_4$) and generate 96 h lethality data. These data, together with other lethality data from organisms exposed to $MgSO_4$ in international studies, were used to calculate Reserve boundary values for $MgSO_4$ using a species sensitivity distribution (SSD) approach.

2.2 Materials and Methods

2.2.1 Experimental organisms

Nymphs of three different mayfly (Ephemeroptera) genera were used for this experiment: *Afronurus barnardi* (Heptageniidae), *Tricorythus discolor* (Tricorythidae), and *Euthraulus elegans* (Leptophlebiidae). These indigenous mayflies were collected from the Kat River, Eastern Cape, South Africa, and are considered an established species for toxicity testing in South Africa (Scherman et al., 2003). These three test species were exposed to increasing concentrations of magnesium sulphate ($MgSO_4$) in three separate experiments.

2.2.2 Experimental systems

The experimental design was based on the recirculating channel system described by Scherman and Palmer (2000), with the following minor adjustments to facilitate a ten day chronic test:

Test solutions were changed on day 4 and 8 to minimise build up of algae and nutrients within channels. Experiments were conducted at a constant temperature in a controlled environment room. Water quality parameters were recorded after each water change. Animals were fed by placing three disks of filter paper used to filter 250 mL Palmiet River water beneath stones in each channel for 6 h prior to each water change.

2.2.3 Experimental design and procedure

The number of organisms per channel per experiment is detailed in Table 2.1. Organism numbers varied between experiments. The organisms were given 36 h to acclimatise to laboratory conditions before test solutions were applied. Nymphs dying before the application of exposure solutions were removed at the start of the test and were not included in the statistical analysis.

Toxicity tests were conducted over a 10 day (240 h) period. LC_{50} values determined after 96 h were considered acute endpoints, while LC_{50} values determined after 240 h were considered chronic endpoints.

Table 2.1 Number of animals per species per channel for 3 experiments

Experiment	Organism number per channel		
	A. barnardi	***T. discolor***	***E. elegans***
Exp1	25	30	35
Exp2	35	35	22
Exp3	20	25	25

Water quality parameters (pH, temperature, EC) were recorded for each channel daily to ensure consistency within the channels. The test endpoint was defined as mortality or immobilisation assessed by prodding the organism and checking for movement. Acceptable control mortality was restricted to 10% for the 96 h period. Percentages reported for mortalities were based on the total number of dead organisms removed from the channels during the experiment and survivors present at the end. Emerged or escaped organisms did not contribute towards the data. Dechlorinated tap water was used as the solvent. Experiment one (Exp1) was undertaken in a different laboratory to experiment two (Exp2) and experiment three (Exp3).

The test animals for Exp1 and Exp2 were collected from a riffle at a minimally impacted reference site in close proximity to Hertzog village, Eastern Cape, South Africa on 19 March (Exp1) and 28 April (Exp2), 2006. Due to high flow conditions in the Kat River on 19 August 2006, animals were collected from the riffle at a reference Site on the Balfour River for Exp3. The Balfour is a relatively unimpacted tributary of the Kat River. Collection was carried out by sweeping selected nymphs off rocks and into buckets of river water with a paintbrush. They were transported to the laboratory in ice-cooled and aerated water by car within three h of collection. Dissolved oxygen (DO), water temperature, pH and electrical conductivity (EC) were measured in the field at the time of collection.

2.2.4 Data analysis

Data were analysed using either Probit or Trimmed Spearman-Kärber (TSK) regression analysis to provide LC_{50} values. Only data unsuitable for Probit analysis (i.e. greater than 10% mortality in the control and deviations from increased mortality with increased concentration assumption) were subjected to TSK analysis. The acute (96 h) LC_{50} values were used in conjunction with acute (<96 h) LC_{50} data on eleven other taxa from the ECOTOX toxicity database (USEPA, 2004) and subjected to a Species Sensitivity Distribution (SSD). The SSD was produced using the Burr Type III regression analysis run on BurrliOz software. The concentration divisor was calculated as the geometric mean of the acute/chronic ratios (ACR) of the LC_{50} values generated in this study. The ACRs were calculated using 96 h and 240 h LC_{50}s. The SSD was used to calculate species protection parameters for 'Natural' (95%), 'Good' (90%) and 'Fair' (80%) conditions as defined by the South African water quality management boundary classification (Warne et al., 2004). The boundary values produced from the SSD were compared to the benchmark boundary values currently in use for Reserve assessments in South Africa (Table 5.1).

2.3 Results

Mapped distributions of the three test species indicate that they inhabit a wide range of South African river systems. *E. elegans* (Figure 2.1) is found country-wide, inhabiting most river systems including the Orange-Vaal, Great Fish, Great Berg, Incomati and Olifants-Klip River systems. *T. discolor* (Figure 2.2) is similarly widely distributed. Both species are found in river systems bordering or flowing through neighbouring countries, suggesting further distribution into Zimbabwe, Mozambique, Lesotho and Swaziland. Both *E. elegans* and *T. discolor* are found in all six of the water quality management regions proposed by Day et al. (1998). *A. barnardi* (Figure 2.3) appears to have a more restricted range, being found mostly in eastern regions including the Incomati, Limpopo, Olifants-Kliprivier, Tugela, Umvoti and Vaal River systems. This species is therefore largely absent from the pure waters of the southern and western coasts, the highly mineralized chloride/sulphate waters of the arid interior and alkaline soda carbonate/temporary hard carbonate waters of the upper Orange/Vaal region (Day et al., 1998).

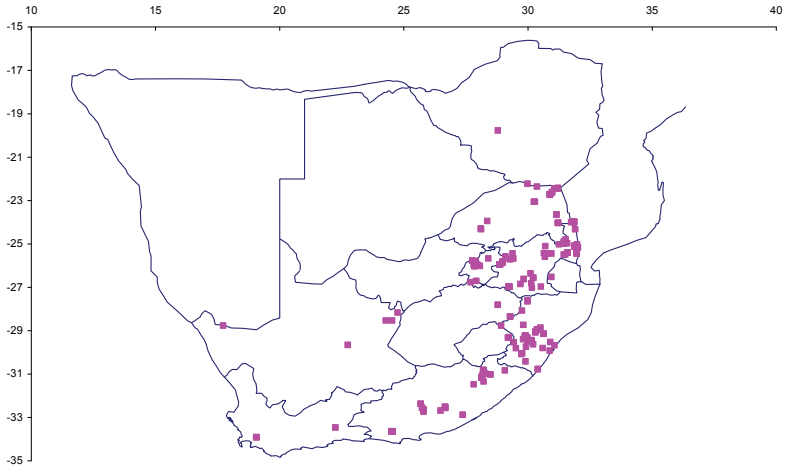


Figure 2.1 Distribution of *E. elegans* in South Africa based on information from the Albany Museum.

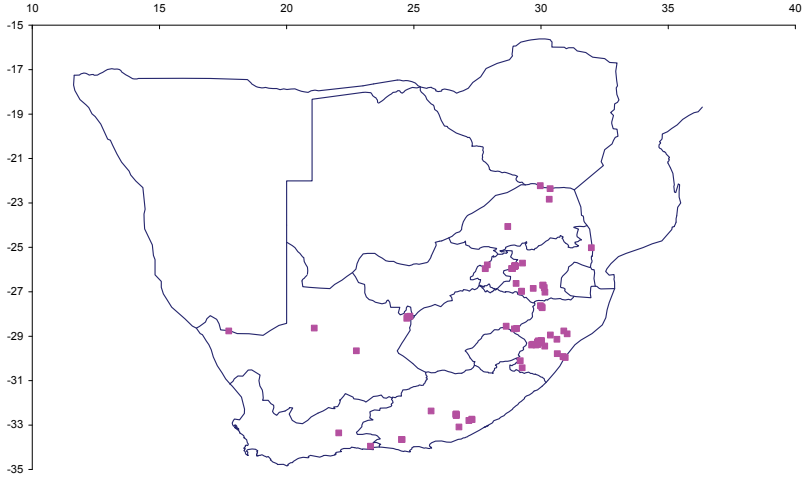


Figure 2.2 Distribution of *T. discolor* in South Africa based on information from the Albany Museum.

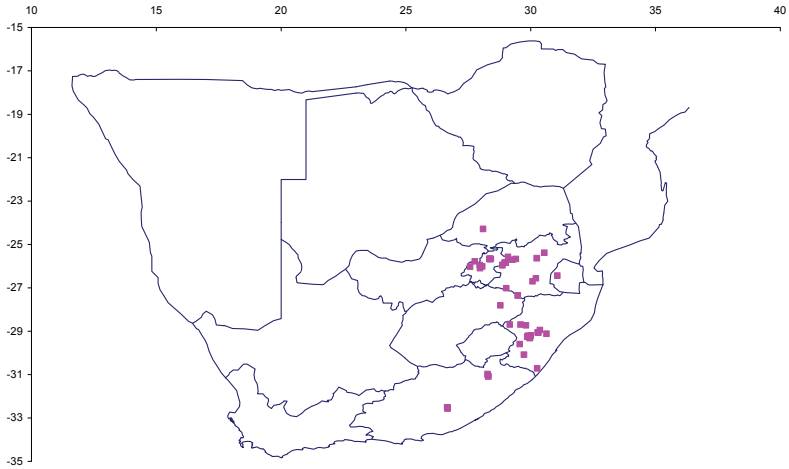


Figure 2.3 Distribution of *A. barnardi* in South Africa based on information from the Albany Museum.

2.3.1 Test conditions

Water quality parameters measured during experiments in the laboratories (Tables 2.2, 2.3 and 2.4) indicate that consistent water temperatures were maintained in all experiments. Coefficients of variation (CV) did not exceed 2.2%.

Table 2.2 Summary of water parameters per channel over 10 days for Exp1

Concentration (g/L)	Mean EC (mS/m)	CV (%)	Mean pH	CV (%)	Mean T (°C)	CV (%)
0.0	58.73	14.36	7.4	4.45	16.3	1.22
1.2	138.21	6.89	7.6	4.80	16.3	1.22
1.6	160.33	7.72	7.6	4.50	16.3	1.22
2.1	190.08	3.99	7.6	4.70	16.3	1.22
2.9	227.87	5.89	7.7	4.46	16.3	1.22
3.8	272.60	3.73	7.6	4.86	16.3	1.22
5.0	326.76	3.87	7.7	4.23	16.3	1.22
6.75	403.12	2.87	7.7	4.17	16.3	1.22
9.0	501.30	4.39	7.8	4.03	16.3	1.22
12.0	609.08	3.99	7.8	4.69	16.3	1.22

Table 2.3 Summary of water parameters per channel over 10 days for Exp2

Concentration (g/L)	Mean EC (mS/m)	CV (%)	Mean pH	CV (%)	Mean T (°C)	CV (%)
0.0	43.28	2.35	7.8	3.66	16.49	2.14
1.5	128.31	2.42	7.9	3.94	16.49	2.14
2.0	148.80	2.78	8.1	1.48	16.49	2.14
2.7	181.93	3.48	8.1	0.96	16.49	2.14
3.5	154.75	32.42	8.2	1.61	16.49	2.14
4.7	621.91	11.04	8.2	1.94	16.49	2.14
6.3	267.65	47.89	8.2	1.76	16.49	2.14
8.4	397.10	3.07	8.2	1.72	16.49	2.14
11.25	493.80	2.65	8.2	1.47	16.49	2.14
15.0	609.24	2.42	8.2	1.51	16.49	2.14

The trends in test channel EC values were different for each experiment. In Exp1 (Table 2.2) the CV for the control solution was high (14.36%), while those of all the other concentrations remained lower than 7.5%. Although slight rises in conductivity occurred over time, no channel in Exp1 experienced a change of more than 50.0 mS/m over 10 days. The control solution in Exp2 (Table 2.3) did not exhibit such high EC variability. Very high CVs occurred in the channels containing 3.5 g/L, 4.7 g/L and 6.3 g/L however, indicating high variation of concentration. Deviations of over 300 mS/m from expected conductivity values were recorded for these channels, with coefficients of variation reaching 47.89% in the 6.3 g/L solution. No uniform pattern of deviation was apparent as values decreased and increased independently and unpredictably. Only these three channels were affected. EC values in Exp2 were consistently lower per concentration compared to those in Exp1. The highest concentration in Exp2, for example, had almost exactly the same EC value as the highest concentration in Exp1 despite being 2 g/L stronger. EC values of control solutions in Exp1 and Exp2 were over four times those at the collection site at the time of collection. Exp3 (Table 2.4) had very consistent EC values in all channels throughout the entire duration of the experiment. CV values stayed below 1% over the 10 day period.

Table 2.4 Summary of water parameters per channel over 10 days for Exp3

Concentration (g/L)	Mean E.C. (mS/m)	CV (%)	Mean pH	CV (%)	Mean T (°C)	CV (%)
0.0	27.51	0.08	7.36	0.05	17.8	0.03
1.5	117.09	0.04	7.41	0.03	17.8	0.03
2.0	143.24	0.05	7.50	0.02	17.8	0.03
2.7	179.00	0.02	7.48	0.02	17.8	0.03
3.5	212.25	0.05	7.50	0.02	17.8	0.03
4.7	261.31	0.05	7.49	0.03	17.8	0.03
6.3	328.84	0.03	7.43	0.02	17.8	0.03
8.4	406.79	0.04	7.45	0.02	17.8	0.03
11.25	508.69	0.03	7.46	0.02	17.8	0.03
15.0	632.48	0.04	7.41	0.02	17.8	0.03

Average pH (Tables 2.2, 2.3 and 2.4) increased slightly with solution concentration, although the difference between the highest concentration and control never exceeded 1 unit of pH in any of the experiments. The pH values in Exp2 were consistently higher than those in Exp1 and Exp3, even at similar EC values.

2.3.2 Organism response – Probit and TSK analysis

Probit results for 96 h tests on *E. elegans* (Table 2.5) provided LC_{50} values which differed by 7.54 g/L between the upper and lower 95% confidence limits. The maximum difference between LC_{50}s from the three experiments was 5.12 g/L. The 95% confidence limit ranges for all LC_{50}s for *E. elegans* were below 2.2 g/L. The LC_{50} value for Exp2 was possibly skewed due to low sample size (Table 2.1) and a trim of nearly 15% of the data contributing to this number. The geometric mean of all LC_{50}s for *E. elegans* was 3.16 g/L.

Table 2.5 LC_{50} values and type of regression analysis for 96h tests for each species by experiment

Experiment	Species	Regression model	Spearman-Kärber trim (%)	LC_{50} (g/L)	95% confidence limits
Exp1	*E. elegans*	PROBIT	0	1.37	(0.12 - 2.29)
Exp2	*E. elegans*	TSK	7.33	6.49	(5.51 - 7.66)
Exp3	*E. elegans*	PROBIT	0	3.56	(2.85 - 4.31)
Exp1	*T. discolor*	PROBIT	0	2.79	(1.99 - 3.57)
Exp2	*T. discolor*	PROBIT	0	10.92	(7.59 - 14.36)
Exp3	*T. discolor*	TSK	18.01	6.95	(5.99 - 8.06)
Exp1	*A. barnardi*	TSK	8.73	7.63	(6.59 - 8.83)
Exp2	*A. barnardi*	TSK	7.89	7.48	(6.65 - 8.41)
Exp3	*A. barnardi*	PROBIT	0	7.56	(6.19 - 9.64)

The LC_{50} values calculated for *T. discolor* also exhibited wide variation. The highest 95% confidence limit was 12.37 g/L higher than the lowest. The LC_{50} calculated for Exp2 had a 95% confidence limit range of 6.77 g/L while those for Exp1 and Exp3 ranged over 1.58 g/L and 2.07 g/L respectively. The geometric mean for all three LC_{50}s for *T. discolor* is 5.96 g/L.

Although both Exp1 and Exp2 for *A. barnardi* required approximately 16% data trims the LC_{50}s for all three experiments were remarkably similar. The LC_{50}s differed by a maximum of 0.15 g/L, and the

95% confidence limits by 3.45 g/L. The geometric mean of the LC_{50}s from all three experiments is 7.55 g/L.

A comparison of LC_{50}s and their 95% confidence limits for all species from all three experiments reveals that they do not differ significantly from one another (Figure 2.4). The large confidence ranges for all species overlapped, and the difference between LC_{50}s did not exceed 10 g/L.

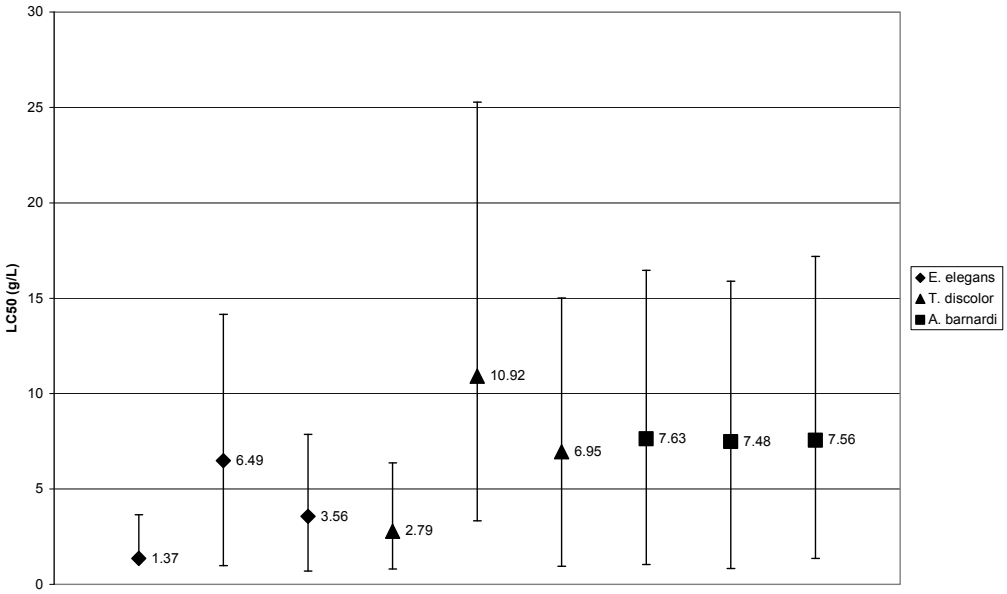


Figure 2.4 Plot of comparative 96 h LC_{50} values for each species for three experiments with 95% confidence values

2.3.3 Species sensitivity distribution

LC_{50} values for eight other taxa were used in conjunction with those calculated by this study. These comprised *Lymnaea sp*. (Pond snail), *Villorita cyprinoides* (Black clam), *Pimephales promelas* (Fathead minnow), *Oryzias latipes* (Medaka/high-eyes), *Lepomis macrochirus* (Bluegill), *Gambusia affinis* (Western mosquitofish), *Daphnia magna* (Water flea) and *Ceriodaphnia dubia* (Water flea) (see Appendix Table A.1). The geometric mean of LC_{50}s was determined wherever multiple results were available. All LC_{50}s were calculated for an acute time period (<96 h). The ACR was calculated as 1.441 and was applied as the concentration divisor (Figure 2.5). The calculated protection concentrations were higher than the current Reserve boundaries for $MgSO_4$ (Table 5.2) at the Good/Fair and Fair/Poor boundaries but almost half that of the Natural/Good boundary value.

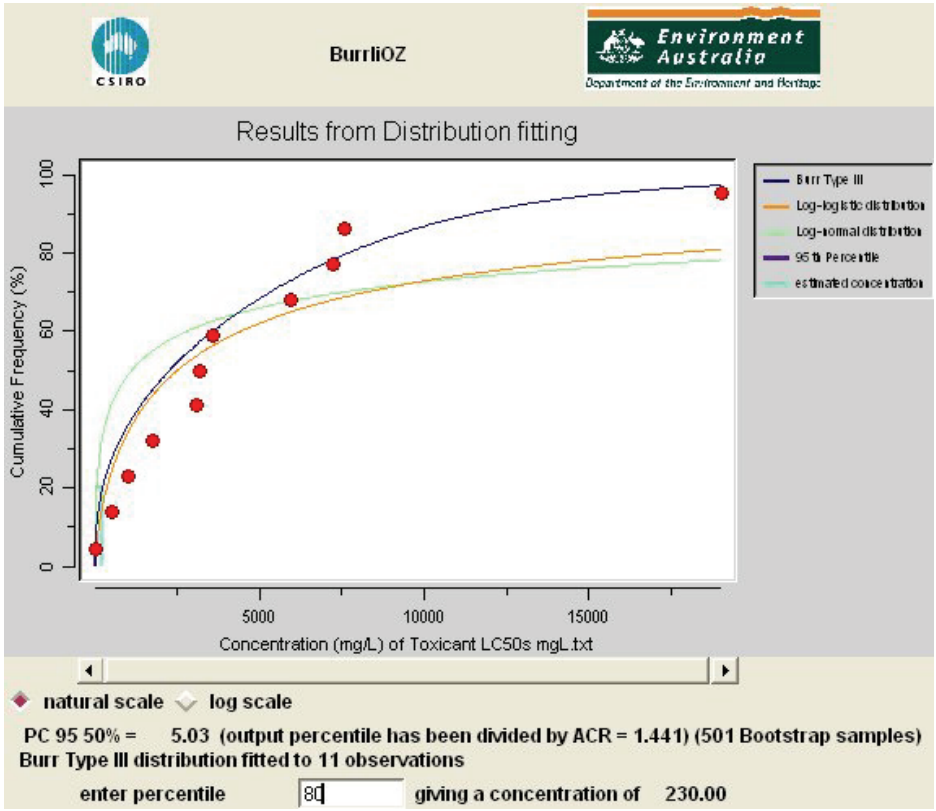


Figure 2.5 Species sensitivity distribution (SSD) for $MgSO_4$

2.4 Discussion

Exp2 exhibited a number of variations from the results provided by Exp1 and Exp3, especially in terms of water quality and mortality rates. The EC readings for the concentrations were consistently lower at the same $MgSO_4$ concentration than those in Exp1. Additionally, the deviations of three mid-range channels from the expected conductivity range in an unpredictable manner reduced the reliability of Probit analyses based upon reduced data sets, necessitating the use of the TSK method.

There was a disparity between the EC values in Exp2 and Exp3 for the same $MgSO_4$ concentrations. The anomalous EC data for the three mid-range channels in Exp2 could explain why such different LC_{50} values resulted from this experiment. These anomalies can possibly be attributed to some form of contamination in the three channels as it was apparent from the beginning of the experiments. Although water for both laboratories originates from the same source there are certainly differences in piping materials and distance the water travels. Due to the divalent property of $MgSO_4$ this inorganic salt reacts readily with other ions when in aqueous solution (Péqueux, 1995). It is possible that differences in piping material between the two laboratories may have resulted in differing ion contents of the tap water. These slight differences could have affected the bioavailability and speciation of the $MgSO_4$ between experiments.

Despite the problems raised by the anomalous results in Exp2, results from Exp3 appear to support LC_{50} results obtained from this experiment. The fact that the LC_{50}s for the three species in all experiments are not significantly different from one another suggests that the responses of these taxa to $MgSO_4$ are fairly uniform. In fact it appears from LC_{50} data recorded by Browne (2005) for mayflies exposed to NaCl and Na_2SO_4 and LC_{50} values obtained in this study for $MgSO_4$ that mayflies respond in a similar manner to these three different salts (Figure 2.6).

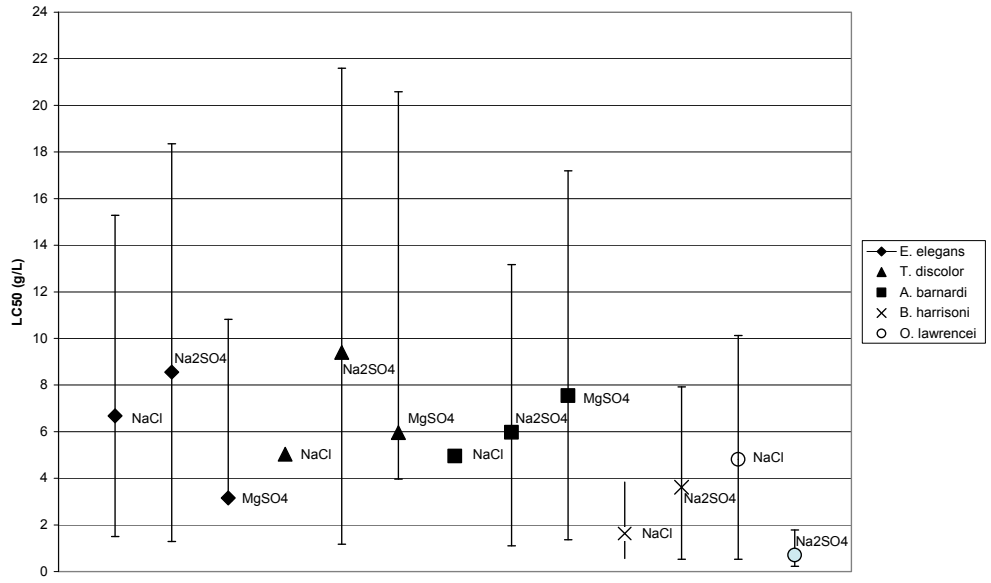


Figure 2.6 Comparison of LC_{50} values and 95% confidence limits between NaCl, Na_2SO_4 and $MgSO_4$ for five species of indigenous mayflies.

It is apparent that the differences in tolerance to $MgSO_4$ between mayfly species are no larger or more significant than the differences in tolerance to multiple salts within each species. There are no trends in tolerance which apply to all species, and although *A. barnardi* appears to indicate that $MgSO_4$ is more toxic than Na_2SO_4 which is in turn more toxic than NaCl, the confidence limits overlap significantly and the trend is not reflected by the other species. This lack of a general trend in tolerance to inorganic salts within or between taxa reiterates the need for increased species and salt specific ecotoxicity tests on indigenous organisms. Without these specific data the accuracy of boundary values based upon LC_{50}s extrapolated from exotic organisms or other chemicals may be drastically reduced (Warne et al., 2005). It is also important to note that natural intraspecies tolerance variation can lead to broad confidence limits being applied. This might reduce the accuracy and relevance of LC_{50}s, especially in cases where only one toxicity test has been undertaken. It must be recognized however, that although an increase in sample size and replication could reduce this margin of uncertainty, the logistical implications of testing more than 400 individuals per species are a limiting factor. In addition it is clear that natural variation in salt tolerance is an important factor in the widespread regional distribution of the taxa involved and is thus probably unavoidable. It is conceivable that significant regional differences in tolerance could exist. This would further the case for the need for extensive site-salt-species specific ecotoxicology in South Africa.

The SSD results provide an interesting contrast to the $MgSO_4$ Reserve boundary values proposed by Jooste and Rossouw (2002). The much lower 95% protection concentration conflicts with the expectation that the current Reserve boundary value for Natural/Good is conservative. The 90% and 80% boundaries are conversely much less conservative than the Reserve boundary values for Good/Fair and Fair/Poor. The assessment of the Kat River, Eastern Cape (Muller, 2005a), indicates that concentrations between 23.6 mg/L (Good) and 48.1 mg/L (Poor) were present within a system that is considered to be in a 'Good' condition for most other water quality parameters. An example from the Leeuspruit (Muller, 2005b) also shows that concentrations of $MgSO_4$ vastly exceeding the Fair/Poor boundary (124 mg/L - 142 mg/L) are found within a system which could be categorized as largely 'Fair' based upon other water chemistry parameters. Such disparities suggest that the boundaries produced in this study have the potential to provide a more realistic representation of background prevalence and ecosystem tolerance of $MgSO_4$ within South African systems.

It should be noted that the SSD, and hence the protective concentration values, in this study are based on preliminary and to some extent limited data. No data for algae, and very little for macroinvertebrates were available. For greater accuracy, multiple ACRs from each group should be combined to provide a more representative divisor. The presence of the highly sensitive estuarine clam *Villorita cyprinoids* in the SSD made a significant difference to the estimated protective concentration values. This exemplifies how such a small taxon sample size can skew the resulting protective concentration values. Hence more toxicology testing of $MgSO_4$ on indigenous organisms from all trophic levels is critical for definition of accurate and relevant boundary values and WQGs for $MgSO_4$ in South Africa.

3 OXYGEN CONSUMPTION IN TWO SPECIES OF FISH IN RESPONSE TO INCREASED CONCENTRATIONS OF SELECTED INORGANIC SALTS

3.1 Introduction

The objective of this experiment was to determine whether a change in dissolved oxygen (DO) could be used as a measure of the physiological response of guppies, *Poecilia reticulata* and zebra fish, *Danio rerio* when exposed to increasing concentrations of sodium chloride (NaCl) and sodium sulphate (Na_2SO_4). By using fish species in toxicity tests a more comprehensive approach to toxicity testing is provided through incorporating another trophic level in addition to that of invertebrates.

3.2 Materials and Methods

3.2.1 Experimental organisms

Two freshwater fish species were used for this experiment following an approval by the Rhodes University Ethical Standards Committee (RUESC). Test species were the guppy, *Poecilia reticulata* and the zebra danio, *Danio rerio*. Both species are exotic to South Africa, however are used globally in toxicity tests (Boisen et al., 2003). Guppies are cultured on large scale for ornamental purposes and were obtained from a local breeder. The danios were obtained from a wholesaler dealing in ornamental fish. These two species were exposed to increasing concentrations of the inorganic salts Na_2SO_4 and NaCl in separate experiments.

3.2.2 Experimental systems

Numerous experimental systems have been used to determine the tolerance of biota to salinity, however static systems are used as a simple standard for rapidly testing many species (Kefford et al., 2003; Kefford et al., 2004). These systems are utilised across the world (Kefford et al., 2004) and were used in this study to determine the effects of salinity on the test species. Tests conducted in this study made use of static systems without replacement (non-renewal). This meant that test organisms were exposed to the same test solution for the duration of the test (USEPA, 1994). Static test systems were favoured as they are simple, cost effective (compared with flow through systems) and require few resources (USEPA, 1994). Some of the major disadvantages are the possible DO depletion due to biological oxygen demand (BOD) and chemical oxygen demand (COD) (USEPA, 1994) as well as the accumulation of waste products. The effects of this were hoped to be controlled by the short experimental time and by purging the fish for 24 h prior to testing. Any effects that occurred as a result of this would be expected to appear in the controls, and treatments could be measured relative to this.

Respirometers were used as static test systems to determine the oxygen consumption of aquatic organisms over time. A pilot study revealed that a plastic respirometer of 2.4 L volume was suitable for the test species as dissolved oxygen (DO) did not drop below 5 mg/L over the experimental period. As with static systems for toxicity tests, static respirometers have the disadvantage of decreased DO levels over time, yet unlike flow through respirometers they are not subject to frequent calibrations, baseline errors and effects of dilution rate (Steffenson, 1989).

3.2.3 Experimental design and procedure

Prior to the start of the experiment, physio-chemical water quality parameters were recorded. These included water hardness (mg/L $CaCO_3$), pH, conductivity (mS/m), temperature (°C) and dissolved oxygen (mg/L). In addition light intensity was recorded. Dechlorinated tap water was used for all experiments. Respirometers were filled completely so as to eliminate air gaps. Thereafter one test specimen was added and the lid was screwed on tightly underwater.

To minimise confounding factors, respirometers were randomly placed on the test bench, with a colour assigned to each experimental period. The colours facilitated faster removal of respirometers at the end of each experimental period (24, 48, 72 and 96 h) when 60 respirometers were removed and water quality parameters measured. The experiment was conducted in a constant environment room where the temperature was maintained at 22 ± 2°C with a photoperiod of 14:10 h light:dark (Slabbert, 2004) to simulate South African summer conditions.

Electrical conductivity was measured using an AMEL 160 conductivity (mS/m) meter, pH was measured using a Cyberscan pH 5000 and DO (mg/L) was measured using a Cyberscan DO 1500.

In the first experiment DO was measured by chemically fixing the oxygen and titrating the sample using the modified Winkler method (Mackereth et al., 1978). Statistical analyses of this data when compared with that of the Cyberscan DO meter revealed no significant difference in trends between the two methods. The Winkler method was therefore discarded in favour of the DO probe as a time saving tool due to the number of samples that required processing every 24 h.

To avoid oxygen depletion within the static systems, acclimation of fish in the respirometers was not undertaken. Static respirometers do not facilitate sampling without the introduction of atmospheric oxygen. For this reason a destructive sampling method was used, where respirometers sampled at the end of each exposure period were not re-introduced into the experiment.

Six sub-lethal concentrations were tested. The control concentration was 0 mg/L with increasing concentrations of 0.5, 1, 2, 4 and 8 g/L for NaCl and 0.375, 0.75, 1.5, 3, 6 g/L for Na_2SO_4. These concentrations were derived using concentrations less than LC_{50} values obtained from the ECOTOX database (USEPA, 2004), as the endpoint of this experiment was oxygen consumption, not mortality. All six concentrations contained fish. The 0 mg/L concentration acted as one control, in addition there were controls for each salt concentration at 0 and 96h, these contained no fish. Each concentration as well as the controls comprised 10 replicates across the four exposure periods.

3.2.4 Data analysis

Dissolved oxygen (DO) data were analysed using Statistica software package and a multifactorial ANOVA (analysis of variance). A multi-stage Neuman-keuls test was used to show significant

differences between treatments. Temperature and pH were also recorded and means, standard deviations and coefficient of variations (CV) were determined.

The lowest value that was not significantly different from the control would indicate the no observed effect concentration (NOEC). This value could be incorporated into water quality guidelines and in doing so help in the refinement of these values.

3.3 Results

3.3.1 Poecilia reticulata

NaCl

For the NaCl experiment a mean pH of 7.73 was determined and a minimum and maximum pH of 7.19 and 8.36 were recorded respectively. A CV value of 3.2% was calculated. A mean temperature of 23.25°C was determined with minimum and maximum temperatures of 22.1°C and 23.6°C being recorded respectively. A CV value of 0.8% for temperature was calculated.

In the test without fish, available DO within the control showed a general decreasing trend from the start of the experiment (0 h) to the completion of the experiment (96 h) (Figure 3.1). A significant (F= 4.03 and p= 0.0037) change in DO was observed for concentrations of 0.5, 1 and 2 g/L.

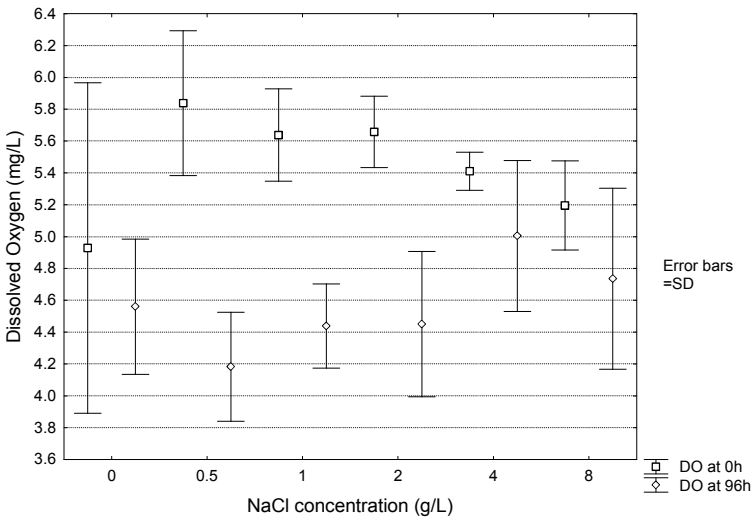


Figure 3.1 Changes in dissolved oxygen (mg/L) at different NaCl concentrations at 0 h and 96 h. These concentrations did not contain fish and measured natural changes on DO over 96 h of exposure.

During the 96 h toxicity test, which contained fish, a significant difference was found across treatments between the 0 g/L concentration and the other treatments and between the 8 g/L concentration and the other treatments at the 0 h time interval (Figure 3.2) (F=3.02, p=0.017). This difference was also reflected with change in DO over time 0 h to 24 h period (Figure 3.3). No significant difference was found between treatments at the 24 h time interval (F=1.21, p=0.314) (Figure 3.2). Significant differences (F=5.45, p=0.0004) were observed for the 48 h time period at the 1 g/L and 8 g/L treatments. At the 72 h time period a significant difference was noted for the 1 g/L concentration (F= 7.32, p=0.00003) while at the culmination of the experimental period (96 h) a significant difference was found at the 2 g/L concentration (Figure 3.2). In addition to the general

decrease in available oxygen from 0 h to 24 h (Figure 3.3), a significant change is also reflected at the 48 h time period for 1 g/L concentration (Figure 3.3).

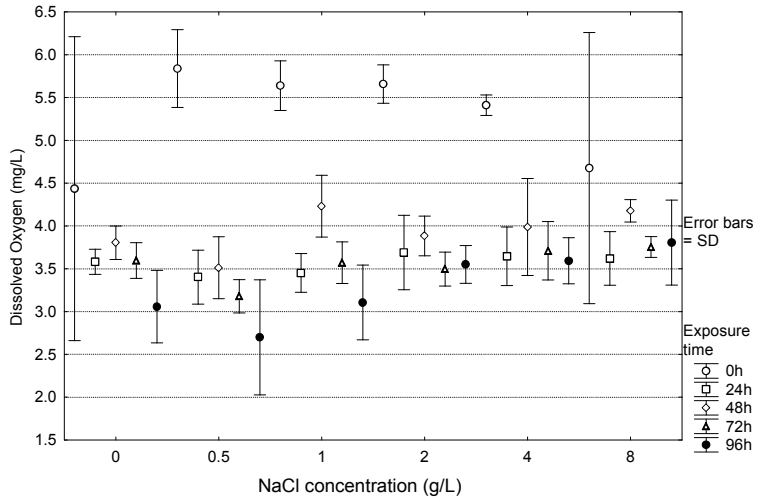


Figure 3.2 Change in dissolved oxygen (mg/L) in response to *Poecilia reticulata* exposed to increasing concentrations of NaCl over 5 exposure times

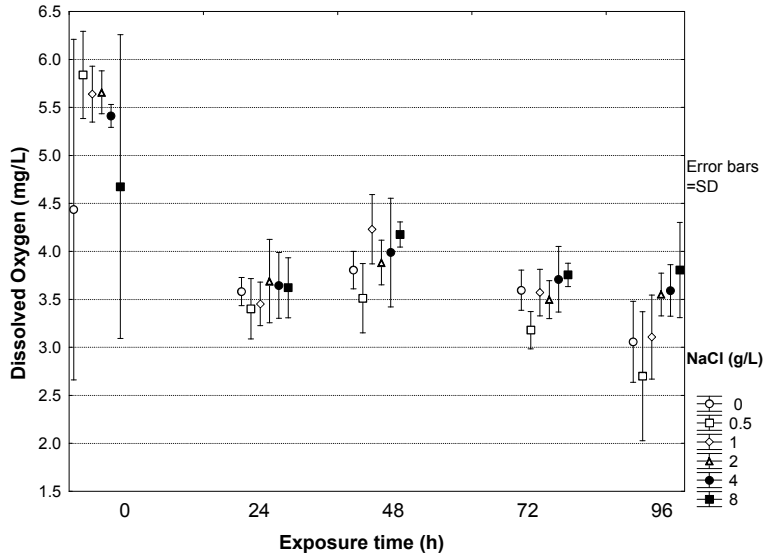


Figure 3.3 Change in dissolved oxygen (mg/L) in response to *Poecilia reticulata* exposed to increasing concentrations of NaCl per concentration over time

Na_2SO_4

For the Na_2SO_4 experiment, a mean pH of 7.35 was determined and a minimum and maximum pH of 6.6 and 7.81 were recorded respectively. A CV value of 2.2% was calculated. A mean temperature of 22.32°C was determined with minimum and maximum temperatures of 21°C and 23.2°C being recorded respectively. A CV value of 2.0% for temperature was calculated.

DO changed significantly ($F=20.43$, $p<0.00005$) from the 0 h to 96 h time period showing a decrease across all concentrations for the controls that contained no fish (Figure 3.4).

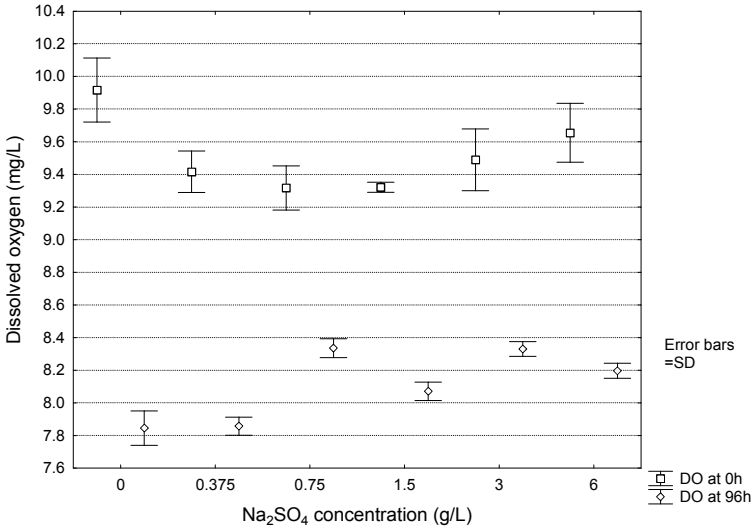


Figure 3.4 Changes in dissolved oxygen (mg/L) at different Na_2SO_4 concentrations at 0 h and 96 h. These concentrations did not contain fish and measured natural changes on DO over 96 h of exposure.

The 96 h toxicity test containing fish showed significant decreases in DO across all concentrations over duration of the experiment. Most notable were the changes from 0 h to 24 h and 48 h respectively ($F=157.78$, $p<0.0005$) (Figure 3.5). These decreases in available DO are also reflected in the change of DO over the exposure time with each concentration (Figure 3.6).

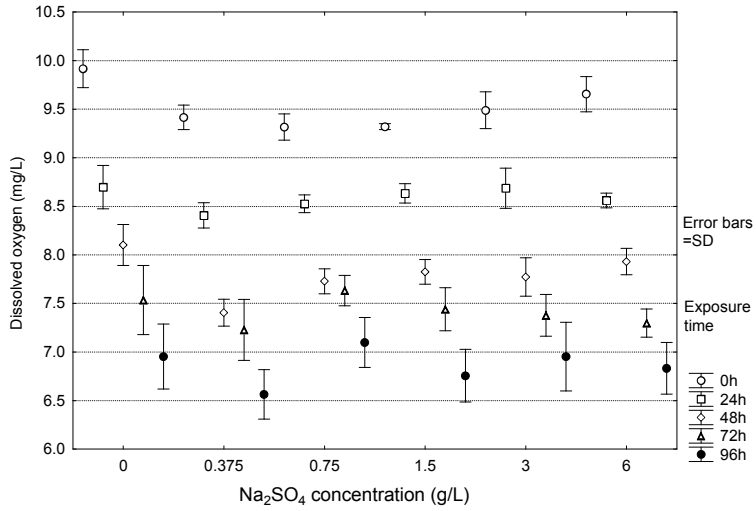


Figure 3.5 Change in dissolved oxygen (mg/L) in response to *Poecilia reticulata* exposed to increasing concentrations of Na_2SO_4 over 5 exposure times

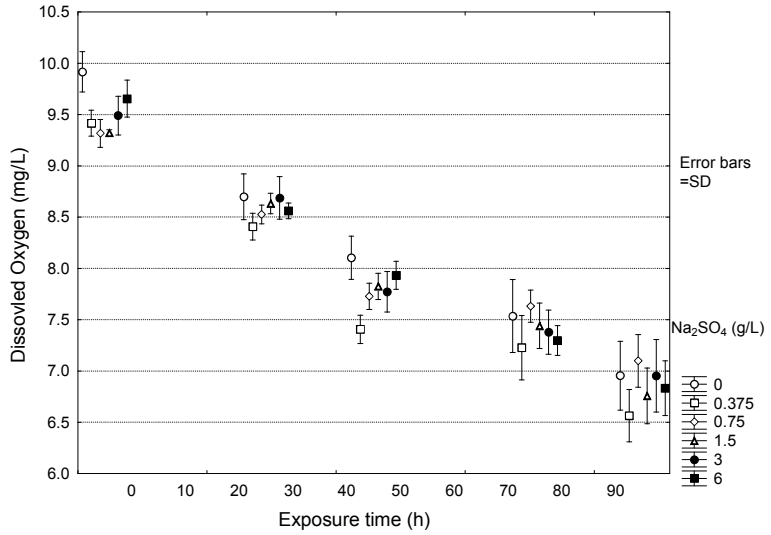


Figure 3.6 Change in dissolved oxygen (mg/L) in response to *Poecilia reticulata* exposed to increasing concentrations of Na_2SO_4 per concentration over time

3.3.2 Danio rerio

NaCl

All DO decreased significantly (F= 21.12, p<0.005) across the controls containing no fish. This was observed for all treatments with NaCl (Figure 3.7).

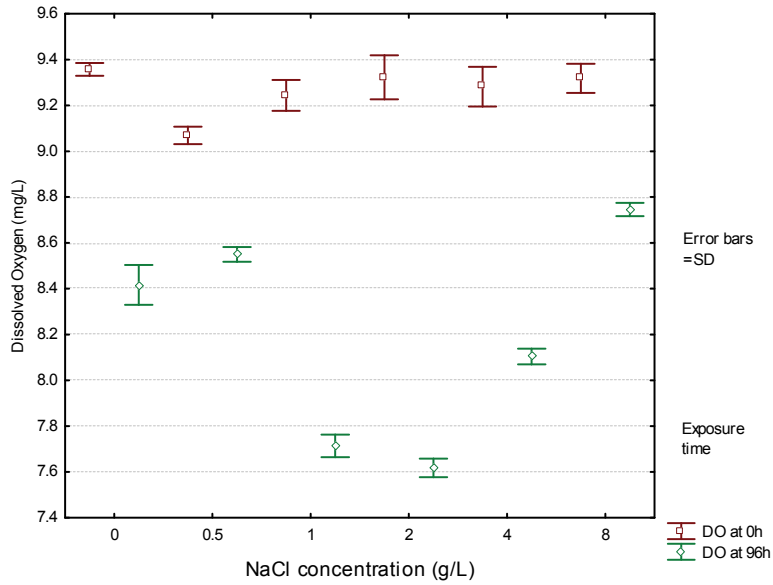


Figure 3.7 Changes in dissolved oxygen (mg/L) at different NaCl concentrations at 0 h and 96 h. These concentrations did not contain fish and measured natural changes on DO over 96 h of exposure.

Similar trends were seen with this experiment (Figures 3.8 and 3.9) as with the Na_2SO_4 experiment with *P.reticulata* (Figures 3.5 and 3.6). A significant decrease in DO was seen over the 96 h exposure period, notably around the 24 h and 48 h periods and 0.5 g/L and 1 g/L concentrations (F= 5.1, p=0.0007 ; F=6.36, p=0.0001) (Figures 3.8 and 3.9).

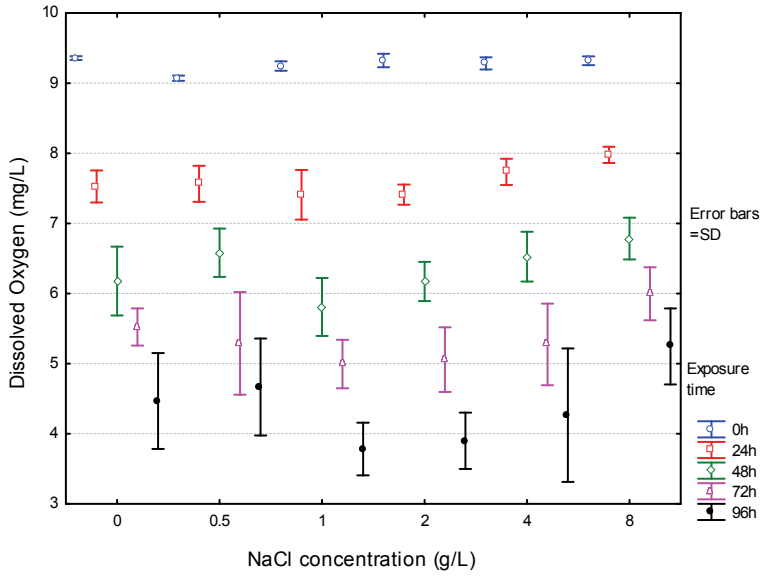


Figure 3.8 Change in dissolved oxygen (mg/L) in response to *Danio rerio* exposed to increasing concentrations of Na_2SO_4 over 5 exposure times

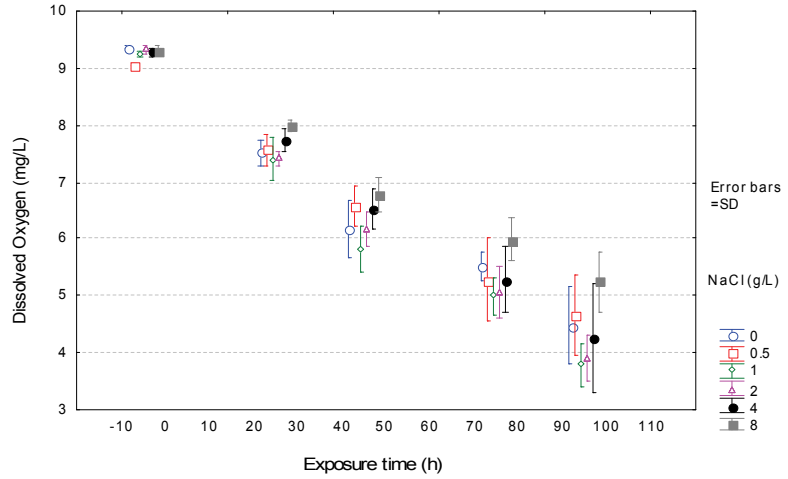


Figure 3.9 Change in dissolved oxygen (mg/L) in response to *Danio rerio* exposed to increasing concentrations of NaCl per concentration over time

Na_2SO_4

As seen with the other experiments, DO significantly decreased from the start of the Na_2SO_4 experiment (0 h) to the end of the experimental period (96 h) ($F=40$, $p<0.0005$) (Figure 3.10). Similar to the other experiments these controls also contained no fish.

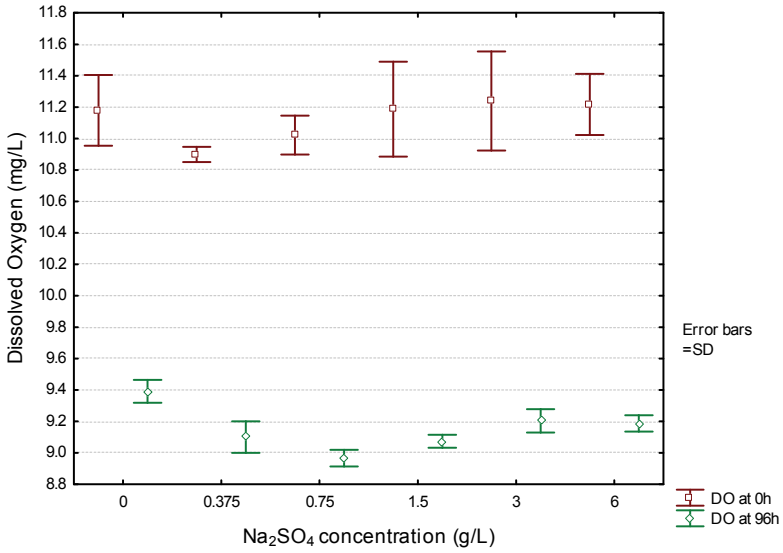


Figure 3.10 Changes in dissolved oxygen (mg/L) at different Na_2SO_4 concentrations at 0 h and 96 h. These concentrations did not contain fish and measured natural changes on DO over 96 h of exposure.

The *D. rerio* experiment also showed a decrease in available DO over the 96 h period. Significant differences were found between 0 h and 24 h and between 0 h and 48 h ($F=180.12$, $p<0.0005$; $F=108.88$, $p=0.005$) (Figures 3.11 and 3.12). These trends were also reflected in Figure 3.12, where a decrease in available DO was seen over concentrations over the 24 h and 48 h time periods, particularly for the 0.375 g/L and 0.75 g/L concentrations.

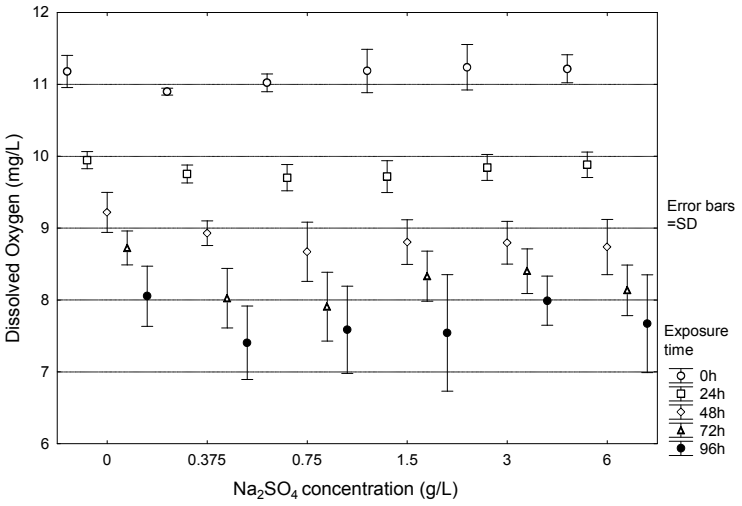


Figure 3.11 Change in dissolved oxygen (mg/L) in response to *Danio rerio* exposed to increasing concentrations of Na_2SO_4 over 5 exposure times

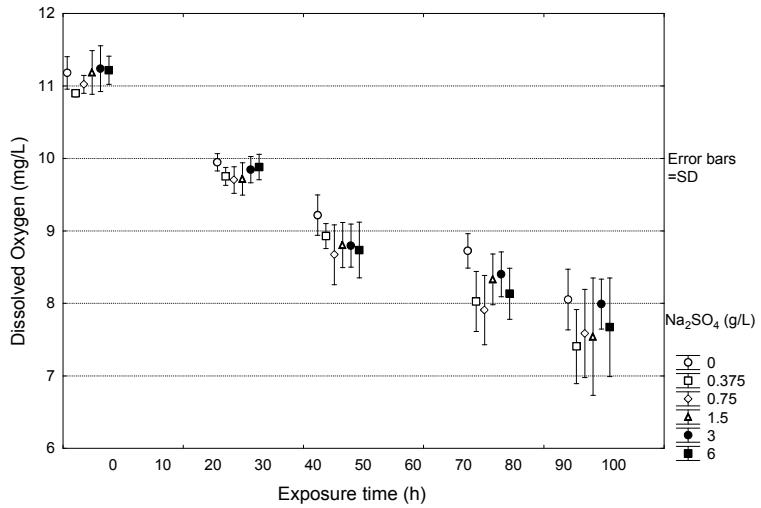


Figure 3.12 Change in dissolved oxygen (mg/L) in response to *Danio rerio* exposed to increasing concentrations of Na_2SO_4 per concentration over time

3.4 Discussion and Conclusion

Temperature and pH were recorded for all concentrations over the 96 h period. From the results the CV values were very low (<3.2%) for both parameters. Based on this it is unlikely that these parameters had a confounding effect on the experiment as changes in DO were measured as relative changes. It has been therefore decided to exclude these parameters in data analysis.

The NaCl experiment using *P. reticulata* as test species revealed that a concentration of 1 g/L was the lowest concentration to show a response, particularly at the 24 and 48 h exposure periods. When testing the same salt on *D. rerio* it was found that the same concentration was the lowest to yield a response, however this occurred at the 48 and 96 h exposure periods. These differences in response times may be explained by differences in the abilities of these species to conform or regulate when exposed to inorganic salt toxicants. Most fishes are osmoregulators, maintaining osmotic balance by regulating their internal osmotic environment to maintain cellular function even when the external environment fluctuates (Helfman et al., 2002). Some species are able to tolerate large osmotic changes in their environment (euryhaline), while others are only able to tolerate small changes (stenohaline). Freshwater fish are hyperosmotic to their environment and therefore gain water while losing salts to the environment, as a result salts need to be actively transported back across the gills to maintain homeostasis (Helfman et al., 2002).

The ability of freshwater fishes to adapt to increasing salt concentrations is species specific and is dependent on several factors, including (but not limited to) gill to body surface ratio, hormonal and endocrine control, oxygen levels and temperature fluctuations (Lagler et al., 1962).

Guppies (*P. reticulata*) are neither catadromous nor anadromous and are only able to adapt to changes in salinity in a gradual manner (Daikoku, 1980). Sudden changes, such as those experienced during a rapid influx of salt toxins may affect the ability of these fishes to osmoregulate and be reflected in the oxygen consumption of these organisms (Daikoku, 1980). Data gathered on euryhaline species showed a wider tolerance to salinity than guppies, reflecting a better developed ability of these species to osmoregulate and therefore to adapt to rapid changes in salinities (Daikoku, 1980)

Oxygen consumption has been used in relation to physiological activity when assessing stress caused by pollutants and, in addition to indicating metabolic rate, it has been used to provide an index for stress through toxin exposure (Grobler et al., 1989; Palanivelu et al., 2005).

These results showed that oxygen consumption could be used as a physiological response variable to stressors such as the inorganic salts Na_2SO_4 and NaCl. These data indicate that the NOEC (no observed effect concentration) for NaCl may be found at the 0.5 g/L concentration for both *D. rerio* and *P. reticulata*. The LOEC for Na_2SO_4 appeared to be at a concentration of 0.375 g/L for both species and seeing that this was the lowest concentration tested a MATC (maximum allowable toxicant concentration) of 0.188 g/L was calculated by dividing the LOEC by two. This indicated that there appeared to be no difference between the sensitivity of the two species, as both responded to the same concentrations, albeit that *D. rerio* appeared to lag behind *P. reticulata* by 24 h.

These sublethal data could prove useful in refining water quality guidelines. In addition, a protocol for testing fish species in this manner has now been established and may be incorporated into future toxicity testing involving the use of oxygen consumption as a physiological response in aquatic organisms. Further analysis could still be done on these data to show how metabolic rate is affected over time with respect to the given salts and the given concentrations. This may explain some variability in the data and the response of the two species. While this study may provide baseline data, it would prove very useful to conduct such tests on indigenous species and provide data that is more environmentally accurate for local conditions.

4 OSMOREGULATORY RESPONSES OF FRESHWATER SHRIMP TO INCREASED CONCENTRATIONS OF SELECTED INORGANIC SALTS

4.1 Introduction

Crustaceans inhabit a wide range of aquatic biotopes (marine, semi-marine, brackish, estuarine, and freshwater) and use a wide variety of different osmoregulatory mechanisms in different salinities. The two main mechanisms split crustaceans into two categories: osmoregulators and osmoconformers (Anger, 2001). Most marine crustaceans are osmoconformers where the internal osmotic pressure equals the external one of the medium (marine environment) which is more or less stable (Péqueux, 1995). Osmoregulators actively keep the internal concentration of body fluids (haemolymph, blood) different from external media which involves a fair amount of energy expenditure in changing environmental conditions. Among osmoregulators there are different mechanisms involved in maintaining the internal osmotic concentration. Hyper-regulators actively replace passively lost ions in dilute media through ion pumps whereas hypo-regulators in hypersaline media actively excrete ions (Anger, 2001). Hyper-hypo regulators are able to maintain their haemolymph osmolality at a relative constant level (hyper-regulation in low salinities and hypo-regulation in high salinities) (Péqueux, 1995). This form of osmoregulation is very common in Decapoda, Branchiopoda, Isopoda, Copepoda, and Mysidacea in particular amongst semi terrestrial and terrestrial forms (Péqueux, 1995).

Effects of increased salinities on crustaceans include decreases in longevity and fecundity in *Daphnia magna* (Martínez-Jerónimo and Martínez-Jerónimo, 2007) and *Branchipus schaefferi* (Sarma et al., 2005). An increase in salinity may also result in limitations of growth rates as shown for *Daphnia carinata* (Hall and Burns, 2002) and *Daphnia magna* (Teschner, 1995, Arner and Koivisto, 1993).

The osmoregulatory capacity (OC) is the difference between the osmolality of haemolymph and that of the external medium (Charmantier et al., 1989) and has been suggested by Lignot et al. (2000) as

a tool for monitoring physiological stress in crustaceans. The freshwater shrimp *Caridina nilotica* has been used as a model indigenous crustacean species in acute and chronic toxicity testing in South Africa (Slaughter et al., 2008). Therefore the physiological endpoint for this experiment was osmoregulatory capacity (OC) of the freshwater shrimp, *C. nilotica* (Decapoda: Atyidae) in response to increasing concentrations of sodium chloride (NaCl) and sodium sulphate (Na_2SO_4).

4.2 Materials and Methods

4.2.1 Experimental organisms

Freshwater shrimp, *C. nilotica* (Crustacea: Decapoda), collected from the Bushmans River in Alicedale, South Africa, were used in this study. These animals are indigenous to southern Africa (Hart 1983) and have been used in toxicity testing (Slaughter et al., 2008). Shrimp were subjected to increasing concentrations of inorganic salts (NaCl and Na_2SO_4) in two separate experiments.

4.2.2 Experimental systems

The respirometer system (see Chapter 3) was adjusted for the freshwater shrimp. A 350 mL plastic respirometer used with one individual shrimp each was determined to suit the experimental requirements best.

4.2.3 Experimental design and procedure

Caridina nilotica were collected the Bushmans River in Alicedale, South Africa, using a SASS net and returned to the laboratory in aerated cooler boxes. Test animals acclimatised in an aerated 40 L glass aquarium for 48 h at 26°C water temperature and a 8:16 h light/dark cycle.

The following concentrations of sodium chloride (NaCl) and sodium sulphate (Na_2SO_4) were made up in 25 L buckets: NaCl: 0.5, 1, 2, 4, 8 g/L; Na_2SO_4: 0.375, 0.75, 1.5, 3, 6 g/L. These concentrations were derived by using less than LC_{50} values from the IWR-UCEWQ toxicity database and are the same concentrations used in Chapter 3 of this report for fish. For each concentration, fifty respirometers (350 mL) were half filled with experimental solution and one animal was added into each of these plastic jars. The respirometers were then fully submerged in experimental solution and closed with a lid under the surface to prevent oxygen from entering. The experiments were conducted at 20°C room temperature and with a 8:16 h light/dark cycle. Electrical conductivity was measured using an AMEL 160 conductivity (mS/m) meter, pH was measured using a Cyberscan pH 5000 and dissolved oxygen (mg/L) was measured using a Cyberscan DO 1500. These water quality parameters were measured prior to the experiment (0 h) and thereafter at 12, 24, 48, 72, and 96 h from respirometers after being removed from the experiment to measure haemolymph osmolality in the shrimp.

Ten shrimp from each concentration were removed from the experiment after 12, 24, 48, 72, and 96 h. As a control, twenty shrimp were taken directly from the 40 L tank at the start of the experiment as a control (0 h). Shrimp length was measured from eye socket to tail tip using a caliper. The tail was then cut off behind the last pleopod with a scalpel. Guts were removed and discarded. A syringe needle was inserted into the heart of the shrimp and 20 units sodium citrate was injected. Any excess fluid coming out of the opening of the tail-cut or through any other openings was collected with another syringe.

Osmolality of the sodium citrate/haemolymph mixture was measured using an Osmometer (Advanced Micro-Osmometer 3320) located at the Department of Zoology, Rhodes University. Sodium citrate without haemolymph was measured as a blank and subtracted from the osmolality reading to get adjusted osmolality.

4.2.4 Data analysis

Data were tested for normality using the Shapiro-Wilk-Test and for homogeneity of variance using the Levene-Test. Significant differences were established using the one-way ANOVA and t-test for normally distributed data sets and the Kruskal-Wallis-Test for non-parametric data sets. The Statistica software package was used for all analysis.

4.3 Results

4.3.1 NaCl

Water quality parameters for 0.5 g/L NaCl at 0 h were not measured due to problematic meters. DO and pH data are normally distributed ($p<0.05$) and homogenous ($p<0.05$). EC data are normally distributed but not homogenous ($F=14.94$, $p=0.00$) due to different concentrations of salt used in the experiments. Coefficient of variance (CV) values for pH and EC were below 7% and CV values for DO were up to 20% (Table 4.1).

Table 4.1 Summary of water quality parameters per concentration (Conc) over 96 h exposure for NaCl measured from random respirometers

Conc (g/L)	Mean pH	Std. Dev.	CV (%)	Mean EC (mS/m)	Std. Dev.	CV (%)	Mean DO (mg/L)	Std. Dev.	CV (%)
0.0	7.08	0.31	4.36	66.24	0.04	6.74	4.10	0.27	6.67
0.5	7.90	0.24	3.10	178.65	0.04	2.50	3.22	0.54	16.66
1.0	8.06	0.15	1.89	316.14	0.11	4.05	4.12	0.63	15.41
2.0	8.30	0.37	4.47	450.85	0.15	3.21	4.18	0.24	5.68
4.0	8.00	0.19	2.41	795.92	0.25	3.09	3.50	0.70	20.07
8.0	8.10	0.15	1.79	1465.97	0.72	4.86	3.96	0.47	12.07

EC values were translated into medium osmolality values by measuring them with the Osmometer (see Table 4.2) and were used as such for further analysis.

Table 4.2 Overview of NaCl concentrations with their respective EC and osmolality values

Concentration (g/L)	EC (mS/m)	Medium Osmolality (mOsm/kg)
0.0	66.24	3
0.5	178.65	21
1.0	316.14	27
2.0	450.85	58
4.0	795.92	108
8.0	1465.97	212

Only DO values of the lowest concentration tested (0.5 g/L) differed significantly from the control (0.0 g/L) (p=0.01) all other concentrations were not significantly different from the control (Figure 4.1). After an exposure time of 24 h all DO values were significantly different from the start of the experiment (0 h) (24 h p=0.01, 48 h p=0.03, 72 h p=0.00, 96 h p=0.00) (Figure 4.2).

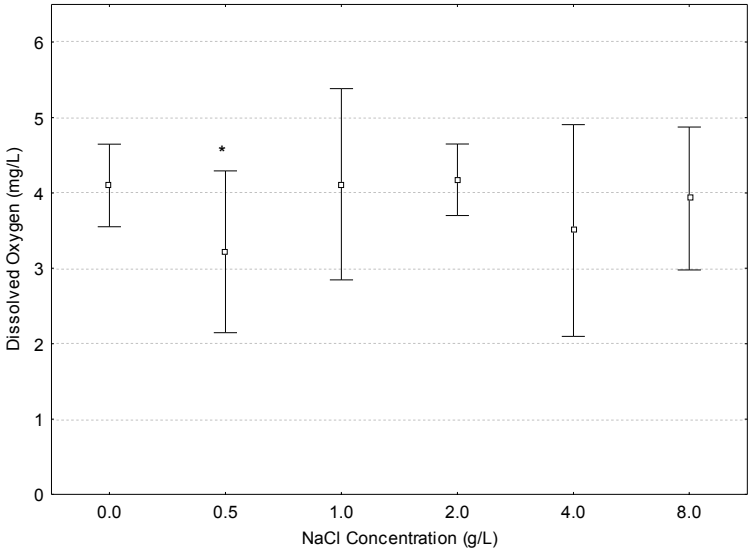


Figure 4.1 Dissolved Oxygen of all exposure times combined over all NaCl concentrations (error bars are standard deviation, significant differences are marked with an *)

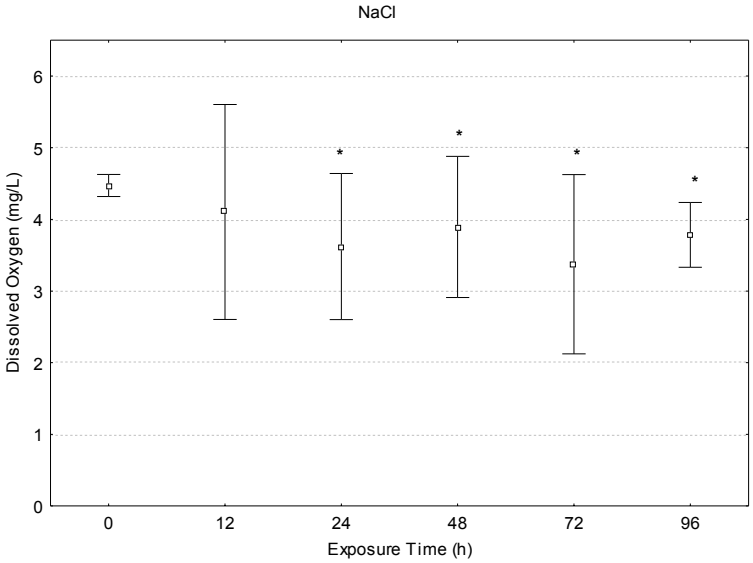


Figure 4.2 Dissolved Oxygen of all NaCl concentrations combined over time (error bars are standard deviation, significant differences are marked with an *)

Haemolymph osmolality data were not normally distributed (W=0.97920, p=0.00) and not homogenous grouped as time (F=6.499995, p=0.00) but homogenous when grouped as concentration (F=1.37213, p=0.25). Only osmolality values of the lowest concentration (0.5 g/L) were significantly different from the control (p=0.01) (Figure 4.3). Haemolymph osmolality values of 12 (p=0.00), 24 (p=0.00), 48 (p=0.00) and 72 h (p=0.00) of exposure differed significantly from 0 h. After 96 h of exposure the haemolymph osmolality was not different from the start of the experiment (p=1.00) (Figure 4.4).

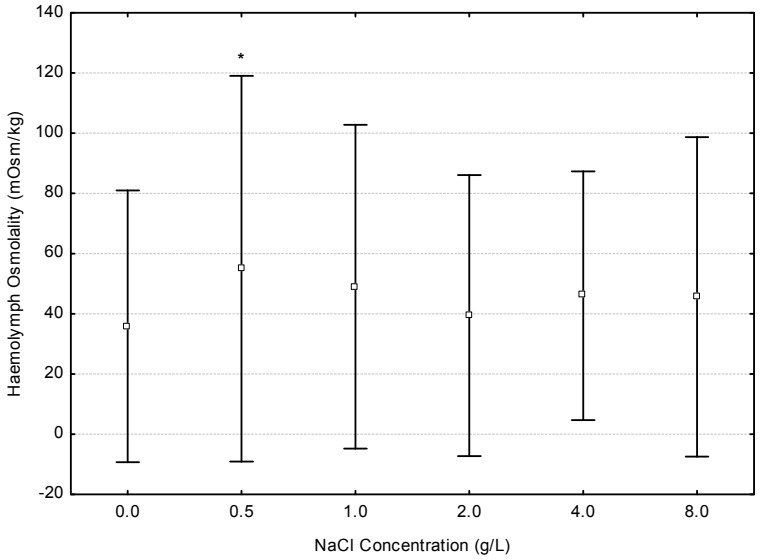


Figure 4.3 Haemolymph osmolality of all exposure times combined over all NaCl concentrations (error bars are standard deviation, significant differences are marked with an *)

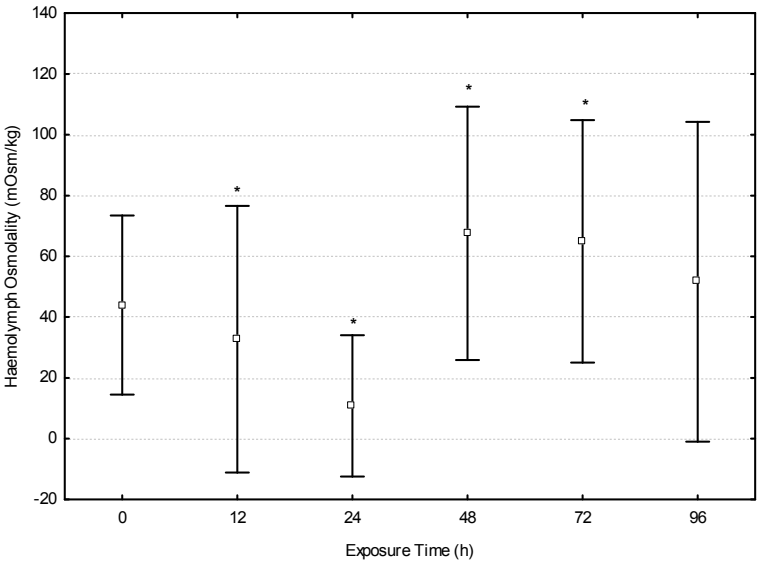


Figure 4.4 Haemolymph osmolality of all NaCl concentrations combined over time (error bars are standard deviation, significant differences are marked with an *)

There were significant differences of haemolymph levels in shrimp between the start of the experiment (0 h) and 12 h in the control (0.0 g/L p=0.0.2) and the highest concentration (8.0 g/L p=0.04) (Figure 4.5). Differences were found between 0 h and 24 h in the control (p=0.00), 1.0 g/L (p=0.02), 2.0 g/L (p=0.00) and 4.0 g/L NaCl (p=0.01). Differences are significant in 0.5 g/L between 0 h and 48 h (p=0.03) and 96 h (p=0.00) and in 1.0 g/L between 0 h and 72 h of NaCl exposure (p=0.02).

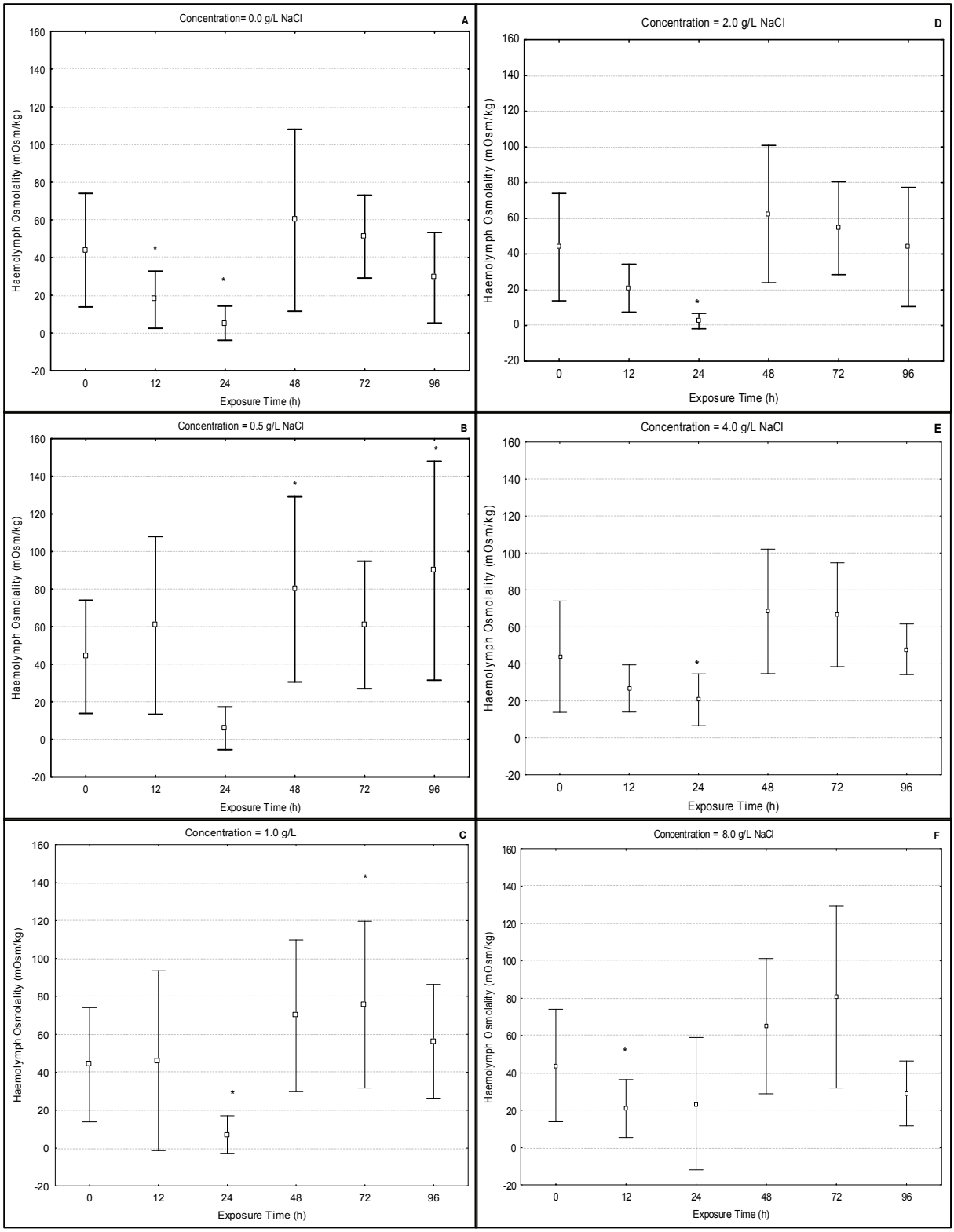


Figure 4.5 Haemolymph osmolality for each NaCl concentration over all exposure times (error bars are standard deviation, significant differences are marked with an *)

Differences between haemolymph levels in the control (0.0 g/L) and the two lowest concentrations of NaCl (0.5 g/L and 1.0 g/L) were significant after 12 h of exposure (p=0.00) and after 96 h (p=0.00 and 0.01 respectively) (Figure 4.6). After 24 h the control differed significantly from 4.0 g/L (p=0.00) and 8.0 g/L (p=0.01) NaCl concentration. After 72 h of exposure 8.0 g/L differed significantly from the control (p=0.02).

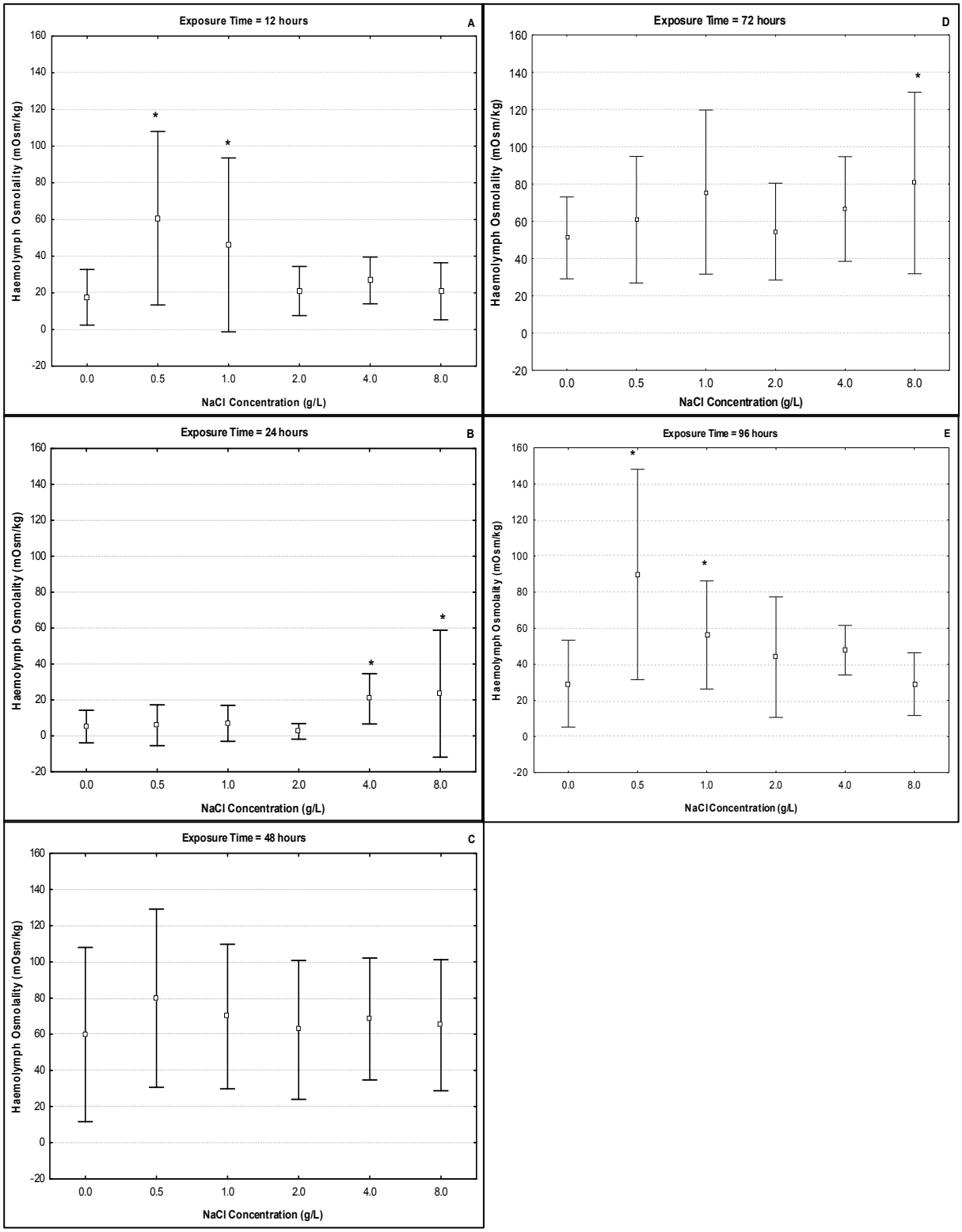


Figure 4.6 Haemolymph osmolality for each exposure time over all NaCl concentrations (error bars are standard deviation, significant differences are marked with an *)

Haemolymph osmolality stayed very constant over different exposure concentrations even beyond the isosmoticity line (Figure 4.7). Only the lowest concentration was significantly different from the control (second line of circles from the left) (see Figure 4.3). The linear fit line (horizontal line) stayed at a constant level with no significant rise or fall (p=0.79).

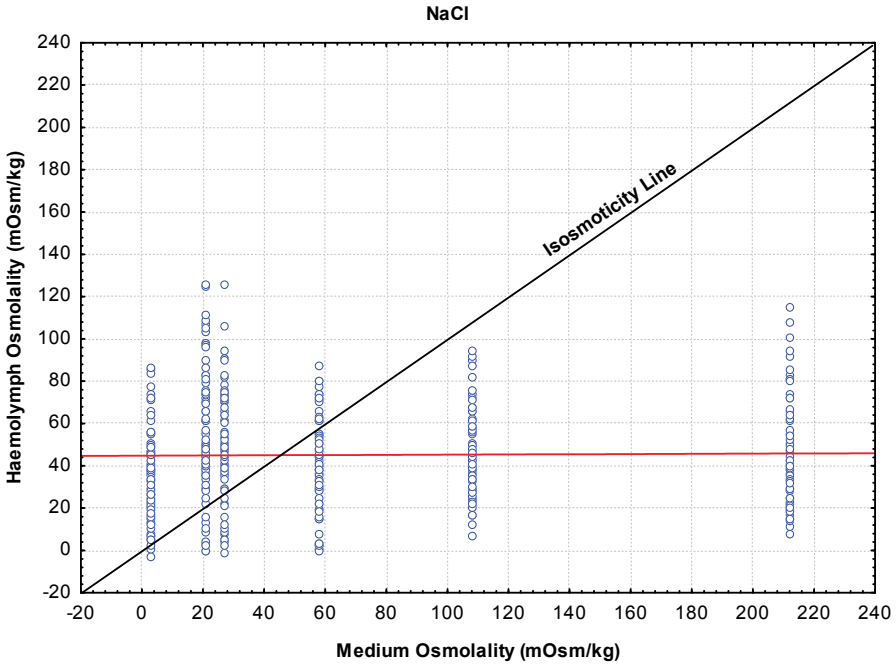


Figure 4.7 Haemolymph Osmolality *vs.* Medium Osmolality of *C. nilotica* exposed to NaCl (Isosmoticity Line at Medium Osmolality =Haemolymph Osmolality)

When calculating the osmoregulatory capacity (OC) of freshwater shrimp with regards to different NaCl concentrations the following formula was used (Charmantier et al., 1989):

Haemolymph osmolality – Medium osmolality = Osmoregulatory Capacity (OC).

A positive OC indicates that the test organism is hyper-regulating, a negative value indicates that the test organism is hypo-regulating and a null value indicates that the test organism is osmo-conforming. The freshwater shrimp, *C. nilotica*, was hyper-regulating up to a NaCl concentration of 1g/L (27 mOsm/kg) and hyporegulating from a NaCl concentration of 2 g/L (58 mOsm/kg) and higher (Figure 4.8). Consequently, it appears that C. nilotica used in this study were hyper-hypo-regulating.

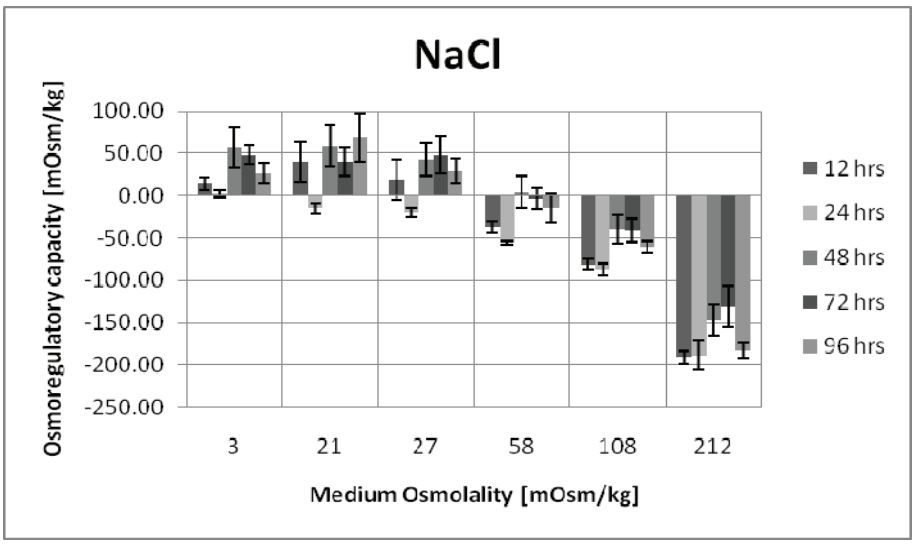


Figure 4.8 Osmoregulatory capacity (OC) at different NaCl concentrations (medium osmolality) for different exposure times

4.3.2 Na_2SO_4

Water quality parameters for 6.0 g/L Na_2SO_4 at 96 h were not measured due to all shrimp having died. The DO and pH data were normally distributed ($p<0.05$) and homogenous ($p<0.05$), while all EC data were normally distributed but not homogenous ($F=4.76$, $p=0.02$) due to different concentrations of salt used in the experiments. Coefficient of variance (CV) values for pH and EC were below 5% and for DO up to 15% (Table 4.3). The EC values were translated into medium osmolality values by measuring them with the Osmometer (see Table 4.4) and were used as such for further analysis.

Table 4.3 Summary of water quality parameters per concentration (Conc) over 96 h exposure for Na_2SO_4 measured from random respirometers

Conc (g/L)	Mean pH	Std. Dev.	CV (%)	Mean EC (mS/m)	Std. Dev.	CV (%)	Mean DO (mg/L)	Std. Dev.	CV (%)
0	7.73	0.18	2.28	53.36	0.01	1.29	4.51	0.67	14.82
0.375	8.19	0.13	1.59	88.75	0.02	1.24	4.56	0.60	13.26
0.75	8.28	0.13	1.54	166.37	0.06	3.34	4.63	0.49	10.52
1.5	8.40	0.14	1.62	287.68	0.05	1.80	4.33	0.46	10.59
3	8.58	0.11	1.30	465.65	0.14	3.04	4.60	0.60	13.03
6	8.74	0.11	1.25	799.64	0.39	4.82	3.95	0.43	10.84

Table 4.4 Overview of Na_2SO_4 concentrations with their respective EC and osmolality values

Concentration (g/L)	EC (mS/m)	Medium osmolality (mOsm/kg)
0.000	53.36	3
0.375	88.75	11
0.750	166.37	14
1.500	287.68	23
3.000	465.65	39
6.000	799.64	77

Values of DO of all concentrations were not significantly different from the control (Figure 4.9), whereas all DO values for different exposure times differed significantly from the start of the experiment at 0 h (all p-values <0.00) (Figure 4.10).

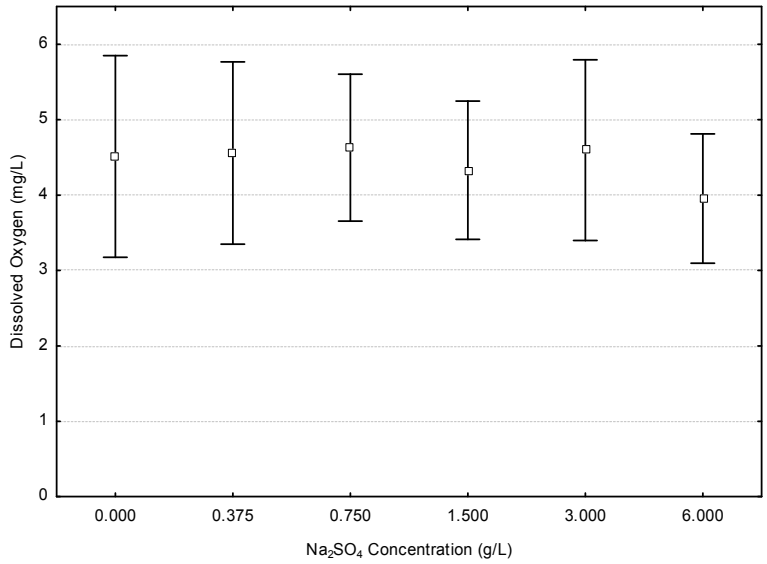


Figure 4.9 Dissolved Oxygen of all exposure times combined over all Na_2SO_4 concentrations (error bars are standard deviation, significant differences are marked with an *)

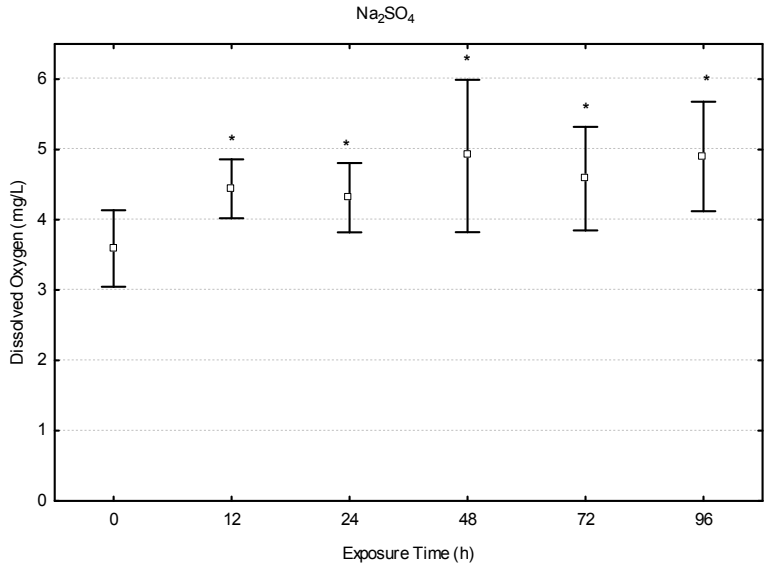


Figure 4.10 Dissolved Oxygen of all Na_2SO_4 concentrations combined over time (error bars are standard deviation, significant differences are marked with an *)

Haemolymph osmolality data were not normally distributed (W=0.91983, p=0.00) but homogenous (F=0.005060, p=0.99). Osmolality values of all concentrations were not significantly different from the control (Figure 4.11). The only values differing from the start of the experiment (0 h) were the 12 h haemolymph osmolality values (p=0.00) (Figure 4.12).

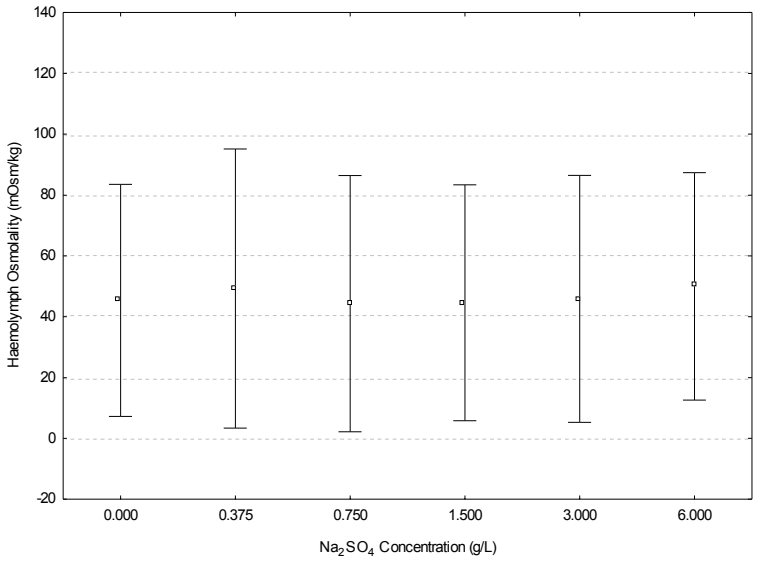


Figure 4.11 Haemolymph osmolality of all exposure times combined over all Na_2SO_4 concentrations (error bars are standard deviation, significant differences are marked with an *)

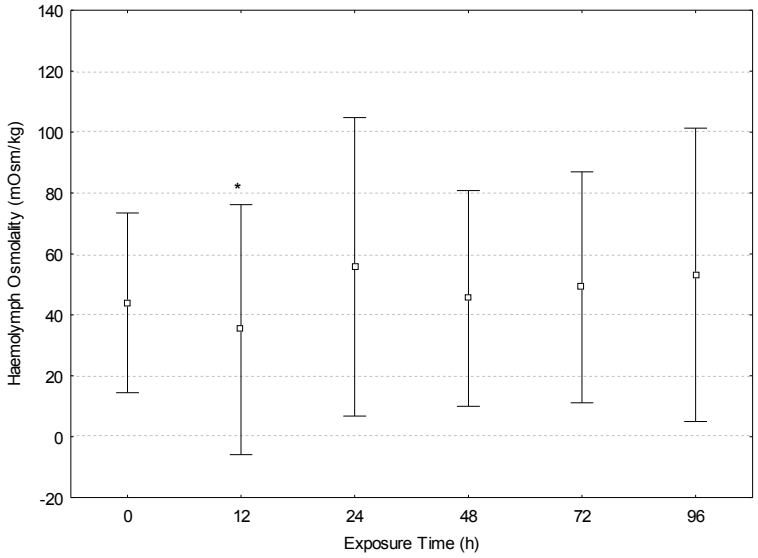


Figure 4.12 Haemolymph osmolality of all Na_2SO_4 concentrations combined over time (error bars are standard deviation, significant differences are marked with an *)

There was a significant difference between haemolymph levels at 0 h and 12 h in the control (0.0 g/L p=0.03) and the two lowest concentrations (0.375 g/L p=0.03 and 0.75 g/L p=0.00) (Figure 4.13). For the highest concentration (6 g/L) there were no 96 h data since all animals died before the end of the experiment.

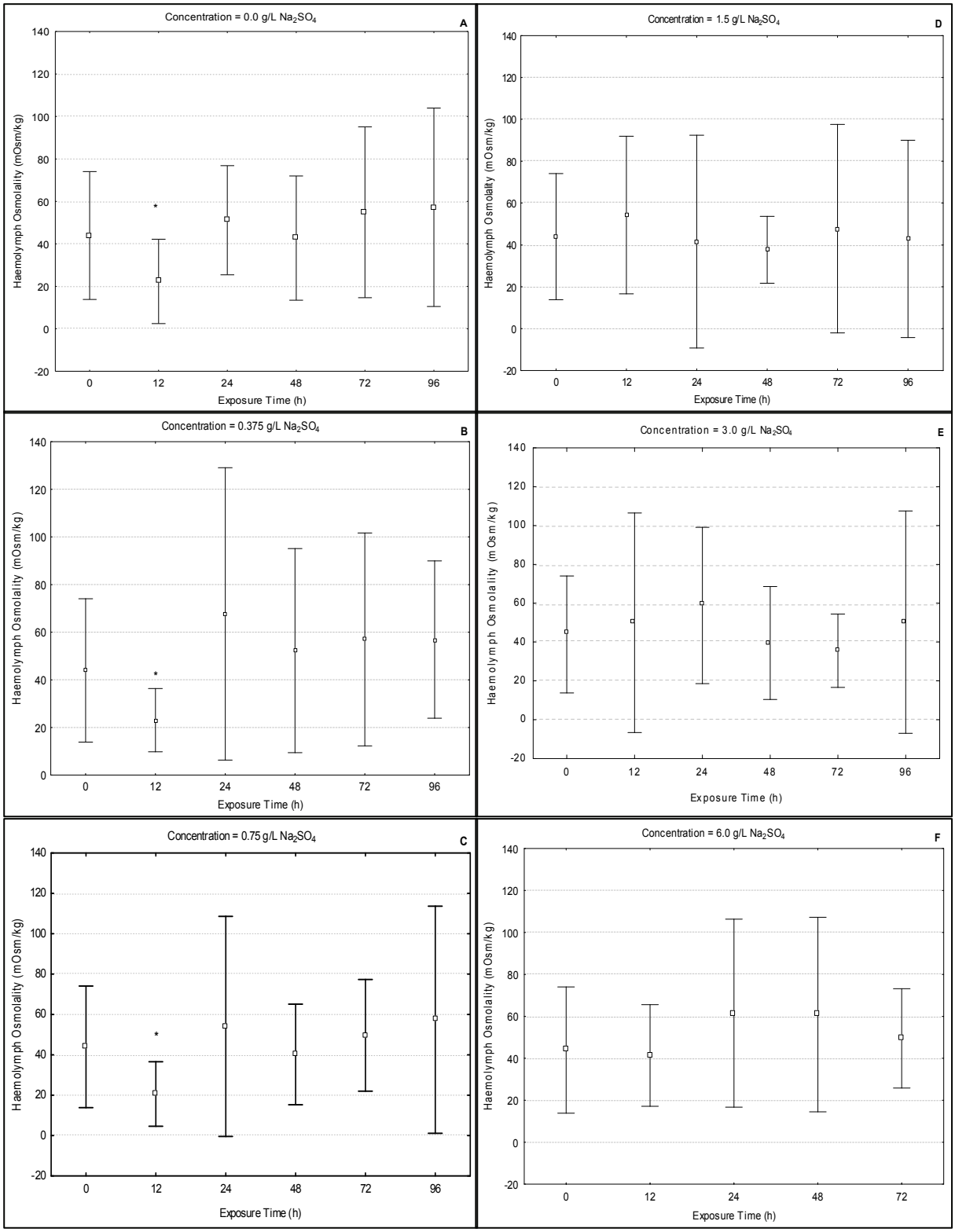


Figure 4.13 Haemolymph osmolality for each Na_2SO_4 concentration over all exposure times (error bars are standard deviation, significant differences are marked with an *)

While there was a significant difference in haemolymph levels after 12 h of exposure between the control (0.0 g/L) and 1.5 g/L (p=0.00) and 3.0 g/L (p=0.03), there are no other significant differences at later exposure times (Figure 4.14).

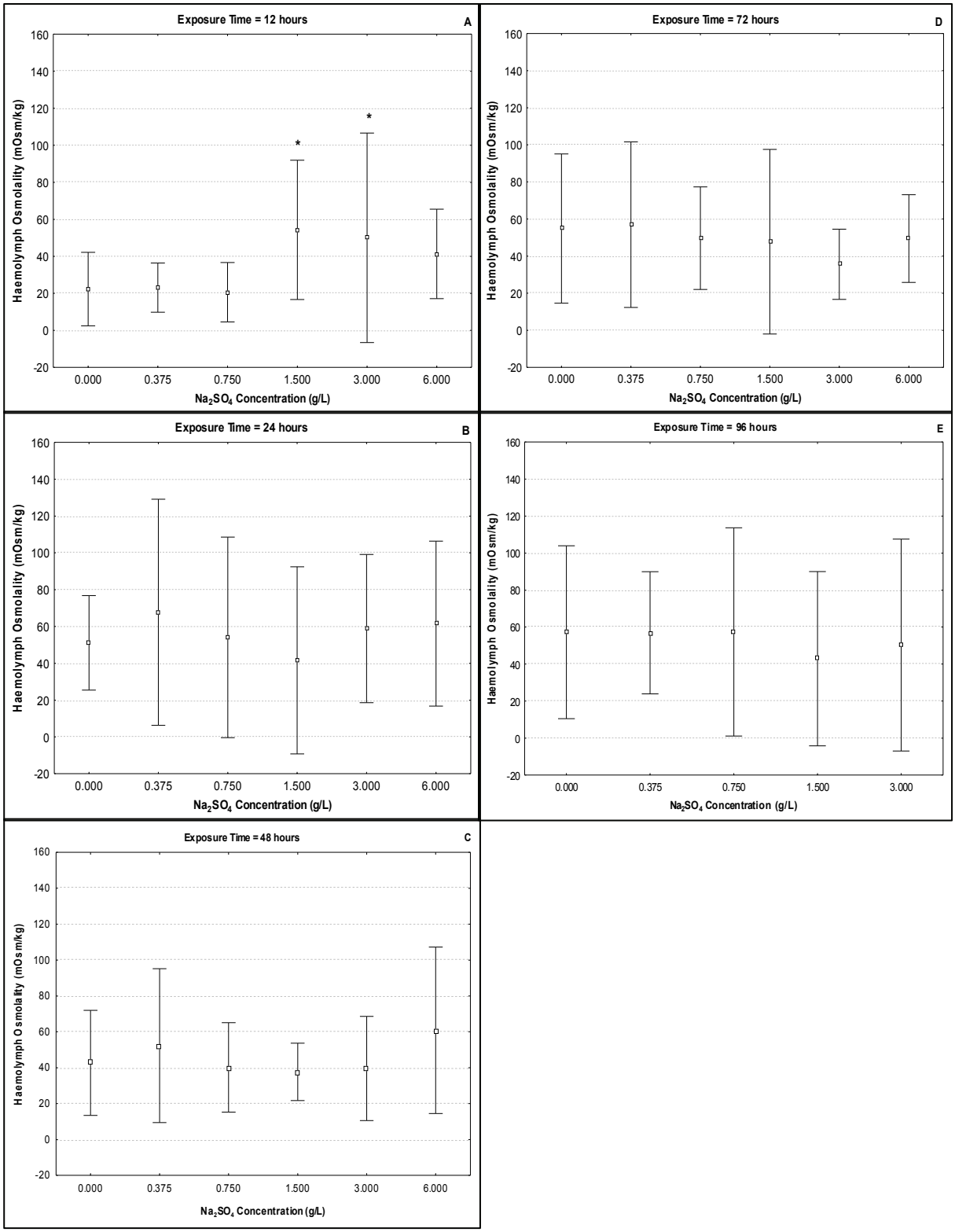


Figure 4.14 Haemolymph osmolality for each exposure time over all Na_2SO_4 concentrations (error bars are standard deviation, significant differences are marked with an *)

The haemolymph osmolality stayed very constant over different exposure concentrations (Figure 4.15). Mortality was 100% in the highest concentration (6.0 g/L) after 96 h of exposure. The linear fit line (horizontal line) rose slightly as the Na_2SO_4 concentration rose but this was not significant (p=0.28).

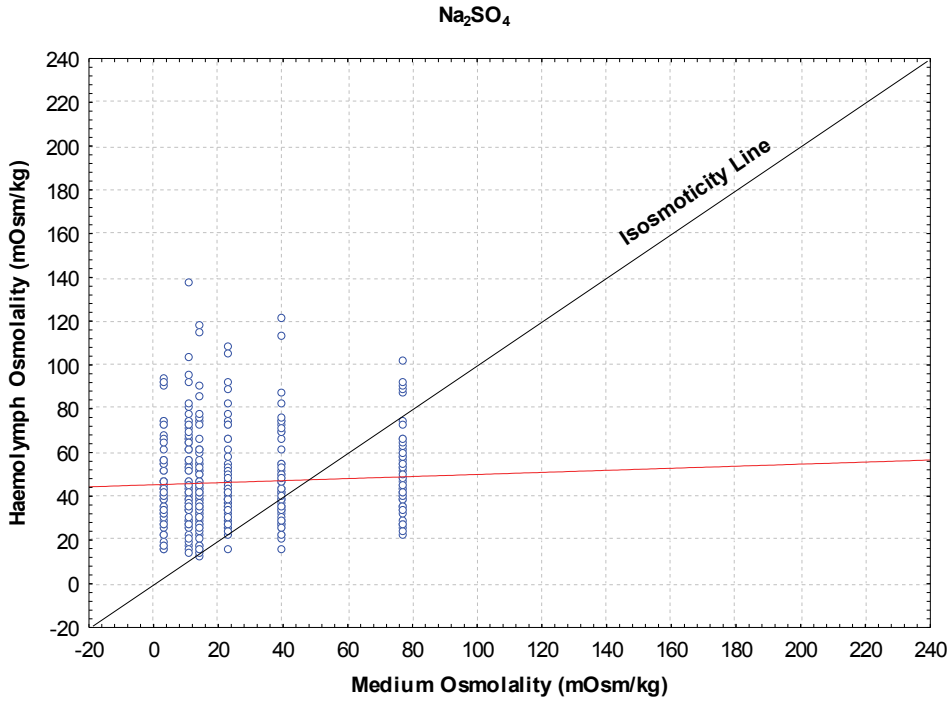


Figure 4.15 Haemolymph Osmolality *vs.* Medium Osmolality of *C. nilotica* exposed to $Na2SO_4$ (Isosmoticity Line at Medium Osmolality =Haemolymph Osmolality)

When calculating the osmoregulatory capacity (OC) of the freshwater shrimp with regards to different Na_2SO_4 concentrations, the results show that *C. nilotica* was hyper-regulating up to a Na_2SO_4 concentration of 3 g/L (39 mOsm/kg) and hyporegulating from a Na_2SO_4 concentration of 6 g/L (77 mOsm/kg) and higher (Figure 4.16). As with exposure to NaCl, *C. nilotica* in this study hyper-hypo-regulated when exposed to increasing concentrations of Na_2SO_4.

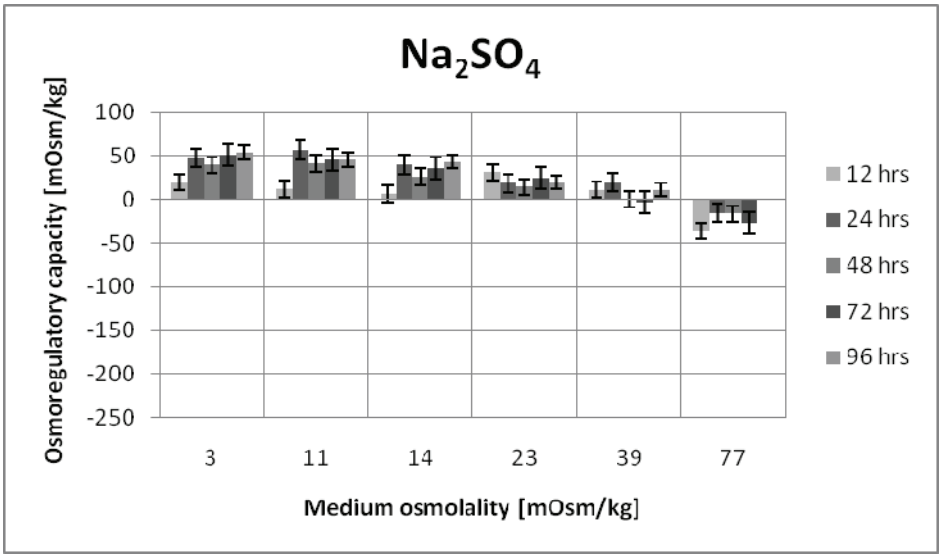


Figure 4.16 Osmoregulatory capacity (OC) at different Na_2SO_4 concentrations (medium osmolality) for different exposure times

4.4 Discussion and Conclusion

Looking at the relationship between oxygen levels in the test solutions and haemolymph osmolality in shrimp exposed to NaCl we found that at the lowest concentration (0.5 g/L NaCl) the DO was lower and the haemolymph osmolality was higher than in the control (Figure 4.1 and Figure 4.3). At this low concentration of NaCl, an increased uptake of Na^+- and Cl^-- ions seems to take place which results in a higher respiration rate and therefore a lower DO value.

DO levels in the NaCl exposure experiments decreased significantly over time (Figure 4.2) whereas DO levels in experiments with Na_2SO_4 were significantly higher over the course of the experiment compared to the control (Figure 4.10). Shrimp exposed to NaCl were very active and higher heart rates were observed compared to control organisms, whereas organisms exposed to Na_2SO_4 were less active with lower heart rates compared to control animals (personal observation). The lower DO values measured during NaCl exposure could be the result of a higher respiration rate due to increased overall activity in the test organisms. Higher DO values in the Na_2SO_4 experiment could be the result of a measurement error of the control value, which seems very low at 3.62 mg/L dissolved oxygen compared to the control value of 4.5 mg/L for the NaCl control.

When treated with Na_2SO_4, shrimp haemolymph osmolality decreased within the first 12 h of exposure in the control and the two lowest concentrations, whereas when exposed to NaCl all treatments showed decreased haemolymph osmolality between 12 and 24 h, except for 0.5 g/L where osmolality increased at 48 and 96 h. Haemolymph osmolality levels dropped when shrimp were exposed to NaCl and Na_2SO_4 during the first 24 h of the experiments, which might be due to the medium osmolality being lower than the haemolymph osmolality from field conditions. Therefore the exposure time of 12 to 24 h could be considered as acclimatisation time and thus only values determined after 96 h are discussed further.

Exposure to the two lowest NaCl concentrations (0.5 g/L and 1 g/L) resulted in a significantly higher haemolymph osmolality value at 96 h, whereas all other exposure concentrations were not different from the control. There were no significant differences in haemolymph osmolality at any exposure periods in the Na_2SO_4 exposure experiments. This might be due to the fact that Na^+- and Cl^--ions can be taken up more readily because of a smaller diameter of the ions, whereas $SO4^-$ -ions are much bigger, divalent (can form two bonds with other ions or molecules), and require more energy for diffusion (Péqueux, 1995).

Plotting shrimp haemolymph osmolality against medium osmolality shows, that the freshwater shrimp, *C. nilotica,* is a hypo-hyper-osmoregulator, since haemolymph osmolality levels remain at around the same mean when hypo- and hyper-regulating. This means that the internal ion concentration of the shrimp is higher at lower external ion concentrations (hyper) and the internal ion concentration is lower in higher external concentrations. The point at which internal and external ion concentrations are equal is called the isosmotic point. For some animals (most of the marine crustaceans for example) the ion concentration budget stays close to the isosmotic point, these are called osmoconformers as opposed to osmoregulators because they conform their internal ion concentration to that of the external medium. This means that osmoconforming organisms are confined to a more or less stable environment and would die in places of high salinity fluctuations like estuaries and ephemeral rivers (Péqueux, 1995).

According to results generated in this study (Chapter 4), there was no evidence of osmotic stress in *C. nilotica*, haemolymph osmolality levels stayed the same when exposed to different concentrations of selected inorganic salts. At 96 h, shrimp exposed to the highest concentration of Na_2SO_4 died, but there was no evidence at 72 h that the osmoregulatory capacity of these organisms was failing.

Hence osmoregulatory capacity (OC) could not be applied as an indicator for osmotic stress in *C. nilotica* exposed to the inorganic salts NaCl and Na_2SO_4.

5 ASSESSING THE USE OF PHYSIOLOGICAL RESPONSES IN MANAGING ENVIRONMENTAL WATER QUALITY

In this chapter, acute lethality data for three indigenous mayfly species (Chapter 2), sublethal physiological response data for two fish species (Chapter 3), and an indigenous shrimp (Chapter 4) are discussed in terms of their usefulness in assessing the Reserve benchmark boundary values for selected inorganic salts. These boundary values for inorganic salts were derived by Jooste and Rossouw (2002) (Table 5.1), whereby acute lethality data (LC_{50}s) from the ECOTOX database maintained by the USEPA were projected to 336 h and the 5th percentile determined as a lethality benchmark, analogous to the Fair/Poor boundary. Similarly, the 5th percentile of available sublethal data was determined as the sublethality benchmark and analogous with the Natural/Good boundary value. The Good/Fair boundary was the mean value between Natural/Good and Fair/Poor values.

In this report, Reserve boundary value results for inorganic salts are reported according to the Natural/Good/Fair/Poor classification system as detailed in Jooste and Rossouw (2002) (Table 5.1). However, current EWQ management encourages the use of the classification system A-F (DWAF, 2008). A conversion table between the two systems is available in DWAF (2008). Electrical conductivity values are also included in this report for comparative purposes (Table 5.2).

Table 5.1 Current Reserve boundary values for inorganic salts in the South African ecological Reserve (Jooste and Rossouw, 2002)

Variable	Categories and associated salt concentration		
	Natural	Good	Fair
$MgSO_4$	<16 mg/L	16-27 mg/L	27-37 mg/L
Na_2SO_4	<20 mg/L	20-36 mg/L	36-51 mg/L
NaCl	<45 mg/L	45-217 mg/L	217-389 mg/L

Table 5.2 Current electrical conductivity boundary values in the South African ecological Reserve (DWAF, 2008)

Variable	Categories and associated electrical conductivity		
	Natural	Good	Fair
Electrical conductivity	<30 mS/m	30.1 - 55.0 mS/m	55.1 – 85.0 mS/m

5.1 Evaluation of the current Reserve benchmark boundary value for $MgSO_4$ using lethality data

An evaluation of the current Reserve boundary values was undertaken by combining indigenous mayfly 96 h LC_{50} data (generated in Chapter 2) with international acute lethality data from the ECOTOX database (USEPA, 2004) and deriving protective concentration values (PCVs) according to methods outlined in Warne et al. (2005). The derivation process involved subjecting the acute data to a SSD and obtaining the 5th, 10th and 20th percentiles of the data. These percentiles are considered to be protective of 95%, 90% and 80% of the organisms used in the derivation process, i.e. the protective concentration (PC). These PCs are analogous of the Natural/Good, Good/Fair and Fair/Poor categories respectively (Table 5.3).

A comparison of the current Reserve boundary value and the PCVs determined in this study show the PCV to be more conservative at the Natural/Good boundary, but less conservative at the Good/Fair boundary and considerably so at the Fair/Poor boundary (Table 5.4). In recent assessments of the water quality component of the ecological Reserve (Scherman, 2009; Scherman, 2010), the $MgSO_4$ boundary value guidelines have been shown to be inconsistent with EC and biotic response data assessed concurrently. This suggests that the salt is either being overestimated by the analytical tool TEACHA (Tool for Ecological Aquatic Chemical Habitat Assessment) which is used to determine the inorganic salt concentrations from the available salt ions found in solution, or that the guideline boundary values may be over-protective. This situation has particularly problematic implications when only desktop analyses of water quality data for water use licenses are undertaken, as biotic response data are generally not available for comparative assessment purposes. Consequently, the PCV derivation approach should be investigated further in order to determine if it may provide more realistic boundary values for $MgSO_4$. Although it is possible to use only acute lethality data in deriving guidelines and then apply an acute to chronic ratio (ARC), further research should investigate the use of chronic/sublethal data only in the derivation of the PCVs (this may include the need to generate these data), as these data are considered to provide more reliable boundary values than the use of acute values and some ARCs.

Table 5.3 Relationship between ecological categories, protective concentrations and linear distribution percentiles as determined using methods outline by Warne et al. (2005).

Category	Level of protection (PC)	Percentile
Natural	>95	$<5^{th}$
Good	>90	$> 5^{th} < 10^{th}$
Fair	>80	$> 10^{th} < 20^{th}$
Poor	<80	$> 20^{th}$

Table 5.4 Protection concentration values (PCVs) for $MgSO_4$ calculated using three indigenous mayfly species and eight other taxa available from ECOTOX database (USEPA, 2004)

$MgSO_4$	Categories and associated salt concentration		
	Natural	Good	Fair
Current Reserve boundary value	16 mg/L	27 mg/L	37 mg/L
PCVs	7.25 mg/L	41 mg/L	230 mg/L

5.2 Evaluation of the current Reserve benchmark boundary values for NaCl and Na_2SO_4 using physiological response data

Oxygen consumption was determined as a sublethal physiological response endpoint in two species of fish exposed to the salts NaCl and Na_2SO_4 (Chapter 3). As sublethal data were used in the derivation of the Natural/Good Reserve boundary values, physiological response data such as the oxygen consumption data measured in *Danio rerio* and *Poecilia reticulata* could be used to evaluate this boundary value. For NaCl, a no observed effect concentration (NOEC) of 500 mg/L was determined for both species. When compared with the sublethal toxicity data used by Jooste and Rossouw (2002) to derive the Reserve boundary values for NaCl (Table 5.5) it is evident that the physiological response of oxygen consumption has the potential to contribute as a sensitive endpoint in the determination of a realistic but protective guideline. The types of sublethal endpoints used in the derivation of the Reserve boundary values (e.g. growth, reproduction etc) are not detailed in Jooste and Rossouw (2002) and thus it is difficult to interpret the significance of the difference in NOEC value obtained for *D. rerio* in the current study as compared to the NOEC listed in Table 5.5.

A NOEC could not be obtained for oxygen consumption as a physiological response in Na_2SO_4 exposed *D. rerio* and *P. reticulata*, although a lowest observed effect concentration (LOEC) could, allowing the calculation of a MATC (maximum allowable toxicant concentration) of 188 mg/L. The MATC (calculated by dividing the LOEC by half) is sometimes, in the absence of a NOEC, used as a sublethal endpoint in guideline derivation. When comparing this endpoint to the NOECs used by Jooste and Rossouw (2002) to derive the Reserve boundary values for Na_2SO_4 (Table 5.5), it is again evident that oxygen consumption can contribute as a sensitive endpoint in the determination of suitable guidelines.

Table 5.5 Sublethal toxicity data used in the derivation of the Natural/Good ecological Reserve boundary values for NaCl and Na_2SO_4 (Jooste and Rossouw, 2002).

NaCl		**Na_2SO_4**	
Organism	**NOEC (mg/L)**	**Organism**	**NOEC (mg/L)**
Anguilla anguilla	14 142	*Anabaena sp.*	384
Anguilla anguilla	30 000	*Cyprinidae sp.*	4 500
Astacus astacus	86	*Daphnia magna*	1 920
Baetis tricaudatus	8 000	*Gambusia affinis*	849
Ceriodaphnia dubia	704	*Myriophyllum spicatum*	2 161
Lemna minor	5 186	*Navicula seminulum*	1 900
Chlorella vulgaris	590	*Oncorhynchus mykiss*	704
Danio rerio	5 031	*Pectinatella gelatinosa*	44 904
Pectinatella gelatinosa	41 366	*Spartina alterniflora*	25
Pimephales promelas	4 000	*Spartina cynosuroides*	1 094
Stenonema modestum	5	*Tricorythus sp.*	7 340

Due to the hyper-hypo-regulatory mechanism employed by freshwater shrimp exposed in this project (Chapter 4), a negative impact on the osmoregulatory mechanism of these animals could not be determined for either salt and consequently NOECs could not be calculated. To successfully evaluate current Reserve boundary values using osmoregulation as endpoint, test organisms whose mechanisms of osmoregulation are measurably impacted by increasing concentrations of inorganic salts should be utilised. As internal haemolymph osmolality levels may vary between taxa, the use of multiple species is also recommended in order to increase confidence in derived guidelines.

5.3 Conclusions and Future Research

The lack of confidence in the $MgSO_4$ Reserve boundary value guidelines has recently led to a review of the guideline and a revision of derivation methods for salts being included as sub-tasks in a Water Research Commission (WRC) / Department of Water Affairs (DWA) proposal for further development of the water quality methods of the ecological Reserve, submitted in August 2010. Results from the current study, particularly the demonstration of the PCV derivation approach, could make a contribution to this project and should be further investigated.

Usually there are very few sublethality data available to derive the Natural/Good Reserve boundary value using the method described by Jooste and Rossouw (2002), leading to lower confidence in the resultant guideline. Although the most reliable PCVs are also derived using sublethality data, it is still possible to utilise acute lethality data in deriving PCVs and apply a default or, preferably, experimentally determined acute-to-chronic ratio. Ultimately, however, sublethal endpoints generated using indigenous aquatic organisms are necessary in order to derive realistic protective guidelines and the generation of these data should be prioritised.

Problematic issues encountered in producing and utilising sublethality endpoints at sub-organism levels in water quality management, such as osmoregulatory capacity, are well documented (Clark et al. 1999; Tannenbaum 2005; Forbes et al. 2006). Issues raised are: the inherent variability of the endpoints measured (mainly related to the assay protocol and the differences in tolerances at low levels of organisation among exposed individuals); complicated time- or dose-dependent responses are frequently measured, but are difficult to explain and to derive endpoints such as NOECs or EC_{50}s from; confounding nonchemical influences such as temperature, nutritional state, reproductive state and lifecycle stage often impact results and; there are unclear or undetermined links between sub-organism endpoints and the fitness of the individual, and especially, fitness of the population and community. These issues need to be considered when undertaking sublethal toxicity tests, and applying these data to guideline derivation.

Lastly, the EWQ management approach to salinity should reconsider the use of electrical conductivity as an additional tool, particularly in combination with biological response data. The process to determine individual salt concentrations (TEACHA) is complex, not well understood and requires salt ion data that is often not available. In addition, the accuracy of the Reserve boundary values for some salts have been questioned (Scherman, 2009; Scherman, 2010). Electrical conductivity, however, is easy to measure and the data are readily available in most cases. Further research should be conducted to determine advantages and limitations of using electrical conductivity data, either alone or in combination with biological data, in EWQ management practices.

6 CAPACITY BUILDING

This project was utilised as an opportunity to develop scientific thinking, experimentation and writing skills in a number of students and early career water scientists based within the Institute for Water Research at Rhodes University. Much of the experimental work was undertaken by undergraduate students, supported by the incumbent IWR research intern, and overseen by the project manager Dr Muller.

6.1 Undergraduate

This project funded a 3rd year project for **Mr Guy Williams** in Zoology who generated the data for Chapter 2 of this report.

6.2 Postgraduate

This project funded the Honours project of **Mr Greg Tutt** who generated the data and contributed substantially to Chapter 3 of this report.

6.3 Staff Development

Three research interns worked in turn on this project whilst undertaking their MSc's/PhDs. This project offered them training in research and scientific writing and broadened their aquatic scientific expertise:

Ms Nosiphiwo Ketse – previously disadvantaged (MSc student and research Intern until 2006)
Mr Andrew Slaughter (PhD student and research Intern until 2008)
Ms Alexandra Holland (PhD student and research Intern since 2008)

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