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Influence of calcium proportion on the toxicity of a saline solution to Ephemeroptera, *Austrophlebioides* sp. AV11

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Abstract

Anthropogenic activities such as coal mining, oil and gas production, application of salts on roads for de-icing and agricultural practices can result in saline discharges to aquatic ecosystems. Salts are components of all natural waters. Salinity is a measure of inorganic ions or salts. It is mainly comprised of major cations (calcium–Ca, potassium–K, magnesium–Mg, sodium–Na) and major anions (chloride–Cl, sulfate–SO_4, bicarbonate–HCO_3). The ionic composition of mine waters can have varying proportions of major cations and anions. Saline discharges to freshwater ecosystems can increase salinity as well as change the ionic composition. Major ions are also essential for the normal functioning of the organisms. Change in salinity or ionic composition of the surrounding water can have a detrimental effect on organisms. While increased calcium concentration is presumed to decrease the toxicity of saline solutions, the effect of calcium proportion of a mine water ionic composition is not well understood. The aim of this study was to investigate the influence of calcium proportion on the toxicity of a saline solution to Ephemeroptera, *Austrophlebioides* sp. AV11. The hypothesis tested was that increased calcium proportion decreases the toxicity of a saline solution to *Austrophlebioides* sp. AV11. The effect of calcium proportion was tested in presence of all the major cations, potassium, magnesium and sodium and the major anions, chloride, sulfate and bicarbonate.

The aim of the study was evaluated using static non – renewal 96 h acute toxicity tests. The saline solution used in the present study was based on the ionic composition of mine water from the study of Prasad et al. (2012) and named as artificial mine water (AMW). The term calcium proportion used here refers to the proportion of calcium in relation to all the major ions measured in milliequivalents (% meq). Solutions with increased calcium proportion were prepared using calcium chloride and calcium sulfate salts together. Calcium proportion was increased above that of AMW two fold (Ca-Cl.SO_4(2)), four fold (Ca-Cl.SO_4(4)) and eight fold (Ca-Cl.SO_4(8)). Additional tests were conducting using calcium chloride and calcium sulfate salts separately to assess the effect of calcium in isolation of the associated anions. Calcium proportion was increased as two fold and four fold designated Ca-Cl(2), Ca-Cl(4) and Ca-SO_4(2) and Ca-SO_4(4).

For the experiments with calcium chloride and calcium sulfate together, there was a statistically significant reduction in toxicity for Ca-Cl.SO_4(2) and Ca-Cl.SO_4(4) solutions compared with AMW, but no difference between Ca-Cl.SO_4(8) and AMW. The 96 h LC50 values were: AMW – 6.0 mS/cm, Ca-Cl.SO_4(2) – 6.9 mS/cm, Ca-Cl.SO_4(4) – 8.1 mS/cm and Ca-Cl.SO_4(8) – 6.2 mS/cm. These results show that both two fold and four fold increase in calcium reduced toxicity by 15 % and 35 % respectively but that an eight fold increase in calcium did not affect toxicity.

The finding of this thesis suggests that calcium concentration beyond 7.5 mM (300 mg/L) has no ameliorative effect or may contribute to increased toxicity to organisms. The observed toxicity associated for solutions Ca-Cl.SO_4(2) and Ca-Cl.SO_4(4) and Ca-Cl.SO_4(8) could be due to the influence of calcium on the permeability of ions and water across the cell membranes. The concentration of calcium has been found to influence the sodium influx in aquatic organisms. Increase in calcium can alter the Ca:Na ratio, which has been found to influence the physiological mechanism.

For experiments which used calcium chloride or calcium sulfate separately, the chloride solutions were more toxic than AMW, while the sulfate solutions showed similar toxicity to the AMW. The 96 h LC50 values were: AMW – 6.0 mS/cm, Ca-Cl(2) – 4.5 mS/cm and Ca-Cl(4) – 5.4 mS/cm, Ca-SO_4(2) – 5.2 mS/cm and Ca-SO_4(4) – 6.1 mS/cm. These solutions did not show a similar effect as observed for solutions with increased calcium proportion using calcium chloride and calcium sulfate together. This can be due to the change in anion ratio that could have interfered in ion exchange mechanisms across cell membranes. It also suggests solutions with both calcium chloride and sulfate together probably had more balanced anions and were less toxic.

The findings of this study will assist in evaluating the toxicity of waters with ionic compositions with a similar relative proportion of calcium. The results suggest that a potential ameliorative effect of calcium should be considered when assessing potential impacts of saline discharges and developing discharge criteria. While this thesis provides the effect of calcium proportion on a complex ionic composition, further research on the effect of Ca:Na ratio and the ratios of other ions on toxicity is needed. This thesis focussed on an Ephemeroptera species; however, the effect of calcium proportion can vary for laboratory cultured organisms that are widely used to evaluate effluent toxicity. The potential for variation in the observed toxicity between laboratory and field collected species needs further investigation.

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Contributions by others to the thesis

Dr. Sue Vink has made a significant contribution in conception and design of the project, critically revising the writing that has assisted substantially in the interpretation of the data.
Dr. Jason Dunlop has significantly contributed in design of experiments, critically revising and assisting in editing the document.
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Keywords

Salinity, ionic composition, calcium, Leptophlebiidae

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List of Abbreviations

EC Electrical conductivity

Electrical conductivity is the measure of conductance of electric current within a solution

IC50 Inhibition concentration 50

An estimated toxicant concentration, that can cause 50 % inhibition or impairment in a qualitative biological function. For eg., 50 % reduction in growth of larval fish relative to the control.

LC10 Lethal concentration 10

An estimated toxicant concentration that can cause death to 10 % of a test population

ECx Effective concentration x

An estimated toxicant concentration that can cause a specified effect (eg., change in respiration rate) in x % of test population

EC50 Effective concentration 50

An estimated toxicant concentration that can cause a specified effect (eg., change in respiration rate) in 50 % of the test population

Chapter 1 Introduction

Anthropogenic activities have resulted in increased salinity of freshwater streams and have become a growing concern globally (Kefford et al. 2003; Mount et al. 1997). Activities such as coal mining (Kennedy et al. 2003), agricultural practices (Hart et al. 1990), de-icing (Evans & Frick 2001) and petroleum oil field industries produce saline waters (Boelter et al. 1992). Freshwater is used in coal mines in preparation of coal, to wash machinery and suppress dust (Gozzard et al. 2009). The used water is stored in dams for reuse to save water. However, this reused water can be high in salts (Gozzard et al. 2009). Agricultural irrigation in some cases cause water tables to rise to the soil surface, and this irrigation water can be high in dissolved salts (Environment Australia 2012; Hart et al. 1990; Hart et al. 1991). Salts are used in temperate regions to de-ice snow from roads for safety during heavy snowfall (Blasius & Merritt 2002; Evans & Frick 2001). When snow melts, the run-off subsequently reaches freshwater streams (Crowther & Hynes 1977). In petroleum oil field industries, saline water is produced during the process of oil production. In some instances, saline water produced due to these various anthropogenic activities can be released to freshwater streams. Consequently, these saline discharges increase salinity and also can change the ionic composition of the freshwater streams (Goodfellow et al. 2000; Hart 2008; Kennedy et al. 2003; Lincoln-Smith 2010; QFCI 2012). Salts are essential components for normal functions in organisms (Kefford et al. 2002). However, a change in salinity or ionic composition in the surrounding media can have detrimental effects on the organisms (Hart et al. 1990; Hart et al. 1991; Lincoln-Smith 2010).

Salinity is one of the attributes of all natural waters. It is the measure of total salts dissolved in water (ANZECC 2000; USEPA 2006). Salts include the major cations calcium (Ca), potassium (K), sodium (Na) and magnesium (Mg) and major anions, chloride (Cl), sulfate (SO_4) and bicarbonate (HCO_3) (ANZECC 2000; Soucek et al. 2011). Salinity is measured as electrical conductivity (EC) or total dissolved solids (TDS) (Goodfellow et al. 2000). Electrical conductivity is the measure of conductance of electric current within a solution (USEPA 2006). The units for salinity can be represented as parts per thousand (‰, per mille) (USEPA 2006), total dissolved solids as milligram per litre (mg/L) and electrical conductivity as milliSiemens per centimetre (mS/cm).

Salinity can be considered as a stressor (ANZECC 2000) or as a toxicant (Kefford et al. 2002). A stressor is defined as ‘any physical, chemical or biological entity that can induce an adverse effect’ (USEPA 2012). While, a toxicant is defined as, ‘an agent that can produce an adverse response (effect) in a biological system, seriously damaging its structure or function or producing death’

(Rand 1995). Although salinity has been considered as a stressor, in high concentrations it has been found to cause death to organisms (Kefford et al. 2002). Saline discharges can be released to an ecosystem and require management of the discharge to protect the ecosystem. Whilst a debate surrounds whether to consider salinity as a stressor or toxicant (Goodfellow et al. 2000), the terminology influences the approaches used to manage salinity in natural waters.

In Australia, the ANZECC (2000) water quality guidelines provide a framework to manage salinity for the protection of ecosystems. These guidelines provide numerical values (commonly referred as trigger values), which when exceeded in the ambient or the receiving waters require management actions. When salinity is considered as a stressor, exceedance of a trigger value could indicate a potential risk to the environment and would require monitoring of salinity in the ambient water. If there is exceedance of the trigger value, the trigger value may be refined for site specific conditions and also evaluate the effect of the discharge. In contrast, when salinity is considered as a toxicant, exceedance of trigger value indicates likely impact on the environment and thus requires management of the toxicant. The ANZECC (2000) guidelines describe a hierarchy for the derivation of trigger values, with the most preferred being local or site specific biological effect data followed by local reference data, while the least preferred is regional reference data. Biological effect data refers to the concentration of a chemical that can cause an adverse effect on an organism. Local reference data refers to the water quality data of a site that has been recorded over a period of time. Regional reference data refers to the data that have been recorded from the reference sites of the region. In the absence of local or site specific information, the default guideline as specified in ANZECC (2000) guidelines is implemented. The present guideline values for salinity are based on local reference data. However, the ANZECC (2000) guidelines indicate a preference for locally relevant biological effect data obtained from ecotoxicity tests. Ecotoxicity tests are preferable because they demonstrate a cause and effect relationship between the concentration of the chemical and response of the organisms. This provides a clear threshold value that can be utilised to derive a guideline value.

Although increased salinity can have a toxic effect on freshwater organisms (Hart et al. 1991), different ions present in a solution have also been found to influence the toxicity (Mount et al. 1997; van Dam et al. 2010). While the presence of ions such as potassium (Freitas & Rocha 2011; Mount et al. 1997) and chloride (Iowa DNR 2009) have been found to increase the toxicity of a saline solution, increased hardness has been reported to decrease the toxicity of saline solutions to freshwater organisms (Davies & Hall 2007; Soucek 2007; Soucek et al. 2011). Water hardness is a

measure of primarily calcium and magnesium concentrations (APHA 2005). Though hardness has been reported to decrease the toxicity of saline solutions to freshwater organisms, it is often reported that increased calcium concentration decreases toxicity.

Dunlop et al. (2011) and Prasad et al. (2014) have also reported similar findings. These two studies evaluated the toxicity of various saline solutions on Ephemeroptera, commonly known as mayflies. In these studies, saline solutions were based on ionic compositions of streams (Dunlop et al. 2011) and dam waters of mine sites (Prasad et al. 2014) of the Fitzroy catchment, Queensland, Australia. In the second study, two different mine water compositions were tested and it was found that one mine water was more toxic than the other. The difference in toxicity between the two solutions was attributed to the difference in calcium proportion of the solutions (Prasad et al. 2014).

The toxicity data from the above mentioned studies suggested that the difference in toxicity was due to the difference in the ionic compositions of the solutions tested. To understand the relationship between toxicity and the proportion of calcium in different solutions, the toxicity data was plotted against calcium proportion. The toxicities of solutions did not correspond to the proportion of calcium as expected. Two solutions presented different toxicity but both had similar calcium proportion. In contrast, two solutions presented similar toxicity but different calcium proportion. While it was postulated calcium proportion can have an ameliorative effect as observed for calcium concentration, it was not evident in the data of the above mentioned studies. This was a key knowledge gap to investigate whether increased calcium proportion would decrease toxicity to Ephemeroptera.

In this thesis, chapter 2 includes a review of literature and describes the aim and objectives of this study. Chapter 3 includes a detailed description of 96 h acute toxicity tests and statistical analyses used to evaluate the results. Chapter 4 describes the results of acute toxicity tests and a comparison of the effect of solutions with increased calcium proportions. Chapter 5 discusses the results of the toxicity tests and the conclusion of this study as well as questions for future research.

Chapter 2 Literature Review

This chapter begins with an outline of methods and approaches for conducting toxicity tests because the results and interpretation of such tests underpin much of the literature discussed subsequently. The toxicity of salinity, the ions that contribute to salinity and various ionic compositions that determine different levels of toxicity to freshwater organisms are discussed. The influence of hardness and calcium concentration on the toxicity of saline solutions are explored, along with the effect of salinity on osmoregulation within organisms. Finally, attributes of Ephemeroptera (Insecta) as test organisms are discussed. The chapter concludes with the aim and objectives of the present study.

2.1 Toxicity tests

Toxicity testing can be used to define effect based thresholds which provide a basis for the management of salinity. Toxicity tests are conducted in a laboratory and can determine cause and effect relationships between the concentration of the toxicant and the effect on test organisms. This provides a numerical threshold that can be used in the derivation of a guideline value for salinity.

Toxicity tests are conducted in a laboratory under temperature and light controlled conditions. Test organisms are introduced into test chambers comprised of different concentrations of the toxicant of interest (Rand 1995). The responses of organisms are observed over a predefined duration of test. During the period of the test, organisms are not fed as per the standard approaches for undertaking static non-renewal tests. Organisms are usually fed for tests with longer duration such as chronic toxicity tests or where the organisms tested are known to have cannibalistic behaviour. For tests where organisms are fed, the debris of food and faecal matter has to be cleaned regularly to avoid discrepancies with dissolved oxygen and increased concentrations of ammonia (ASTM E729-96 2007; USEPA 2002). The effect or end point measured is a biological response such as mortality, immobilisation, inhibition of growth rate, reproduction and/or feeding. Responses are compared to a control solution which does not include the toxicant. Toxicity tests are classified as acute toxicity or chronic toxicity tests based on the duration of the test and the end point (APHA 2005; Rand 1995).

Acute toxicity tests are typically of a short duration. The standard test duration for fish and macroinvertebrates is 96 h, and for organisms with shorter life spans, such as daphnids, it is 48 h. This standard exposure time of 96 h or less was found to ‘cover the period of acute lethal action’ (Rand 1995). The most common response assessed in acute toxicity testing is mortality because it

represents a clear and certain response (APHA 2005; Rand 1995). When mortality is not easily determined, effects such as immobilisation (e.g., daphnids and midge larvae) and growth (e.g., algae) are considered as suitable end points (Rand 1995). Chronic toxicity tests are long term tests that include a partial life cycle test or complete life cycle test, usually '10 % life span of the organism' (APHA 2005). Chronic toxicity tests in addition to mortality consider effects on different life stages of organisms such as growth rate, reproduction, development of sex products, maturation as end points. Various concentrations of toxicant used in the tests are plotted on x–axis and responses as percentage effect such as mortality are plotted on the y–axis. This yields a typical sigmoidal curve (Figure 2.1).

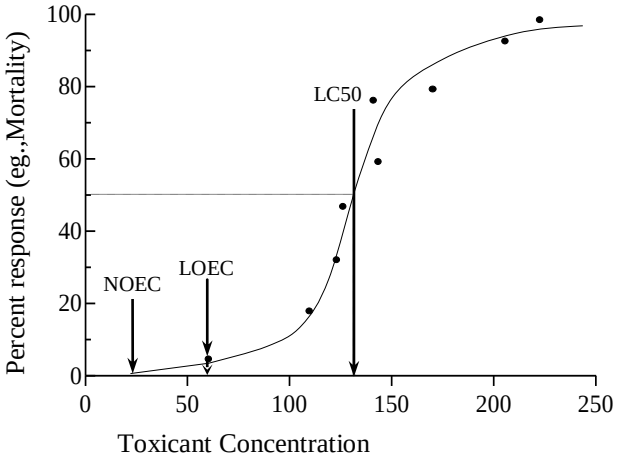

Figure 2.1: A typical dose response curve showing different points on the curve that represent different values that are labelled as no observed effective concentration (NOEC), lowest observed effective concentration (LOEC), lethal concentration 50 (LC50).

Usually the dose response curve is elicited using statistical packages which provide point estimates and hypothesis testing. Point estimates provide effect concentrations for corresponding percent response (e.g., LC1 to LC99). Hypothesis testing is facilitated using 'No Observed Effective Concentration' (NOEC) and 'Lowest Observed Effective Concentration' (LOEC) indices that are determined using statistical tests such as Dunnett's test, Bonferroni's test, Many-One Rank test or Wilcoxon rank-sum test. Definitions for NOEC, LOEC and LC50 are presented in Table 2.1.

Table 2.1: Definition of No observed effective concentration (NOEC), Lowest observed effective concentration (LOEC), Lethal concentration 50 (LC50).

Acronym	Full form	Definition
NOEC	No Observed Effective Concentration	Highest toxicant concentration in which the values of the measured response are not statistically significantly different from those in the control
LOEC	Lowest Observed Effective Concentration	Lowest toxicant concentration in which the values of the measured response are statistically significantly different from those in the control
LC50	Lethal Concentration 50	Estimated toxicant concentration that produces 50 percent of mortality in the test population

Effective concentration (e.g., EC50) is used where the endpoint is other than mortality. Although any LCx or ECx values can be used to determine the toxicity of a chemical, LC50 is the most commonly used because it provides the greatest degree of accuracy and certainty. NOEC and LOEC are commonly elicited in chronic toxicity tests in addition to lethal concentrations (APHA 2005; Rand 1995). Warne and van Dam (2008) insist that it is not appropriate to use LOEC and NOEC values. A major limitation with the use of LOEC and NOEC values is that they are limited to the concentrations of the toxicant used in the experimental designs, yet the effect observed in a toxicity test can be beyond the concentrations used in the experiments. For this reason, the EC5 or EC10 are recommended where the effect of toxicity can be extrapolated beyond the concentrations of the toxicant used in the experiments. However, ANZECC (2000) guidelines reports LOEC and NOEC but recommend the use of EC5 and EC10. However, in this thesis, 96 h LC50 is reported as it provides a suitable endpoint value to understand the toxicity of the saline solution with various calcium proportions on Ephemeroptera.

Acute toxicity tests provide rapid and reproducible dose response curves that help to identify and estimate the effect of a given chemical (Macek et al. 1978). Static acute toxicity tests are tests in which the same test solution is used throughout the duration of the test and is never changed (USEPA 2002). Static acute toxicity tests provide an initial means to derive estimates of the upper limit of a range of concentrations producing a toxic effect. Such approaches are used to evaluate the relative toxicity of chemicals and for the preliminary understanding of the nature of dose response relationship (Macek et al. 1978). Results of acute toxicity tests assist in the establishment of dilution levels of chemicals or effluents prior to discharge into water bodies (Chapman 1995). The LC50 and the LC10 values derived from toxicity tests provide an indication of the concentration of the chemical that can cause a harmful effect on organisms for a defined percentage of organisms. These values can be used as a basis to derive ecosystem protection trigger values.

2.2 Toxicity of salinity

Increased salinity of freshwater affects all organisms (Hart et al. 1991). Changes in salinity can cause a direct toxic effect to aquatic organisms and can result in the loss of sensitive species (James et al. 2003). This can modify community structure and biodiversity of an ecosystem (Nielsen et al. 2003). To restore the ecosystem from harmful effects, management of saline discharges is necessary. While assessing the abundance and richness of taxa indicates the effect of salinity on aquatic organisms, it is necessary to define a numerical value to manage salinity. Laboratory toxicity tests can provide a numerical value. Many laboratory studies have found saline solutions

can cause death above certain thresholds (Bacher & Garnham 1992; Goetsch & Palmer 1997; Kefford et al. 2002). For instance, Bacher & Garnham (1992) conducted acute toxicity tests on the cladoceran, *Daphnia carinata* and the shrimp, *Paratya australiensis* with marine salt (raw sea salt). The 48 h EC50 for *D. carinata* was 2.5 g/L and 96 h LC50 for *P. australiensis* was 4.5 g/L. This gives a clear indication of the threshold value for a species tested.

2.2.1 Effect of ionic composition on toxicity of solutions

While increased salinity measured as EC or TDS can cause toxicity to freshwater organisms, salinity is a combination of different ions. The variation in the combination of these ions can influence the toxicity (Goetsch & Palmer 1997; Kefford 2000). Goetsch & Palmer (1997) reported differences in toxicity to the mayfly *Tricorythus* sp. between two saline solutions with similar EC. The study evaluated the effect of sodium sulfate and sodium chloride salts at a similar range of conductivity levels. The toxicity of sodium sulfate was greater than sodium chloride with a 96 h LC50 of 2 mS/cm for sodium sulfate and 96 h LC50 of 4–8 mS/cm for sodium chloride. Similarly, Kefford (2000) reported a difference in toxicity to *D. carinata* between two saline solutions. In that study, the two solutions tested were raw lake water and simulated lake water prepared using marine salt (Ocean Nature® salt). Toxicity tests were carried out with a dilution series of these two solutions within similar EC range. Test results showed there were differences in toxicity between raw lake water (48 h LC50 of 8.5 mS/cm) and simulated lake water (48 h LC50 12.3 mS/cm). Another study by Mount et al. (1997) tested 2900 saline solutions made with salts of sodium, potassium, calcium and magnesium with their associated anions of bicarbonate, chloride and sulfate on *Ceriodaphnia dubia*, *Daphna magna* and *Pimephales promelas.* Test solutions included both single salt and combinations of two salts. The study reported 24 h and 48 h LC50 values for *C. dubia* and *D. magna* and 24 h, 48 h and 96 h LC50 values for *P. promelas*. Evaluation of the effect of each ion suggested that potassium was the most toxic ion for all the three species. Magnesium was the second most toxic ion for *P. promelas* whereas bicarbonate was the second most toxic ion for *D. magna* and *C. dubia*. There was no observable difference in toxicity for chloride and sulfate among the three species. The toxicity of a given ion in a single salt toxicity test decreased in the presence of another salt in the double salt toxicity tests. For example, potassium was found to be the most toxic ion in single salt tests. However, the presence of sodium chloride in addition to potassium chloride decreased the 48 h LC50 of potassium from of 330 to 460 mg/L for *C.dubia*. Overall this study suggested that sodium and calcium ions did not contribute significantly to toxicity; instead the toxicity of these ions was attributed to the associated anions. The relative toxicity of various ions was $K > HCO_3 \approx Mg > Cl > SO_4$. In a similar fashion, Freitas & Rocha

(2011) also reported that potassium was the toxic ion when compared with sodium for the cladoceran, *Pseudosida ramosa* with an observed 48 h LC50 of 560 mg/L for sodium and 18 mg/L for potassium.

A difference in toxicity has been reported for complex saline mixtures. Lincoln Smith et al. (2010) reported differences in toxicity of three different mine waters and marine salt (Ocean Nature® Salt) to *Atalophebia* sp. (Ephemeroptera). The 96 h LC50 responses were: mine water 1– > 1.7 mS/cm (ca.), mine water 2– > 2.6 mS/cm, mine water 3– > 2.3 mS/cm (ca.) and marine salt– > 5.9 mS/cm (ca.). Thus, mine water 1 was the most toxic solution tested and marine salt was the least toxic. Differences in the toxicity of the solutions were attributed to variation in the magnesium concentration in solutions. Likewise, magnesium has also been found to increase toxicity of salinity for a pulmonate snail (van Dam et al. 2010). For instance, van Dam et al. (2010) evaluated the toxicity of magnesium sulfate and sodium sulfate to the pulmonate snail, *Amerianna cumingi*. The 96 h IC50 (inhibition concentration 50) was 64 mg/L for magnesium sulfate whereas the 96 h IC50 was > 500 mg/L for sodium sulfate. This suggested that the ion contributing most to toxicity was magnesium. This is similar to the finding of Mount et al. (1997) described above in which magnesium was the second-most toxic ion after potassium for *P. promelas*. A solution that contains a high concentration of chloride can be more toxic than a solution that contains a high concentration of sulfate to some invertebrates (Iowa DNR 2009). This difference in toxicity of solutions due to the presence of ions that can increase toxicity has led to the creation of discharge criteria for chloride concentration in USA (Iowa DNR 2009) and Canada (Environment Canada 2011) and sulfate concentration in USA (IPCB 2015). The above examples illustrate that while increased salinity can cause toxicity, the type of ions present in solutions can contribute to increased toxicity of solutions. Although high concentrations of some ions can increase toxicity of solutions, water hardness has been found to decrease the toxicity of saline solutions to some organisms (Davies & Hall 2007; Soucek 2007; Soucek et al. 2011).

2.2.2 Effect of hardness on toxicity of solutions

Hardness generally represents the concentrations of calcium and magnesium ions in water. There are two types of hardness observed based on the anion associated with calcium and magnesium, namely temporary hardness and permanent hardness. Temporary hardness is caused by bicarbonates of calcium and magnesium. It can be removed by boiling the water. On boiling the water, the bicarbonates precipitate as insoluble carbonates as the water evaporates. The other type of hardness is the permanent hardness which is due to the presence of chloride and sulphates associated with

calcium and magnesium ions. These salts are not precipitated by boiling the water (Williams 2001). Hardness is expressed as calcium carbonate in milligrams per litre. Hardness can influence the toxicity of chemicals to aquatic organisms.

A study by Soucek (2007) showed that increased hardness was associated with decreased toxicity of sulfate on an amphipod, *Hyalella azteca*. This study tested a hardness range of < 100 to 600 mg $CaCO_3$/L with a Ca:Mg ratio of 1.41:1 molar ratio. Test solutions also included chloride at a constant concentration of 25 mg/L. Results revealed that an increase in hardness from 100 to 500 mg $CaCO_3$/L decreased the toxicity of sulfate from 96 h LC50 of < 1900 to > 4000 mg/L. However, the LC50 value at a hardness of 600 mg $CaCO_3$/L was less than that for a hardness of 500 mg $CaCO_3$/L. Similarly, Soucek et al. (2011) found that an increase in hardness decreased acute toxicity of chloride to *C. dubia*. An increase in hardness between the ranges of 25 to 800 mg $CaCO_3$/L decreased the toxicity of chloride from 48 h LC50 of 980 to 1840 mg/L. A similar finding was also reported in the study of Davies & Hall (2007) on the toxicity of sulfate to *H. azteca*. This study reported that an increase in hardness from 25 to 250 mg $CaCO_3$/L decreased toxicity of sulfate from a 96 h LC50 of 570 to 5260 mg/L. These examples suggest hardness decreased toxicity of saline solutions to the organisms. However, hardness is a combination of calcium and magnesium ions. Often magnesium is found to cause toxicity and hence it is possible calcium has an ameliorative effect on toxicity.

2.2.3 Influence of calcium concentration on toxicity of solutions

Previous research has demonstrated that increasing the ratio of Ca:Mg can decrease the toxicity of saline solutions. Davies & Hall (2007) reported that an increase in Ca:Mg molar ratio decreased the toxicity of sulfate to *H. azteca* and *D. magna*. Similarly, Dwyer et al. (1992) reported the influence of Ca:Mg ratio on the toxicity of trace elements and a saline mixture to *Morone saxatilis* (striped bass) and *D. magna*. The study included reconstituted water based on the ionic composition of the study site and commercial marine salt (Instant Ocean® salt). Tests were conducted by diluting the stock reconstituted water and the marine salt solution between the ranges of 34–100 %. The reconstituted water was tested at three levels of hardness between the ranges of 870 to 4200 mg $CaCO_3$/L. The commercial marine salt solution had a hardness of 4200 mg $CaCO_3$/L. These solutions were tested with and without the addition of trace metals. Results showed that toxicity for solutions with or without the trace metals did not differ to *D. magna*; but the toxicity differed for *M. saxatilis* (Table 2.2).

Table 2.2: Influence of Ca:Mg molar ratio on acute toxicity of sulfate to *Daphnia magna, Hyallela azteca* and reconstituted ocean water to *Morone saxatilis.*

Species	Test duration	Hardness (mg $CaCO_3$/L)	Ca:Mg (molar ratio)	LC50		Reference
Daphnia magna	48 h	25	0.7	1190	mg SO_4/L	Davies et al. (2007)
			7.0	1980		
		100	0.7	3200		
			7.0	4390		
Hyallela azteca	96 h	100	0.7	2100		
			7.0	2720		
Morone saxatilis	96 h	4200	0.03[a]	49 – 100	% ROW_t[b]	Dwyer et al. (1992)
			0.22[a]	>100		

[a]calculated ratios from the original reference

[b]ROW_t reconstituted ocean water with trace metals

These data suggest an increased calcium concentration as Ca:Mg molar ratio from 0.7 to 7.0 decreased the toxicity of sulfate to *D. magna* at a constant hardness of 25 mg $CaCO_3$/L and 100 mg $CaCO_3$/L. Increased calcium concentration also decreased the toxicity of sulfate to *H. azteca*. Similarly, Dwyer et al. (1992) reported increased calcium concentrations decreased toxicity of ROW_t (reconstituted ocean water with trace metals) to *M. saxatilis*. Results of the toxicity tests were expressed as percentage dilution of the test waters. Although hardness of the reconstituted water of highest hardness and the marine salt were similar, it was observed that the calcium concentrations in these two solutions were different and that could have contributed to the difference in toxicity.

Another study by van Dam et al. (2010) also found that increased calcium concentration as Ca:Mg molar ratio decreased the toxicity of magnesium sulfate to *Lemna aequinoctialis*, *Hydra viridissima* and *Amerianna cumingi*. Test species were exposed to a range of calcium concentrations at constant magnesium concentrations. There was complete elimination of toxicity to *L. aequinoctialis* and *H. viridissima* at high Ca:Mg mass ratios of 1:9 and 1:10 whereas 10 to 30 % of toxicity existed for *A. cumingi*. The study considered Ca:Mg mass ratio of 1:9 as the ratio that had an effective amelioration on magnesium sulfate toxicity. Thus increased calcium concentration was found to influence the toxicity of other ions in saline solutions. The toxicity of various ions including the influence of calcium on toxicity of other ions can be due to the physiological role of these ions in the organisms.

2.3 Physiology of ions

All major ions sodium, potassium, magnesium, calcium, chloride, bicarbonate and sulfate are essential for normal functions in organisms (API 1998; Kefford et al. 2002). Sodium, potassium and chloride ions are components of cells and body fluids that have an essential role in electrical and osmotic balance (Press 2008). Sodium and potassium are distributed in an uneven concentration across the cell membrane creating an electric potential gradient (Ochiai 2010). This facilitates the normal function of muscle and nerve tissue (Shaw 1958). Magnesium is an important ion in structural stabilisation of proteins, nucleic acids and cell membranes by surface binding as well as an ion that plays a vital role in enzyme activities and protein synthesis (Cowan 1995). The anions chloride, sulfate and bicarbonate play an important role in the acid balance in cellular fluids (Ochiai 2010). Calcium plays a vital role in cell permeability, structure, cell proliferation and cell death as well as cell activation (Rubin et al. 1985). Calcium has been found to have a high affinity to bind to negative charges on the surface of cells and influences the excitability and the permeability of the cell membranes. Calcium is involved in the excitation of cells where excitation refers to the contraction of muscle fibre. The stimulus from a neuron to muscle fibre influences the concentration of calcium inside and outside the cell membrane that changes the electrical potential across the cell membrane. This assists in the contraction of muscle fibre (Sperelakis 2012). While calcium promotes cellular adhesion and cellular activity, high levels of free calcium ions are found to inhibit this function. It is necessary for cells to maintain a low level of cellular calcium for efficient functioning of the cells. Cells maintain a low level of cellular calcium by buffering mechanisms of energy dependent ion pumps and intracellular binding. Calcium is bound to anions, macromolecules and intracellular organelles, namely mitochondria and the endoplasmic reticulum. These stores provide the buffering mechanism as well as the reservoir for calcium mobilisation during activation (Rubin et al. 1985). While all the major ions are essential for the normal functions in the organisms, calcium seems to play a key role in most of the physiological processes. The appropriate concentrations of calcium and all the other major ions are essential for the normal functions in organisms and thus regulation of these ions is necessary. Mechanisms such as osmoregulation assist in the regulation of the ions between the external media and the internal media in aquatic organisms.

2.3.1 Osmoregulation

Osmoregulation is a mechanism that maintains the concentration of solutes and water within an organism (Rankin & Davenport 1981). Anatomical structures such as body surface, respiratory organs (e.g., gills) and excretory organs (e.g., malpighian tubules) help to maintain osmoregulation in aquatic organisms (Beadle 1969). Freshwater organisms maintain a higher internal ionic concentration when compared to their surrounding water. This is maintained by active uptake of ions and excretion of dilute urine (Hart et al. 1991). When there is an increase of salinity in the external media, cells continue to uptake more ions and lose water until a threshold level is reached. Above this threshold level, cells continue to lose more water and eventually cells and the organism dies (Hart et al. 1991).

The impact of increasing salinity in the external media on osmoregulation was assessed in experiments on a freshwater Trichopteran, *Limnephilus stigma* (Sutcliffe 1961). The experiments were conducted using laboratory tap water and sea water. Organisms were placed in tap water and then varying dilutions of sea water. Osmotic pressure of the medium and that of the haemolymph were compared. Concentrations of salts were represented as mM NaCl for both inside the haemolymph and external media (Figure 2.2). The diagonal line represents the iso-osmotic line and the point at which the concentration of external medium and haemolymph is same. As the concentration of salts in the external media increased, there was an increase in the concentration of salts in the haemolymph until it reached the iso-osmotic line. The point where the concentration of salts in the haemolymph meets the iso-osmotic line is the threshold point above which cells die and results in death of the organisms. As the concentrations of salts were increased in the external media, survival of the larvae decreased. For instance, it was observed there was 50 % mortality at 120 mM NaCl within 3 days, 75 % mortality in 170 mM NaCl within 3 days and very few survived for more than 2 days at 220 mM NaCl external concentration (Sutcliffe 1961).

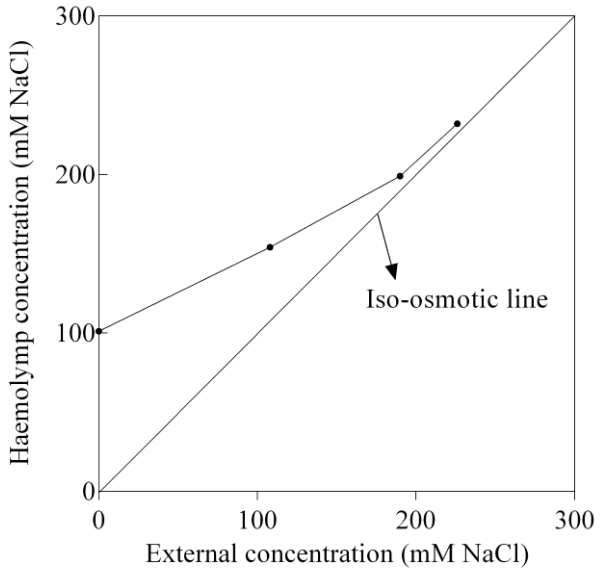

Figure 2.2: Relationship between the concentration of salt within the haemolymph and the external media in a Trichopteran, *Limnephilus stigma*; concentration of salt is expressed as mM NaCl (Sutcliffe 1961).

Regulation of major ions has been found to be dependent on the concentration of individual ions in the external media (Shaw 1958). Shaw (1958) reported uptake of sodium in crayfish *Astacus pallipes* required a minimum of 1 mM Na in the external medium. Sutcliffe (1971b) reported uptake of potassium in an amphipod *Gammarus pulex* required an external concentration of 0.010–0.015 mM K in starved organisms and 0.005 mM K in fed organisms. Vickers (1961) and Keys & Willmer (1932) described regulation of chloride in a teleost guppy *Lebistes reticulatus* and the common eel *Anguilla vulgaris* respectively. These organisms have specialised cells on the gills, called the 'chloride secretory cells' (Keys & Willmer 1932) that excrete chloride when there is increased chloride concentration in the external media.

While the concentration of individual ions directly influences the regulation of a particular ion, the concentration of one ion can also influence the regulation of another ion (Shaw & Stobbart 1963; Sutcliffe 1971a). Sutcliffe (1971a) investigated the influence of calcium concentration on influx of sodium ion on three species of amphipods, namely *G. pulex*, *Gammarus zaddachi* and *Gammarus duebeni*. Sodium influx in these organisms was assessed across a range of calcium concentrations. Calcium was added as concentrated solution of calcium carbonate or calcium chloride for experiments with *G. pulex*, and calcium chloride for *G. duebeni* and *G. zaddachi*. Sodium influx in *G. pulex and G. duebeni* was determined using a range of calcium concentrations between 1–10 mM and in *G. zaddachi* using a range of calcium concentration of 1–53 mM. A decrease in sodium influx was observed for calcium concentrations between 2–10 mM for *G. pulex*, and there was no change for *G. duebeni* for calcium concentration of up to 10 mM. However,

sodium influx in *G. zaddachi* was markedly increased between calcium concentrations of 2–4 mM, then fluctuated in response between 7–10 mM and decreased between calcium concentrations of 20–53 mM. The effect of calcium ion on sodium influx in *G. pulex* was consistent above calcium concentrations of 2 mM regardless of the anion used for calcium concentration.

Calcium concentration has been found to influence the permeability to water and ions across the cell membrane in a freshwater fish *Fundulus kansae* (Potts & Fleming 1970). The rate constant decreased with increased calcium concentration. The effect of various calcium concentrations on the permeability was investigated using tap water that constituted 2.5 mM sodium and 1.0 mM calcium. The other solutions used included 10 % tap water (0.1 mM calcium), 100 % tap water (1.0 mM calcium), tap water with 5 mM calcium and 100 % tap water with 10 mM calcium. Calcium was added as calcium chloride to increase calcium concentration. The permeability to water was determined as the rate constant. Increased calcium concentration from 0.1 to 10 mM decreased the rate constant from 2.0 to 0.9/h. This suggests that calcium concentration influences the permeability to water across the gill surface.

Therefore, elevated levels of salinity in freshwater streams affect the osmoregulation of the organisms and can eventually cause death. Additionally, regulation of individual major ions in the organisms is dependent on their concentration in the surrounding water. Also, calcium concentration in the surrounding media has been found to affect sodium regulation. Thus, not only does increased salinity affect osmoregulation, concentrations of individual ions in the surrounding media affect osmoregulation and likely contribute to the toxicity in aquatic organisms.

2.4 Ephemeroptera as test organisms

Ephemeroptera have been found to be one of the more ecologically sensitive macroinvertebrate taxa used to assess the water quality of freshwater streams (Chessman 2003). Chessman (2003) designed the Stream Invertebrate Grade Number – Average Level (SIGNAL) index, which grades invertebrate orders and families from 1 to 10 according to their sensitivity to ecosystem impact, in which a grade of 1 indicates highly tolerant taxa and 10 highly sensitive taxa. According to this scoring system, Ephemeroptera were given a grade of 9 which indicates the sensitivity of these taxa to toxicants. Additionally, Ephemeroptera is abundant in all freshwaters around Australia (Williams 1980) and can be used in toxicity tests. Various species of this order have often been recommended as test organisms in standard protocols such as ASTM E729-96 (2007) and APHA (2005). The abundance of Ephemeroptera, Plecoptera and Trichoptera commonly represented as EPT index is

used to evaluate the water quality of a stream. Their presence and abundance in a stream provides an indication of stream health and is used in bio monitoring protocols. Sensitivity of Ephemeroptera to salinity has also been reported in various studies.

A study by Short et al. (1991) investigated the impact of saline discharges from oil field operations to a stream on the distribution and abundance of fish and macroinvertebrate fauna. This study found that Ephemeroptera were absent in regions with salinities greater than 2 ‰ (≈ 2.9 mS/cm) and there was decreased species richness with increased salinity. Fish were found to be least sensitive. Similarly, Kennedy et al. (2004) evaluated the effect of a saline discharge from a coal processing effluent to a fresh water stream. This study observed a significant reduction in Ephemeroptera abundance within the conductivity range of 2–3 mS/cm and complete absence of the group at a conductivity range of 3–8 mS/cm. The same study also used laboratory microcosm experiments to test simulated effluent on the Ephemeroptera, *Isonchyia* and the cladoceran, *C. dubia.* Ephemeroptera were more sensitive than *C. dubia* to the effluent with a LOEC of ca. 1.6 mS/cm for *Isonchyia* survival and 3.7 mS/cm for *C. dubia* 7 day survival and fecundity.

Toxicity tests on various macroinvertebrates with marine salt have also found that Ephemeroptera are sensitive to salinity. Kefford et al. (2003) studied the effect of marine salt to various macroinvertebrates by using 72 h acute toxicity tests with 15 taxa from different orders. The 72 h LC50 for all taxa ranged from 5.5–76 mS/cm; the most salt sensitive were Baetidae (Ephemeroptera) with 72 h LC50 of 5.5–6.2 mS/cm and the most tolerant were macrocrustaceans (Decapoda, Isopoda and Amphipoda) with 72 h LC50 of 38–76 mS/cm. Similarly, Dunlop et al. (2008) evaluated the effect of salinity using 72 h acute toxicity tests with marine salt on 102 macroinvertebrates. This study also found that Ephemeroptera was one of the most sensitive taxa with 72 h LC50 of 10.9 mS/cm and Isopoda was the tolerant taxa with 72 h LC50 of > 55 mS/cm. This study also reported that the most sensitive taxon of Ephemeroptera was from the genus *Austrophlebioides* (Family Leptophlebiidae) with 72 h LC50 of 6.9 mS/cm.

In addition to their sensitivity to salinity, Ephemeroptera have also been found to be sensitive to varying ionic compositions. For instance, Kunz et al. (2013) studied the response of Ephemeroptera species, *Centroptilium triangulifer* to three different reconstituted waters based on coal mine drainage. Two compositions were representative of alkaline mine drainage with elevated magnesium, calcium, potassium, sulfate and bicarbonate and the third was representative of neutralised mine drainage with elevated sodium, potassium, sulfate and bicarbonate. The tests were

conducted with percentage dilutions of the reconstituted waters. The survival in a 35 day exposure for *C. triangulifer* showed toxic effect for the two alkaline mine drainage compositions with NOEC of < 33 % , < 50 % and no toxic effect with NOEC of > 100% for the neutral mine drainage composition. Collectively, not only Ephemeroptera was detrimentally affected by increased salinity, but this insect group was also one of the most sensitive to both salinity and different ionic compositions.

In Australia, the effects of various ionic compositions on Leptophlebiidae mayflies were assessed by Dunlop et al. (2011) and Prasad et al. (2014). In the study of Dunlop et al. (2011), saline solutions based on the ionic composition of the streams of the Fitzroy Catchment, Queensland, Australia were tested. This study suggested that these solutions were toxic at low concentrations and that the ion causing toxicity was found to be magnesium, hence a solution with low magnesium was tested (Fitzroy Composition Low Magnesium – FCLMg). In the study of Prasad et al. (2014), the test solutions were based on the ionic composition of dam water of the mine sites in the Fitzroy Catchment. Two different mine water compositions were tested and were named AMW1 and AMW2. The 96 h LC50 values between AMW 1 and AMW 2 suggest there is difference in the toxicity of the two solutions, AMW 1 was more toxic than AMW 2. There was overlap of the confidence intervals for the 96 h LC50 values that suggest a high degree of uncertainty in the data. The ionic composition of AMW 1 and AMW 2 differed and was likely the cause of difference in toxicity. Across the two studies, there were differences in the 96 h LC50 values for the solutions tested (Figure 2.3).

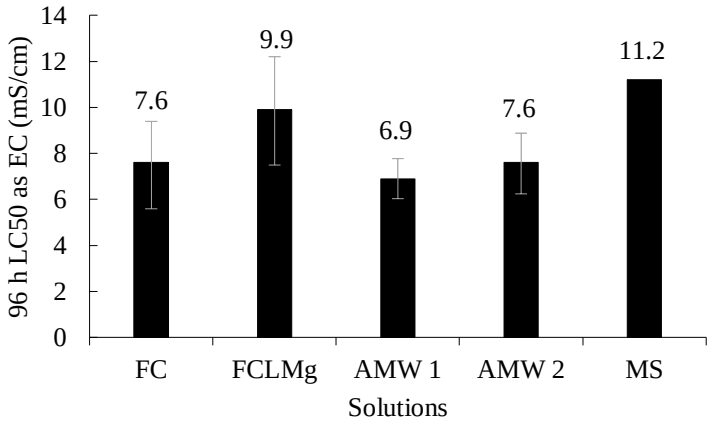

Figure 2.3: Response of Leptophlebiidae to Fitzroy composition (FC), Fitzroy composition low magnesium (FCLMg), Artificial mine water 1 (AMW 1), Artificial mine water 2 (AMW 2), Marine salt (MS) represented as LC50 (mS/cm). Note: The LC50 value for marine salt represented here is the average of 13.2, 8.7 and 11.7. The toxicity of all the solutions is 96 h LC50 value except for MS which is 72 h LC50 value. The error bars represent the 95 % confidence intervals.

The difference in toxicity among the solutions was likely attributed to the variation in ionic compositions (Table 2.3). The composition of solutions was based on different proportions of all major ions present in the natural waters (stream of Fitzroy or dam water of mine site). Thus, the solutions were represented in % meq proportion of ions. The difference in toxicity was hypothesised to be due to the difference in the proportion of calcium relative to all other ions in the solutions (Prasad et al. 2014).

Table 2.3: Ionic composition of saline solutions based on ionic compositions of; streams of Fitzroy catchment – Fitzroy composition (FC) and Fitzroy composition low magnesium (FCLMg); mine water of dams of the mine in the Fitzroy catchment – Artificial Mine Water 1 (AMW 1) and Artificial mine water (AMW 2); and marine salt (MS).

Test Solutions	Na	Ca	Mg	K	HCO_3	Cl	SO_4	References
	% meq							
Fitzroy Composition (FC)	18	16	14	0.4	18	26	5	Dunlop et al. (2011)
FC Low Magnesium (FCLMg)	25	23	0.7	0.4	18	26	5	
Artificial Mine Water 1 (AMW 1)	39	2	8	0.4	3	32	15	Prasad et al. (2012)
Artificial Mine Water 2 (AMW 2)	23	11	15	0.4	12	25	13	
Marine Salts (MS)	38	2	9	0.8	0.2	45	5	Dunlop et al. (2011)

The proportions of calcium and the toxicity of solutions were plotted in Figure 2.4. The data plotted showed high variability between and within tests and suggested calcium proportion did not correspond to the toxicity of the solutions. The marine salt (MS) and artificial mine water 1 (AMW 1) consisted of similar calcium proportion of 2 % meq[1] but presented widely different toxicities. Likewise, AMW 2 and FC consisted of different calcium proportion but presented similar toxicity. In contrast, FCLMg had the highest calcium proportion of 23 % meq but the toxicity of this solution was lower than FC, AMW 1, AMW 2 and higher than MS. Thus, the toxicity data did not correlate with the calcium proportion. While literature suggested increased calcium concentration would decrease toxicity for saline solutions on aquatic organisms, a similar trend was not observed for calcium proportion on toxicity for Ephemeroptera. This purpose of the hypothesis in the present study was to test whether increased proportion of calcium would decrease the toxicity of a saline solution to Ephemeroptera.

[1] % meq is percent milliequivalent; % meq of an ion is the proportion of that ion out of the sum of all the major ions in the solution as percentage. % meq ion = (meq/L ion / sum of all ions in meq/L)*100; meq/L = mmol/L/charge on the ion. Eg., % meq Ca = (Ca meq/L /(sum (Ca+Na+K+Mg+Cl+HCO_3+SO_4) meq/L)*100

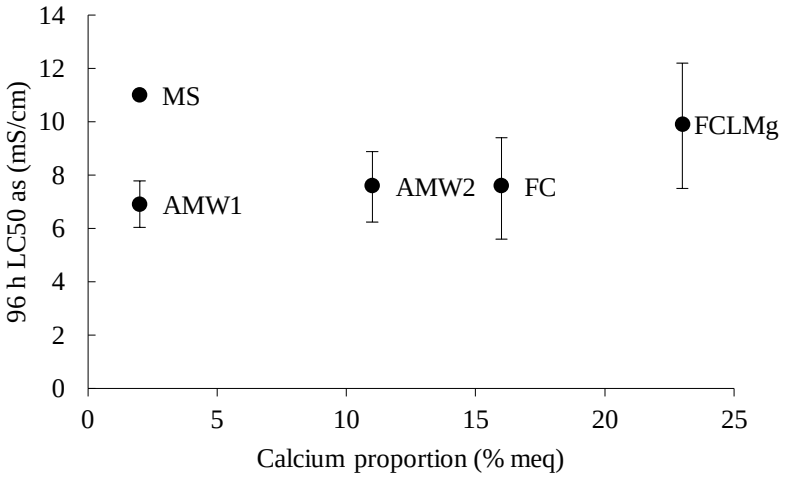

Figure 2.4: Comparison of the response of Letpophlebiidae represented as 96 h LC50 (mS/cm) against relative calcium proportion of the major ions (% meq) in FC, FCLMg, AMW, AMW 2 and MS (72 h LC50 for MS); error bars represent 95 % confidence intervals.

The same toxicity data was compared with $Ca:HCO_3$ ratio (Figure 2.5). The toxicity of solutions FC, FCLMg, AMW 1, AMW 2 and MS appeared to correspond to the $Ca:HCO_3$ meq ratio. AMW 1 which showed the highest toxicity had lowest $Ca:HCO_3$ ratio. AMW 2 and FC had similar toxicity and with similar $Ca:HCO_3$ meq ratios. Marine salt possessed the lowest toxicity and the highest $Ca:HCO_3$ meq ratio. FCLMg had a lower toxicity than AMW 1, AMW 2 and FC but higher than MS which was also consistent with $Ca:HCO_3$ meq ratio. This solution had a $Ca:HCO_3$ ratio higher than AMW 1, AMW 2 and FC but lower than MS. These data demonstrate a possible relationship between the toxicity of saline solutions to Leptophlebiidae and the $Ca:HCO_3$ ratio.

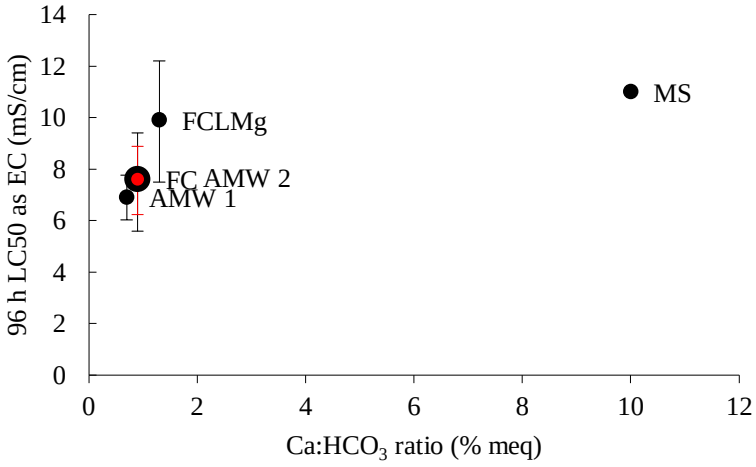

Figure 2.5: Comparison of the response of Leptophlebiidae represented as 96 h LC50 (mS/cm) to $Ca:HCO_3$ ratio in FC, FCLMg, AMW 1, AMW 2 and MS (Note: 72 h LC50 for MS); error bars represent 95 % confidence intervals.

2.5 Aim and Objectives

The aim of this study was to investigate the influence of calcium proportion on the toxicity of a saline solution to a species of Ephemeroptera.

The objectives were:

1. To determine whether increased proportion of calcium at a constant $Ca:HCO_3$ ratio decreased the acute toxicity of a saline solution.
2. To establish whether increased proportion of calcium in a geometric sequence of two fold, four fold and eight fold increases would decrease the toxicity of the saline solution using chloride and sulfate salts of calcium.
3. To verify whether increased proportion of calcium in isolation of the associated anions using calcium chloride and calcium sulfate salts separately would change the toxicity of the saline solution.

The findings of this thesis would assist to gain knowledge on the effect of calcium proportion based on an ionic composition similar to an effluent discharge in the natural environment. In Australia, the ANZECC (2000) guidelines suggest that salinity should not exceed 1000 mg/L (1.5 mS/cm) in freshwater streams. However, the criterion value is varied based on the type of the ecosystem (eg., upland river, lowland river, freshwater lakes and reservoirs, wetlands). Nevertheless, the ionic composition in freshwater varies and it is necessary to evaluate the effect of specific ionic compositions. Currently, the trigger value is not based on the site specific ionic composition. Toxicity test can provide threshold value for the derivation of a trigger value for specific ionic composition. One of the examples of this is the derivation of water quality guidelines for magnesium specifically for a mine discharge in Northern Australia (van Dam et al. 2010). The study evaluated the toxicity of magnesium in relation to the influence of calcium. Similarly, the findings of this thesis will be applicable in the regulation of saline discharges with ionic compositions that consist of similar calcium proportion of ions to protect freshwater ecosystems from harmful effects of salinity.

Chapter 3 Methods

This chapter includes a description of the ecological importance of the test organisms used in this study and an overview of their abundance in the selected site of collection. The sampling site along with brief description of the method of collection of mayfly for the tests is provided. The chapter further explains the toxicity tests and provides a detailed description of the test solutions used in the present study. The chapter concludes with a description of the statistical analyses used in the present study.

3.1 Test organisms

Ephemeroptera are insects. The nymph stage of their life cycle is aquatic and the adult stage is short lived and terrestrial. They are abundant in all freshwaters around Australia (Williams 1980). Ephemeroptera occupy an important trophic level in ecosystems as the nymphs are collector – gatherers or detritivores, which eat fine organic matter, dead and decaying plants and animals, thus recycling and regenerating of nutrients in ecosystems (Ingram et al. 1997). Mayflies in turn form part of the diet of fish and predatory insect larvae (Ingram et al. 1997; Peters & Campbell. 1991) and occupy an essential place in the ecosystem. They are typically found under stones and in swift currents (Williams 1980). Family Leptophlebiidae are one of the families of Ephemeroptera that are dominant both worldwide and in Australia (Australia Bureau of Flora Fauna 1988). They are also abundant in south east Queensland (Phil Suter, pers. comm., 2013). The species collected in the present study was identified as *Austrophlebioides* sp. AV11 (courtesy Dr. Phil Suter, LaTrobe University, Wodonga). The species has been recognised as a separate undescribed species by Christidis & Dean (2008) and the notation of the species is represented as *Austrophlebioides* sp. AV11 (see Figure 3.1). This taxon is a member of the Family Leptophlebiidae.

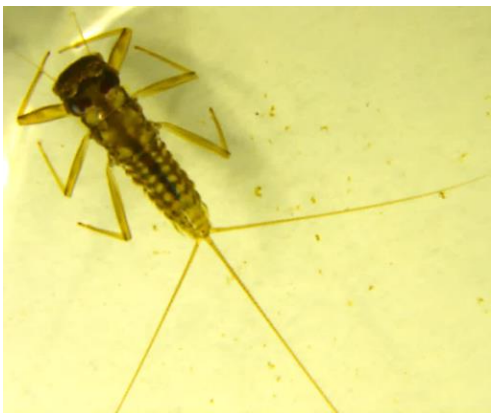

Figure 3.1: Nymph of *Austrophlebioides* sp. AV11.

3.2 Sampling site

Austrophlebioides sp. AV11 was collected at Mt. Barney Creek, classified as a reference site in the Queensland Water Quality Guidelines (QWQG 2009). This site is located 150 km southwest of Brisbane with latitude -28.2373 S and longitude 152.7427 E (Figures 3.2 & 3.3). This site is not impacted by any major human activity and has an unmodified flow regime (QWQG 2009). It was unlikely the organisms were exposed to variations in water quality prior to collection. This site was recorded for abundance of Leptophlebiidae that have previously been collected during the Autumn (March to May) and Spring (September to November) (BoM 2014) seasons in routine bio-monitoring (Dave Logan, Healthy Waterways, Brisbane; Stephen Moore, Department of Science, Information Technology, Innovation and the Arts, Brisbane). *Austrophlebioides* sp. AV11 nymphs were collected by washing rocks and picked live in sorting trays (Figures 3.4 & 3.5). While the instars of the nymphs were unable to be recognised during the collection, individuals of relatively similar size were picked. They were transferred to containers supplied with aeration (Figure 3.6). The organisms were transported to the laboratory and the experiments were carried out the following day. The duration between the collection of organisms in the field and the experiments was approximately 24 hours.

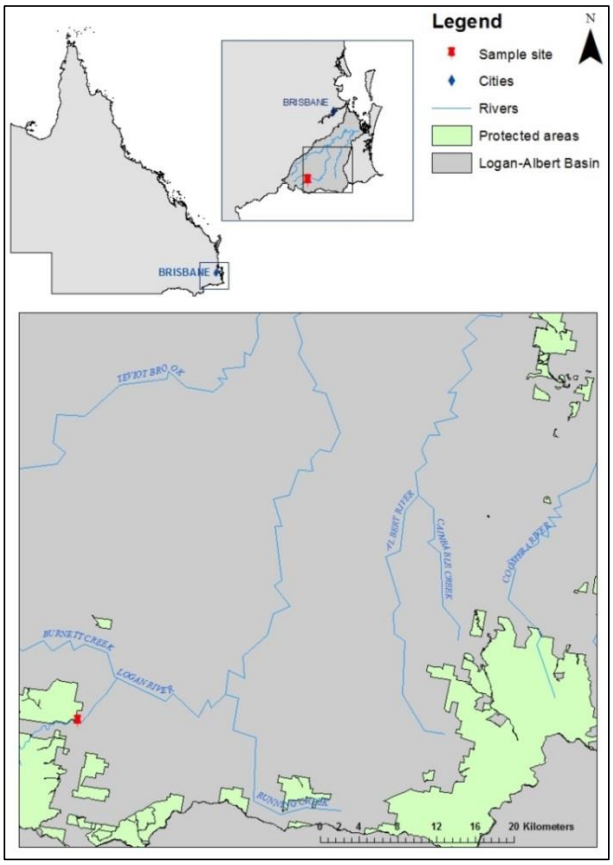

Figure 3.2: Location of sample site, major city, major rivers, protected areas and the catchment area of the sampling site - Logan Albert basin.

Courtesy: Alexandra Wolhuter

Figure 3.3: Mt Barney creek, sampling site.

Figure 3.4: Washing rocks to collect *Austrophlebioides* sp. AV11.

Figure 3.5: Sorting of *Austrophlebioides* sp. AV11.

Figure 3.6: Containers aerated for transportation.

3.3 Toxicity Tests

Acute toxicity tests with 96 hour duration were carried out in accordance with standard methods (APHA 2005; ASTM E729-96 2007). Static non-renewal exposure was used where organisms were exposed to the same test solution throughout the duration of the test. The experiments were carried out in a temperature and light controlled room. The light was run on a 12:12 h light and dark cycle (APHA 2005; ASTM E729-96 2007). The temperature was maintained at 20ºC ± 2ºC during experiments. The estimated standard deviation of temperature was between the ranges of 0.14 to 0.65 (details are provided in Appendix Table A.7) among the different experiments. The chambers were aerated to ensure that the dissolved oxygen (DO) was maintained between 60–100 % saturation. Aeration avoids depletion of DO during the course of experiments that could have an influence on toxicity results. Figure 3.7 shows an experimental set up.

Figure 3.7: Experimental set up.

Toxicity tests comprised of two tests with a reference toxicant test and toxicant solution run simultaneously. Each reference toxicant test and toxicant solution included a control and a series of dilutions of the test solutions. Each experiment constituted three replicates for each toxicant solution toxicity test and two or three replicates for the reference toxicant test. Eight to twelve individuals of *A.* sp. A11 were introduced into each test containers. For each experiment a range of dilutions from the stock solution were created. The dilutions were based on the measurement of electrical conductivity. Tests were conducted in food grade plastic containers. Organisms were introduced into test solutions and observed for mortality every 24 h for the duration of 96 h of the test. An organism was considered to be dead when there was no response to gentle prodding. Any organism that emerged or missing was also recorded. There were two or three that went missing or one or two emerged in most of the tests. Emerged or missing organisms were subtracted from the total number of organisms that were exposed to the treatments. Details of all the tests are presented in Appendix Table A.5 for reference toxicant and Appendix Table A.6 for toxicant solution. Organisms used in the experiments were preserved in 90 % ethanol at the end of the experiments and were submitted for identification to the taxonomist.

3.4 Test solutions

3.4.1 Control

A control is a solution that contains no toxicant that is tested in the toxicity test but is a treatment that duplicates all the conditions of exposure of the toxicity test (APHA 2005). Creek water was used as the control to evaluate the survival of organisms and the test acceptability.

3.4.2 Reference toxicant

A reference toxicant test was simultaneously conducted with each toxicity test to make comparisons between test results (Environment Canada 1990). Additionally it was used as a positive control to determine the health and sensitivity of the test organisms (Rand 1995; USEPA 2002) as the organisms were collected in the field (USEPA 2002). Although there are various chemicals that are recommended as reference toxicants (Environment Canada 1990; USEPA 1999), sodium chloride and potassium chloride are often recommended reference toxicants for toxicity tests that include saline solutions (USEPA 1999). The mine water composition used in the present study was sodium chloride dominant and therefore, sodium chloride was chosen as the reference toxicant.

A stock solution of sodium chloride was prepared using analytical grade sodium chloride (Merck®) dissolved in Millipore ultrahigh purity water (UHP). A range of dilutions from this stock solution were prepared to use in the toxicity tests using UHP water. To each of the dilution test water, sodium hydroxide (25 mg/L) and the buffer monobasic phosphate (272 mg/L) were added (Adelman & Smith Jr 1976) to maintain pH above 6 to avoid the potential for pH related toxicity.

3.4.3 Toxicant solutions

Artificial mine water 1 from Prasad et al. (2012) was used as the base solution and subsequently referred to as AMW. The ionic composition of this solution is represented in Table 3.1

Table 3.1: Ionic composition of Artificial Mine Water (AMW) as described in Prasad et al. (2012) in percent milliequivalents

Solution Description	Ca	Mg	Na	K	HCO_3	Cl	SO_4
	% meq						
Artificial Mine Water (AMW)	2	8	39	0.4	3	32	15

The calcium proportion was increased above the proportion of calcium in artificial mine water (AMW) solution using calcium chloride and calcium sulfate salts together. The calcium proportion was increased at a constant $Ca:HCO_3$ ratio in a geometric sequence as two fold ($Ca\text{-}Cl.SO_4(2)$), four fold ($Ca\text{-}Cl.SO_4(4)$) and eight fold ($Ca\text{-}Cl.SO_4(8)$) increase above the $Ca:HCO_3$ ratio of AMW (Table 3.2). Subsequently, to distinguish the effect of the anions associated with calcium, additional solutions with increased calcium proportion using only calcium chloride or only calcium sulfate were prepared separately. Calcium proportion was increased as two fold and four fold the ratio of $Ca:HCO_3$ above that of AMW. Solutions with calcium chloride were named as Ca-Cl(2) and Ca-Cl(4) and solutions with calcium sulfate were named as $Ca\text{-}SO_4(2)$ and $Ca\text{-}SO_4(4)$. Ratios mentioned here are nominal ratios which are the expected ratios.

Table 3.2: Calcium proportion in artificial mine water (AMW) and in solutions with increased calcium proportion above that of AMW as constant Ca:HCO_3 ratio, Ca:HCO_3 represented as nominal proportions.

Salts used to increase calcium concentration	Solution ID	Ca	HCO_3	Ca:HCO_3	Increased calcium proportion
		% meq			
No increase of calcium	AMW	2	3	0.7	-
Calcium chloride and calcium sulfate	Ca-Cl.SO_4 (2)	4.2	3	1.4	2 fold
	Ca-Cl.SO_4 (4)	8.4	3	2.8	4 fold
	Ca-Cl.SO_4 (8)	16.8	3	5.6	8 fold
Calcium chloride	Ca-Cl (2)	4.2	3	1.4	2 fold
	Ca-Cl (4)	8.4	3	2.8	4 fold
Calcium sulfate	Ca-SO_4 (2)	4.2	3	1.4	2 fold
	Ca-SO_4 (4)	8.4	3	2.8	4 fold

Saline solutions were prepared using analytical grade chemicals dissolved in Merck® with Millipore Ultra High Purity (UHP) water. The salts $CaCl_2.2H_2O$, NaCl, $NaHCO_3$, $MgCl_2.6H_2O$, KCl were used for all the solutions. For sodium sulfate, either hydrous or anhydrous forms of salt were used for different solutions. For solutions Ca-Cl.SO_4(4), Ca-Cl.SO_4(8), Ca-Cl(2) and Ca-Cl(4), hydrous form $Na_2SO_4.10H_2O$, was used and for solutions AMW, Ca-Cl.SO_4(2), Ca-SO_4(2) and Ca-SO_4(4), anhydrous form Na_2SO_4 was used. A stock solution was prepared to a calculated EC of approximately 14 mS/cm for each type of solution. The stock solution was mixed overnight and then filtered through 0.45 µM Millipore Durapore membrane filters by vacuum filtration. To conduct the experiments, the stock solution was diluted into a series of lower treatments between the EC range of 1.2 mS/cm to 14 mS/cm using the UHP water.

Stock solutions were prepared on the basis of the relative proportions of the ions. This was to represent the ionic proportions in the mine water. The stock solution was prepared to a calculated EC of 14 mS/cm to avoid the solubility issue of the calcium salts. Calcium was increased as proportion of ions above that of AMW while the EC was maintained to approximately 14 mS/cm across all the stock solutions. While the relative proportion of anions was kept consistent, the proportion of cations varied particularly sodium. Increased calcium proportion compromised the proportion of sodium ion. The calcium proportion within each solution type was constant and its ratio in relation to other ions was constant. The dilutions from the stock solution within each solution type had constant proportion of ions and also constant ratios of ions.

3.5 Water quality

Water quality parameters DO, EC, pH and temperature were recorded at the beginning, 48 h and 96 h during the test. Solutions were subsampled for cations and anions measurement and stored at 4º C as per the requirements of APHA (2005) until the analyses were carried out. Chemical analyses of ions were carried out at the School of Agricultural and Food Sciences, The University of Queensland in accordance with standard methods (APHA 2005). Calcium, potassium, sodium, magnesium and sulfate as sulphur were analysed by inductively coupled plasma–optical emission spectroscopy (Varian Vista Pro® USA). Chloride was analysed by Flow injection analysis (AQ^{2+} Automated Discrete Analyser, Seal® Analytical Inc. USA). Alkalinity was analysed by Gran titration (Metrohm 902 Titrando, Metrohm® USA). Samples were run in duplicate for every 5 % of samples and a method blank for every 20 % of samples. For each of the analyte, a standard was run for every 20 % samples to comply with QA/QC protocols. The EC and pH were measured using TPS Model 901–CP. The pH probe was calibrated with buffers 7 and 4 and the EC probe was calibrated with standard solution of 2.76 mS/cm prior to the measurement of pH and EC of the solutions during experiments.

3.6 Data analyses

3.6.1 Water quality data

Field observations of water quality parameters DO, EC, pH and temperature as well as the chemical data for the stream water were summarised using descriptive statistics using Graphpad Prism v.6. Descriptive statistics provide minimum, maximum, mean, median, standard deviation and standard error of all the data. This assists to evaluate any variation in the water quality parameters.

The ionic compositions of the test solutions were plotted as piper diagrams using Geochemist's Workbench (GSS 8.0.12, Craig N, Bethke & Aqueous solutions LLC, USA). Piper diagrams show the distribution of relative proportion of ions in the solutions but do not provide the actual concentration of the ions. All the concentrations of ions are converted as milliequivalents and plotted as percentages of cation or anion in respective trilinear diagrams. A piper diagram plots the cation and anion proportions as two trilinear diagrams and a combined quadrilateral diagram at the top. The bottom left trilinear is assigned to cation and the right trilinear is assigned to anion proportion. Each cation and anion trilinear diagrams make up for 100 % of cation or anion proportion in the solutions.

3.6.2 Reference toxicant

The 96 h LC50 value for the reference toxicant for each test was determined by fitting probit regression using Toxcalc v5.01 (Tidepool Scientific LLC. USA). The 96 h LC50 values of all the tests were used to calculate the cumulative mean and two standard deviations between 96 h LC50 values of successive tests. These values were then plotted as a cusum chart in MS Excel (Microsoft®). The x–axis represent the test number and y–axis represents the 96 h LC50 value of NaCl in mg/L. Additionally, the cumulative mean, plus or minus two standard deviations were plotted as upper and lower limits (Environment Canada 1990; USEPA 2002).

3.6.3 Toxicant solutions

Three separate analyses were used to interpret toxicity test results. These were 1) derivation of 96 h LC50 values, 2) comparison between the 96 h LC50 values and 3) comparison between the dose response curves. The regression curves for the comparison between the 96 h LC50 values and the comparison between the dose response curves are shown as separate graphs. These were prepared using Graphpad prism v6.05 (Graphpad Software, USA).

The point estimates of LC50 values for 96 h tests were determined by fitting a log regression variable slope model. Concentrations of solutions were log transformed to reduce positive skewness and render more symmetrical distribution prior to regression analyses (Sparks 2000).

The toxicity of AMW was compared to the toxicity of each solution with increased calcium using an *f* test and log regression model (Motulsky & Christopoulos 2003). The *f* test provides a useful and robust analysis because it uses sums of squares to compare the variability between two different data sets. The *f* ratio between the two data sets is used to calculate the *p* value to evaluate the significant difference between the two data sets. The purpose of the statistical test was to evaluate the significant difference between the toxicity of the solution with increased calcium and AMW. The *f* ratio was used to calculate the *p* value, where the $p \leq 0.05$ was used to evaluate the significant difference.

Toxicity data between AMW and each solution with increased calcium proportion was compared for both 96 h LC50 values and the overall dose response curves. Single parameter log regression model was used to compare the 96 h LC50 values between AMW and each solution with increased calcium proportion. A four parameter log regression model (Top, bottom, logEC50 and Hillslope) was used to compare the overall dose response curves between AMW and each solution with increased calcium proportion. However, for the four parameter regression curve, the bottom of the

curve was constrained to 0 to fit the criteria of the model. The comparisons between 96 h LC50 values and dose response curves provided the *f* ratio. The *f* ratio was used to calculate the *p* value, where $p \leq 0.05$ was used to evaluate the significant difference.

The statistical output was given as numerical values of the *f* test, *p* value and significant difference for both 96 h LC50 and dose response comparisons. Additionally, the model gave a graphical output for the comparison of 96 h LC50 values and dose response curves that are represented as separate graphs. When there was statistical difference, there were two distinct curves for each solution and when there was no statistical difference, there was overlap of curves for both the solutions. Comparison of both the 96 h LC50 values and the dose response curves enhances the evaluation of the effect of the additional calcium.

The trend in the data was analysed using Regression analysis in MS Excel. The regression analysis provided a R^2 value that was used to interpret the trend in the data. The 96 h LC50 value for each ion in each solution type was determined by fitting a probit regression using Toxcalc v5.01. The toxicity of individual ions in solutions AMW, Ca-Cl.SO_4(2), Ca-Cl.SO_4(4) and Ca-Cl.SO_4(8) were compared with the calcium concentration in the solutions at 96 h LC50 values. The graphs were plotted in MS Excel. The x–axis represents calcium concentration at the 96 h LC50 value and the y−axis represents the 96 h LC50 values of AMW and Ca-Cl.SO_4(2), Ca-Cl.SO_4(4) and Ca-Cl.SO_4(8).

Chapter 4 Results

This chapter begins with a summary of water quality data of the stream and the test solutions. Further results of the chemistry of all the test solutions are provided. Chapter 4 continues to give a detailed report on the statistical results of the toxicity tests and concludes with the interpretation of the results of the toxicity tests.

4.1 Water Quality Data

4.1.1 Field water quality

The electrical conductivity (EC), pH, dissolved oxygen (DO) and temperature were recorded in the field during collection of the mayfly nymph (Appendix Table A.1) and summarised below (Table 4.1).

Table 4.1: Minimum, maximum, median, mean, standard deviation, standard error of means and number of sample values of EC, pH, DO and temperature of stream water during the period of collection of *Austrophlebioides* sp. AV11.

Description	EC µS/cm	pH	DO mg/L	Temperature° C
Minimum	55.3	7.4	8.3	12.9
Maximum	106.3	8.5	10.6	22.6
Median	94.2	8.0	9.1	18.1
Mean	86.1	8.0	9.2	17.5
Standard Deviation	20.3	0.34	0.8	4.1
Standard Error of Mean	7.2	0.12	0.2	1.4
Number of samples	8	8	9	9

A wide range of temperature was recorded with the lowest being 12.9ºC and the highest 22.6ºC. This was most likely due to the collection of the organisms during different seasons. There was also some variation recorded for electrical conductivity with a minimum of 55.3 µS/cm and a maximum of 106.3µS/cm and a standard deviation of 20.3. There was little variation observed for pH with a standard deviation of 0.34. The DO of freshwater ranges between 14.6 mg/L at 0ºC to 9.1 mg/L at 20ºC and 7.5 mg/L at 30ºC (Rand 1992). The range of DO concentrations of 8.3–10.6 mg/L recorded in the field in the present study was closer to the DO mentioned in the literature. These data suggest there was little variation for pH and DO, slight variation for EC but a wide range for temperature during the period of collection of the organisms. The water quality parameters were recorded to ensure there was no substantial change in water quality during the period of collection of organisms in the field that would influence the toxicity test results.

4.1.2 Stream water chemistry

Stream water samples were collected prior to the collection of test organisms for each experiment. Every sample was analysed for major cations and anions (Appendix Table A.2) and summarised (Table 4.2). Concentrations of all major ions were low and indicated that organisms were not previously exposed to high levels of salinity at the point of collection throughout the experimental period.

Table 4.2: Minimum, maximum, median, mean, standard deviation, standard error of means and number of sample values of major cations and anions of the stream for the period of collection of *Austrophlebioides* sp. AV11.

Description	Ca	K	Mg	Na	SO_4	Cl	HCO_3
	mg/L						
Minimum	3.0	0.4	1.6	0.0	1.8	4.9	13.0
Maximum	5.2	1.0	2.9	2.8	6.4	9.3	69.5
Median	3.9	0.8	2.3	1.8	2.9	6.5	56.0
Mean	4.0	0.7	2.2	1.8	3.2	6.6	49.8
Standard Deviation	0.7	0.2	0.4	0.9	1.4	1.3	18.8
Standard Error of Mean	0.2	0.07	0.1	0.3	0.5	0.5	7.1
Number of samples	8	8	8	8	8	8	7

4.1.3 Chemistry of test solutions

Artificial mine water was prepared as per the composition in Prasad et al. (2012) and Prasad et al. (2014). The ionic composition of the artificial mine water measured by subsequent laboratory analysis was close to the required ionic composition (Table 4.3). The ionic composition for all the solutions was based on the proportion of major ions present in the AMW. The proportion of ions of all the stock solutions was calculated to obtain the expected proportion of calcium and other ions. The measured proportion of ions is provided in Table 4.3. There was a reduction between the calculated and the measured concentrations of ions in the stock solution due to precipitation. The calculated and measured concentrations of ions for the stock solutions is provided in Appendix Table A.3 and the concentration of the ions for all the solutions is provided in Appendix Table A.4. The measured EC and concentration of all major ions suggest there was decrease in sodium concentration for solution Ca-Cl.SO_4(8) (Table 4.4).

Table 4.3: Measured ionic proportions of AMW, solutions with increased calcium proportion using calcium chloride and calcium sulfate together and separately.

Salts used to increase calcium proportion	Solution ID	Ca	K	Mg	Na	SO_4	Cl	HCO_3
		% meq						
No increase in calcium proportion	AMW	2.0	0.3	8.8	37.2	16.7	32.9	2.0
Calcium chloride and calcium sulfate	Ca-Cl.SO_4(2)	3.8	0.4	9.8	35.8	18.9	29.7	1.7
	Ca-Cl.SO_4(4)	5.9	0.3	9.3	32.7	18.3	32.2	1.2
	Ca-Cl.SO_4(8)	13.3	0.4	11.3	24.6	13.6	36.1	0.8
Calcium chloride	Ca-Cl(2)	1.6	0.4	10.2	36.0	18.2	31.5	2.1
	Ca-Cl(4)	6.9	0.3	9.1	32.7	16.0	30.9	1.0
Calcium sulfate	Ca-SO_4(2)	3.2	0.4	8.4	36.4	19.3	30.7	1.4
	Ca-SO_4(4)	4.8	0.3	8.0	35.0	21.6	29.4	0.8

Table 4.4: Measured EC and concentration of all major ions for AMW and solutions with increased calcium proportions using calcium chloride and calcium sulfate together and separately.

Solution ID	Measured EC (mS/cm)	Ca	K	Mg	Na	SO_4	Cl	HCO_3
		mg/L						
AMW	15.1	160	53	410	3360	3160	4590	490
Ca-Cl.SO_4 (2)	14.7	300	56	470	3310	3640	4240	430
Ca-Cl.SO_4 (4)	18.5	430	48	410	2760	3230	4190	270
Ca-Cl.SO_4 (8)	12.5	880	51	450	1870	2160	4240	150
Ca-Cl (2)	13.8	110	48	420	2830	3000	3830	440
Ca-Cl (4)	15.5	590	56	470	3210	3270	5150	250
Ca-SO_4 (2)	14.5	250	54	390	3260	3610	4240	340
Ca-SO_4 (4)	14.9	390	54	400	3300	4250	4280	200

The proportion of calcium was between a range of 20–30 % for solutions AMW, Ca-Cl.SO_4(2), Ca-Cl.SO_4(4) and 50 % for Ca-Cl.SO_4(8) (Figure 4.1). AMW, Ca-Cl.SO_4(2), Ca-Cl.SO_4(4) had sodium proportion of 80–70 % and Ca-Cl.SO_4(8) had 50 %. All the solutions had a magnesium proportion of 20 %. The range of proportions for chloride was between 60–70 %, and sulfate 30–40 %; and bicarbonate was approximately 10 % for all the solutions. As seen in Figure 4.1, there was reduction in the sodium, potassium and magnesium ion proportion in the solution with the highest calcium proportion, Ca-Cl.SO_4(8).

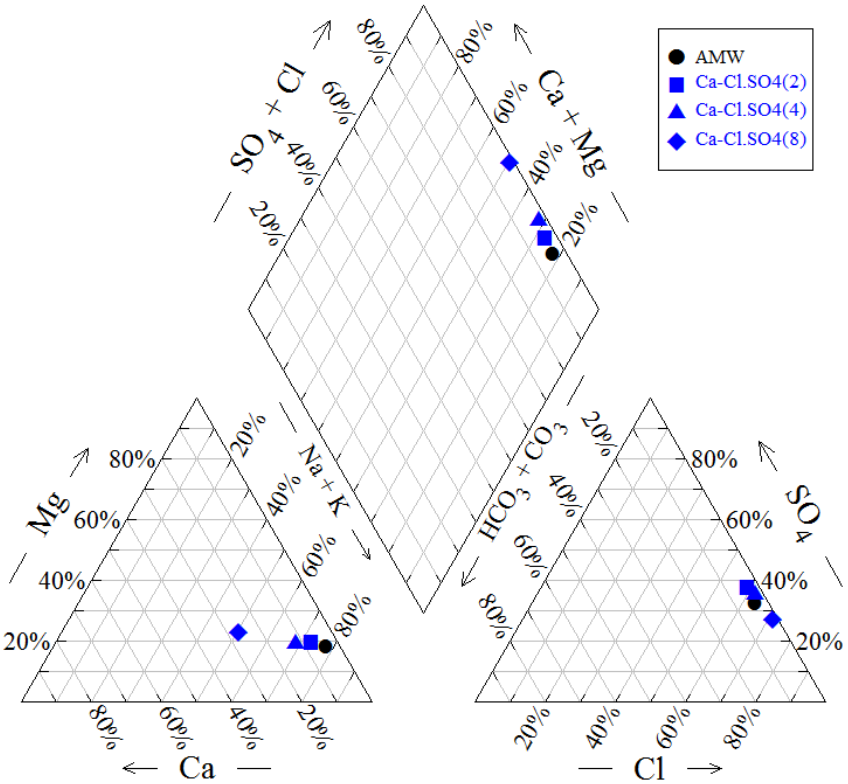

Figure 4.1: Piper diagram showing measured concentrations of ions in AMW, Ca-Cl.SO$_4$(2), Ca-Cl.SO$_4$(4) and Ca-Cl.SO$_4$(8).

Solutions with increased calcium proportion using only calcium chloride (Figure 4.2) had calcium proportions between 10–20 %, magnesium approximately 85–95 %, sodium and potassium between 70–80 %, sulfate between 30–40 %, chloride around 60–70 % and bicarbonate approximately 10 % for both solutions.

Solutions with increased calcium proportion using only calcium sulfate had calcium proportions between 15–20 %, magnesium approximately 90 %, sodium and potassium 75 %, sulfate between 40–45 %, chloride approximately 60 % and bicarbonate approximately 10 %. The difference in the proportions of ions was narrow for solutions with increased calcium using either calcium chloride or calcium sulfate when compared against the solution with increased calcium using calcium chloride and calcium sulfate together.

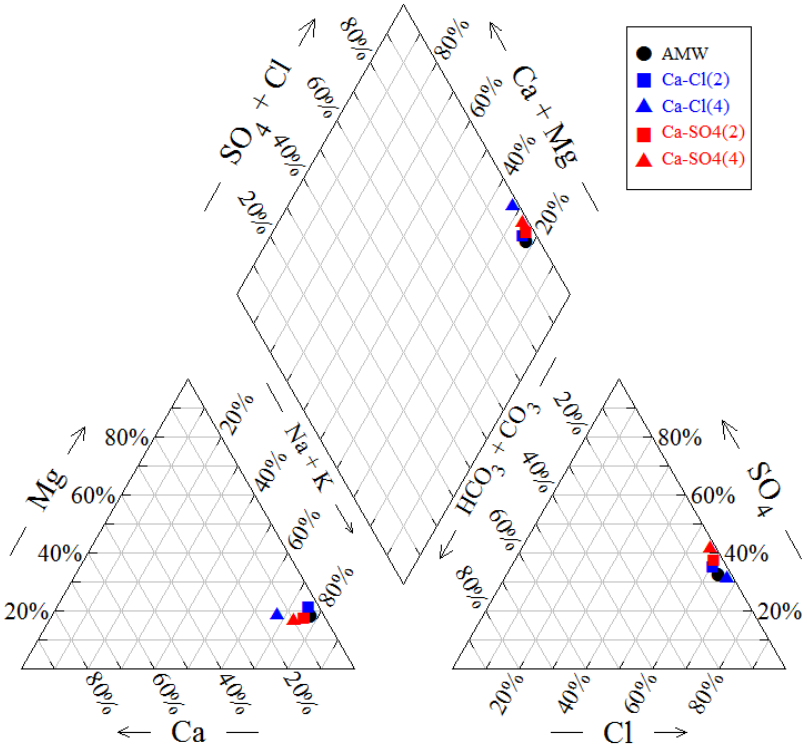

Figure 4.2: Piper diagram showing measured concentrations of ions in AMW, Ca-Cl(2), Ca-Cl(4); Ca-SO_4(2) and Ca-SO_4(4).

4.2 Reference toxicant test

Survival of test organisms in the control for the reference toxicant test was more than 90 % and hence all tests met the acceptability criteria (USEPA 2002). The cumulative mean and the 96 h LC50 values of NaCl fell within the upper and lower limits of two standard deviations (Figure 4.3). This indicated the organisms collected were healthy and met the additional acceptable criteria for the toxicity tests (USEPA 2002).

The 96 h LC50 values to sodium chloride varied between the ranges of 95−221 mg/L. These values were lower when compared to the sensitivity of other organisms as reported in the literature. The 96 h EC50 for mayfly, *Tricorythus* sp. was between 2200 mg/L and 4500 mg/L (Goetsch et al. 1997), the 48 h LC50 for the cladoceran, *Ceriodaphnia dubia* was 1960 mg/L, the 48 h LC50 for the cladoceran, *Daphnia magna* was 4770 mg/L, the 96 h LC50 for fathead minnow, *Pimephales promelas* was 6890 mg/L (Mount et al. 1997). However, the study by Goetsch et al. (1997) used the river water for dilution and the study of Mount et al. (1997) used moderately hard reconstituted water for dilution series. The difference between the toxicity of sodium chloride reported in the literature and that reported here is likely due to the hardness of the test solutions. In the present study, *Austrophlebioides* sp. AV11 was found to be sensitive to sodium chloride when compared to other organisms. In the present study, only sodium chloride was present in the dilutions. This could be the reason for the high sensitivity observed here for sodium chloride.

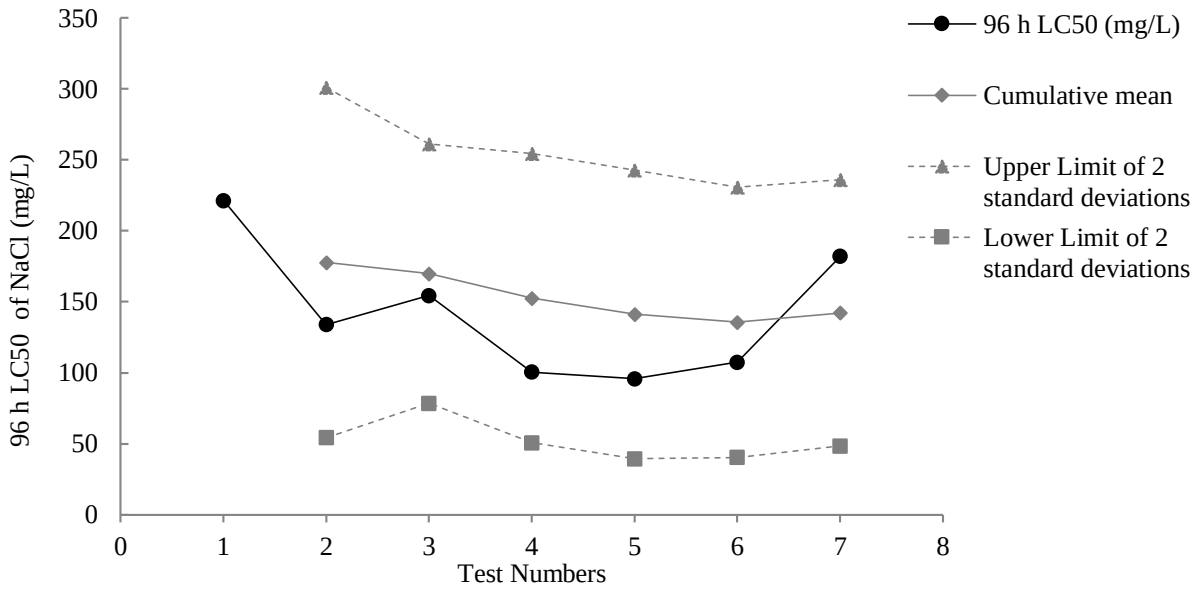

Figure 4.3: Cusum chart for reference toxicant NaCl showing 96 h LC50 values for all tests, cumulative mean of 96 h LC50, two standard deviations as upper and lower limit.

4.3 Toxicity tests

Survival of organisms in the controls for all toxicity tests was 100 % and hence met the acceptability criteria for toxicity tests (USEPA 2002). The 96 h median LC50 values along with 95 % confidence intervals (Table 4.5) suggest that Ca-Cl.SO_4(4) had the lowest toxicity with 96 h LC50 of 8.1 mS/cm. The toxicity of solutions with increased calcium proportion were compared with the toxicity of AMW (Figures 4.4 to 4.6).

Table 4.5: 96 h LC50 values for artificial mine water (AMW) and solutions with increased calcium proportion of total ions using calcium chloride and calcium sulfate, only calcium chloride and only calcium sulfate along with lower 5th and upper 95th % confidence intervals.

Salts used to increase calcium proportion	Solution ID	96 h LC50	Lower limit	Upper limit
		EC mS/cm		
No increase	AMW	6.0	5.8	6.2
$CaCl_2$ and $CaSO_4$	Ca-Cl.SO_4(2)	6.9	6.1	7.7
	Ca-Cl.SO_4(4)	8.1	7.0	9.2
	Ca-Cl.SO_4(8)	6.2	5.5	7.0
$CaCl_2$	Ca-Cl(2)	4.5	3.8	5.4
	Ca-Cl(4)	5.4	5.0	5.9
$CaSO_4$	Ca-SO_4(2)	5.2	4.2	6.4
	Ca-SO_4(4)	6.1	5.6	6.6

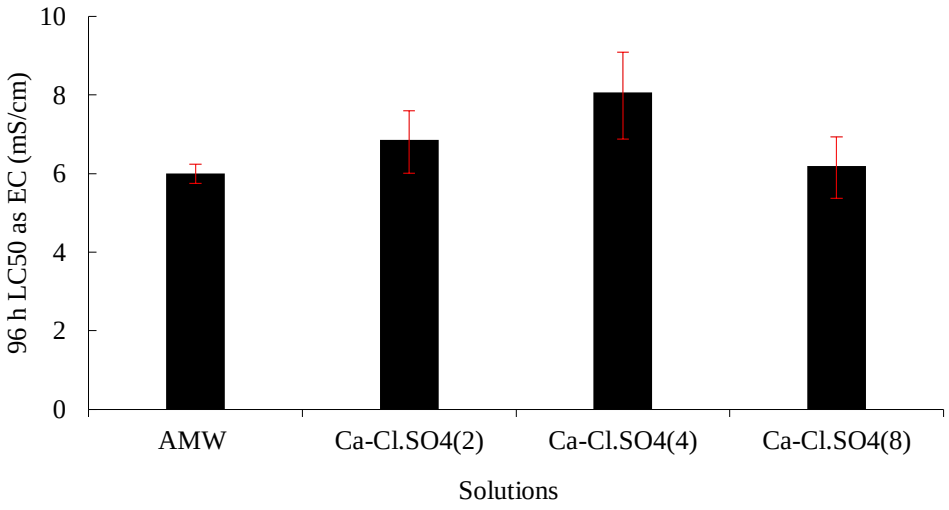

Figure 4.4: Comparison of 96 h LC50 as EC (mS/cm) between AMW, Ca-Cl.SO_4(2), Ca-Cl.SO_4(4) and Ca-Cl.SO_4(8); error bars represent 95 % confidence intervals.

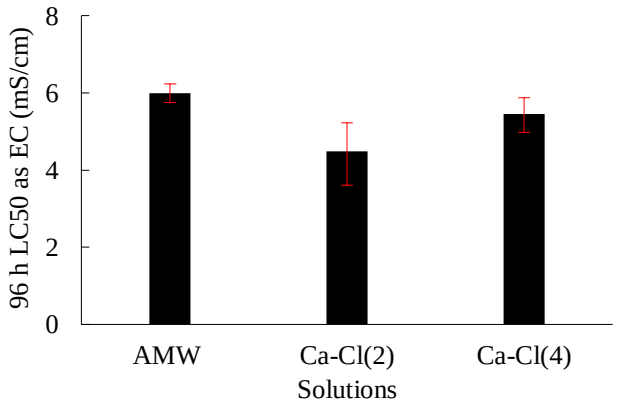

Figure 4.5: Comparison of 96 h LC50 as EC (mS/cm) between AMW, Ca-Cl(2) and Ca-Cl(4); error bars represent 95 % confidence intervals.

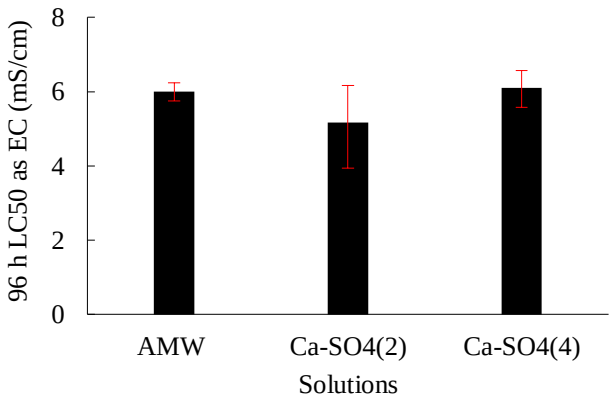

Figure 4.6: Comparison of 96 h LC50 as EC (mS/cm) between AMW, Ca-SO_4(2) and Ca-SO_4(4); error bars represent 95 % confidence intervals.

The data suggests there was decreased toxicity for solutions with increased calcium proportion Ca-Cl.SO_4(2), Ca-Cl.SO_4(4) and there was no observable difference in toxicity for the solution Ca-Cl.SO_4(8) when compared with AMW (Figure 4.4). There was increased toxicity for solution Ca-Cl(2), Ca-Cl(4) when compared to toxicity of AMW (Figure 4.5). There was increased toxicity for solution Ca-SO_4(2) and no difference in toxicity for solution Ca-SO_4(4) when compared to toxicity of AMW (Figure 4.6). The difference between toxicity of AMW and each solution with increased calcium proportion was further analysed for statistically significant differences.

4.4 Comparison of toxicity of AMW and solutions with increased calcium proportion

The toxicity of AMW was compared with the toxicity of each of the solutions with increased calcium proportion. The details of the comparison has been provided under separate headings for the solutions with increased calcium using calcium chloride and calcium sulfate together and separately. There were two distinct curves for each solution when there was a statistically significant difference, $p \leq 0.05$ and there was overlapping of the curves for both the solutions when there was no significant difference.

4.4.1 Comparison of the toxicity between AMW and solutions with increased calcium using calcium chloride and calcium sulfate together

Results of the *f* test for both 96 h LC50 and dose response curve showed significant differences between solutions Ca-Cl.SO_4(2) and Ca-Cl.SO_4(4) compared to AMW, but there was no significant difference for the solution Ca-Cl.SO_4(8) when compared with the toxicity of AMW (Table 4.6). Also, there were two distinct curves for both comparisons between 96 h LC50 and dose response curves for these solutions (Figure 4.7 & 4.8) that suggest there was significant difference between the toxicity of AMW and the solutions Ca-Cl.SO_4(2) and Ca-Cl.SO_4(4). The 96 h LC50 (Table 4.5) suggest that there was decreased toxicity for these solutions when compared with the toxicity of AMW. There was overlap of curves for both comparisons between 96 h LC50 and dose response curve for the solution Ca-Cl.SO_4(8) (Figure 4.9). These graphs and the results of the *f* test (Table 4.6) suggest that there was no significant difference between the toxicity of the solution Ca-Cl.SO_4(8) and AMW

Table 4.6: Results of *f* test, *p* value and statistical difference for comparison of 96 h LC50 value and dose response curves between AMW and Ca-Cl.SO_4(2), AMW and Ca-Cl.SO_4(4), AMW and Ca-Cl.SO_4(8). The numbers within brackets for *f* test represent degrees of freedom.

Comparison / Test results	AMW and Ca-Cl.SO_4(2)		AMW and Ca-Cl.SO_4(4)		AMW and Ca-Cl.SO_4(8)	
	96 h LC50	Dose response curve	96 h LC50	Dose response curve	96 h LC50	Dose response curve
***f* test**	5.5 (1, 38)	9.3 (3, 36)	16.8 (1, 38)	15.5 (3, 36)	0.3 (1, 38)	0.3 (3, 36)
***p* value**	0.02	0.0001	0.0002	< 0.0001	0.6	0.8
Significant difference	Yes	Yes	Yes	Yes	No	No

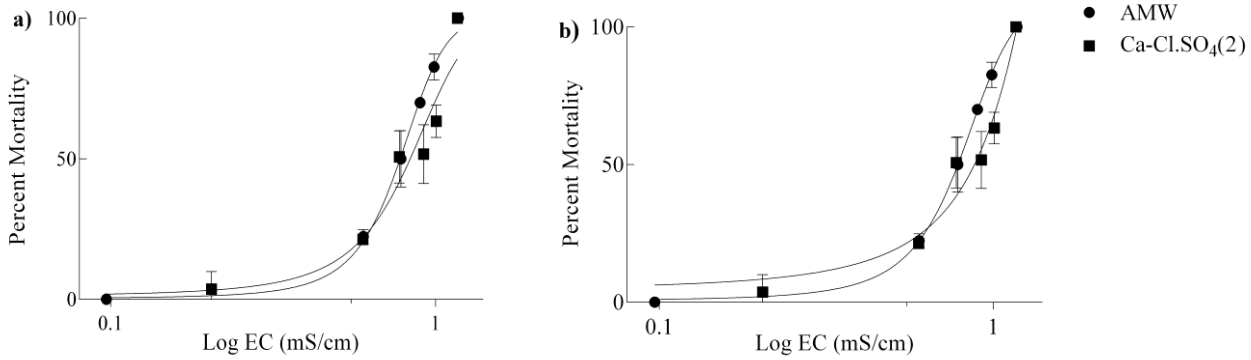

Figure 4.7: Comparison of toxicity between AMW and Ca-Cl.SO_4(2); a) single parameter log regression for the comparison of 96 h LC50 and b) four parameter log regression for comparison of dose response curves; the error bars represent standard deviation.

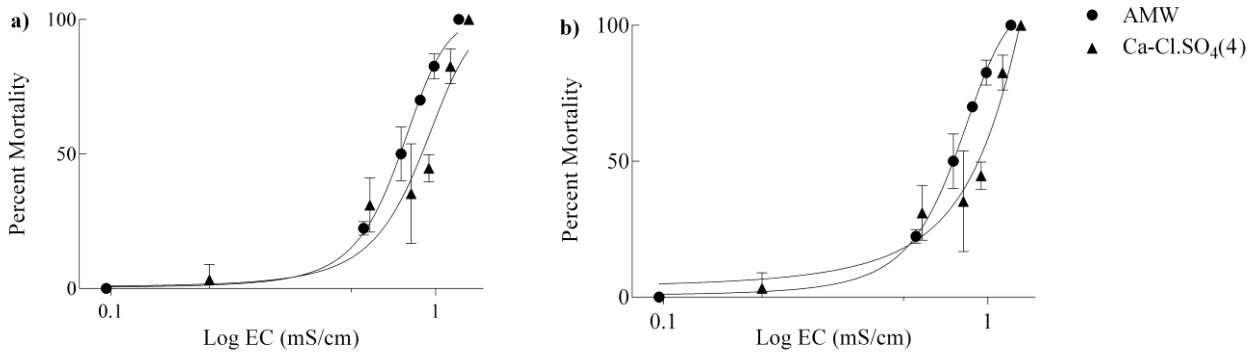

Figure 4.8: Comparison of toxicity between AMW and Ca-Cl.SO_4(4); a) single parameter log regression for the comparison of 96 h LC50 and b) four parameter log regression for comparison of dose response curves; the error bars represent standard deviation.

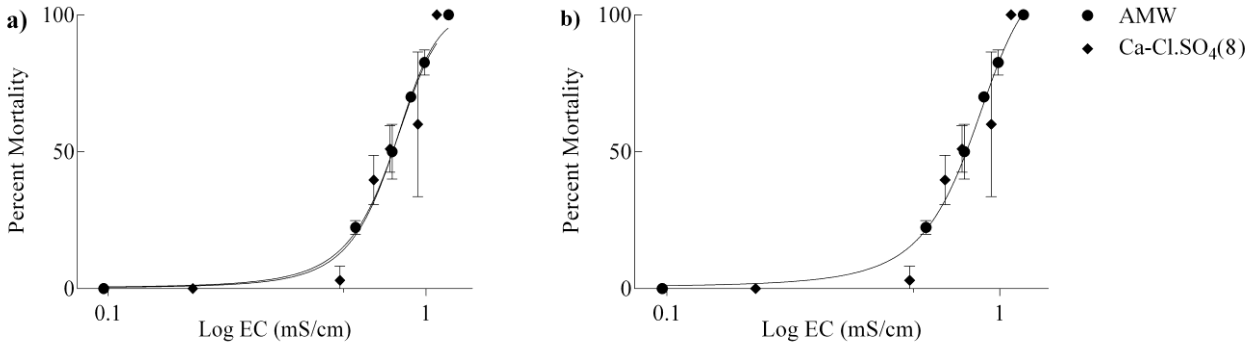

Figure 4.9: Comparison of toxicity between AMW and Ca-Cl.SO_4(8); a) single parameter log regression for the comparison of 96 h LC50 and b) four parameter log regression for comparison of dose response curves; the error bars represent the standard deviation.

4.4.2 Comparison of the toxicity between AMW and solutions with increased calcium using only calcium chloride

A significant difference was observed for the both 96 h LC50 and dose response curve for the solution, Ca-Cl(2) when compared with AMW (Table 4.7). There were two distinct curves for both 96 h LC50 and dose response curves for this solution (Figure 4.10 a, b) that suggests there was

significant difference between the toxicity of AMW and Ca-Cl(2). The 96 h LC50 (Table 4.4) suggest that there was increased toxicity for this solution when compared with the toxicity of AMW. This could be as a consequence of a decrease in the calcium concentration in the test compared with that expected in the solution (Appendix Table A.3). The solution Ca-Cl(4) showed a different response. While there was significant difference between the 96 h LC50 values, there was no significant difference between the dose response curves. The comparison of 96 h LC50 values showed two distinct curves (Figure 4.11 a) but comparison of the dose response curves showed overlap of the curves (Figure 4.11 b). The 96 h LC50 (Table 4.5) suggest there was a difference between the toxicity of AMW and Ca-Cl(4).

Table 4.7: Results of *f* test, *p* value and statistical significant difference for comparison of 96 h LC50 value and dose response curves between AMW Ca-Cl(2), AMW and Ca-Cl(4).

Comparison / Test results	AMW and Ca-Cl(2)		AMW and Ca-Cl(4)	
	96 h LC50	**Dose response curve**	**96 h LC50**	**Dose response curve**
***f* test**	14.5 (1, 38)	4.7 (3, 36)	5.0 (1, 38)	1.9 (3, 36)
***p* value**	0.0005	0.007	0.03	0.1
Significant difference	Yes	Yes	Yes	No

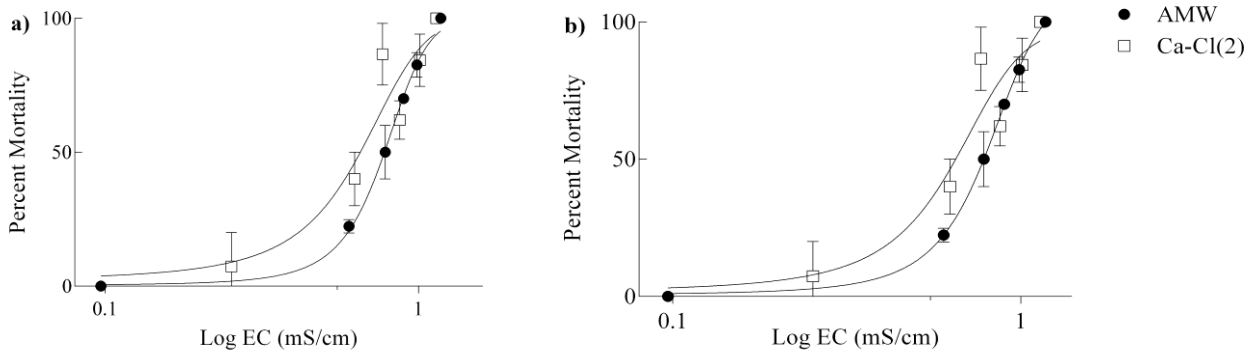

Figure 4.10: Comparison of toxicity between AMW and Ca-Cl(2) using calcium chloride; a) single parameter log regression for the comparison of 96 h LC50 and b) four parameter log regression for comparison of dose response curves; the error bars represent standard deviation.

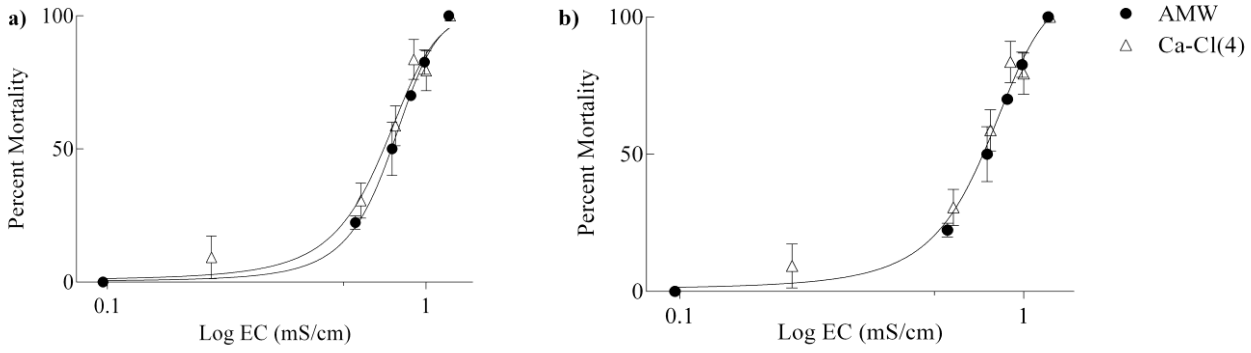

Figure 4.11: Comparison of toxicity between AMW and Ca-Cl(4); a) single parameter log regression for the comparison of 96 h LC50 and b) four parameter log regression for comparison of dose response curves; the error bars represent standard deviation.

4.4.3 Comparison of the toxicity between AMW and solutions with increased calcium using only calcium sulfate

Results of the *f* test for both 96 h LC50 and the dose response curve showed no significant difference between solutions Ca-SO_4(2), Ca-SO_4(4) and that of AMW (Table 4.8). Although there were two distinct curves for the solution Ca-SO_4(2) (Figure 4.12 a), there was an overlap for the major part of the curve which suggests there was no significant difference between the 96 h LC50 values of Ca-SO_4(2) and AMW (Figure 4.12 b). There was overlap of curves for both 96 h LC50 and dose response for solution Ca-SO_4(4) and AMW (Figure 4.13 a & b) which suggests there was no significant difference between the toxicity of Ca-SO_4(4) and AMW.

Table 4.8: Results of *f* test, *p* value and statistical significant difference for comparison of 96 h LC50 value and dose response curves between the toxicity of AMW and Ca-SO_4(2) and Ca-SO_4(4). The *f* ratio was used to calculate the *p* value which defines the significant difference.

Comparison / Test results	AMW and Ca-SO_4(2)		AMW and Ca-SO_4(4)	
	96 h LC50	**Dose response curve**	**96 h LC50**	**Dose response curve**
***f* test**	2.9 (1, 38)	2.8 (3, 36)	0.1 (1, 38)	0.2 (3, 36)
***p* value**	0.09	0.05	0.7	0.8
Significant difference	No	No	No	No

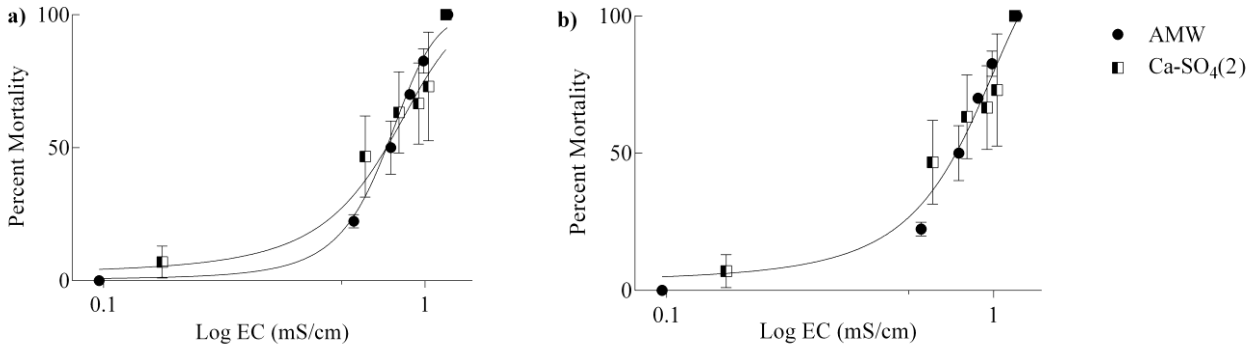

Figure 4.12: Comparison of toxicity between AMW and Ca-SO_4(2); a) single parameter log regression for the comparison of 96 h LC50 and b) four parameter log regression for comparison of dose response curves; the error bars represent standard deviation.

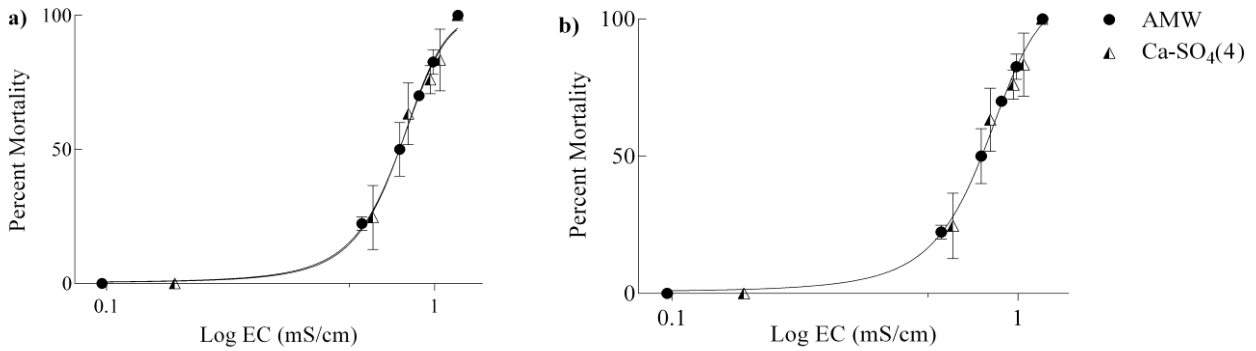

Figure 4.13: Comparison of toxicity between AMW and Ca-SO_4(4); a) single parameter log regression for the comparison of 96 h LC50 and b) four parameter log regression for comparison of dose response curves; the error bars represent standard deviation.

The ionic strength of the solutions was measured as EC and the results were represented in terms of EC. The solutions used in this study comprised of all major cations and anions. The calcium proportion was increased as a constant Ca:HCO_3 ratio. Since calcium was increased at equal proportion of HCO_3, the ratio of calcium to sodium varied between tests. Therefore, in addition to the discussion of the effect of calcium, toxicity of Ca:HCO_3 ratio and Ca:Na ratio are also discussed. As the solutions comprise of major cations and anions, the relation of the toxicity of individual ions to calcium concentration in each solution type is also presented. Additionally, toxicity is commonly interpreted in terms of concentrations and thus the data is also provided in terms of concentrations of ions.

4.4.4 Effect of calcium to bicarbonate ratio

As the calcium proportion was increased above that of AMW using a consistent Ca:HCO_3 ratio, the toxicity data was compared with Ca:HCO_3 ratio. Molar ratios were used to relate to the toxicity. The R^2 was 0.046, which suggests that increase in the Ca:HCO_3 ratio did not decrease in toxicity of the solutions. Looking at the results from Table 4.5, the solutions Ca-Cl.SO_4(2) and Ca-Cl.SO_4(4) had lower toxicity compared to AMW. The Ca:HCO_3 molar ratio was 1.1:1 for Ca-Cl.SO_4(2) and 2.5:1 for Ca-Cl.SO_4(4) compared to the Ca:HCO_3 ratio of 0.5:1 for AMW. On the other hand, the solution Ca-Cl.SO_4(8) showed no difference in toxicity compared to AMW with highest Ca:HCO_3 ratio of 8.8:1 (Figure 4.14).

The solutions Ca-Cl and Ca-SO_4 also showed a non-linear trend with R^2=0.015 and 0.17 respectively. The toxicity results from Table 4.5 suggest that the solution Ca-Cl(2) had higher toxicity than AMW. However, this solution had a lower calcium concentration than expected and the ratio was 0.4:1 similar to AMW. The solution Ca-Cl(4) showed increased toxicity that had a Ca:HCO_3 ratio of 3.6:1. Similarly, the toxicity results from Table 4.5 suggest that the solution Ca-SO_4(2) had higher toxicity compared to AMW and had a Ca:HCO_3 ratio of 1:1. The Ca-SO_4(4) solution also showed higher toxicity with a Ca:HCO_3 ratio of 2.9:1.

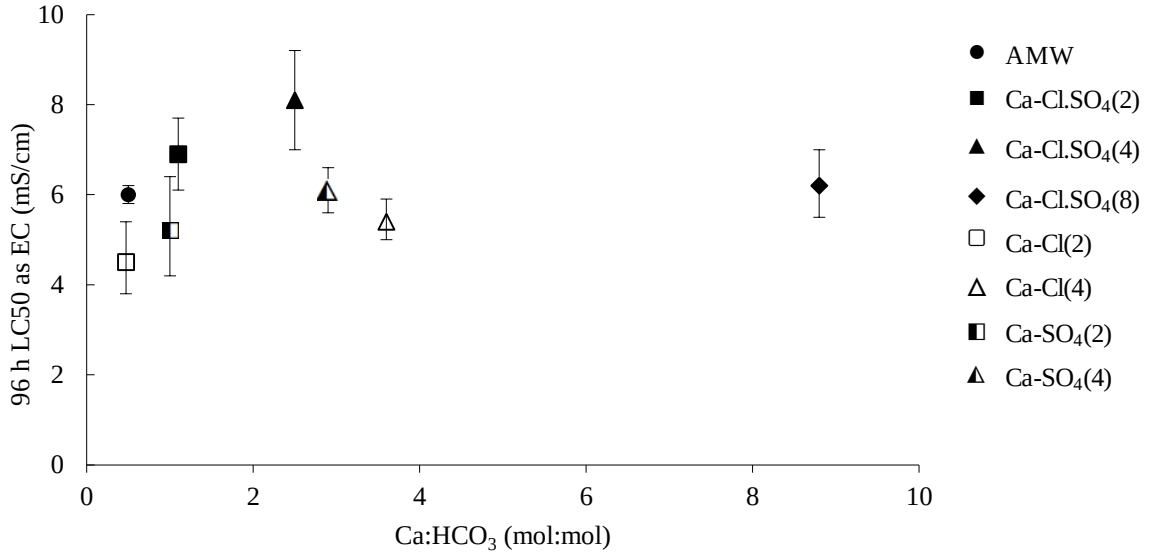

Figure 4.14: Comparison of the Ca:HCO_3 ratio against the toxicity of AMW, Ca-Cl.SO_4(2), Ca-Cl.SO_4(4) and Ca-Cl.SO_4(8); Ca-Cl(2) and Ca-Cl(4); Ca-SO_4(2) and Ca-SO_4(4); error bars represent 95 % confidence intervals.

The toxicity data of all the solutions were compared with their respective Ca:HCO_3 ratio. The comparison suggest that Ca-Cl.SO_4(2) and Ca-SO_4(2) have similar ratio but the toxicity of Ca-Cl.SO_4(2) is lower when compared to Ca-Cl.SO_4(2). Ca-Cl.SO_4(4) has lower Ca:HCO_3 ratio when compared to Ca-Cl(4) and Ca-SO_4(4) but has lower toxicity. Ca-Cl(4) has higher Ca:HCO_3 compared to Ca-SO_4(4) and has higher toxicity. This suggests that in addition to the Ca:HCO_3 ratio, the ratio of other ions in the solutions may contribute to the toxicity. The difference in toxicity observed to a greater extent appears to relate to the calcium concentration in the solutions.

4.4.5 Effect of calcium concentration and sodium concentration on toxicity of solution

Response of organisms as percent mortality against calcium concentration (Figure 4.15 a) indicate the shift in response with increased calcium concentration for solutions using calcium chloride and calcium sulfate together. In contrast, the percent mortality against sodium concentration (Figure 4.15 b) suggested Ca-Cl.SO_4(8) had lower sodium concentration but had a higher toxicity. There was no observable difference in response for solution Ca-Cl.SO_4(2) when compared to AMW but the solution Ca-Cl.SO_4(4) showed decreased toxicity. The Ca-Cl.SO_4(4) had a decreased sodium concentration compared with AMW and had a lower toxicity.

The percent mortality against calcium concentration for solutions Ca-Cl (Figure 4.16 a) and Ca-SO_4 (Figure 4.17 a) also showed similar pattern as observed for the solutions Ca-Cl.SO_4. There was not much difference in the calcium concentration for solution Ca-Cl(2), however, the solution Ca-Cl(4) had higher calcium concentration and the percent mortality shifted. The sodium concentration in solutions Ca-Cl(2) and Ca-Cl(4) (Figure 4.16 b) was lower and the toxicity appears to be higher.

The sodium concentration in solutions Ca-SO_4(2) and Ca-SO_4(4) (Figure 4.17 b) was similar and there was no difference in toxicity observed.

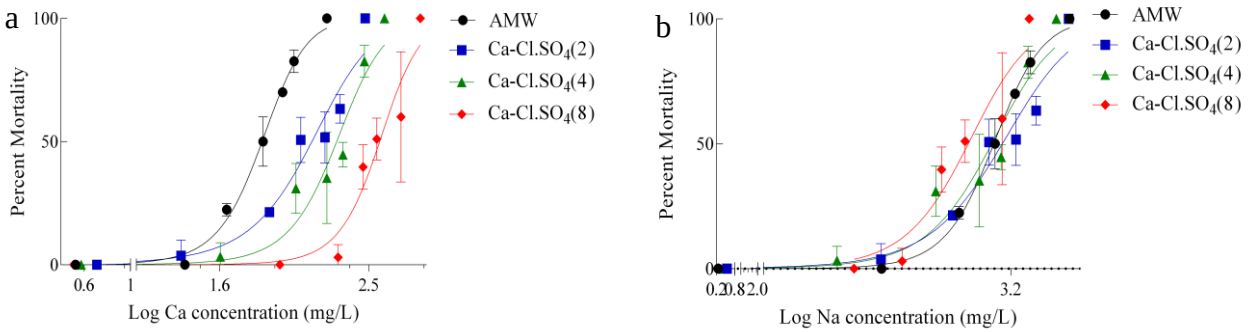

Figure 4.15: Comparison of the percent mortality of the solutions AMW, Ca-Cl.SO_4(2), Ca-Cl.SO_4(4) and Ca-Cl.SO_4(8) against a) calcium concentration (mg/L) and b) sodium concentration; error bars represent standard deviation.

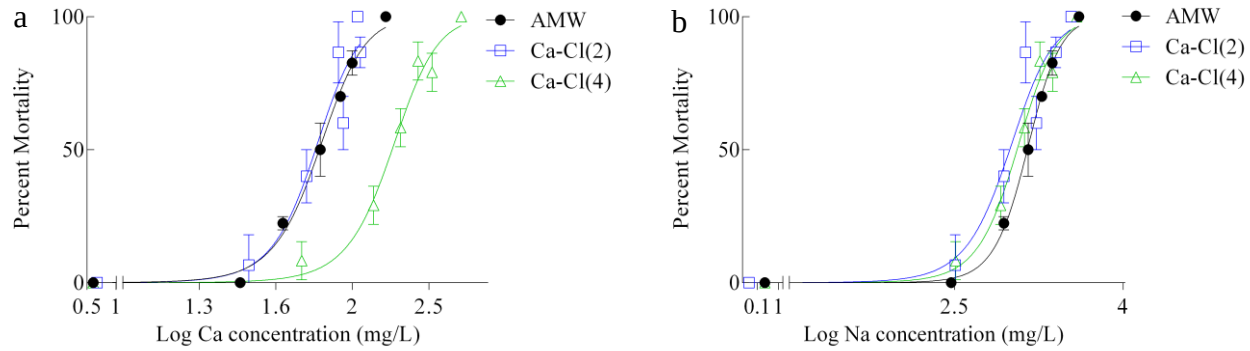

Figure 4.16: Comparison of the percent mortality of the solutions AMW, Ca-Cl(2) and Ca-Cl(4); against a) calcium concentration (mg/L) and b) sodium concentration; error bars represent standard deviation.

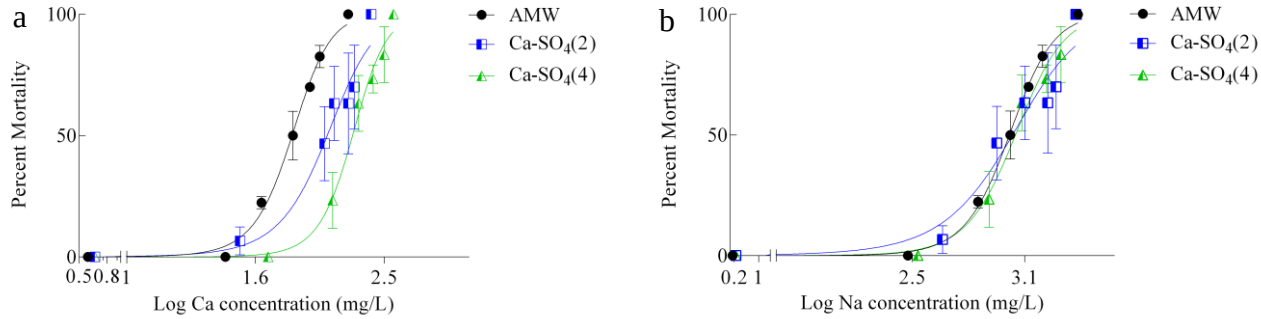

Figure 4.17: Comparison of the percent mortality of the solutions AMW, Ca-SO_4(2) and Ca-SO_4(4); against a) calcium concentration (mg/L) and b) sodium concentration; error bars represent standard deviation.

The toxicity data of all the solutions were also compared to the calcium concentration at 96 h LC50 value (Figure 4.18) and sodium concentration at 96 h LC50 value (Figure 4.19). The toxicity of Ca-Cl.SO_4(2), Ca-Cl.SO_4(4) was lower compared to AMW and had higher calcium concentration. However, the solution Ca-Cl.SO_4(8) had similar toxicity that had the highest calcium concentration. In contrast, Ca-Cl.SO_4(8) had a lower sodium concentration to AMW. Ca-Cl.SO_4(2) has slightly higher sodium concentration to AMW but had a lower toxicity. Solutions Ca-Cl.SO_4(4) had a decreased sodium concentration compared with AMW and had the least toxicity. Solutions Ca-Cl

had higher calcium concentration and showed higher toxicity than AMW. The sodium concentration in the solutions was lower than AMW. Solutions Ca-SO_4 too had higher calcium concentration and showed higher toxicity than AMW. The sodium concentration in the solutions was lower than AMW.

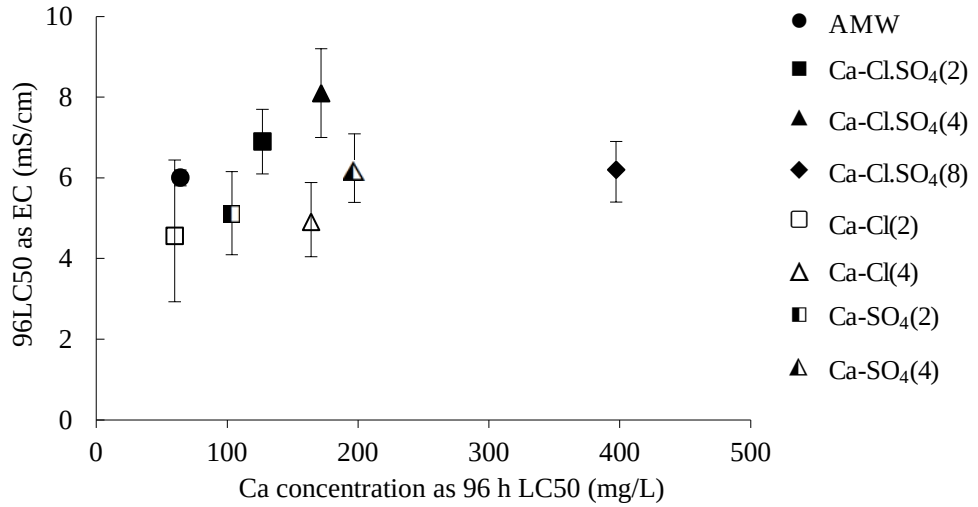

Figure 4.18: Comparison of the calcium concentration as 96 h LC50 (mg/L) against the toxicity of AMW, Ca-Cl.SO_4(2), Ca-Cl.SO_4(4) and Ca-Cl.SO_4(8); Ca-Cl(2) and Ca-Cl(4); Ca-SO_4(2) and Ca-SO_4(4); error bars represent 95 % confidence intervals.

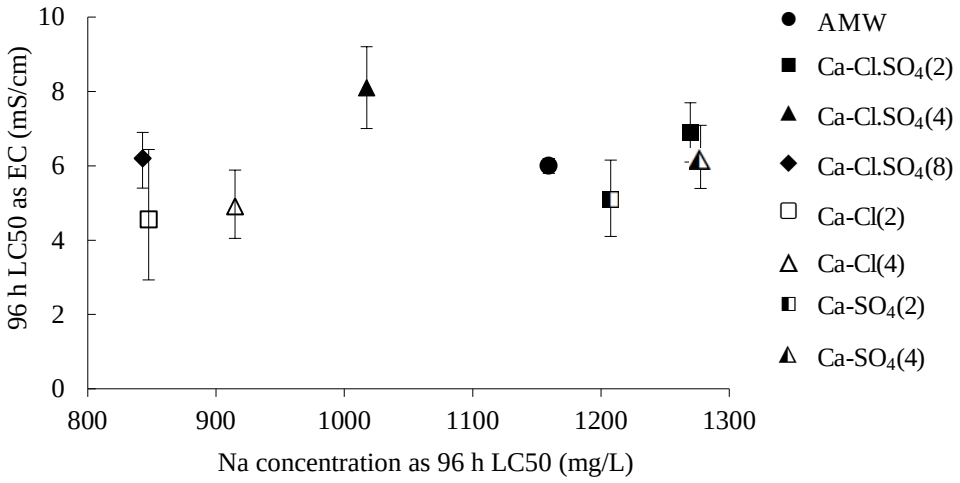

Figure 4.19: Comparison of the sodium concentration as 96 h LC50 (mg/L) against the toxicity of AMW, Ca-Cl.SO_4(2), Ca-Cl.SO_4(4) and Ca-Cl.SO_4(8); Ca-Cl(2) and Ca-Cl(4); Ca-SO_4(2) and Ca-SO_4(4); error bars represent 95 % confidence intervals.

4.4.6 Relationship of calcium concentration with individual ions

Since the toxicity of calcium was tested in the presence of all the major ions, the concentration of calcium was compared to the toxicity of individual ions. Although calcium concentration varied within each solution type, the calcium proportion remained consistent within each solution type. Because the calcium concentration varied within each solution type, calcium concentration at the

96 h LC50 value for each solution type was used to compare the toxicity of the individual ions for each solution.

The toxicity for calcium ion shows a linear decrease with a R^2=1. However, the toxicity for other cations and anions showed a non-linear regression from R^2=0.30 for sulfate to 0.84 for bicarbonate. Chloride ion showed a linear trend with R^2=0.98. As calcium concentration was increased, there was an increase of 96 h LC50 for calcium ion that suggested decreased toxicity (Figure 4.20 a). In contrast, for sodium ion there was decreased toxicity for solution Ca-Cl.SO_4(2), but increased toxicity for solutions Ca-Cl.SO_4(4) and Ca-Cl.SO_4(8). The 96 h LC50 of potassium was similar across all the solutions, but the 96 h LC50 of magnesium showed varying result among the solutions (Figure 4.20 b). The 96 h LC50 for magnesium was higher for solution Ca-Cl.SO_4(2) and Ca-Cl.SO_4(8), but lower for solution Ca-Cl.SO_4(4) which suggests toxicity of magnesium was higher in Ca-Cl.SO_4(4). The 96 h LC50 for bicarbonate was similar among all the solutions (Figure 4.20 c). The 96 h LC50 for both chloride and sulfate ions for the solutions Ca-Cl.SO_4(2) and Ca-Cl.SO_4(4) suggested a similar pattern, but there was variation for the solution Ca-Cl.SO_4(8). Ca-Cl.SO_4(2) had a higher 96 h LC50 value for both chloride and sulfate ions and a lower 96 h LC50 value for Ca-Cl.SO_4(4). However, for Ca-Cl.SO_4(8), the 96 h LC50 of chloride was higher than that of the 96 h LC50 of sulfate. In addition to the toxicity of individual ions, the toxicity of sum total of all the ions as total dissolved solids (TDS) was compared against calcium concentration (Figure 4.20 d). TDS was calculated using the equation in APHA (2005)[2]. The R^2 was low with a value of 0.0015 that suggested there was no decrease in toxicity as TDS with increased calcium concentration. However, the 96 h LC50 suggest, there was decreased toxicity for solution Ca-Cl.SO_4(2), but increased toxicity for solutions Ca-Cl.SO_4(4) and similar toxicity for Ca-Cl.SO_4(8) compared to AMW.

[2] TDS = 0.6 (alkalinity*)+Na^++K^++Ca^{2+}+Mg^{2+}+Cl-+SO_4^{2-}+SiO_3^{2-}+NO_3^-+F^-. Since this is a laboratory prepared solution, there is no silica, nitrate and fluoride in the calculation of TDS.

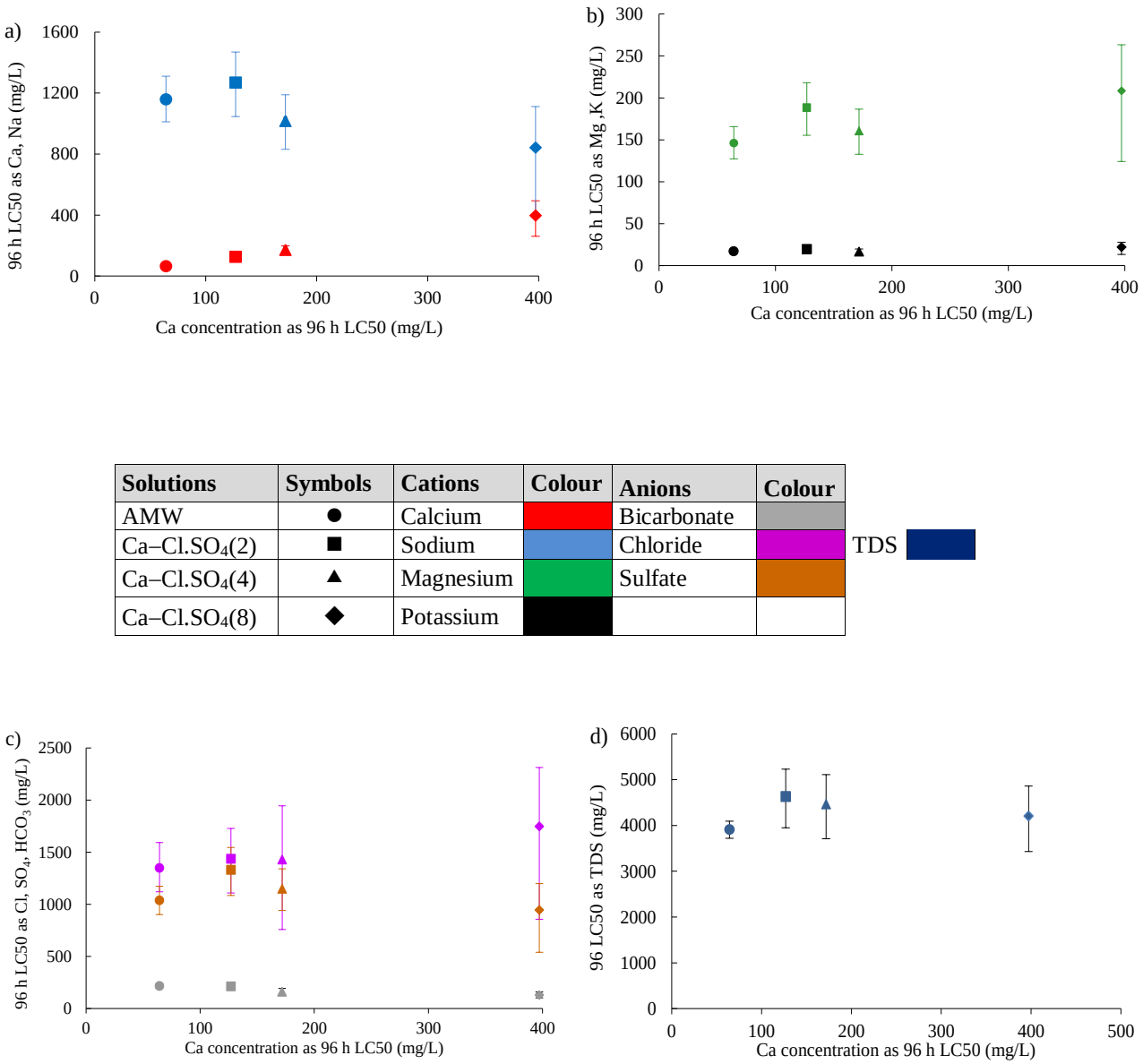

Figure 4.20: Relationship between calcium concentration as 96 h LC50 (mg/L) and a) 96 h LC50 (mg/L) of calcium and sodium, b) 96 h LC50 (mg/L) of potassium and magnesium, c) 96 h LC50 (mg/L) of bicarbonate, chloride and sulfate and d) Total dissolved solids (TDS)(mg/L) for AMW, Ca-Cl.SO$_4$(2), Ca-Cl.SO$_4$(4) and Ca-Cl.SO$_4$(8), error bars represent 95 % confidence intervals.

Although the 96 h LC50 of individual ions are compared with the calcium concentration, the response of the organisms cannot be attributed only to one particular ion. The toxicity observed is due to the collective concentrations of all the ions present in the solution. The response can be due to additive or antagonistic effects of different ions present in the solution.

Chapter 5 Discussion

5.1 Influence of increased calcium proportion on the toxicity of saline solution

Solutions with increased calcium proportion prepared using calcium chloride and calcium sulfate together showed difference in toxicity when compared with AMW. Ca-Cl.SO_4(2) and Ca-Cl.SO_4(4) showed decreased toxicity when compared to AMW and Ca-Cl.SO_4(8) had similar toxicity to AMW. Solutions Ca-Cl and Cl-SO_4 did not show the decreased toxicity as observed for the solutions Ca-Cl.SO_4. Although the solution Ca-Cl(2) showed a significant difference in toxicity when compared with AMW, there was increased toxicity. However, this solution had a lower calcium proportion than expected. Solution Ca-Cl(4) did not show a significant difference in toxicity from AMW. Solutions with increased calcium proportion using calcium sulfate suggested no observable change in toxicities compared with the toxicity of AMW.

The toxicity of the solutions from the present study was compared to the toxicity of the solutions from the studies of Dunlop et al. (2011) and Prasad et al. (2014). The comparison was made using calcium proportion in the solutions. The solutions from all these studies were based on the relative ionic proportion. In addition, the toxicity data from the present study were also compared to those studies in the literature and is discussed as calcium concentration.

5.1.1 Comparison of results from present study and from studies of Dunlop et al. (2011) and Prasad et al. (2014)

The relationship between toxicity data versus proportion of calcium in the solutions in the present study was similar to that observed in the previous studies from Dunlop et al. (2011) and Prasad et al. (2014). There was no consistent increase or decrease in toxicity with increase in proportion of calcium in the solutions (Figure 5.1). For instance, solutions AMW 2 and FC from the previous studies had similar toxicity as EC but had different calcium proportion. Similarly, the toxicity of the solutions AMW and Ca-Cl.SO_4(8) from the present study was similar but had different calcium proportion. In contrast, solutions AMW 1, MS, AMW and Ca-Cl(2) have similar calcium proportion but vary in toxicity. Solution AMW in the present study was the ionic composition of AMW 1 in the study of Prasad et. al. (2014) and therefore the calcium proportion is similar. A lack of consistent response between the solutions tested in the present study and those tested in the studies of Dunlop et al. (2011) and Prasad et al. (2014) may be attributed to the variation in the ratios of cations and anions. The ratios between the ions can influence the ion exchange across the gill membrane resulting in toxicity. An ion ratio of 3:2 of Na:K has been shown to influence the

activity of the Na-K-ATPase on the gills of a marine crustacean, *Mysidopsis bahia* (Towle DW 1993). The change in ratio of ions can modify such mechanisms and can cause toxicity.

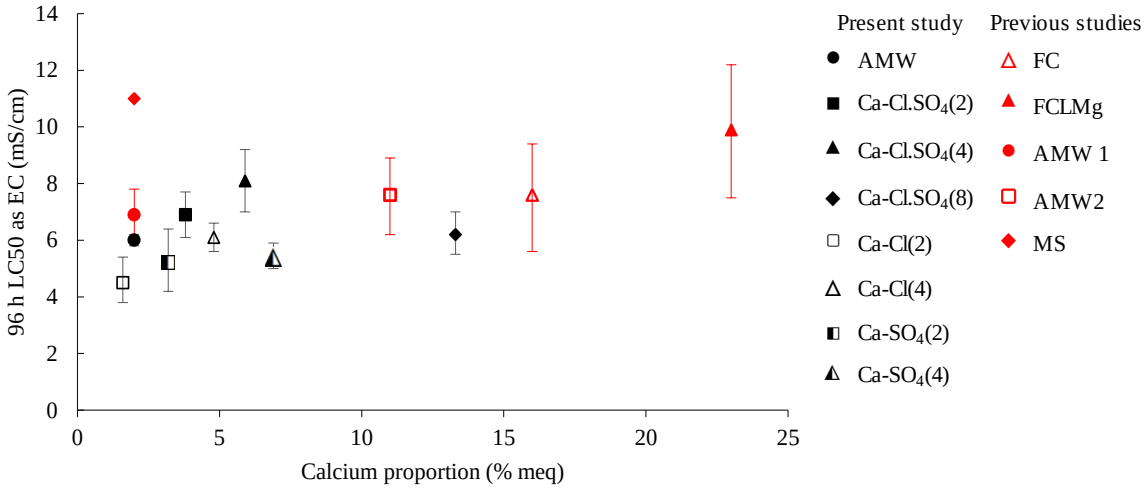

Figure 5.1: Comparison between calcium proportion (% meq) and the toxicity from present study (AMW, Ca-Cl.SO$_4$(2), Ca-Cl.SO$_4$(4) and Ca-Cl.SO$_4$(8)); data from the studies of Dunlop et al. 2011 (FC, FCLMg and MS) and Prasad et al. 2014 (AMW 1 and AMW 2); the error bars represent 95 % confidence intervals.

The comparison of toxicity data against Ca:HCO$_3$ (% meq) ratio from the present study did not agree with the studies of Dunlop et al. (2011) and Prasad et al. (2014) (Figure 5.2). While it was observed in the previous studies that increased ratio of Ca:HCO$_3$ would decrease the toxicity of test solutions as EC, such an effect was not found in the present study.

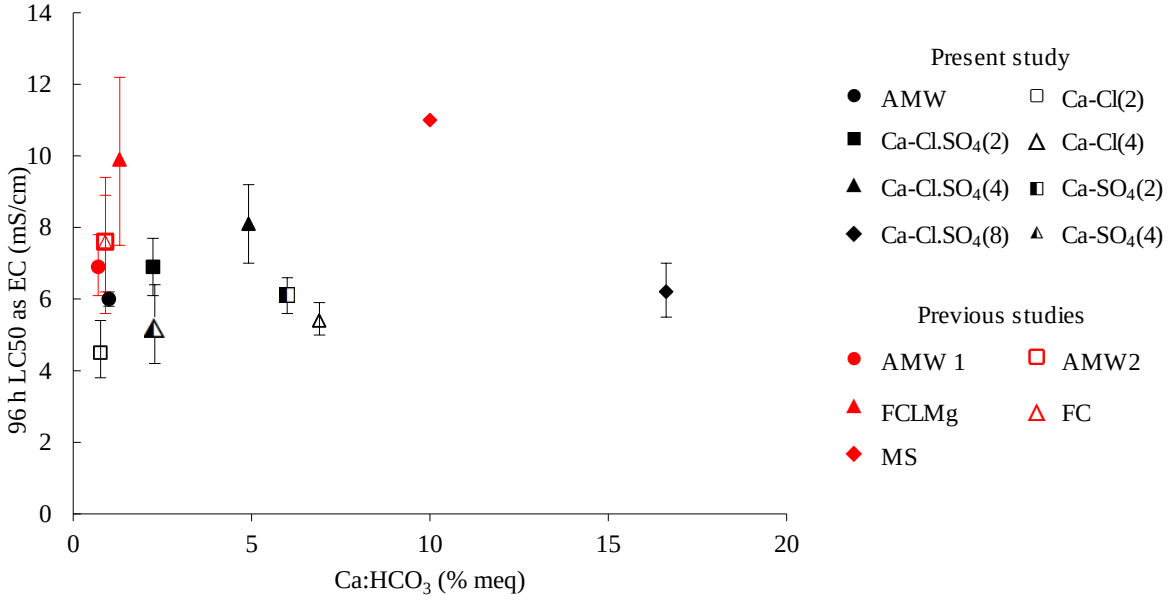

Figure 5.2: Comparison of Ca:HCO$_3$ meq ratio and the toxicity from present study (AMW, Ca-Cl.SO$_4$(2), Ca-Cl.SO$_4$(4) and Ca-Cl.SO$_4$(8)) and from the studies of Dunlop et al. 2011 (FC, FCLMg and MS) and Prasad et al. 2014 (AMW 1 and AMW 2); the error bars represent 95 % confidence intervals.

FC, AMW 2 and Ca-Cl.SO$_4$(4) that had similar toxicity did not have similar Ca:HCO$_3$. The ratio for FC and AMW 2 was similar whereas the ratio for Ca-Cl.SO$_4$(4) was higher than FC and AMW 2. Ca-Cl.SO$_4$(8) that had highest Ca:HCO$_3$ ratio did not show decreased toxicity to AMW.

MS that had lower Ca:HCO_3 ratio than Ca-Cl.SO_4(8) and a lower toxicity than Ca-Cl.SO_4(8). This suggests that while high Ca:HCO_3 ratio can decrease toxicity, a Ca:HCO_3 ratio greater than that of MS may not decrease the toxicity of a saline solution. This could be due to the effect of calcium concentration on membrane permeability for ions.

5.2 Comparison of anion ratio of the solutions in the present study

Increased calcium proportion using only calcium chloride and calcium sulfate did not show a significant difference in toxicity when compared to AMW as observed for solutions with calcium chloride and calcium sulfate together to *Austrophlebioides.* sp. AV11. The difference in toxicity observed was probably due to the change in the anion concentration in the solutions (Figure 5.3). Figure 5.3 suggests that the anion ratio varies when a particular anion is added. The anion ratio is often found to influence the ion exchange mechanisms in organisms. This could be the likely reason for the pattern of toxicity observed for solutions with increased calcium using calcium chloride and calcium sulfate separately.

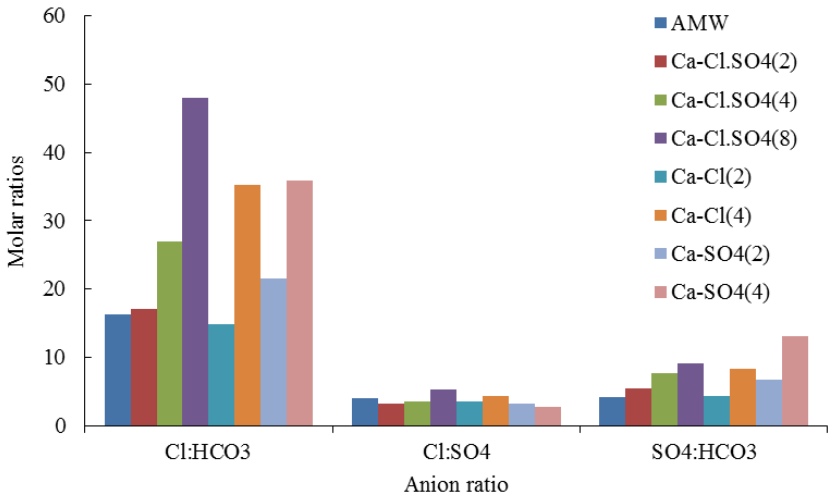

Figure 5.3 Comparison of molar ratio of anions for solutions AMW, Ca-Cl.SO_4(2), Ca-Cl.SO_4(4) , Ca-Cl.SO_4(8), Ca-Cl(2), Ca-Cl(4) , Ca-SO_4(2) and Ca-SO_4(4).

While Shaw (1960a) found that the anions associated with sodium did not have any difference on the influx of sodium ions in freshwater crayfish *A. papilles* (Shaw 1960a), the concentration of chloride and bicarbonate ions influenced the Cl/HCO_3 exchange across the epithelia of gills in the crab (Towle 1993). The gill epithelia in freshwater fish regulate the acid base balance; the change in the anion ratio can influence this mechanism (Perry et al. 2003). A Na:H ionic ratio of 2:1 is necessary for the exchange of Na^+/H^+ on the gills of crustaceans (Towle 1993). Thus when the concentration of one ion is increased, it alters the ratios of ions in the solutions and this can interfere in ion exchange mechanisms. The anion ratio could be the reason for the lack of ameliorative effect

observed for the solutions when calcium proportion was increased using calcium chloride and calcium sulfate separately.

5.3 Comparison of toxicity data from the present study with the literature

Although increased calcium concentration has been found to decrease toxicity in saline solutions, results from the present study and other studies have shown that high calcium concentrations do not show significant effect on toxicity. A recent study by Mount (Research Aquatic Biologist, Dulith, USEPA, pers. Comm., 2013) has shown that high concentrations of calcium did not have any significant effect on the toxicity of sodium chloride on *Ceriodaphnia dubia*. The 48 h LC50 for NaCl decreased beyond the calcium concentration of 7.5 mM (300 mg/L). The curve continued with a sharp downward bend to a calcium concentration of 20 mM (795 mg/L). Those results agree with the present study, the 96 h LC50 for Ca-Cl.SO_4(2) was 3 mM (127 mg Ca/L) and Ca-Cl.SO_4(4) was 4 mM (172 mg Ca/L) that showed decrease in toxicity to *A.* sp. AV11, whereas the 96 h LC50 for Ca-Cl.SO_4(8) was 10 mM (397 mg/L) and this solution showed highest toxicity. This suggests that calcium concentration beyond 7.5 mM (300 mg/L) has no ameliorative effect or may contribute to increased toxicity to organisms. However, solutions Ca-Cl and $CaSO_4$ showed a different toxicity pattern in relation to the calcium concentration. Solution Ca-Cl(2) had 96 h LC50 of 1 mM Ca and Ca-Cl(4) had 4 mM Ca. Solutions Ca-SO_4(2) had 96 h LC50 of 3mM and Ca-SO_4(4) had 4 mM Ca. Nevertheless, the toxicity of the Ca-Cl solutions was higher than that of AMW and the toxicity of solutions Ca-SO_4 were similar to AMW. The solution Ca-SO_4(4) and Ca-Cl(4) had similar 96 h LC50 calcium concentrations of 4 mM and both did not show significant difference in toxicity to AMW. This suggests calcium can have variable influences on the toxicity to organisms. Calcium concentration is known to increase or decrease the permeability of cell membranes to water and other ions in freshwater organisms (Cuthbert & Maetz 1972). A study by McWilliams & Potts (1978) in the brown trout, *Salmo trutta* found the transepithelial membrane potential (TEP) was negative in a calcium free media, but was shifted to positive with a calcium concentration of 2.0 mM (calcium added as nitrate salt); there was no significant change above this concentration. On the other hand, Potts & Fleming (1970) found there was a decrease in the rate constant of permeability to water across the gills in the freshwater fish, *Fundulus kansae* for a calcium concentration range between 0.1 to 10 mM. These studies suggest that concentration of calcium influences the permeability of the cell membrane in fish. In the present study, decreased toxicity was observed for solutions Ca-Cl.SO_4(2) and Ca-Cl.SO_4(4) compared to AMW and had a calcium concentration of 3.2 mM and 4.3 mM respectively. Whereas Ca-Cl.SO_4(8) that had a higher calcium concentration of 9.9 mM had similar toxicity to AMW. The range of calcium concentration from 1.6 to 4.3 mM could have an effect on the permeability of water across the gill membranes. There is

no clear evidence that mayfly and fish share similar osmoregulatory mechanisms. However, the histological structures of the epithelia of mayfly and fish are similar. Mayfly gills comprise of chloride secretory cells similar to those found in eels. These structures play an important role in osmoregulation (Wichard et al. 1972). There could possibly be a similar physiological process taking place on the gill surface of the mayfly species *Austrophlebioides* sp. AV11 used in the present study that resulted in decreased osmotic stress for Ca-Cl.SO_4(2) and Ca-Cl.SO_4(4).

In mammalian physiology, increase in free calcium in plasma or injection of calcium into nerve tissue is found to activate sodium and potassium channels across the membrane subsequently initiating the changes in cell behaviour (Matthews 1986). While the calcium ion has been found to play a vital role in cell membrane permeability and stability of the membrane, high concentrations of free calcium can cause lysis of the cell and cell death (Rubin et al. 1985).

The marked decrease in toxicity for the solution Ca-Cl.SO_4(4) may be due to the influence of the concentration of calcium on the influx of sodium. A study by Shaw (1960 b) of the freshwater crayfish *Astacus pallipes* found that there was no effect observed on sodium influx for calcium concentration lower than 1 mM but there was a reduction in sodium influx of about 60 % of the initial rate at a calcium concentration of approximately 2 mM. However, the study did not show consistent results for the change in influx of sodium ions for the calcium concentration range of < 1 mM to 5 mM used. The occasional effect of calcium ion was probably because calcium concentrations were sufficient to block the transporting sites. Increase of calcium concentration can have similar effect on the influx of sodium in *A.* sp. AV11. However, the response may not be consistent between different calcium concentrations.

Furthermore, in a freshwater amphipod, *Gammarus pulex*, calcium concentration in a range between 2–10 mM decreased the influx of sodium ions. However, in a marine amphipod, *Gammarus zaddachi*, sodium influx increased for the calcium concentration range between 2−4 mM, the influx was inconsistent for the range between 7–10 mM and the influx decreased between the range of 20–53 mM (Sutcliffe 1971 a). This suggests that apparently, calcium concentration between 2–10 mM likely influences sodium influx in both freshwater and marine organisms. The 96 h LC50 for calcium for the solutions Ca-Cl.SO_4(2) and Ca-Cl.SO_4(4) 96 h LC50 were 3.2 mM and 4.3 mM respectively. Probably these are the concentrations that could have influence on the sodium influx in *A.* sp. AV11. This could be the probable reason for the decreased toxicity observed for the solutions Ca-Cl.SO_4(2) and Ca-Cl.SO_4(4). The 96 h LC50 for calcium for Ca-Cl.SO_4(8) was 9.9 mM and apparently showed no difference in toxicity for the increase in calcium. Literature evidence suggest that calcium concentrations in the range 2–10 mM increase the

influx of sodium in amphipods, while higher concentrations (ca. 20–50 mM) result in reduced influx of sodium. Additionally, an increase in sodium influx results in a reduced calcium toxicity. In the experiments reported here, the Ca-Cl.SO_4(2) and Ca-Cl.SO_4(4) solutions had calcium concentrations in the range 7–10 mM and a reduced toxicity compared with AMW, while the Ca-Cl.SO_4(8) solution had a calcium concentration around 22 mM and a toxicity comparable to AMW.

5.3.1 Influence of calcium to the ratios of other ions on the toxicity of solutions

Although the hypothesis of the present study was that increased calcium would decrease the toxicity of saline solution, such an effect was not observed for solution Ca-Cl.SO_4(8). The observed toxicity pattern could be due to the proportion of other ions. With increased calcium proportion in solutions in the present study, there was decreased sodium proportion (Figure 4.1). Solution with high calcium proportion Ca-Cl.SO_4(8) had lower sodium proportion. For the comparison of response of the mayfly, the responses are explained against calcium concentrations and sodium concentrations for each solution AMW, Ca-Cl.SO_4(2), Ca-Cl.SO_4(4) and Ca-Cl.SO_4(8) (Figure 4.17 to 4.20). When calcium concentration is increased, it likely alters the ratios of other ions.

In addition to the increased calcium concentration, the Ca:Na ratio may also affect the response of the organism. Goodfellow et al. (2000) found toxicity decreased for a solution with Ca:Na mass ratio of 15:1 compared to a solution with a mass ratio of 1:20 on the test organisms. In this present study, there was decreased toxicity for Ca-Cl.SO_4(2) and Ca-Cl.SO_4(4) that had a Ca:Na mass ratio of 1:11 and 1:6 respectively when compared to the toxicity of AMW that had Ca:Na ratio of 1:20. This suggests when calcium concentration is increased, it alters the Ca:Na ratio. The increased Ca:Na molar ratio in Ca-Cl.SO_4(8) may be a contributing factor for the toxicity observed.

In addition to the calcium concentration, the ratio of Ca:Na also appears to influence the sodium influx in aquatic organisms. Sutcliffe (1971a) suggested a decrease in the influx of sodium in an amphipod, *Gammarus pulex* at a Ca:Na molar ratio of 47:1 whereas (Shaw 1960b) suggested there was no change in the influx of sodium at a Ca:Na ratio of 100:1 in crayfish *A. papilles.* This suggests that when calcium concentration is increased, it also alters the ratio of other ions and that could probably influence the osmoregulation in the aquatic organisms. These studies have suggested that influence of Ca:Na ratio on sodium influx requires further investigation. A similar mechanism can probably influence the sodium influx in mayflies.

Although the range of Ca:Mg ratio in the present study and in the study of Davies & Hall (2007) was similar, the response observed in both these studies differed. In the study of Davies & Hall

(2007), as the Ca:Mg ratio increased, there was a linear decrease in toxicity of sulfate for both species. In the present study, while the toxicity as EC of solutions Ca-Cl.SO_4(2) and Ca-Cl.SO_4(4) decreased, there was no change in toxicity of solution Ca-Cl.SO_4(8) on *Austrophlebioides* sp. AV11. This could be due to the difference in the concentrations of calcium used in both of these studies. The study of Davies & Hall (2007) increased calcium concentration as Ca:Mg molar ratio and found decreased toxicity of sulfate to *Hyallela azteca* and *Daphnia magna*. The study used calcium concentrations in the range of 0.4–0.9 mM (17 to 36 mg/L) for *H. azteca* and 0.4–0.9 mM (17 to 37 mg/L) for *D. magna*, and a Ca:Mg molar ratio between 0.7 to 7.0. In the present study calcium concentration used was in the range of 4–22 mM (161 to 879 mg/L) and Ca:Mg molar ratio was between 0.2 and 1.2. Additionally, the study of Davies & Hall (2007) used single salts; and calcium sulfate and magnesium sulfate salts were used to modify the Ca:Mg ratio, whereas in the present study, it was a complex mixture of all the ions. There may be an interaction of all the ions in the solution that have contributed to the different pattern of toxicity observed in the present study when compared with the study of Davies & Hall (2007). Also Davies & Hall (2007) used laboratory cultured organisms that could have different response to the solutions used in the present study.

5.4 Conclusion

This study found that increased calcium proportion decreased toxicity of the saline solution by using calcium chloride and calcium sulfate together whereas using calcium chloride and calcium sulfate separately did not decrease the toxicity of the saline solution. Although increased calcium proportion was found to decrease the toxicity, there was no effect observed for increased calcium proportion above a threshold level. There was decreased toxicity for Ca-Cl.SO_4(2) and Ca-Cl.SO_4(4) with 96 h LC50 of 6.9 mS/cm and 96 h LC50 of 8.1 mS/cm respectively when compared to AMW with 96 h LC50 of 6.0 mS/cm for AMW, the toxicity was similar for Ca-Cl.SO_4(8) with 96 h LC50 of 6.2 mS/cm compared to AMW. The calcium proportion for Ca-Cl.SO_4(2) was 3.8 % meq, for Ca-Cl.SO_4 (4) it was 5.9 % meq and for AMW it was 2.0 % meq. However, Ca-Cl.SO_4(8) had the highest calcium proportion of 13.3 % meq but showed similar toxicity to AMW. The concentration of calcium at 96 h LC50 values for the solutions were: AMW – 1.6 mM, Ca-Cl.SO_4(2) − 3 mM, Ca-Cl.SO_4(4) − 4 mM and Ca-Cl.SO_4(8) − 10 mM. These results suggest that increased calcium proportion of 11.3 % above that of the AMW composition do not likely decrease the toxicity of the saline solution to *Austrophlebioides* sp. AV11. While the calcium concentration of 3 mM and 4 mM likely decreased toxicity, calcium concentration of 10 mM did not show effect on toxicity. So it is necessary to consider the proportion of calcium of ions as it defines the ionic composition and the calcium concentration to evaluate the toxicity of a saline

discharge. Calcium increased in lower increments is likely to have an ameliorative effect on a complex saline solution to *Austrophlebioides* sp. AV11 but not in higher increments. The solutions with increased calcium proportion using calcium chloride and calcium sulfate separately showed higher toxicity and this could be due to the ratio of anions. Anion ratio is known to influence the acid base balance and also the ion exchange mechanisms across the cell membrane. This suggests using a balanced anion ratio in addition to calcium would be necessary to evaluate the toxicity of solution.

Calcium plays a vital role in physiological mechanisms such as exchange of ions across the cell membrane and influences the sodium pump. This indicates that high calcium concentrations may interfere in the ion exchange mechanism across the cell membrane that can result in toxicity. Although calcium is found to decrease toxicity, the concentration of calcium needs to be considered for its effect. This needs further investigation as it can differ among various organisms other than the one used in the present study. Additionally increase of calcium concentrations can likely alter the ratios of other ions that play a vital role in osmoregulation.

Whilst salinity can cause toxic effect on organisms, it is well known that ionic composition plays an important role to define the toxicity of a solution. Many studies have used single salts or double salts or marine salt mixtures to evaluate the toxicity of ions on organisms. Experiments on saline solutions with single salts or double salts are also beneficial to understand the toxicity of individual ions, the studies on marine salts are beneficial to understand the toxicity of saline solutions of major ion composition; but freshwater ecosystems comprise of all major ions and widely differ in ionic composition (ANZECC 2000). Additionally, some industrial discharges can vary in ionic composition that can result in changes to the ionic composition of the receiving water. The mine water composition used in the present study was a specific ionic composition and it is recognised that the toxicity of calcium evaluated can differ for other ionic compositions. As the saline discharges from anthropogenic activities can vary in ionic composition, it will be useful to understand the effect of calcium for different ionic compositions.

Current trigger values for salinity in ANZECC (2000) are specified as EC which limits and does not account for effect of ionic compositions. As EC is an integrative measure of ions present in solution there is uncertainty associated with its application to scenarios with varying ionic compositions. The findings of this thesis clearly indicate that the ionic composition with varying calcium proportion can influence the toxicity to mayfly. Therefore it is essential to consider the calcium proportion of ions in the derivation of discharge criteria or a guideline value.

It was necessary to evaluate the influence of calcium on the toxicity of the ionic composition of mine water to the locally relevant taxa, Ephemeroptera. Ephemeroptera is one of the indicators used to assess the water quality of a freshwater stream. Thus the response of Ephemeroptera to the ionic compositions evaluated in the present study can assist to assess water quality. However, the other insects groups such as Plecoptera and Trichoptera are also sensitive and are used in the assessment of water quality of fresh water streams (Chessman 2003). As calcium may influence organisms differently, it may be necessary to evaluate its effect on these organisms to gain an understanding of calcium influence on invertebrates more broadly.

It is likely when calcium concentration is increased it can alter the ratios of the other ions such as sodium. This has to be taken into account to evaluate the toxicity of saline solutions. As observed in this study in addition to the knowledge on the effect of increased calcium concentration on the toxicity of the solutions, it will be interesting for further research to test the effect of Ca:Na ratio on the toxicity of saline solutions on aquatic organisms.

5.5 Further research

Calcium was increased as calcium proportion using a particular ionic composition in the present study. Increased calcium proportion on the basis of other ionic compositions can result in different toxicity. It would be interesting to evaluate whether calcium has similar toxicity pattern or differs depending on the ionic proportion of other ions. Further research on increased calcium proportions based on different ionic composition will be worth investigating.

This research was focussed on environmentally relevant organisms. Laboratory cultured standard organisms are extensively used for toxicity tests in the evaluation of effluent toxicity. Further investigation of the influence of calcium on these organisms will be useful to assess the sensitivity of the standard organisms.

In addition to Ephemeroptera, Plecoptera and Trichoptera are two other insect groups that are used as bioindicators. The calcium proportion of the ionic compositions used in this study could have varying influences on these organisms. Further research on the influence of calcium on these organisms can assist in the assessment of water quality of freshwater ecosystems.

When calcium is increased, it alters the ratio of other ions. Increase of calcium can alter the Ca:Na ratio and affect freshwater organisms. Toxicity studies on varying the Ca:Na ratio needs further research. Investigation of the Ca:Na ratio on the toxicity of freshwater organisms can assist in

assessing the water quality for ionic compositions with varying Ca:Na ratio. Additionally the toxicity studies on varying ratios of calcium to other cations and anions needs to be investigated.

As calcium influences sodium influx, research on the physiology of the calcium on Ephemeroptera or freshwater organisms need to be further investigated. While there is limited knowledge on the physiology of the effect of mayfly larvae, studies on the physiology of calcium in Ephemeroptera will be useful. This could also help in understanding the mechanism that causes toxicity for Ephemeroptera.

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Appendix

Table A.1:Report of DO, temperature, EC and pH in the field at the time of collection of *Austrophlebioides* sp. AV11

Test ID	Collection Date	DO mg/L	Temperature ºC	EC µS/cm	pH
MB1	25/03/2013	8.6	21.4	105.4	8.11
MB2	15/04/2013	8.7	21.1	92.7	7.84
MB3	20/05/2013	9.3	12.9	55.3	7.87
MB4	05/06/2013	9.9	14.2	60.0	8.16
MB5	24/06/2013	9.84	13.5	Not recorded	Not recorded
MB6	05/08/2013	10.6	12.9	73.7	7.37
MB7	04/09/2013	9.1	18.1	95.6	8.54
MB8	23/09/2013	8.6	20.6	100.1	7.97
MB9	08/10/2013	8.3	22.6	106.3	8.14

Table A.2: Chemistry of major cations and anions of the stream water

Test ID	Start Date	Ca	K	Mg	Na	SO_4	Cl	HCO_3
		mg / L						
MB1	26/03/2013	3.1	0.5	1.7	2.6	6.4	4.9	13.0
MB2	15/04/2013	3.0	0.4	1.6	1.8	1.8	9.3	Not analysed
MB3	21/05/2013	4.1	0.9	2.3	2.2	3.0	5.8	56.0
MB4	06/06/2013	4.3	0.6	2.4	1.1	2.5	7.0	62.9
MB5	25/06/2013	3.7	0.8	2.0	0.0	2.9	6.0	45.3
MB7	05/09/2013	4.5	0.8	2.5	1.9	3.1	7.0	42.1
MB8	24/09/2013	3.8	0.9	2.3	1.7	2.7	6.0	69.5
MB9	08/10/2013	5.2	1.0	2.9	2.8	2.9	7.0	59.8

Table A.3 a: Calculated and measured proportions of ions in the solutions

Solution ID	Calculated (mg/L)							Measured (mg/L)						
	Ca	Mg	Na	K	HCO_3	Cl	SO_4	Ca	Mg	Na	K	HCO_3	Cl	SO_4
AMW	2.9	7.4	39.2	0.4	3.4	29.5	17.4	2.0	8.7	37.1	0.5	2.0	32.9	16.7
Ca-Cl.SO_4(2)	4.8	8.3	36.3	0.4	3.4	28.3	18.5	3.8	9.7	35.7	0.5	1.7	29.7	18.8
Ca-Cl.SO_4(4)	7.6	8.0	33.8	0.4	3.5	28.3	18.4	5.9	9.3	32.7	0.4	1.2	32.2	18.3
Ca-Cl.SO_4(8)	21.5	7.9	20.1	0.4	3.5	26.9	19.8	13.3	11.3	24.6	0.5	0.8	36.0	13.6
Ca-Cl(2)	3.7	8.7	37.1	0.4	3.8	28.2	18.2	1.5	10.2	35.9	0.5	2.1	31.5	18.2
Ca-Cl(4)	8.3	7.7	33.5	0.3	3.1	31.5	15.6	6.9	9.1	32.7	0.4	1.0	33.9	16.0
Ca-SO_4(2)	5.2	8.3	35.8	0.5	3.7	33.3	13.3	3.2	8.4	36.4	0.5	1.4	30.7	19.3
Ca-SO_4(4)	8.0	7.8	33.6	0.4	3.5	31.2	15.5	4.8	8.0	35.0	0.4	0.8	29.4	21.6

Table A.3 b: Calculated and measured concentrations of ions in the solutions

Solution ID	Calculated (mg/L)							Measured (mg/L)						
	Ca	Mg	Na	K	HCO_3	Cl	SO_4	Ca	Mg	Na	K	HCO_3	Cl	SO_4
AMW	245	372	3802	62.8	871	4412	3515	161	413	3362	53.2	487	4593	3160
Ca-Cl.SO_4(2)	412	426	3558	62.8	871	4284	3793	304	471	3309	55.6	429	4242	3638
Ca-Cl.SO_4(4)	678	426	3448	62.8	944	4458	3905	433	411	2758	48	268	4195	3227
Ca-Cl.SO_4(8)	1976	437	2120	62.8	980	4383	4374	879	448	1871	51.5	152	4237	2157
Ca-Cl(2)	299	426	3486	62.8	944	4089	3578	106	420	2834	48.5	444	3834	2998
Ca-Cl(4)	762	426	3553	62.8	871	5153	3448	588	467	3214	56.2	251	5149	3275
Ca-SO_4(2)	372	354	2924	62.8	799	4198	2264	249	393	3257	53.9	339	4244	3611
Ca-SO_4(4)	604	354	2924	62.8	799	4198	2823	394	396	3305	54.3	205	4282	4249

Table A.4: Chemical analyses of the test solutions used in 96 h acute toxicity tests

Test ID	Solution ID	Sample ID	Nominal EC (mS/cm)	EC (mS/cm)	Ca (mg/L)	K (mg/L)	Mg (mg/L)	Na (mg/L)	SO_4 (mg/L)	Cl (mg/L)	HCO_3 (mg/L)
MB8	AMW	ETCa_156	Control	0.1	4	1	2	2	3	6	69
		ETCa_157	1	1.3	27	5	37	308	310	151	82
		ETCa_158	3.5	4.0	42	11	94	748	645	764	145
		ETCa_159	5.5	6.1	65	17	147	1176	1050	1393	211
		ETCa_160	7.5	7.9	84	23	198	1538	1385	1930	290
		ETCa_161	9.5	9.7	99	28	243	1903	1765	2732	320
		ETCa_162	15.0	15.1	161	53	413	3362	3160	4593	487
MB9	Ca-Cl.SO_4(2)	ETCa_171	Control	0.1	5	1	3	3	3	7	60
		ETCa_172	1.2	1.6	37	5	46	306	322	191	85
		ETCa_173	3.5	4.0	71	10	103	692	695	685	125
		ETCa_174	5.5	5.9	108	17	163	1091	1113	1276	191
		ETCa_175	7.5	8.3	156	25	234	1558	1657	1918	256
		ETCa_176	10.0	10.1	199	33	307	2064	2220	2623	285
		ETCa_177	14.7	14.7	304	56	471	3309	3638	4242	429
MB3	Ca-Cl.SO_4(4)	ETCa_108	Control	0.1	4	1	2	2	3	6	56
		ETCa_109	1.2	1.6	39	3	33	196	234	290	75
		ETCa_110	3.5	4.2	101	9	91	565	632	715	113
		ETCa_111	6.0	6.9	161	16	155	962	1076	1269	153
		ETCa_112	8.0	9.0	208	21	195	1276	1420	1896	187
		ETCa_113	12.0	12.9	300	31	288	1840	2087	2823	216
		ETCa_114	16.0	18.5	433	48	411	2758	3227	4195	268
MB5	Ca-Cl.SO_4(8)	ETCa_122	Control	0.1	4	1	2	0	3	6	45
		ETCa_123	1.2	1.5	81	5	55	232	271	245	55
		ETCa_124	3.0	3.4	193	10	102	385	436	774	92
		ETCa_125	4.5	4.8	294	16	152	604	669	1182	114
		ETCa_126	6.0	5.9	375	21	193	805	875	1629	128
		ETCa_127	10.0	8.8	593	34	321	1295	1482	2915	151
		ETCa_128	14.0	12.1	879	52	449	1871	2157	4237	152
MB4	Ca-Cl(2)	ETCa_115	Control	0.1	4	1	2	1	3	7	63
		ETCa_116	1.5	1.8	29	5	51	329	342	239	84
		ETCa_117	4.0	4.2	55	12	115	748	746	757	184
		ETCa_118	6.0	5.9	82	18	172	1119	1104	1211	210
		ETCa_119	8.0	7.4	87	22	219	1381	1445	1719	292
		ETCa_120	12.0	10.2	111	34	318	2055	2105	2782	308
		ETCa_121	16.0	13.8	106	49	420	2834	2998	3834	444

Table A.4: Chemical analyses of the test solutions used in 96 h acute toxicity tests (cont'd)

Test ID	Solution ID	Sample ID	Nominal EC (mS/cm)	EC (mS/cm)	Ca (mg/L)	K (mg/L)	Mg (mg/L)	Na (mg/L)	SO_4 (mg/L)	Cl (mg/L)	HCO_3 (mg/L)
MB8	Ca-Cl(4)	ETCa_156	Control	0.1	4	1	2	2	3	6	69
		ETCa_163	1.2	1.6	52	5	48	331	320	230	54
		ETCa_164	3.5	4.2	134	11	104	706	654	902	79
		ETCa_165	5.5	6.3	203	17	162	1095	1034	1643	148
		ETCa_166	7.5	8.2	270	23	217	1483	1419	2388	176
		ETCa_167	10.0	10.0	343	31	284	1922	1836	3090	178
		ETCa_168	15.5	15.5	588	56	467	3214	3275	5149	251
MB7	Ca-SO_4(2)	ETCa_143	Control	0.1	5	1	3	2	3	7	42
		ETCa_144	1.2	1.4	32	7	44	470	384	248	56
		ETCa_145	4.0	4.5	107	15	117	969	979	871	150
		ETCa_146	6.0	6.7	126	21	167	1456	1472	1547	206
		ETCa_147	8.0	9.0	162	31	232	2065	2116	2280	206
		ETCa_148	10.0	10.6	181	36	280	2352	2463	2794	246
		ETCa_149	14.0	14.5	249	54	393	3257	3611	4244	339
MB7	Ca-SO_4(4)	ETCa_143	Control	0.1	5	1	3	2	3	7	42
		ETCa_150	1.2	1.5	46	5	40	348	396	232	54
		ETCa_151	4.0	4.5	123	13	99	870	984	888	112
		ETCa_152	6.0	6.8	196	21	159	1393	1594	1469	143
		ETCa_153	8.0	9.3	260	31	236	2024	2354	2243	153
		ETCa_154	10.0	11.0	324	39	298	2547	3020	2821	199
		ETCa_155	14.0	14.9	394	54	396	3305	4249	4282	205

Table A.5: Raw data of 96 h Acute toxicity test for the Reference toxicant NaCl to *Austrophlebioides* sp. AV11

Test ID	Reps	Measured EC (mS/cm)	Average EC (mS/cm)	NaCl mg/L	Number at start	Number missing &/or emerged	Actual Number in treatments	Number Dead	Number Alive
MB2	1	0.057	0.06	11	12	0	12	0	12
	2	0.055			13	1	12	1	11
	3	0.057			14	0	14	0	14
	1	1.29	1.29	338	10	0	10	6	4
	2	1.29			12	3	9	7	2
	3	1.30			12	1	11	10	1
	1	1.98	1.98	518	10	0	10	8	2
	2	1.98			10	2	8	5	3
	3	1.98			10	0	10	6	4
	1	3.19	3.18	1020	10	0	10	8	2
	2	3.18			10	0	10	7	3
	3	3.18			9	0	9	6	3
	1	5.78	5.78	2274	12	0	12	11	1
	2	5.77			12	0	12	11	1
	3	5.78			12	0	12	12	0
	1	8.69	8.70	3819	9	0	9	9	0
	2	8.71			10	0	10	9	1
	3	8.71			10	0	10	10	0
	1	13.12	13.11	6479	10	0	10	10	0
	2	13.11			10	0	10	10	0
	3	13.11			10	0	10	10	0
MB3	1	0.063	0.06	8	11	0	11	0	11
	2	0.062			10	0	10	0	10
	1	0.74	0.74	151	10	1	9	5	4
	2	0.74			10	0	10	6	4
	1	1.30	1.30	396	10	0	10	10	0
	2	1.30			10	2	8	6	2
	1	2.03	2.02	719	10	0	10	10	0
	2	2.02			10	0	10	10	0
	1	3.27	3.27	1393	10	0	10	10	0
	2	3.26			10	0	10	10	0
	1	6.02	6.03	2833	10	0	10	10	0
	2	6.03			10	0	10	10	0
	1	14.12	14.12	7079	10	0	10	10	0
	2	14.11			10	0	10	10	0

Table A.5: Raw data of 96 h Acute toxicity test for the Reference toxicant NaCl to *Austrophlebioides* sp. AV11 (cont'd)

Test ID	Reps	Measured EC (mS/cm)	Average EC (mS/cm)	NaCl mg/L	Number at start	Number missing &/or emerged	Actual Number in treatments	Number Dead	Number Alive
MB4	1	0.072	0.07	8	10	0	10	0	10
	2	0.072			10	0	10	0	10
	1	0.53	0.53	58	10	1	9	2	7
	2	0.54			10	0	10	4	6
	1	0.79	0.79	151	10	0	10	3	7
	2	0.79			10	0	10	7	3
	1	1.23	1.23	324	10	0	10	7	3
	2	1.23			10	0	10	5	5
	1	2.0	2.02	705	9	1	8	6	2
	2	2.0			10	0	10	9	1
	1	3.2	3.22	1373	10	0	10	10	0
	2	3.2			10	0	10	10	0
	1	5.82	5.82	2833	9	0	9	9	0
	2	5.82			11	0	11	11	0
MB5	1	0.062	0.06	6	13	0	13	0	13
	2	0.062			10	0	10	0	10
	3	0.063			10	0	10	0	10
	1	0.53	0.53	58	10	1	9	6	3
	2	0.53			11	0	11	5	6
	3	0.53			12	1	11	7	4
	1	0.78	0.77	146	10	0	10	5	5
	2	0.77			10	1	9	6	3
	3	0.77			10	1	9	5	4
	1	1.22	1.22	324	10	0	10	6	4
	2	1.22			10	0	10	8	2
	3	1.22			11	0	11	9	2
	1	2.03	2.04	705	9	0	9	5	4
	2	2.05			10	0	10	7	3
	3	2.05			10	0	10	8	2
	1	3.16	3.18	1373	10	0	10	10	0
	2	3.19			10	0	10	10	0
	3	3.18			10	1	9	8	1
	1	7.62	7.67	4027	10	0	10	9	1
	2	7.68			10	0	10	10	0
	3	7.70			10	0	10	10	0

Table A.5: Raw data of 96 h Acute toxicity test for the Reference toxicant NaCl to *Austrophlebioides* sp. AV11 (cont'd)

Test ID	Reps	Measured EC (mS/cm)	Average EC (mS/cm)	NaCl mg/L	Number at start	Number missing &/or emerged	Actual Number in treatments	Number Dead	Number Alive
MB6	1	0.062	0.06	7	8	0	8	0	8
	2	0.062			8	0	8	0	8
	3	0.063			8	0	8	0	8
	1	0.53	0.53	58	6	0	6	5	1
	2	0.53			5	0	5	0	5
	3	0.53			5	0	5	4	1
	1	0.78	0.77	146	7	0	7	5	2
	2	0.77			7	0	7	6	1
	3	0.77			7	1	6	5	1
	1	1.22	1.22	324	6	1	5	3	2
	2	1.22			6	0	6	3	3
	3	1.22			6	0	6	3	3
	1	2.03	2.04	705	5	0	5	4	1
	2	2.05			5	0	5	4	1
	3	2.05			7	0	7	6	1
	1	3.16	3.18	1373	6	0	6	5	1
	2	3.19			7	0	7	5	2
	3	3.18			7	0	7	6	1
	1	7.62	7.67	4027	5	0	5	5	0
	2	7.68			5	0	5	4	1
	3	7.70			6	0	6	6	0
MB7	1	0.073	0.07	8	10	0	10	0	10
	2	0.074			10	0	10	1	9
	3	0.074			10	0	10	0	10
	1	0.52	0.52	58	8	0	8	5	3
	2	0.52			8	1	7	3	4
	3	0.52			8	0	8	4	4
	1	0.75	0.75	146	8	0	8	5	3
	2	0.75			8	0	8	5	3
	3	0.75			8	0	8	5	3
	1	1.19	1.21	324	8	0	8	6	2
	2	1.23			8	0	8	6	2
	3	1.20			8	0	8	5	3
	1	7.68	7.69	4027	8	0	8	8	0
	2	7.66			8	0	8	8	0
	3	7.73			8	0	8	8	0

Table A.5: Raw data of 96 h Acute toxicity test for the Reference toxicant NaCl to *Austrophlebioides* sp. AV11 (cont'd)

Test ID	Reps	Measured EC (mS/cm)	Average EC (mS/cm)	NaCl mg/L	Number at start	Number missing &/or emerged	Actual Number in treatments	Number Dead	Number Alive
MB8	1	0.072	0.07	8	7	0	7	0	7
	2	0.071			7	0	7	0	7
	3	0.071			7	0	7	0	7
	1	0.51	0.51	78	7	0	7	3	4
	2	0.51			7	1	6	3	3
	3	0.51			7	0	7	4	3
	1	0.72	0.72	146	7	0	7	5	2
	2	0.72			7	1	6	3	3
	3	0.72			7	0	7	4	3
	1	1.17	1.17	325	7	0	7	5	2
	2	1.17			7	1	6	6	0
	3	1.17			7	0	7	5	2
	1	7.42	7.44	4027	7	0	7	7	0
	2	7.44			7	0	7	7	0
	3	7.46			7	0	7	7	0
MB9	1	0.082	0.08	10	12	0	12	0	12
	2	0.080			11	0	11	0	11
	3	0.081			12	0	12	0	12
	1	0.53	0.53	78	10	2	8	2	6
	2	0.53			10	0	10	5	5
	3	0.53			10	1	9	3	6
	1	0.77	0.77	149	10	0	10	3	7
	2	0.77			10	0	10	5	5
	3	0.77			10	0	10	6	4
	1	1.22	1.22	325	9	0	9	5	4
	2	1.22			10	0	10	6	4
	3	1.22			10	0	10	5	5
	1	7.60	7.60	3992	10	0	10	10	0
	2	7.59			10	0	10	10	0
	3	7.60			10	0	10	10	0

Table A.6: Raw data of 96 h acute toxicity tests for the test solutions to *Austrophlebioides* sp. AV11

Test ID	Test Solutions	Reps	Nominal EC mS/cm	Measured EC mS/cm	Average Measured EC mS/cm	Number at start	Number missing &/or emerged	Actual Number in treatments	Number Dead	Number Alive
MB3	Ca-Cl.SO_4(4)	1	Control	0.0631		10	0	10	0	10
		2	Control	0.0621	0.062	12	0	12	0	12
		3	Control	0.0620		10	0	10	0	10
		1	1.2	1.59		10	0	10	1	9
		2	1.2	1.59	1.59	10	0	10	0	10
		3	1.2	1.59		11	1	10	0	10
		1	3.5	4.23		10	0	10	4	6
		2	3.5	4.24	4.24	10	1	9	3	6
		3	3.5	4.24		10	0	10	2	8
		1	6	6.94		11	1	10	3	7
		2	6	6.93	6.93	10	0	10	2	8
		3	6	6.93		10	1	9	5	4
		1	8	8.99		10	0	10	4	6
		2	8	8.99	8.99	10	0	10	5	5
		3	8	9.00		10	1	9	4	5
		1	12	12.90		10	0	10	8	2
		2	12	12.89	12.89	9	0	9	7	2
		3	12	12.88		10	0	10	9	1
		1	16	18.47		10	0	10	10	0
		2	16	18.46	18.47	10	0	10	10	0
		3	16	18.49		10	0	10	10	0
MB4	Ca-Cl(2)	1	Control	0.0742		10	0	10	0	10
		2	Control	0.0721	0.073	10	0	10	0	10
		3	Control	0.0721		10	0	10	0	10
		1	1.5	1.79		10	1	9	0	9
		2	1.5	1.79	1.79	10	0	10	0	10
		3	1.5	1.79		10	1	9	2	7
		1	4	4.22		10	0	10	3	7
		2	4	4.21	4.22	10	0	10	5	5
		3	4	4.22		10	0	10	4	6
		1	6	5.85		10	0	10	8	2
		2	6	5.84	5.86	10	0	10	10	0
		3	6	5.88		10	0	10	8	2

Table A.6: Raw data of 96 h acute toxicity tests for the test solutions to *Austrophlebioides* sp. AV11 (cont'd)

Test ID	Test Solutions	Reps	Nominal EC mS/cm	Measured EC mS/cm	Average Measured EC mS/cm	Number at start	Number missing &/or emerged	Actual Number in treatments	Number Dead	Number Alive
MB4	Ca-Cl(2)	1	8	7.43	7.43	10	0	10	7	3
		2	8	7.43		10	0	10	6	4
		3	8	7.43		10	1	9	5	4
		1	12	10.26	10.24	11	0	11	8	3
		2	12	10.23		10	0	10	9	1
		3	12	10.22		10	0	10	9	1
		1	16	13.77	13.75	10	0	10	10	0
		2	16	13.72		10	0	10	10	0
		3	16	13.77		10	0	10	10	0
MB5	Ca-Cl.SO_4(8)	1	Control	0.064	0.064	10	0	10	0	10
		2	Control	0.064		10	0	10	0	10
		3	Control	0.064		10	0	10	0	10
		1	1.2	1.5	1.53	10	1	9	0	9
		2	1.2	1.5		10	0	10	0	10
		3	1.2	1.5		10	0	10	0	10
		1	3	3.5	3.44	10	0	10	0	10
		2	3	3.4		11	0	11	1	10
		3	3	3.4		10	0	10	0	10
		1	4.5	4.8	4.84	10	0	10	5	5
		2	4.5	4.8		10	1	9	3	6
		3	4.5	4.9		11	0	11	4	7
		1	6	5.9	5.90	10	0	10	5	5
		2	6	5.9		10	0	10	6	4
		3	6	5.9		8	1	7	3	4
		1	10	8.7	8.76	10	0	10	7	3
		2	10	8.8		10	0	10	8	2
		3	10	8.7		10	0	10	3	7
		1	14	12.1	12.08	11	0	11	11	0
		2	14	12.1		10	1	9	9	0
		3	14	12.1		9	0	9	9	0

Table A.6: Raw data of 96 h acute toxicity tests for the test solutions to *Austrophlebioides* sp. AV11 (cont'd)

Test ID	Test Solutions	Reps	Nominal EC mS/cm	Measured EC mS/cm	Average Measured EC mS/cm	Number at start	Number missing &/or emerged	Actual Number in treatments	Number Dead	Number Alive
MB7	Ca-SO_4(2)	1	Control	0.0736		12	0	12	0	12
		2	Control	0.0734	0.074	11	0	11	0	11
		3	Control	0.0735		12	0	12	0	12
		1	1.2	1.41		10	0	10	1	9
		2	1.2	1.41	1.42	9	0	9	0	0
		3	1.2	1.42		9	0	9	1	8
		1	4	4.50		10	0	10	3	7
		2	4	4.43	4.48	10	0	10	5	5
		3	4	4.51		10	0	10	6	4
		1	6	6.72		10	0	10	5	5
		2	6	6.72	6.74	10	0	10	6	4
		3	6	6.77		10	0	10	8	2
		1	8	8.97		9	1	8	4	4
		2	8	9.03	9.01	10	0	10	8	2
		3	8	9.04		10	0	10	7	3
		1	10	10.53		10	0	10	5	5
		2	10	10.57	10.57	10	0	10	8	2
		3	10	10.62		10	1	9	8	1
		1	14	14.41		10	0	10	10	0
		2	14	14.52	14.49	10	0	10	10	0
		3	14	14.55		10	0	10	10	0
MB7	Ca-SO_4(4)	1	Control	0.0735		12	0	12	0	12
		2	Control	0.0732	0.073	12	0	12	0	12
		3	Control	0.0735		12	1	11	0	11
		1	1.2	1.45		9	0	9	0	9
		2	1.2	1.45	1.45	10	0	10	0	10
		3	1.2	1.45		10	0	10	0	10
		1	4	4.45		10	1	9	3	6
		2	4	4.45	4.46	10	1	9	1	8
		3	4	4.47		10	0	10	3	7
		1	6	6.78		10	0	10	5	5
		2	6	6.77	6.78	10	0	10	7	3
		3	6	6.78		10	0	10	7	3
		1	8	9.33		10	0	10	7	3
		2	8	9.36	9.34	10	0	10	8	2
		3	8	9.33		10	1	9	7	2

Table A.6: Raw data of 96 h acute toxicity tests for the test solutions to *Austrophlebioides* sp. AV11 (cont'd)

Test ID	Test Solutions	Reps	Nominal EC mS/cm	Measured EC mS/cm	Average Measured EC mS/cm	Number at start	Number missing &/or emerged	Actual Number in treatments	Number Dead	Number Alive
MB7	Ca-SO_4(4)	1	10	10.95		10	0	10	9	1
		2	10	10.94	10.95	10	0	10	7	3
		3	10	10.97		10	0	10	9	1
		1	14	14.96		10	0	10	10	0
		2	14	14.92	14.94	10	0	10	10	0
		3	14	14.95		10	0	10	10	0
MB8	AMW	1	Control	0.07		12	0	12	0	12
		2	Control	0.07	0.07	12	0	12	0	12
		3	Control	0.07		12	0	12	0	12
		1	1	1.247		10	0	10	0	10
		2	1	1.252	1.25	10	1	9	0	9
		3	1	1.252		10	0	10	0	10
		1	3.5	3.98		10	1	9	2	7
		2	3.5	3.98	3.98	10	2	8	2	6
		3	3.5	3.99		10	0	10	2	8
		1	5.5	6.05		10	0	10	5	5
		2	5.5	6.07	6.06	10	0	10	6	4
		3	5.5	6.07		10	0	10	4	6
		1	7.5	7.87		10	0	10	7	3
		2	7.5	7.87	7.88	10	0	10	7	3
		3	7.5	7.91		10	0	10	7	3
		1	9.5	9.72		10	0	10	8	2
		2	9.5	9.72	9.75	10	2	8	7	1
		3	9.5	9.80		10	0	10	8	2
		1	15	15.05		10	0	10	10	0
		2	15	15.06	15.06	10	0	10	10	0
		3	15	15.08		10	0	10	10	0
MB8	Ca-Cl(4)	1	Control	0.071		10	0	10	0	10
		2	Control	0.071	0.071	10	0	10	0	10
		3	Control	0.071		10	0	10	0	10
		1	1.2	1.62		7	0	7	1	6
		2	1.2	1.63	1.63	8	1	7	1	6
		3	1.2	1.63		7	0	7	0	7
		1	4	4.20		8	0	8	2	6
		2	4	4.21	4.21	8	1	7	2	5
		3	4	4.21		8	0	8	3	5

Table A.6: Raw data of 96 h acute toxicity tests for the test solutions to *Austrophlebioides* sp. AV11 (cont'd)

Test ID	Test Solutions	Reps	Nominal EC mS/cm	Measured EC mS/cm	Average Measured EC mS/cm	Number at start	Number missing &/or emerged	Actual Number in treatments	Number Dead	Number Alive
MB8	Ca-Cl(4)	1	6	6.32	6.32	8	0	8	5	3
		2	6	6.32		8	0	8	5	3
		3	6	6.33		8	0	8	4	4
		1	8	8.20	8.22	8	0	8	7	1
		2	8	8.23		8	0	8	6	2
		3	8	8.24		8	0	8	7	1
		1	10	9.99	10.01	8	0	8	6	2
		2	10	10.02		8	0	8	6	2
		3	10	10.03		8	0	8	7	1
		1	15	15.51	15.51	8	0	8	8	0
		2	15	15.51		8	0	8	8	0
		3	15	15.52		8	0	8	8	0
MB9	$Ca\text{-}Cl.SO_4(2)$	1	Control	0.080	0.081	12	0	12	0	12
		2	Control	0.082		12	0	12	0	12
		3	Control	0.080		12	0	12	0	12
		1	2	1.61	1.60	10	0	10	0	10
		2	2	1.61		10	1	9	1	8
		3	2	1.60		10	0	10	0	10
		1	3	3.97	3.96	10	0	10	2	8
		2	3	3.96		10	1	9	2	7
		3	3	3.96		10	1	9	2	7
		1	4	5.95	5.94	10	1	9	5	4
		2	4	5.94		10	1	9	5	4
		3	4	5.94		10	0	10	4	6
		1	5	8.34	8.32	10	0	10	4	6
		2	5	8.30		11	0	11	6	5
		3	5	8.31		10	0	10	6	4
		1	6	10.15	10.14	10	0	10	7	3
		2	6	10.14		10	0	10	6	4
		3	6	10.14		10	0	10	6	4
		1	7	14.73	14.70	10	0	10	10	0
		2	7	14.68		10	0	10	10	0
		3	7	14.68		10	0	10	10	0

Table A.7: Data for average, minimum, maximum, standard deviation and standard error of temperature for the duration of each experiment.

Test ID	Start Date	End Date	Temperature ºC	Minimum Temperature	Maximum Temperature	Standard Deviation	Standard Error
MB1	26/03/13	30/03/13	22.5	21.9	23.3	0.30	0.02
MB2	15/04/13	19/04/13	22.2	22.2	22.4	0.14	0.01
MB3	21/05/13	25/05/13	20.7	19.9	21.3	0.33	0.02
MB4	06/06/13	10/06/13	20.7	20.3	21.1	0.19	0.01
MB5	25/06/13	29/06/13	19.6	18.4	20.8	0.65	0.04
MB6	06/08/13	10/08/13	20.3	19.7	22.7	0.45	0.03
MB7	05/09/13	09/09/13	21.1	20.5	21.6	0.28	0.02
MB8	24/09/13	29/09/13	21.5	21.0	22.2	0.22	0.01
MB9	08/10/13	13/10/13	21.3	20.6	21.8	0.21	0.01