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Characterizing the chronic toxicity of ion mixtures to *Ceriodaphnia dubia* using two experimental designs

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CHARACTERIZING THE CHRONIC TOXICITY OF ION MIXTURES TO *CERIODAPHNIA DUBIA* USING TWO EXPERIMENTAL DESIGNS

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by
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ABSTRACT

Total Dissolved Solids, commonly referred to as TDS, is a measure of all organic and inorganic substances that pass through a 2-µm filter and are naturally found in aquatic environments. Anthropogenic activities such as agricultural irrigation, road salt runoff, hydraulic fracturing, and coal-fired power plant effluents can increase TDS concentrations of freshwater ecosystems ultimately increasing salinity. Aquatic organisms actively manage the ion balance between their external and internal environments. Freshwater organisms use energy to pump sodium in from the environment, while losing it through passive diffusion. If the external ion concentration changes significantly, these organisms must allocate more energy towards ionoregulation, reducing energy for other important functions such as reproduction. Eight-day static renewal exposures were conducted to characterize the chronic effects of chloride, sulfate and bicarbonate as single anions and in binary mixtures to *Ceriodaphnia dubia*. The results of the individual anion toxicity tests were used to design binary mixture bioassays. Two experimental approaches were used to test the hypothesis that binary anion mixtures were additive in their combined toxic effects: Dose Addition and Slope Analysis. Reproductive effects (EC_{50}s) indicated that the relative toxicity of single anions was SO_4^{2-} (0.0108M) > HCO_3^- (0.0136M) > Cl^- (0.0163M). The slope of the overall concentration-response relationship for each anion identified a similar trend, with SO_4^{2-} and HCO_3^- having similar slopes (-4776,-4884 respectively) while Cl- was significantly less (-3074). The Dose Addition approach utilized binary mixtures expected to result in

an EC_{50} and then tested if the results were significantly different from expected. This experimental design identified mixtures containing chloride as additive and the bicarbonate/sulfate mixture as greater-than-additive. The Slope Analysis approach compares the slope of the single contaminant concentration-response relationship with the slope of the concentration-response relationship for that contaminant in the presence of a constant background concentration of the second contaminant. This approach identified most ion combinations as being greater-than-additive. In general, the Slope Analysis experimental design was a more efficient and straight-forward approach.

DEDICATION

I would like to dedicate this work to my Mama and Daddy for providing me with the utmost love and support throughout this project.

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All of my ENTOX friends and colleagues, especially Austin Wray, Lauren Sweet and Erica Linard, also deserve special thanks for their help and support throughout the years. I have truly and undeniably appreciated everything they have done for me.

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CHAPTER ONE

INTRODUCTION

Total Dissolved Solids, commonly referred to as TDS, is a measure of all dissolved inorganic and organic ions in solution that can pass through a 2-µm filter (U.S. EPA, 1997). Inorganic ions can either be cationic (e.g. Ca^{2+}, Na^{+}, K^{+}, Mg^{2+}) or anionic (e.g. Cl^{-}, SO_4^{2-}, PO_4^{3-}, HCO_3^{-}), leading to electrostatic interactions that can be measured as conductivity (U.S. EPA, 1997). By combining all ions into one parameter and not considering them as individual constituents, conductivity and total dissolved solids measurements infer that all ions act similarly both in their chemical activity and in their effects on biological systems. However, recent research suggests that the toxicity of TDS is dependent on the specific ionic composition, i.e. each ion produces its own toxicity (Mount et al., 1997; Kunz et al., 2013). Because of this, grouping all ions together into one measurement such as conductivity or TDS may not be the best approach for expressing toxic burden (Kennedy et al., 2005; Mount et al., 1997; Soucek et al., 2011).

The majority of ion toxicity data available represents acute endpoints, most often mortality. In one particular study performed by Mount et al., the acute effects of over 2,900 ion combinations were assessed using *Ceriodaphnia dubia*, *Daphnia magna*, and *Pimephales promelas*. Overall, the individual ion acute toxicities were summarized as $K^{+} > HCO_3^{-} \approx Mg^{2+} > Cl^{-} > SO_4^{2-}$, with no significant effects from either Na^{+} or Ca^{2+}. Further studies have indicated the importance of water hardness on decreased ion toxicity (Soucek et al., 2011; Soucek and Kennedy, 2005). However, it has been questioned whether hardness or a general increase in cation concentrations are responsible for this decreased toxicity (Soucek et al., 2011; Mount et al., 1997).

Currently, the chronic toxicity of ion mixtures has not been thoroughly characterized. Classic approaches to characterizing mixture toxicity have employed a Dose Addition approach that relies on point estimates from individual contaminant concentration-response relationships to develop mixture concentrations expected to result in a particular effect (e.g. EC_{20} of contaminant A + EC_{30} of contaminant B = EC_{50} of mixture). This approach may suffer increased variation around both the expected effects and the point estimates produced for each individual component within the mixture, ultimately adding to the difficulty of discriminating between additive and non-additive interactions (e.g. the 95% confidence intervals around an estimate of the EC_{10} may be ± 100%). Finding an approach that provides a quantitative interpretation of results while minimizing and incorporating error and variation may lead to more accurate characterization of the effects of chemical mixtures.

The goal of this study was to characterize the chronic toxicities of the sodium salts of Cl^-, SO_4^{2-} and HCO_3^- as single anions and in binary mixtures. Point estimates derived from single anion concentration-response relationships were used in subsequent mixture exposures. Two experimental designs, a Dose Addition approach and a Slope Analysis Approach, were employed to test the hypothesis that these ions act in a strictly additive manner. Bioassays were performed multiple times, termed test repeats, to create large, robust datasets while increasing statistical power. The overall goal of this research was to characterize the chronic toxicity of these ion mixtures. A secondary goal was to test the utility of the Slope Analysis experimental design to quantify mixture toxicities and compare it to the utility of the Dose Addition experimental design.

CHAPTER TWO

LITERATURE REVIEW

Total Dissolved Solids

Geologic materials, such as rocks and sediments, are largely comprised of a specific composition of ions. For example, limestone is made from a combination of calcium and carbonate ($CaCO_3$). Over many years, as water comes into contact with limestone and other rocks, these molecules break apart into ions allowing water molecules to form hydration shells around each ion. When this occurs, the ions are said to be dissolved and will have little affinity for reforming these minerals while in solution. These ions greatly contribute to the salinity of aquatic systems.

As defined by the U.S. EPA, Total Dissolved Solids, commonly referred to as TDS, is any dissolved ion in solution that can pass through a 2-µm filter. These ions may possess either a positive charge, such as Ca^{2+}, Mg^{2+}, Na^{+} or K^{+}, or a negative charge: Cl^{-}, SO_4^{2-}, PO_3^{4-}, HCO_3^{-}. This charge allows for electrostatic interactions which can be measured as conductivity in microsiemens per centimeter (µS/cm). Increases in TDS (mg/L) and conductivity are both positively correlated to the total number of ions in solution, making both useful tools for measuring overall ion concentrations. One limitation of both, however, is their inability to indicate ionic composition.

Sources of TDS Contamination

Freshwater systems typically have very low ion concentrations and possess ionic compositions that are dependent on the surrounding geologic material. For example, a typical freshwater stream in the Central Appalachian Mountains may have ~21 mg/L

TDS and conductivity of ~62 μS/cm, with sulfate and bicarbonate dominating ionic composition. However, increases in both conductivity and total dissolved solids, as well as a change in the overall ionic composition have been reported in areas impacted by mining operations (Timpano et al., 2011; Pond et al., 2011). For example, Timpano et al. reported a mean TDS of 406 mg/L of twenty-two mining impacted sites in Virginia with a large increase of sulfate, calcium and potassium. Pond et al. also showed similar trends for mining impacted sites located in West Virginia, but to a much higher degree with a mean TDS of 1,165 mg/L and sulfate dominating. Certain individual sites also showed a large spike in bicarbonate and sodium concentrations (Pond et al., 2011).

Other anthropogenic activities also lead to increased TDS in freshwater streams. For example, direct application of salts to roads during snow and ice events is a fairly common practice in many states. This is done in order to lower the freezing point of water and prevent ice buildup on roadways. However, once the snow melts, it can then carry the salt with it to nearby streams as runoff. It has been reported that the United States applies roughly 10-million tons to roads every year, most often as sodium chloride (NaCl) (U.S. EPA, 1999). According to Coris et al. (2010), conductivity in impacted Wisconsin streams can be as high as 30,800 μS/cm with chloride concentrations exceeding 11,200 mg/L. At all sites monitored, chloride concentrations exceeded the U.S. EPA acute (860 mg/L) and chronic (230 mg/L) water quality criteria.

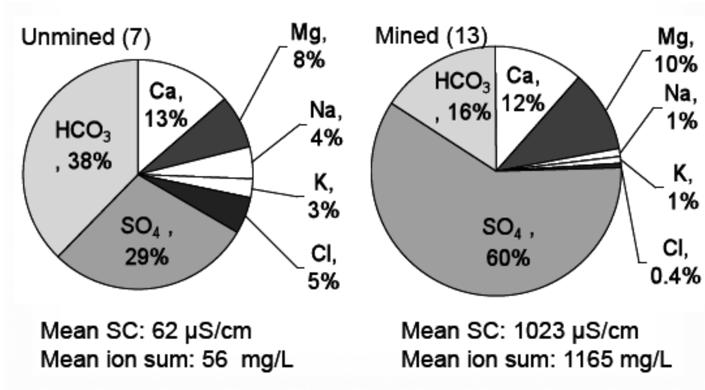

Figure 2.1. The effects of mountaintop removal mining on increased ion concentrations (Pond et al., 2008).

Importance of Ionic Composition

In 2013, a study performed by Kunz et al. identified ionic composition as being an important indicator for ion toxicity. Reconstituted waters simulating three sites impacted by mountaintop removal mining were created. All three sites demonstrated similar conductivities (1,800 µS/cm – 2,100 µS/cm), but exhibited different ionic compositions. The first two sites had elevated concentrations of Mg^{2+}, Ca^{2+}, K^{+}, SO_4^{2-} and HCO_3^{-}, while the third site had elevated Na^{+}, K^{+}, SO_4^{2-} and HCO_3^{-}. Their results indicated that only the third site had a significant acutely toxic effect to *Ceriodaphnia dubia*, while the first two did not. They ultimately concluded that although elevated TDS is comparable to increased toxicity, it is more beneficial to identify major ions present and report toxicity in regards to their individual concentrations.

However, this is not the first reported incidence of ionic composition ultimately determining the toxicity of TDS impacted freshwater systems. Dwyer et al. studied the effects of irrigation drainage waters on *Daphnia magna* and the survival of *Morone* *saxatilis*, striped bass. They were able to demonstrate that the toxicity of high conductivity waters was directly related to the ionic composition.

The importance of ionic composition may be a best explained by the ionoregulatory capability of freshwater organisms (Figure 2.2). The internal environment of a freshwater organism has a greater concentration of ions relative to the external environment. Because of this, these organisms must actively uptake ions at the gills. Specifically, it has been proposed that fish gills contain two types of cells known as Mitochondrial Rich (MR) cells that are important in acid-base regulation and ion uptake and transport. In this gill model, there are two types of MR cells known as PNA^{-}MR, also called an acid-secreting cell, and PNA^{+}MR, or base-secreting cell. The PNA^{+}MR cells are important in the active uptake of ions from the external environment, and PNA^{-} MR cells transport these ions from the cell into the internal environment, or blood, of the fish (Perry et al., 2003). This type of active uptake requires energy. When the ion concentration of the external environment increases, the energy exerted toward ionoregulation must also increase, leading to decreased energy allocated to other important biological functions, such as reproduction, food consumption and mobility (Elphick et al., 2011).

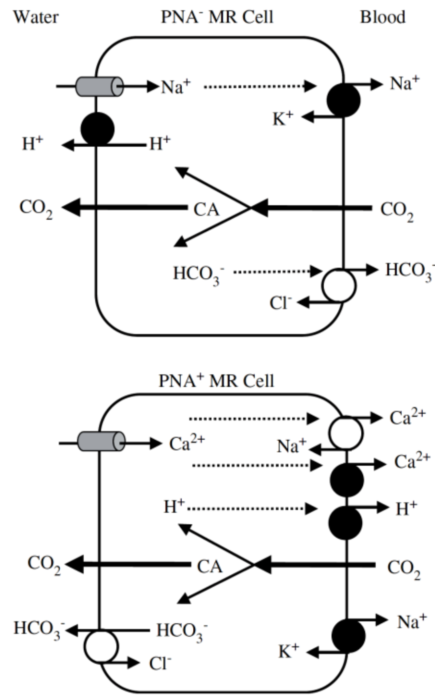

Figure 2.2. The proposed mechanism for ion uptake/acid-base regulation in the freshwater trout gill. Solid circles represent active transport mechanisms, while unfilled circles represent ion exchange mechanisms that are driven by ion gradients. Gray shaded cylinders represent diffusion channels (Perry et al., 2003).

Acute Toxicity of Total Dissolved Solids

The majority of TDS studies involve acute endpoints, such as mortality and survival. One of the largest and most well-known investigations was performed by Mount et al. in 1997. This team of researchers was able to determine the acute effects of over 2,900 ion combinations to *Ceriodaphnia dubia*, *Daphnia magna*, and *Pimephales promelas*, fathead minnow. From this data they were able to establish relative ion toxicities: $K^+ > HCO_3^- \approx Mg^{2+} > Cl^- > SO_4^{2-}$ in terms of mg/L. Both Na^+ and Ca^{2+} did not significantly contribute to the toxicity of these combinations.

Subsequent studies have examined the potential interactions of binary mixtures on an array of organisms. In particular, increasing chloride concentrations seemed to decrease sulfate toxicity to *Hyalella azteca* and *Ceriodaphnia dubia* when exposed in moderately hard water (Soucek and Kennedy, 2005). In this regard, toxicity was identified based on sulfate concentrations alone. The authors note that chloride concentrations were high enough to exert a toxic effect of their own, meaning less sulfate was required to produce toxicity. Because of this, the authors conclude that both ions act in an additive manner. In the opposite aspect, varying sulfate concentrations were not shown to have a statistically significant effect on chloride toxicity in *C. dubia* (Soucek et al., 2011). From this, the authors further concluded that chloride and sulfate, again, acted in an additive manner.

From these studies, it was also suggested that water hardness ($CaCO_3$ mg/L) plays a large role in ameliorating the toxicity of both chloride and sulfate to *H. azteca* and *C. dubia* (Soucek and Kennedy, 2005; Soucek et al., 2011). A significant reduction in mortality was shown by both species when hardness, as $MgCl_2$ and $CaCl_2$, increased (between 400 and 800 mg/L $CaCO_3$). Mount et al. also found a similar effect in solutions containing Ca^{2+} and Mg^{2+}. Although their final conclusions indicate that this toxicity may not be necessarily be due to water hardness. Mount et al. hypothesized this effect was caused by multiple cations being present, regardless of increasing hardness. For example, both NaCl and $CaCl_2$ had similar toxicities when expressed as Cl^- mg/L. However, the toxicity of these salts combined was significantly less than either salt individually. The same trend was shown by combinations including K^+ and Mg^{2+}. The reasons for decreased toxicity of sodium-dominated TDS caused by Ca^{2+} and Mg^{2+} are still largely unknown. Hypotheses include competition for binding sites and reduction of gill cell permeability (Soucek et al., 2011; Potts and Fleming, 1970).

Chronic Toxicity of Total Dissolved Solids

Emphasis has been placed on describing the individual chronic toxicities of chloride, sulfate and bicarbonate as a result of their importance in acute toxicity assays (Mount et al., 1997). By using sodium as a standard cation across treatments, each of the previously described effects on *C. dubia* reproduction were analyzed as just the anion effect because sodium does not significantly contribute to acute toxicity.

Currently, the Effective Concentrations at which there is a 50% decrease in reproduction (EC_{50}) for each anion individually has been determined. The relative toxicity of these three ions has shown similar responses in comparison to acute data: $HCO_3^- > Cl^- > SO_4^{2-}$ in terms of mg/L (Lasier and Hardin, 2010; Elphick et al., 2011*a*; Elphick et al., 2011*b*; Farag and Harper, 2014). Oftentimes, researchers are interested in determining these point estimates for the purpose of creating water quality guidelines for environmental exposures. However, identifying single anion toxicities is not entirely environmentally relevant since these ions always occur in mixtures.

One study performed by Lasier and Hardin aimed to identify the chronic toxicity of chloride, sulfate and bicarbonate to *C. dubia* in three types of water: low hardness/low alkalinity, low hardness/moderate alkalinity, and moderate hardness/moderate alkalinity. Their experimental design involved a three-factor experiment using chloride, sulfate and bicarbonate at four levels, or concentrations, using their respective EC_6, EC_{12}, and EC_{25}

values and one treatment without, creating a full factorial framework. To describe their results, Lasier and Hardin plotted percent of control reproduction verses the total dissolved solids in mg/L (Figure 2.3) then employed an Analysis of Variance (ANOVA) to determine significant anion interactions. Their results indicate a significant interaction between chloride and sulfate, stating that mixtures with lower chloride concentrations when compared to sulfate had a lower toxic response. Other significant interactions were not discovered. Although a significant interaction was reported for chloride and sulfate, the authors ultimately concluded that these anions act in an additive manner.

To our knowledge, this is the only available report indicating the chronic toxicity of these three anions in mixtures. Although this study encompassed many factors, it seemed to overlook key components that could indicate anion interactions. The most prominent of these components is the use of total dissolved solids (mg/L) to determine the interactions of specific anions. Combining anions together as one lump sum may negate any detection of potential interactions. Finding a way to identify the individual toxicity of one anion when in the presence of another may be vital to determining potential interactions

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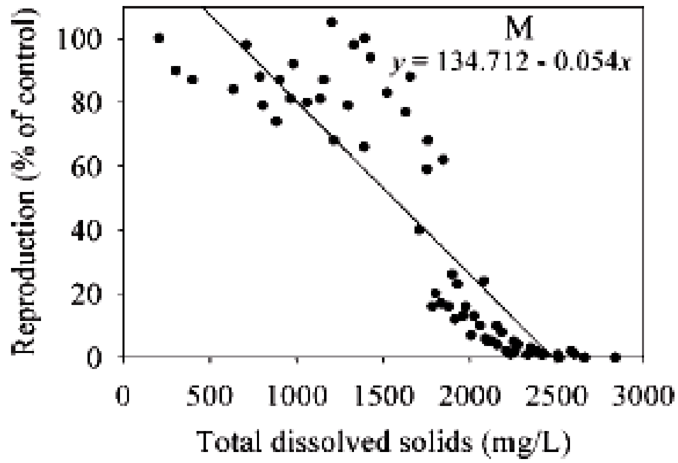

Figure 2.3. Graphical results obtained from Lasier and Hardin, depicting the effects of overall total dissolved solids (mg/L) to the percent decline in reproduction in moderately hard water.

Mixture Designs and Analysis

It is common in environmental toxicology to examine the toxicity of single contaminants in a laboratory setting. However, most aquatic contaminants occur in mixtures and not as single compounds. Over many years, the importance of identifying approaches best suited for estimating the toxicity of mixtures, including potential interactions of compounds, has long been stressed.

Currently, two approaches are most commonly used to estimate and predict the effects of contaminants in mixtures. These two approaches are known as Dose Addition and Independent Action (Bliss, 1939; Finney, 1942). The difference between the two approaches lies within their assumptions. Dose Addition assumes that all compounds within a mixture act in a similar manner, typically having similar concentration-response relationships. On the other hand, Independent Action relies on the assumption that all compounds act in a dissimilar manner, therefore, have different modes-of-action.

Because both assumptions rely on the compound's mode-of-action, they most accurately describe the additive effects of compounds. Inaccuracy in interpreting results and predicting outcomes can occur if compounds have either a greater-than-additive, or less-than-additive response (Feron and Groten, 2002; Barata et al., 2012). With this in mind, researchers compared the accuracy of a Dose Addition approach to a mixture containing compounds with dissimilar modes-of-action, and the Independent Action approach to a mixture with similarly acting compounds. Overall, the final conclusions showed that the Dose Addition approach tended to overestimate the toxicity of the mixture. Because an overestimation is generally protective of multiple species, many governmental agencies have deemed the Dose Addition approach acceptable for situations where the chemicals in a mixture are unknown (ATSDR, 2004; U.S. EPA, 1986). However, overestimations of toxicity can lead to over protective situations and is not always feasible in regards to regulatory practices. Because of this, there is an increased need for an approach that may accurately assess the interaction of components in a mixture.

One of the most basic ideas to estimate chemical interactions is to analyze the slopes of a mixtures concentration-response line. Simply put, if the slope for the mixture concentration-response line is similar to that of a component's single concentration-response line, the compounds are said to behave additively. However, if the slope of a concentration-response line changes when combined with another component, those compounds are said to have a non-additive effect. This non-additivity is based on the change in slope (Shaw et al., 2006). If the slope becomes increasingly shallow, the compounds are said to have a less-than-additive effect. If compounds are greater-than-additive, the slope would be steeper, or more severe (Gennings et al., 2005). This concept of using slopes to determine the interactive behavior of chemicals has been well studied in the pharmacology field making it an excellent candidate for environmental toxicology mixture assessments (Goodman and Gilman, 2001).

CHAPTER THREE

MATERIALS AND METHODS

***Ceriodaphnia dubia* Culturing**

Ceriodaphnia dubia were initially purchased from Aquatic Biosystems (Fort Collins, Colorado, USA) and then cultured in-house at Clemson University Institute of Environmental Toxicology (CU-ENTOX) for four months prior to test initiation. All *C. dubia* were cultured using ultrapure (18 MΩ·cm resistivity) reconstituted moderately hard water prepared following the U.S. EPA procedure for synthetic freshwater and made with reagent grade chemicals (96 mg/L $NaHCO_3$, 60 mg/L $CaSO_4$•$2H_20$, 60 mg/L $MgSO_4$, and 4.0 mg/L KCl) (U.S. EPA, 2002). Selenium (2 µg/L), as sodium selenite (Na_2SeO_4), was also added as an essential trace element. This specific reconstituted moderately hard water was also used in the preparation of test solutions.

Organisms were separated into individual cultures, which allowed health and reproductive success to be monitored. During this time, one <24-hours old *C. dubia* was placed into a 30-mL plastic cup containing 15-mL culture water. Organisms were kept in a temperature controlled chamber (25°C) with a 16:8 light/dark cycle. Water renewals were performed daily and organisms fed a 1:1 mix of *Pseudokirchneriella subcapitata* (~3.0 x 10^7 cells/mL) and YCT (trout chow, yeast, and CEROPHYL®). *Pseudokitcheneriella subcapitata* were cultured at CU-ENTOX using standard methods described by the U.S. EPA (U.S. EPA, 2002). Individuals producing ≥ 15 neonates over 3-broods in 7-days were considered fit, and young from these organisms were used in testing.

Test Solutions

ACS Certified chemicals were purchased from Fisher Scientific, Atlanta, GA, USA: sodium chloride (CAS 7647-14-5), sodium sulfate anhydrous (CAS 7757-82-6), and sodium bicarbonate (CAS 144-55-8). All salts were kept in a drying oven held at 60°C to prevent water adsorption. All stock solutions were made separately and kept in a refrigerator until use. Test solutions were made using reconstituted moderately hard water supplemented with selenium approximately 24 hours prior to use and kept in a temperature controlled chamber. A small aliquot of each test solution, including the control, was removed for validation of ion concentrations using ion chromatography (see below).

Experimental Procedure and Design

Basic experimental procedures followed the framework described by the U.S. EPA for short-term chronic estimation toxicity testing (U.S. EPA, 2002). One organism (*C. dubia*) was placed in a 30-mL plastic cup containing 15-mL of test solution and fed 250-µL a 1:1 mix of *Pseudokirchneriella subcapitata* and YCT. Each treatment group, including the controls, contained 10 replicates. Test chambers were placed on test boards and kept in a temperature controlled (25 °C) chamber with a 16:8 light/dark cycle. Water renewals were performed every 24 hours with mortality and reproduction being recorded. Experiments were carried out for 8 days and total neonates produced per organism were calculated. Results were accepted if the control group maintained 80 percent survival and 60 percent produced 3 broods containing $\geq$ 15 neonates.

Single anion bioassays were conducted twice (n=2) providing two concentration-response curves or test repeats, for each anion (Repeat A and Repeat B). The data were compiled together and Effective Concentrations, specifically the EC_{10}, EC_{20}, EC_{30}, EC_{40}, EC_{50} and EC_{70}, were estimated. These EC values were then used in binary mixture designs.

Binary mixture bioassays consisted of two experimental designs. The first mimicked a typical Dose Addition method where the combined effect of two anions, assuming additivity, was expected to result in a 50 percent decline in reproduction. That is to say, one anion decreased in concentration across treatments while the other increased (Table 1). Individuals for all treatments were normalized as a percent of control and then compared to the expected (EC_{50}) effect. This design was completed for each combination of anions three times (n=3) resulting in three test repeats (A, B, C).

Table 3.1. Point estimate combinations for the Dose Addition Approach. Across treatments, one anion (anion A) decreased in EC values while the second anion (anion B) increased.

	Treatments						
	Control	**1**	**2**	**3**	**4**	**5**	**6**
Anion A (EC_x)	0	50	40	30	20	10	0
Anion B (EC_x)	0	0	10	20	30	40	5

The second experimental design, or Slope Analysis approach, consisted of one fixed anion at its respective EC_{30} in all treatments while increasing a second anion (EC_{10}, EC_{30}, EC_{50} and EC_{70}) across treatments (t=6). One treatment contained only the EC_{30} of the fixed anion and was identified as the positive control. This experimental design was also completed three times (n=3) resulting in three test repeats (A, B, C).

Statistical Analysis

All statistical analyses were performed using JMP ® 10.0.0. Duplicates of single anion experiments were completed. The datasets from each experiment were combined to create one large dataset with twenty replicates. Linear regression methods were employed for each single anion concentration-response. Inverse predictions were made for each test repeat to estimate Effective Concentrations with 95 percent confidence intervals (α=0.05).

In the first binary mixture design, triplicate experiments were performed for each mixture and were combined into one dataset. The number of neonates produced by each replicate was divided by the average neonates produced by the control and then multiplied by one-hundred to determine the percent neonates produced relative to the control. An Analysis of Variance (ANOVA) was performed, to determine the treatments significantly different from both the expected effect with 95 percent confidence intervals.

Triplicate test repeats were also performed in the second binary mixture design, creating one dataset with thirty replicates. The percent of control for each replicate was determined similarly to that of the dose addition design. The only difference being the use of the positive control, which eliminated effects produced by the fixed anion. Linear regressions were then used to determine the slope of each mixture concentration-response line. An Analysis of Covariance (ANCOVA) was performed to determine significant differences between the slopes for mixtures and the single anion concentration-response line for the increasing anion.

Originally, data were plotted using semi-log plots. However, R^2 values for concentration-response curves decreased, hence, an arithmetic scale was used for both the x- and y-axis.

Analytical Analysis

In order to validate nominal ion concentrations, a small aliquot of each test solution at 0 (initial) and 24 hours (final) was obtained every day and filtered using a 0.45-µm filter. Samples were then diluted with an appropriate dilution factor in ultrapure water (18 MΩ resistivity) so concentrations were within the detection limit of each instrument. All analyses were performed at Clemson University Institute of Environmental Toxicology.

Chloride and sulfate were analyzed using a Dionex Ion Chromatography System (DX-500) equipped with an electrochemical detector. A Dionex IonPac™AS22 column was used with an isocratic eluent that consisted of 4.5 mM Na_2CO_3 and 1.4 mM $NaHCO_3$. A Shimadzu TOC-V Carbon Analyzer with an inorganic carbon analyzer was used to measure total inorganic carbon in the system, of which bicarbonate concentrations were determined.

Nominal concentrations were used in statistical procedures if measured ion concentrations were within 10 percent of the expected concentrations.

CHAPTER FOUR

RESULTS

Assessment of Organism Health

Although significant effort was made to maintain culture health throughout testing, the lifetime of this research spanned approximately seven months and raised the possibility of a shift in organism health during testing. In order to assess culture health, the variability in control reproduction was examined across all bioassays. Standard methods require 60% of surviving adults to produce an average of fifteen neonates across three-broods for test acceptability (U.S. EPA, 2002). Controls in all bioassays met this criterion. The average neonates produced per live adult across these assessments was 37.9 (Figure 3.1). Other studies report control reproduction slightly less than the current assessment (LaRocca et al., 1994; Manar et al., 2010; Naddy et al., 1995). An analysis of the average reproduction of controls throughout this research indicated no trend in regression line and a slope not significantly different from zero suggesting no overall change in culture health.

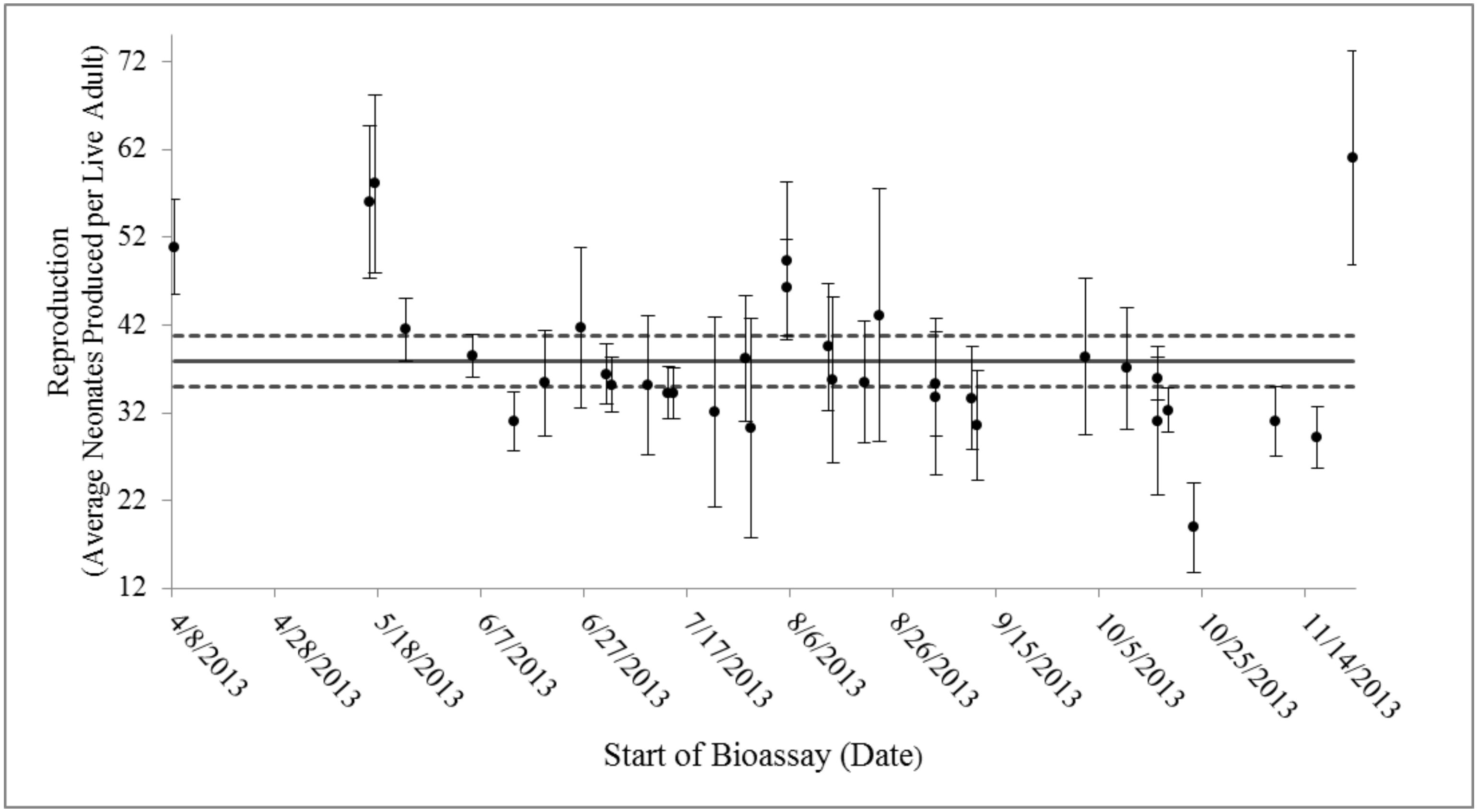

Figure 4.1. Reproduction of control organisms throughout lifetime of research. Data points represent average neonates produced per live adult ($\bar{x}$ ± standard deviation). Overall average neonates produced (solid line) is plotted; with 95% confidence intervals (dotted lines).

Single Anion Toxicity

Linear regressions produced for each anion concentration-response curve were used to estimate Effective Concentrations (EC_{50}) for each anion. The estimated EC_{50} values based on molarity describe the relative toxicity of these anions as $SO_4^{2-} > HCO_3^- > Cl^-$ (Table 3.1). These values are similar to what has been previously described in the literature.

Slopes from the same linear regressions were used to describe the intensity of the response (Figure 3.2). Both bicarbonate and sulfate had statistically similar slopes $(-4777,\ -4884$, respectively), indicating that across a range of concentrations, they had similar effects on reproduction. The slope for chloride was significantly less (-3074), suggesting a decreased toxicity relative to sulfate and bicarbonate.

Table 4.1. Point estimates (molarity) for decreased *C. dubia* reproduction for chloride, sulfate and bicarbonate.

Anion	**EC_{10}**	**EC_{30}**	**EC_{50}**	**EC_{70}**	**EC_{90}**
Chloride	0.0033 (0.00090, 0.0052)	0.0098 (0.0083, 0.0111)	0.016 (0.0151, 0.0176)	0.023 (0.0212, 0.0248)	0.029 (0.0270, 0.0324)
Sulfate	0.0027 (0.0018, 0.0034)	0.0067 (0.0062, 0.0073)	0.011 (0.0103, 0.0114	0.015 (0.0143, 0.0157)	0.019 (0.0181, 0.0201)
Bicarbonate	0.0052 (0.0040, 0.0062)	0.0094 (0.0085, 0.0103)	0.014 (0.0124, 0.0151)	0.018 (0.0161, 0.0201)	0.022 (0.0197, 0.0252)

Numbers in parentheses indicate 95 percent confidence intervals for the individual point estimate (α=0.05).

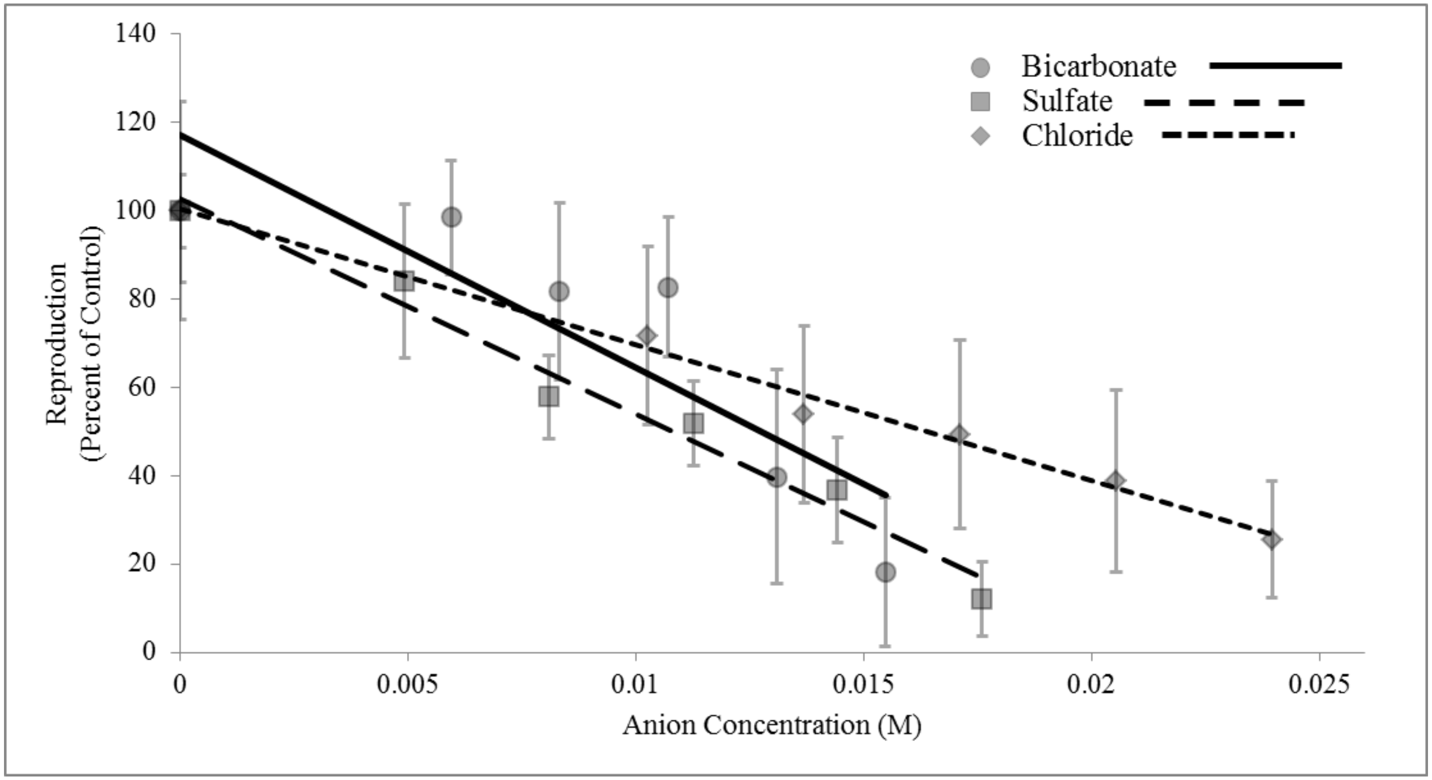

Figure 4.2. Influence of single anion concentrations on *C. dubia* reproduction. Data are shown for chloride (⧱), sulfate (⧮), and bicarbonate (⧲) ($\bar{x}$ ± standard deviation). Linear regressions for each data set are identified in the legend. Point estimates for the effects of each anion are shown in Table 3.1.

Chronic Toxicity of Binary Mixtures Using Dose Addition Approach

Mixtures of sulfate and chloride tested using the Dose Addition experimental design followed an additive trend with all treatments exhibiting a similar response around the expected EC_{50} value (Figure 3.3). Solutions containing bicarbonate and chloride also followed an additive trend (Figure 3.5). Although slight differences were estimated between treatments, all responses were within the 95% confidence intervals indicating further their additive nature.

All bicarbonate positive controls in bicarbonate/sulfate mixtures produced greater effect than expected based on the expected EC_{50} (Figure 3.4). All treatments combining bicarbonate and sulfate yielded similar results to bicarbonate individually, producing significantly greater effects than an EC_{50}. The actual effect for the positive sulfate control was expected. This effect did not decrease with the removal of bicarbonate and the addition of sulfate. Because bicarbonate generated such an extreme effect alone, the expected additive response would not be an EC_{50} across treatments. All treatments were normalized to the actual bicarbonate response to determine a line of expected additivity, as shown by the solid line in Figure 3.4. The response in mixture treatments still fell below the line of additivity, indicating the potential for greater-than-additive effects.

Because the three test repeats were performed at different times and were consistent, organism health was determined to not be a contributing factor to the severe effect by bicarbonate alone. Instead, it was believed to be caused by inherent variation in bicarbonate effects on reproduction. Multiple acute studies have estimated a lethal concentration to 50% of the population (LC_{50}) between 699 and 740 mg/L (0.011, 0.012

M, respectively) (Mount et al., 1997; Harper et al., 2014). This study determined an EC_{50} of 0.014 M (827 mg/L) which was not significantly different from previously described LC_{50} values. Typically, *C. dubia* possess a similar coloration to the ingested food substance, usually brown. However, those exposed to bicarbonate lacked any coloration and were translucent. This could indicate reduced food consumption. It was also noted that mortality in adults typically occurred following the release of the first brood, giving them a much lower total neonates produced than others in the same treatment, increasing variability.

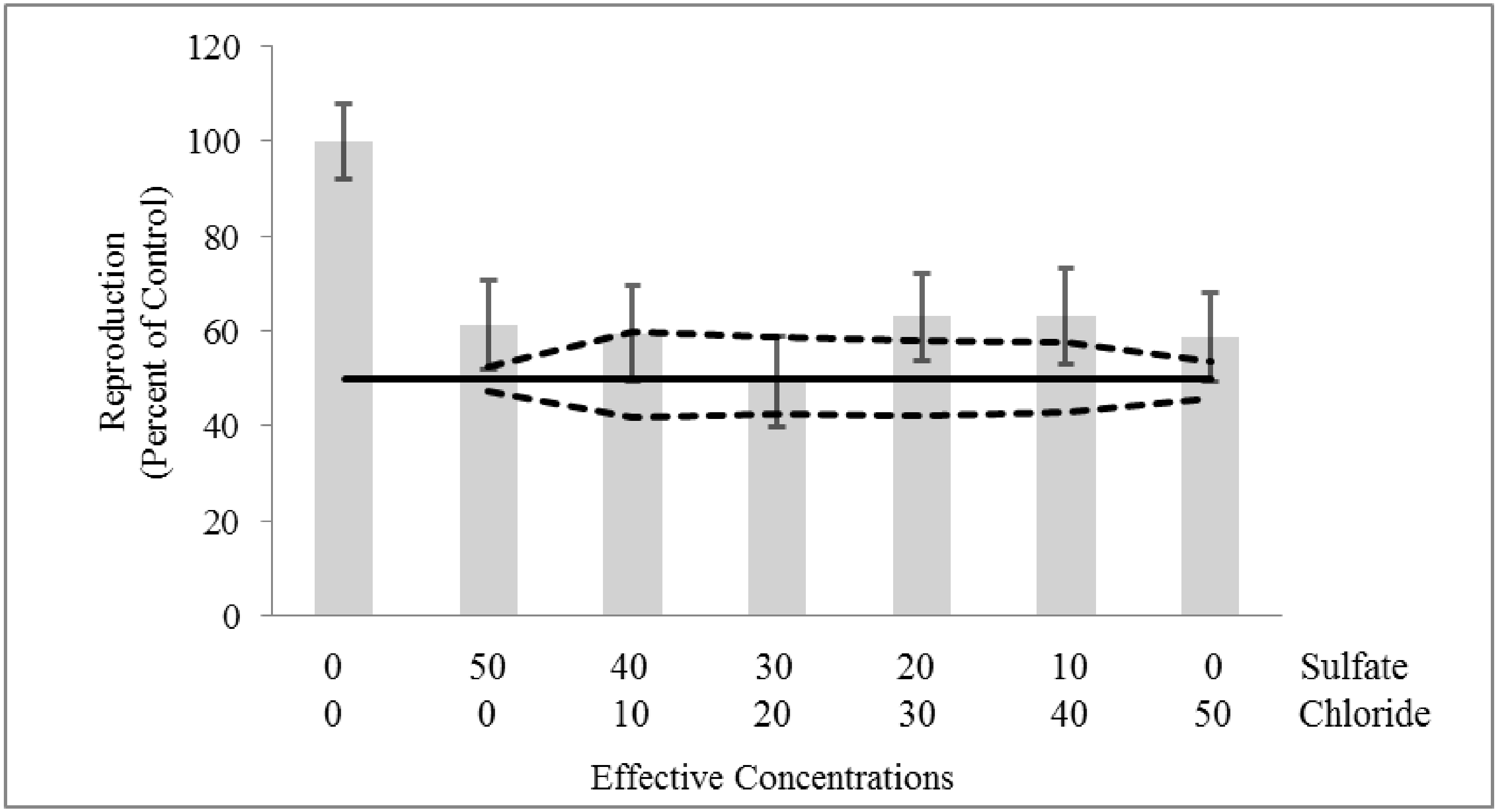

Figure 4.3. The effect of binary mixtures on *C. dubia* reproduction using a dose addition approach with chloride and sulfate. Bars indicate the mean percent of neonates produced relative to the control (± 95% confidence intervals) (n=3). Asterisk (*) represent statistically different means ($p < 0.05$). Dotted lines represent combined 95% confidence intervals for each effective concentration in the mixture. The solid line represents the normalized expected effect (50%) for additivity.

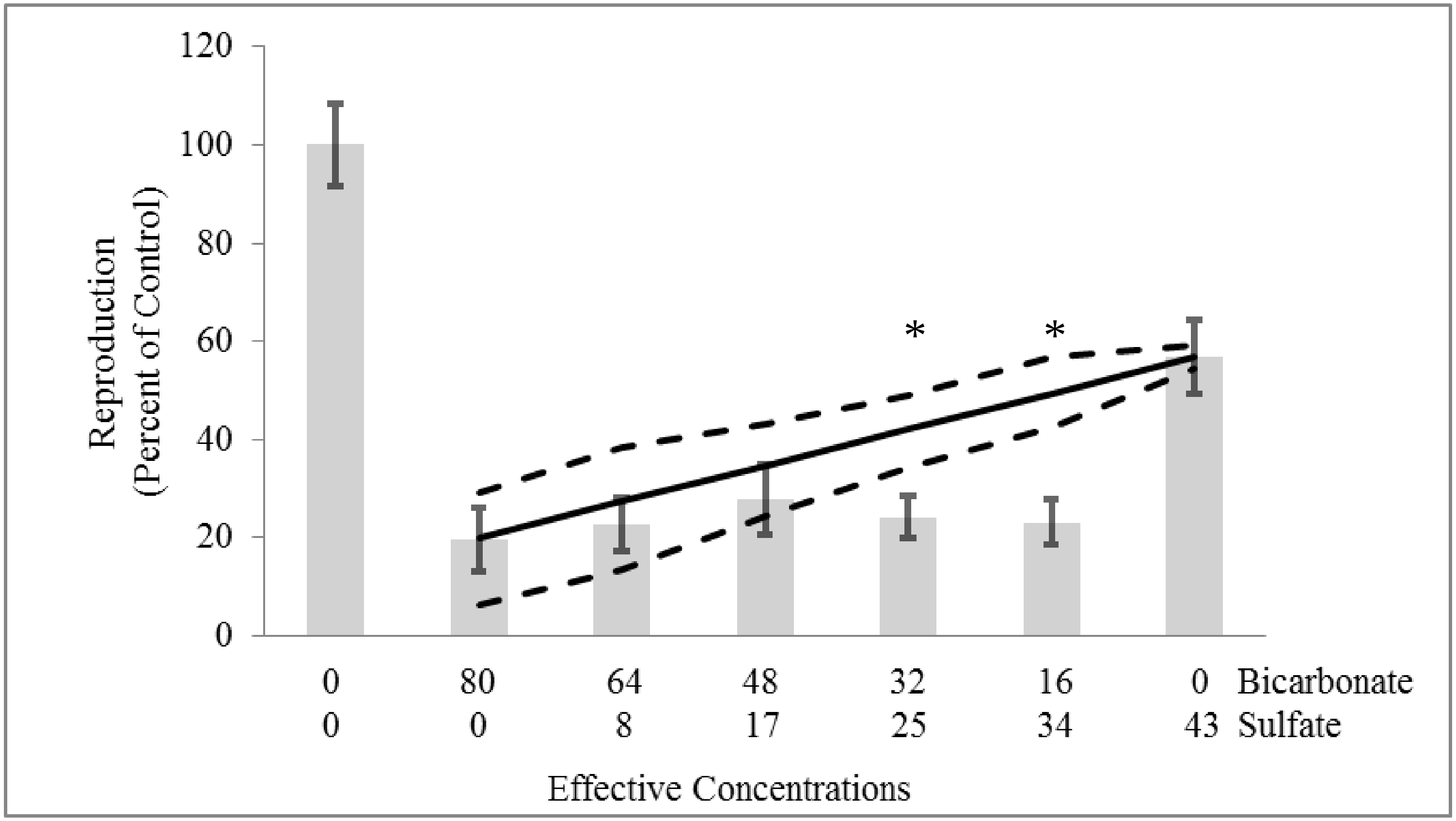

Figure 4.4. The effect of binary mixtures on *C. dubia* reproduction using a dose addition experimental design with bicarbonate and sulfate. Bars indicate the mean percent of neonates produced relative to the control (± 95% confidence intervals) (n=3). Asterisk (*) represents statistically different means ($p < 0.05$). Dotted lines represent normalized 95% confidence intervals for each effective concentration in the mixture. The solid line represents the normalized expected effect (50%) for additivity.

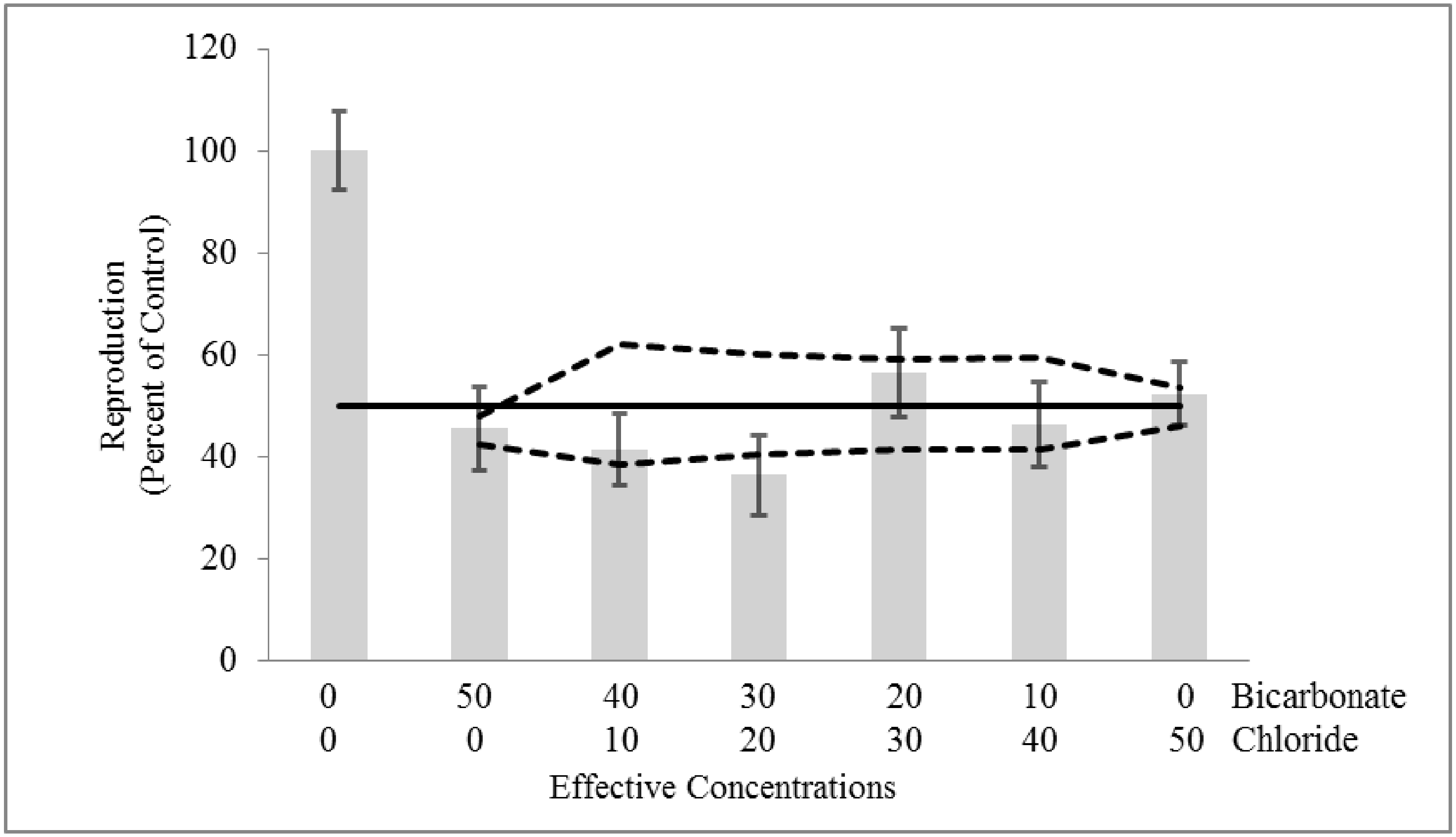

Figure 4.5. The effect of binary mixtures on *C. dubia* reproduction using a dose addition approach with bicarbonate and chloride. Bars indicate the mean percent of neonates produced relative to the control (± 95% confidence intervals) (n=3). Asterisk (*) represents statistically different means ($p < 0.05$). Dotted lines represent 95% confidence intervals for each effective concentration. The solid line represents the normalized expected effect (50%) for additivity.

Chronic Toxicity of Binary Mixtures using Slope Analysis Approach

Combinations with increasing sulfate showed significantly steeper concentration-response slopes when compared to the single sulfate response curve, indicating greater-than-additive interactions of sulfate with bicarbonate (p-value < 0.0001; Figure 3.6), and sulfate with chloride (p-value = 0.0012; Figure 3.6). Further, the concentration-response curve of sulfate in the presence of the bicarbonate EC_{30} was steeper than that for sulfate in the presence of the chloride EC_{30} (Figure 3.6), suggesting a greater interaction between these two ions. Similarly, the dose response for bicarbonate was steeper in the presence of either the EC_{30} of sulfate or the EC_{30} of chloride than alone, indicating greater-than-additive effects (p-value = 0.001 with SO_4^{2-} p-value = 0.0165 with Cl^- Figure 3.7). However, the responses of these two mixtures were not significantly different from each other.

Mixtures containing chloride with fixed sulfate (p-value = 0.4469) had similar concentration-response slopes compared to the single chloride response slope (Figure 3.8). In fact, the response for fixed sulfate with increasing chloride mimicked that of chloride alone. This was opposite for mixtures containing fixed chloride and increasing sulfate. Closer examination of the data revealed that the slope was driven by sulfate concentrations higher than its EC_{30}. This could potentially indicate a critical point at which sulfate deviates from an additive interaction with chloride to a greater-than-additive interaction. This same response was not seen with combinations of sulfate and bicarbonate, suggesting that the bicarbonate response might overshadow the sulfate response. This conclusion is further supported by both mixtures with increasing bicarbonate concentrations. Both mixtures produced a greater response than sulfate alone, but were not significantly different from one another. This suggests increased bicarbonate toxicity when in the presence of another anion.

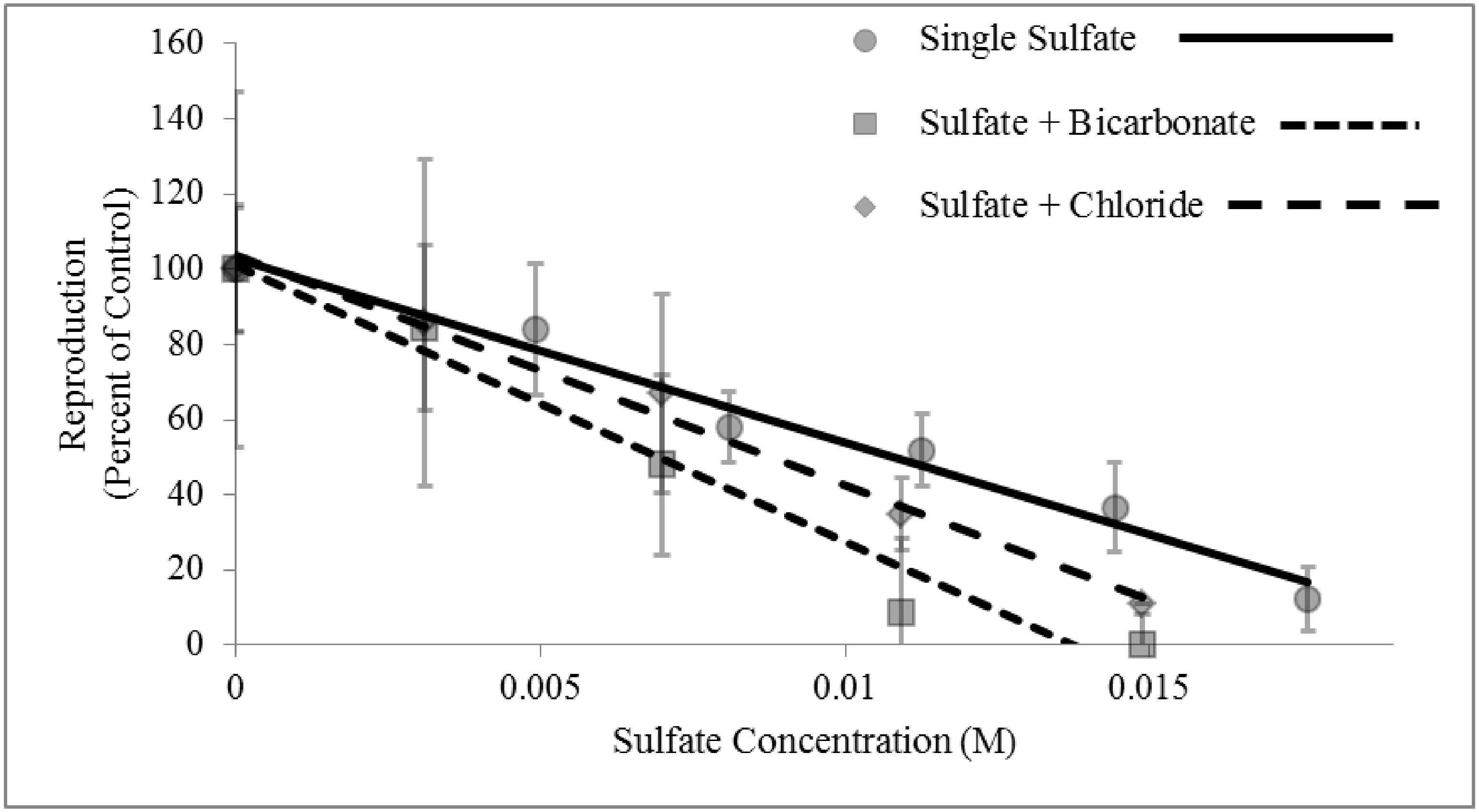

Figure 4.6. The influence of bicarbonate and chloride on sulfate toxicity to *C. dubia* reproduction using a slope analysis approach. Data are shown for single sulfate (⧲), EC_{30} of bicarbonate with increasing sulfate (⊡), and the EC_{30} of chloride with increasing sulfate (◇) ($\bar{x}$ ± standard deviation) (n=3). The linear regressions for each data set are identified in the legend.

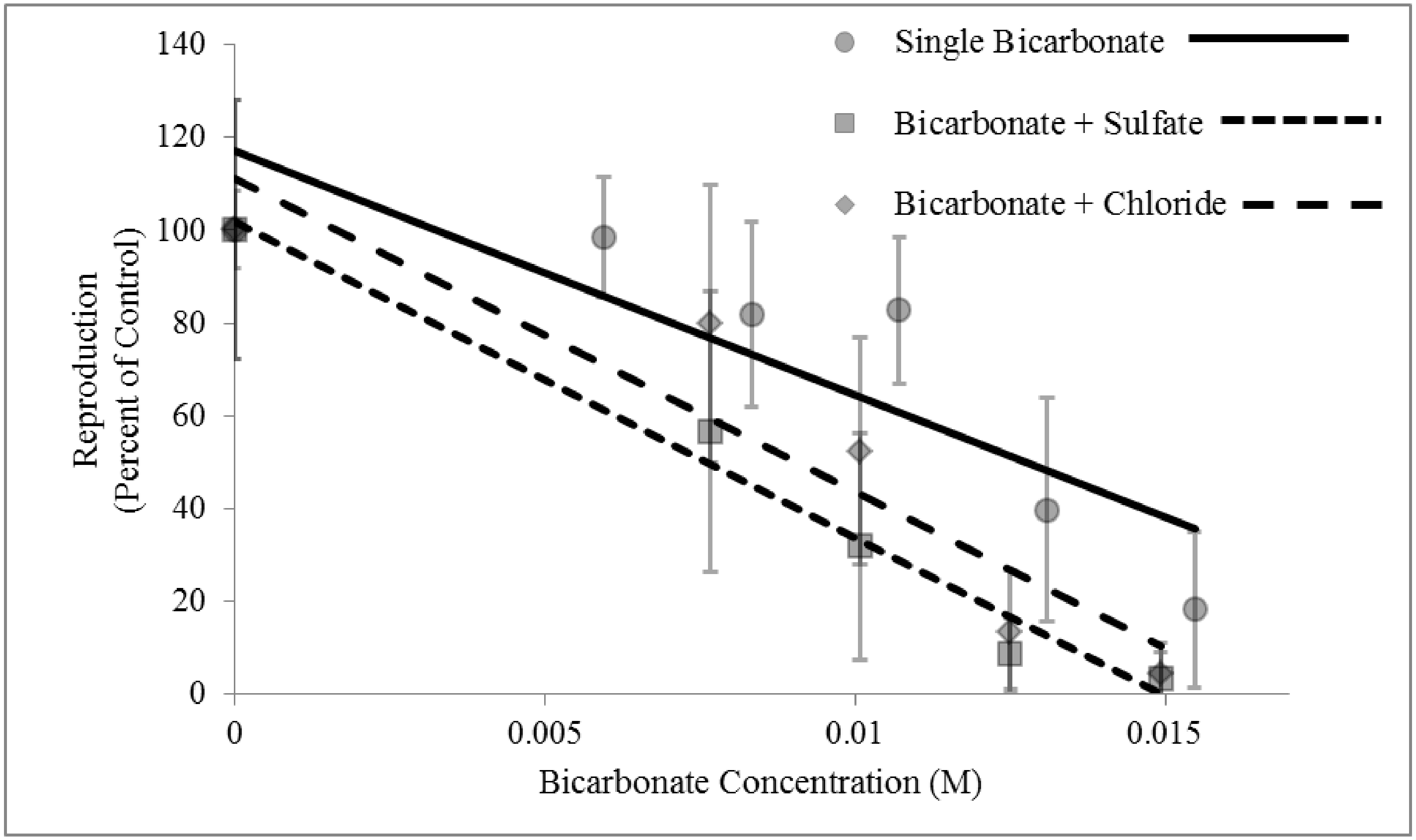

Figure 4.7. The influence of sulfate and chloride on the toxicity of bicarbonate to *C. dubia* reproduction using a slope analysis approach. Data are shown for single bicarbonate (⧲), EC_{30} of sulfate with increasing bicarbonate (⧮), and EC_{30} of chloride with increasing bicarbonate (⧰)($\bar{x}$ ± standard deviation) (n=3). The linear regressions for each data set are identified in the legend.

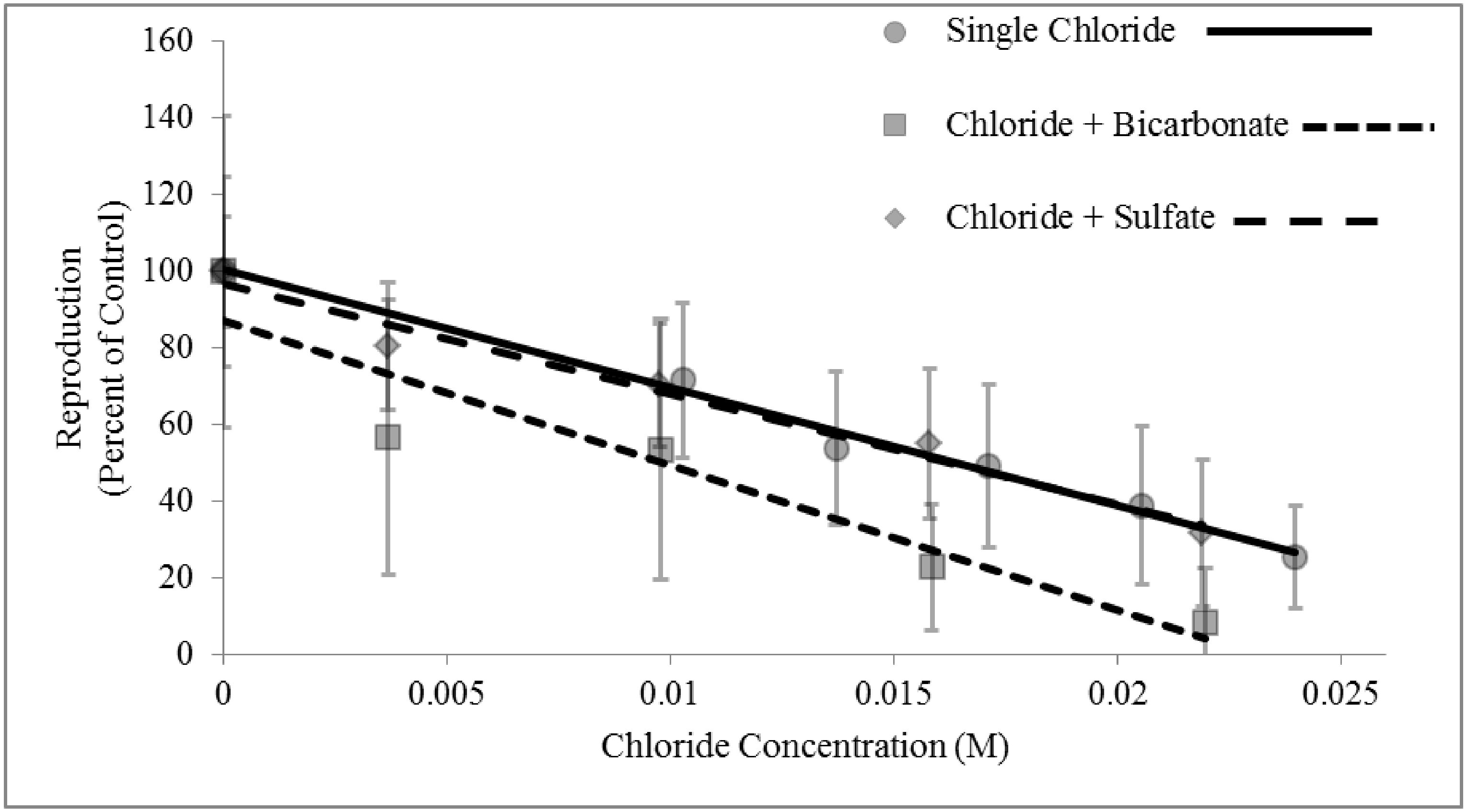

Figure 4.8. The influence of sulfate and bicarbonate on the toxicity of chloride to *C. dubia* reproduction using a slope analysis approach. Data are shown for single chloride (⧲), EC_{30} of bicarbonate with increasing chloride (⧳), and EC_{30} of sulfate with increasing chloride (⧱)($\bar{x}$ ± standard deviation) (n=3). The linear regressions for each data set are identified in the legend.

CHAPTER FIVE

DISCUSSION

Maintaining organism health throughout the experimental process was critical to increase confidence in the results and generate accurate point estimates from single anion concentration-response lines. Minimizing error in point estimates for single anion concentration-response curves is imperative when conducting bioassays on contaminant mixtures. Large confidence intervals associated with point estimates can lead to high variation within treatments and possibly result in low statistical power and an inability to identify responses significantly different from additive.

In the present study, organism health was relatively consistent over the lifetime of this research and as evident by control reproduction far exceeding the minimum requirement for a successful bioassay. Also, estimations of EC values in the present study resulted in 95 percent confidence intervals within 15 percent of the point estimate possibly due to increased replicates in each treatment (n=30). Only EC_{10} estimates were significantly larger, especially for chloride. Other studies have shown similar trends, especially those estimating sub-lethal effects. For example, Van Gestel and Hensbergen (1997) examined the effects of cadmium and zinc on *Folsomia candida* (white springtail arthropod) reproduction. Equitoxic mixtures (EC_{50}:EC_{50}; EC_{10}:EC_{10}) of cadmium and zinc were administered and reproduction was assessed. Non-additive effects were shown for mixtures containing EC_{50}s; however, a trend towards additivity was noticed at lower concentrations (EC_{10}). The authors note large variability around EC_{10} estimates, reaching upwards of an 80% deviation from the point estimate. The conclusion of additive effects when using EC_{10} estimates could have resulted from the large variation around their point estimates essentially precluding any other conclusion. Fortunately, in the present study, mixture treatments covered a wide range of concentrations (EC_{10} – EC_{70}). Because confidence intervals were much tighter towards medium and higher concentrations, variation was greatly reduced.

The EC values estimated for chloride, sulfate and bicarbonate as single anions were similar to what has been previously identified by previous research. Typically, these values have been reported in mg/L because of their useful in water quality practices. However, mass to volume ratios do not accurately represent the actual exposures of a compound to an organism. Instead, organisms are exposed to individual ions within a salt molecule. Because of this, expressing exposure as molarity seemed more appropriate.

These ions are very important in cellular functions such as creating electrochemical gradients within cells. Typically, for freshwater organisms, the concentration inside the hemolymph is greater than the external environment. These organisms rely on a system of pumps and transporters that require energy to move ions across their gills (Ahearn et al., 1998). If the concentration of these ions in the external environment exceeds what is physiologically tolerable for the organism, then the osmoregulatory function or acid-base balancing capabilities of the gill may become dysfunctional. More energy would be needed to counteract the increased ionic strength of their surroundings; this would leave less energy for other important biological functions such as reproduction. However, the exact mechanisms of total dissolved solids and the toxicity of these specific anions in particular are still largely unknown.

Estimating decreased reproduction to identify anion interactions may be indicative of high energy stress conditions. In these results, the reproductive effect of individual ions was greater for sulfate and bicarbonate than for chloride as indicated by slope and EC_{50} values. As binary mixtures, the Dose Addition approach identified additive interactions between all chloride containing mixtures. This was different from the results estimated by the Slope Analysis approach. Slope Analysis identified additivity for chloride, in the presence of the sulfate EC_{30}. However, a greater-than-additive effect occurred when chloride was fixed over increasing concentrations of sulfate. Closer examination of data revealed that when paired with chloride, lower concentrations of sulfate appear to be additive. It was not until sulfate surpassed its EC_{30} that a greater-than-additive effect occurred. In the reverse combination, sulfate was fixed at its EC_{30} concentration and had not reached this critical point. Because of this, sulfate and chloride appeared to act in an additive manner. Once sulfate reached the critical concentration higher than its EC_{30}, it may have competed with chloride at the HCO_3^-/Cl^- exchanger on the gill, reducing chloride uptake. As such, the critical concentration of sulfate may have caused a chloride deficiency, ultimately leading to greater-than-additive effects. Currently, however, the toxic mechanism of excess sulfate is unknown and more research should be completed to determine these effects.

Limitations of the Dose Addition approach may have contributed to the differences in results pertaining to chloride mixtures. Typically, Dose Addition approaches are best suited for compounds with similar concentration-response relationships. However, the concentration-response for chloride was significantly different from both sulfate and bicarbonate. Because of this difference, the Dose Addition approach may have underestimated the effects of combinations including chloride, but accurately estimated sulfate and bicarbonate combinations. The ability for sulfate and bicarbonate to act in a greater-than-additive manner was further highlighted by the Slope Analysis approach.

The difference between experimental designs may also indicate that these ions have different biological effects, indicating that they have different modes of action. Currently, the exact mechanisms for excess ions are largely unknown. Determining the mode of action for each ion individually may identify the way in which these ions interact and contribute to mixture toxicity. Understanding modes of action is particularly important for the development of predicative models to determine the overall toxicity of an environmental exposure.

Earlier work performed by Lasier and Hardin, concluded that binary mixtures of chloride, sulfate and bicarbonate all acted in an additive manner. Because their experimental approach employed the use of a full factorial design with mixtures containing the EC_6, EC_{12} and EC_{25}, it is likely that these point estimates had large 95 percent confidence intervals (95% confidence intervals for point estimates were not published). If so, the variation may have resulted in low statistical power with a low probability of concluding anything but additivity.

Also important may be the way reproduction was assessed. For this particular study, reproduction was normalized to the negative control in single anion experiments, and to the positive control in mixture studies. The latter was done to eliminate the effects of the secondary anion to the initial primary anion. Eliminating these effects resulted in a comparison between the mixture concentration-response line and the single concentration-response line for the increasing anion. The results in the current research were indicative of this divisional normalization approach.

The mixture data was also normalized using an additive normalization approach. The difference in neonate production between the single anion negative control and the mixture positive control was determined. This difference was then added to the total neonates produced per replicate in the mixture treatments. This type of normalization estimated that these anions exhibit additive and less-than-additive behaviors.

One reason for this difference may have been due to the normalization technique. The positive control, to which the mixture treatments were normalized to in a divisional technique, was less than the amount of neonates produced in the negative control for the single anion treatments. Dividing the mixture treatments by a lower number resulted in a change in slope for the mixture concentration-response relationship. Because the mixture slopes may have been influenced by the divisor, the results of this research may be inaccurate. This phenomenon needs more investigation to determine the appropriate way to normalize these data for analysis.

This research was indicative of a static exposure scenario, similar to that experienced in an effluent receiving stream. These results also have wide applicability, especially during constant ion exposure situations. However, some contamination sources occur in an episodic manner such as road runoff from salt application. For these exposures, increased ion concentrations may only last for a few days before diluting, particularly when salt is applied during extended intervals. Because of this, it may be beneficial to determine the chronic effects of chloride, sulfate and bicarbonate in a periodic exposure.

CHAPTER SIX

SUMMARY AND CONCLUSIONS

The objectives of this study were to not only to characterize the toxicity of chloride, sulfate and bicarbonate, but also to compare two different experimental designs for conducting mixture toxicity tests.

The results indicate a relative toxicity of $HCO_3^- > SO_4^{2-} > Cl^-$, estimated from both point estimates (EC_{50}s) and slopes of the concentration-response lines for reproduction as a function of molar units. These results were further reinforced by previous data that suggests similar trends for both chronic and acute exposures, with bicarbonate being the most toxic of the three. Differences between the results of the two experimental designs were also obvious in this research. The Dose Addition approach identified additivity for both mixtures containing chloride, with sulfate and bicarbonate being greater-than-additive. However, the Dose Addition approach is best suited for contaminants with similar concentration-response relationships. Because the concentration-response for chloride was significantly different from sulfate and bicarbonate, the Dose Addition approach may have inaccurately estimated these interactions.

The Slope Analysis identified most ion combinations as being greater-than-additive. In general, the Slope Analysis experimental design yielded results that were more efficiently analyzed.

As a result, the Slope Analysis approach may have more utility in explaining the interactions of contaminants in mixtures. Future studies are needed to test the accuracy of this experimental design using binary mixtures known to produce additive, greater-than-additive and less-than-additive effects.

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