Sulphate toxicity to freshwater organisms
and
molybdenum toxicity to rainbow trout (*Oncorhynchus mykiss*)

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We accept this thesis as conforming
to the required standard

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Abstract

The current "Ambient Water Quality Guidelines for Sulphate (Singleton 2000)" is largely based on studies that have serious flaws that do not accurately assess the toxicity of sulphate. Replication of the principle studies used in the current sulphate guideline rational indicates that the original studies were either flawed in their methodology or the results were misinterpreted. The authors used organisms representing a number of trophic levels and examined sulphate toxicity to the amphipod *Hyalella azteca* (Davies 2002a), the cladoceran *Daphnia magna* (Davies 2002b), the stripped bass (*Morone saxatilus*) (Davies 2002c) and the aquatic moss (*Fontinalis antipyretica*) (Davies 2002d). These replicated studies can be found in the appendices of this report. These studies, as well as others in the literature, indicate that organisms exhibit reduced toxicity from sodium sulphate exposure in waters of increasing hardness and more specifically, waters with increasing calcium content. The incorporation of these new studies into the available literature indicate that in consideration of the relatively low toxicity of sulphate, the current guideline is overly conservative and proposes unnecessarily strict discharge limits. Therefore, a new guideline, taking into consideration the water hardness of the receiving waters is proposed. In waters of hardness (as CaCO₃) of less than 50 mg/L, a maximum allowable discharge that should be sufficient in the protection of ecosystem integrity is proposed at 200 mg/L sulphate. In the range of hardness of 50 to 100 mg/L and in waters of over 100 mg/L hardness, allowable discharge limits of 300 and 400 mg/L sulphate respectfully are proposed.

Two experiments examining the toxicity of molybdenum to rainbow trout (*Oncorhynchus mykiss*) indicate that the results of an earlier study which reported a 29 day LC50 to be 0.79 mg/L molybdenum (Birge et al. 1980) highly exaggerated the toxicity of molybdenum (Davies 2002e). An experiment mimicking the experimental conditions of Birge et al. (1980) study did not cause sufficient mortality to calculate an LC50 up to molybdenum concentrations of 400 mg/L. A second study, which used standard methodology, also did not cause sufficient mortality up to concentrations of 1500 mg/L molybdenum to calculate an LC50.
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Chapter I

Introduction

The polyatomic sulphate anion is composed of a single sulphur atom with four covalently bonded oxygen atoms. This results in the anion having a charge of negative 2 with a chemical formula of $\text{SO}_4^{2-}$ (oxidation state +6). Sulphate occurs naturally in both marine and freshwater systems.

Marine environments have relatively high sulphate concentrations of approximately 2650 mg/L or 27.6 mmol/L in the open oceans (Garrels & MacKenzie 1971). However, much of that sulphate is complexed with other ions naturally occurring in seawater. The percentage concentrations of the dominant sulphate species are as follows: “free” $\text{SO}_4^{2-}$ (39 to 46%), Na$\text{SO}_4$ (26 to 40%), Mg$\text{SO}_4$ (15 to 22%), Ca$\text{SO}_4$ (3 to 5%) and K$\text{SO}_4$ (0.3 to 2%) (Pytkowicz & Kester 1971). This is in contrast to freshwater systems, which generally have far lower sulphate levels with most lakes and rivers in British Columbia having sulphate concentrations between 3 to 30 mg/L. However, some saline lakes in the interior of BC can have sulphate concentrations in the thousands of mg/L (Singleton 2000). Although sulphate ion pairing occurs in freshwater, the amount of complexation is far less in comparison to marine environments due to the far lower concentrations of cations. It has been estimated that sulphate complexes account for about 5 and 2 percent of the calcium and magnesium in solution (Millero 1975 in Nriagu 1978). However, the amount of ion pairing is largely governed by the relative concentrations of cations in solution.

Sulphate can enter freshwater environments from a variety of sources. The burning of fossil fuels, the smelting of ores, and the erupting of volcanoes release sulphur dioxide which can be transformed to sulphuric acid in the atmosphere. The sulphuric acid precipitates from the atmosphere and then dissociates in surface waters to form a sulphate ion and two protons. In poorly buffered areas, this can have deleterious effects on aquatic habitats by causing a drop in $\text{pH}$ which affects gill permeabilities of organisms and influences the speciation and solubility of dissolved metals (Gundersen et al. 1994).
Sulphate can also be produced through the aerobic oxidation of hydrogen sulphide or elemental sulphur by bacteria of the genera *Thiobacillus, Sulfolobus, Chromatia* and *Chlorobia* (Nriagu, 1978). Physical processes can introduce sulphate into freshwater systems through sea spray and ocean water seepage into coastal groundwater systems. Furthermore, the weathering of sulphate containing ores with minerals such as Anhydrite (CaSO₄), Gypsum (CaSO₄·2H₂O), Barite (BaSO₄), Hexahydrite (MgSO₄·6H₂O) and Mirabilite (Na₂SO₄·10H₂O) to name of few, can add substantial amounts of naturally occurring sulphate to surface and ground waters. Depending on physical and chemical conditions affecting solubilities, the reverse reaction with the formation of these minerals can also occur.

Sulphate can be reduced in freshwater environments by anaerobic micro organisms of the genera *Desulfovibrio* and *Desulfotomactum* by using sulphate as an electron acceptor and converting it into hydrogen sulphide (Nriagu, 1978). Furthermore, sulphur is a necessary nutrient for all organisms and can be incorporated into tissues. Sulphate can be used as a source of sulphur for such aerobic organisms as vascular plants, algae, and bacteria which all have the ability to take up, reduce, and assimilate sulphate into amino acids and convert sulphate into ester sulphate compounds (Howarth et al. 1992). Sulphur is needed in such small amounts that it is rarely a limiting nutrient in aquatic environments.

Sulphates are a frequent contaminant found in industrial wastewaters, domestic sewage and mine wastes. Anthropogenic sources of sulphate can account for 20 to over 90 percent of the sulphur in some surface waters (Nriagu 1978).

**Sulphate from industry**

Sulphate is often the major contaminant from mine water and can be the dominant contributor to salinity in the vicinity of waste water discharges (Bowell 2000). Sulphate concentrations in tailings ponds at mine sites can vary depending upon the dominant cation species some of which tend to precipitate with sulphate once its maximum solubility has been reached. Generally, mines are dealing with sulphate concentrations of
around 2000 mg/L due to calcium and sulphate precipitation as gypsum (Bliss, pers. comm.). Due to higher solubilities of other sulphate salts, sulphate concentrations can be found above this concentration; however, it is relatively uncommon to be substantially above this level due to the prevalence of calcium in the environment. Acid mine drainages can have extremely high sulphate levels in the tens of thousands of milligrams per litre (Howarth et al. 1992).

There are physical, biological and chemical methods that can be utilized to reduce sulphate concentrations in wastewaters. The cost, effectiveness and the quantity of land required for these methods usually have an inverse relationship with each other. For example, constructed wetlands are a biological method of treatment which offers the lowest cost for treatment. However, it also has the disadvantage of requiring relatively large amounts of land and one of the lowest treatment efficiencies. In contrast, technologies with high capital costs such as reverse osmosis or ion exchange technologies have high capital costs but can remove most of the sulphate from waste waters in relatively small treatment plants; however, they are also approximately five times more expensive to operate (Bowell 2000). Therefore, required treatment efficiencies and the land use conditions at a mine site can dictate the level and type of treatment strategy that a mine can utilize. All treatment methods have a cost associated with them, and this will consequently influence the type of treatment that a mine will decide to use. As these costs impact a mine’s ability to remain competitive in a global market place it is imperative that allowable discharge levels of sulphate are strict enough that sufficient environmental protection is maintained but is not overly conservative so that industry has to take on a unreasonably high level of treatment burden which is unnecessary in light of the relatively low toxicity of sulphate.
Research Objectives

1. Offer insight into sulphate toxicity in various contexts through a study of the available literature;
2. Investigate and discuss water chemistry characteristics that can influence the toxicity of sulphate and subsequently the results of toxicity tests investigating sulphate toxicity;
3. Critique and replicate specific bioassays used in the current “Ambient water quality guidelines for sulphate (Singleton 2000)” which are inconsistent with other published data and to assess their validity in the use in water quality guideline development;
4. Incorporate new data not used in the current sulphate guideline and determine whether the current guideline is scientifically defensible; and,
5. If justified, recommend a new sulphate guideline for the protection of aquatic life to be used in British Columbia.
Chapter II

Sulphate toxicity in context

The toxicity of a substance is influenced by a variety of biotic and abiotic factors. Different species at different life stages can have vastly different tolerances for substances. This can be seen in sulphate toxicity studies where fish tend to have a much greater tolerance for sulphate than do lower organisms. In general, invertebrate species show much higher sensitivity to sulphate than do fish species (Beak Consultants Incorporated 1997). For example, Mount et al. 1997, reported 48-hour LC50 for fathead minnow (Pimphales promelas) and Ceriodaphnia to be >7960 and 3080 mg/L Na2SO4 respectively. The identification of sensitive species by using a wide range of organisms in toxicity testing that comprise a range of trophic levels is fundamental in order to establish an understanding of whether the introduction of a contaminant will have an adverse effect on an ecosystem.

Factors affecting sulphate toxicity

pH

pH can be an important consideration in certain toxicological studies. For example, the toxicity of aluminum to aquatic organisms is related to its solubility and chemical speciation which are both largely dependant on pH (Deke et al. 1994). However, sulphate is a weak base with a pKa of 1.92 (Oxtoby & Nachtrieb 1990). Therefore, waters with a pH above 5.0 will have approximately 99.9 percent of the sulphate in the SO4^2- form and less than 0.1 percent in the HSO4^- form. Therefore, the influence of sulphate toxicity due to speciation caused by the direct effects of pH is unlikely.

Complexation and water hardness effects on ion toxicity

The toxicity profile of common ions is difficult to quantify in produced waters as they are present with other chemicals in a complex mixture (Tiege et al. 1997). For example, the relationship between calcium, sulphate and bicarbonate is complex with concentrations of
each ion influencing precipitation reactions, complexation, and bioavailability (Pillard et al. 2000).

By comparison, various chemical characteristics of exposure water also influence the toxicity of metals because of associations among chemical constituents. These affect bioavailability, competition among different chemicals for sites of uptake, and/or changes in the physiological susceptibility of the metal (Hamelink et al. 1994). The reduction in the toxicity of some metals to aquatic organisms in waters of increasing water hardness has been well established (Jayaraj et al. 1992) and is much better documented in the literature in comparison to hardness effects on ion toxicity. This has influenced the development of water quality criteria in British Columbia with the allowable discharges of silver, zinc, manganese and copper partially dependant upon the water hardness of the receiving waters (BC Ministry of the Environment, Lands and Parks (1987, 1996, 1999, 2001)).

Designing bioassays to assess the toxicity of common ions presents challenges to experimental design. For instance, ions such as Na⁺, Ca²⁺, Cl⁻, and others are required at minimum levels to support aquatic life (Mount et al. 1997). Therefore, complete absence of these ions, as would be ideal in a fully controlled experiment, is not possible. Furthermore, ions cannot be added individually as a cation or anion, but as a salt; therefore, the effect of the associated counter-ion must also be considered (Ho & Caudle 1997). Sulphate can be added to a test solution with a variety of associated cations. Calcium sulphate (CaSO₄), magnesium sulphate (MgSO₄), potassium sulphate (K₂SO₄) and sodium sulphate (Na₂SO₄) are a few common salts that have been used in toxicity tests to simulate waters of high sulphate content.

Tests which have utilized these salts consistently show different toxicity to these sulphate salts. For example, Mount et al. (1997) reported 48-hour LC50s for D. magna for CaSO₄, MgSO₄, K₂SO₄, and Na₂SO₄ to be >1391, 1452, 397 and 3097 mg/L SO₄ respectively. This suggests that toxicity of some sulphate salts is not always due to the sulphate anion but rather to the associated cation. To complicate matters further, the relatively high toxicity of K₂SO₄, in comparison to CaSO₄ may not simply be due to the toxicity of the
potassium cation but may also be an amelioration effect of sulphate toxicity from the associated calcium cation in CaSO_4. In this instance, possibly both of these mechanisms are at work. Regression models were designed by Mount et al. (1997) to specifically address this problem. Regression modeling of over 2900 ion solutions using *Ceriodaphnia, D. magna* and Fathead minnows indicated that the relative ion toxicity of these solutions were K^+ > Mg^{2+} > Cl^- > SO_{4}^{2-} (based on mass). The Na^+ or Ca^{2+} cations associated with the anions Cl^- and SO_{4}^{2-} were not considered significant variables in the regression. However, the toxicity of the potassium and magnesium salts was attributed to the cation rather than the associated sulphate anion. Therefore, the use of K_2SO_4 or MgSO_4 to test for sulphate toxicity would in fact be testing for the toxicity of the much more toxic cation rather than the relatively less toxic sulphate anion. Mount et al. (1997) went further and tested various salts in combination with each other. His results indicated that a solution with more than one salt (for example, NaCl and CaCl_2) was less toxic (on the basis of Cl^- concentration) than either of the solutions tested alone.

This further complicates ion toxicity studies as there is substantial evidence indicating that sulphate toxicity is reduced in waters with increasing hardness (Figure 1). Furthermore, calcium as a component of water hardness appears to be more important in reducing sulphate toxicity than magnesium, the other anion that contributed to water hardness (Figure 2).
Figure 1. Declining toxicity of sodium sulphate to various organisms in waters of increasing water hardness. ■ and ▲ correspond to 48 and 96 hour LC50s for D. magna (Davies 2001b) and H. azteca (Davies 2001a) respectfully while ● indicates 7 day EC50 trout embryo (Oncorhynchus mykiss) test (Pacific Environmental Science Centre (PESC) 1996).

Figure 2. Reduction in toxicity of sodium sulphate in waters of increasing calcium content to D. magna (Davies 2001b). Waters have identical water hardness of 100 mg/L (as CaCO₃). Error bars are 95 percent fiducial limits.

As sulphate cannot be added without an associated cation, adding sulphate singly to solution is not an option. Even if this were possible, the addition of an abundance of
negatively charged ions would create a charge imbalance in the test water, which could disrupt the natural ion flow between the test organisms and aquatic media. This in itself could cause stress on the organisms and influence the results of the toxicity tests. Furthermore, sulphate is found in an environmental context not by itself but with other ions in solution.

\[ \text{Na}_2\text{SO}_4 \] is likely the best candidate to be used to assess the toxicity of sulphate in the aquatic environment for the following reasons:

1. It is very soluble and does not readily precipitate out of solution; thus maintaining relatively consistent sulphate exposure levels for the duration of the test;
2. Although the lack of Na\(^+\) has a role in ion-deficiency toxicity, studies have shown that it is not generally a major contributor to toxicity (Mount et al. 1997); and,
3. Lakes with naturally occurring high levels of sulphate in the interior of British Columbia have sodium as the dominant cation (Hall, pers. comm. 2002).

A difficulty in assessing sulphate toxicity using sodium sulphate as the associated sodium ion can cause osmotic stress on the test organism. Sodium sulphate is 33 percent sodium by mass; however, by molarity it is 67 percent. Therefore, a 1000 mg/L exposure of sodium sulphate will cause an exposure of 670 and 330 mg/L of sulphate and sodium respectfully. However, changing these values to molarity causes an exposure of 7.0 and 14.4 mmols/L of sulphate and sodium respectfully. The osmotic pressure exerted by a solution is a function of the concentration of the solute components rather than their mass (Potts & Parry 1964). Therefore, in instances where toxicity is caused by osmotic stress, it is likely that the toxicity has been influenced by the presence of the more than double concentration of sodium ions rather than simply by the sulphate ions alone. However, as all the other sulphate salts are more toxic and sodium sulphate may overestimate the toxicity of sulphate, it is still the best sulphate salt to use to assess sulphate toxicity.
Types of ion toxicity

Salinity is a measure of the concentration of dissolved salts found in a solution (Tarbuck & Lutgens 1987). Most studies that investigate the effects of salinity on organisms usually use solutions with high levels of sodium chloride. However, toxicity from different salts can be comparable depending upon the mode of salt toxicity. In general, high salinity is detrimental to the health of freshwater plants and animals because the cells of the organism have either: 1) a lack of water due to osmotic leaching from water with high ion content; or, 2) cells have taken up ions to toxic concentrations in their cells (or both) (Hart et al. 1991). The mechanisms of salt toxicity are usually divided into two main groups; osmotic stress and specific ion toxicity (Blomqvist 1999). Osmotic stress leads to inhibition of water uptake due to decreased osmotic potential between the organism and aquatic medium. Specific ion toxicity occurs when the ions have entered the cells of organisms and have adverse effects on normal cellular functions (Blomqvist 1999).

Much of the research examining the mode of toxicity that sulphate causes has been in the soil sciences examining the effects of various salts on plants, specifically the effects of sodium chloride (Egan & Ungar 1998). For example, Egan and Ungar (1998) investigated the effects of K₂SO₄, KCl, NaCl and Na₂SO₄ on the growth of the terrestrial weed, Atriplex prostrata and found the toxicities of the following to be K₂SO₄ > KCl > NaCl = Na₂SO₄ when exposed by comparable osmotic potentials. At the end of the 5 week test, all plants exposed to K₂SO₄ and 40 percent of the plants exposed to KCl were dead. However, no detrimental impacts on survival were observed in either the NaCl or Na₂SO₄ treatments. This toxicity was attributed to the specific ion toxicity caused by the potassium ion of the salts. However, another study examining the germination of the same weed indicated that seed germination was more sensitive to osmotic stress than to specific ion toxicity (Egan et al. 1997). Therefore, in that instance, the sodium salts were more inhibitory to germination than were the potassium salts.
The trend of specific ion toxicity observed in the Egan et al. (1998) study is consistent with the trend observed in the toxicity to invertebrates and fish where relative ion toxicity for *Ceriodaphnia*, *D. magna* and Fathead minnows is $K^+ > Cl^- > SO_4^{2-}$ (Mount et al. 1997).

**Toxicity of sodium sulphate in comparison to sodium chloride**

Although salinity can refer to any combination of dissolved inorganic salts, in the literature salinity studies frequently use solutions which the dominant salt is sodium chloride. The reasoning behind this is presumably that it is generally the dominant salt where saline conditions are encountered and is the dominant salt in seawater (85 percent by mass).

In contradiction to most studies, some studies have reported that $Na_2SO_4$ is more toxic to some species in comparison to $NaCl$. Goetsch & Palmer (1997) reported that the Ephemeropteran mayfly (*Tricyrythus* sp.) was more sensitive to sodium sulphate with LC50s for $Na_2SO_4$ of 660 mg/L sulphate and an LC50 for $NaCl$ in the range of 2200 and 4500 mg/L chloride.

However, the majority of studies report the toxicity of sodium sulphate and sodium chloride to be quite similar when compared on a mass basis. Stanley (1974) who studied the effects of metals and salts on the Eurasian watermilfoil (*Myriophyllum spicatum L.*) showed that $Na_2SO_4$ has lower toxicity than $NaCl$ with the exception of its affect on shoot length (see Table 5, pg. 25). It was hypothesised that the increased toxicity, seen in the reduction in shoot length was due to the comparatively large size of the sulphate ion which reduced its transport rate across cell membranes which is required to equalize osmotic gradients. It was hypothesized that a slower acclimation period would likely reduce the observed toxicity. Furthermore, Mount et al. (1997) conducted toxicity tests using fathead minnow, *Ceriodaphnia*, and *D. magna* subjecting them to a variety and combination of different salts. *Ceriodaphnia* exhibited the highest sensitivity to the salts and Fathead minnows the least. The results reported indicate that sodium sulphate is less
toxic than sodium chloride when reported on a mass basis; however, due to the relatively high molecular weight of the sulphate molecule, sodium sulphate is actually more toxic than sodium chloride when converted into molar units (Table 1). The high sensitivity of Ceriodaphnia to sodium chloride compared to sodium sulphate was also reported by Warne & Schifko (1999) which reported 48-hour LC50 concentrations of 2123 and 3150 mg/L for NaCl and Na₂SO₄ respectfully. Furthermore, the tests using equal mass amounts of the two salts indicate that the toxicity of the sum total of the salts was relatively close to the toxicity of the single salt tests. However, the salt combination tests use different molar mass amounts and it is difficult to make any claims regarding the relative contribution that either salt adds to toxicity.

Table 1. Toxicity of NaCl, Na₂SO₄ and equal mass amounts of NaCl and Na₂SO₄ to Ceriodaphnia, Daphnia magna and fathead minnow (Pimphales pronelas). Values are LC50 values for the test duration. Values in brackets are sum totals of ratio amounts (Mount et al. 1997).

<table>
<thead>
<tr>
<th></th>
<th>Ceriodaphnia</th>
<th>Daphnia magna</th>
<th>Fathead minnow</th>
</tr>
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<tbody>
<tr>
<td><strong>Mg/L</strong></td>
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<td>48-hour</td>
<td>96-hour</td>
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<td>4770</td>
<td>6390</td>
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<tr>
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<td>2850/2850</td>
<td>3045/3045</td>
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<tr>
<td></td>
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<td>(5700)</td>
<td>(6090)</td>
</tr>
<tr>
<td><strong>Mmols/L</strong></td>
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</tr>
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</tr>
<tr>
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<td>32.2</td>
<td>56.0</td>
</tr>
<tr>
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<td>26.3/10.8</td>
<td>48.8/20.1</td>
<td>52.1/21.43</td>
</tr>
</tbody>
</table>

The data reported by Mount et al. (1997) suggest that Na₂SO₄ and NaCl both cause toxicity by a similar mechanism. This is gained through the observation that the total mass amounts of the Na₂SO₄ and NaCl used in the double salt exposures are comparable to the LC50 values of the single salt tests. Therefore, this suggests that any environmental guideline proposed should be relatively conservative in order to be
protective of environments which also have significant amounts of chloride which may add to toxicity.

Conductivity and total dissolved solids (TDS) are measures that incorporate the total ion content of freshwater (Mount et al. 1997). However, although there is a strong correlation between these parameters for a given ion content, they are both relatively ambiguous terms regarding the specific ion content of the water. For example, waters with a relatively toxic salt such as potassium sulphate will cause toxicity at low conductivity and TDS levels; however, salt of lower toxicity such as sodium sulphate need much higher conductivity and TDS to stimulate a toxic response. However, conductivity and TDS may be a good parameter to use to assess the total toxicity of waters where the dominant salts have relatively low toxicity such as sodium sulphate and sodium chloride.

**Amelioration of toxicity of sodium sulphate**

The toxicity caused by waters high in sodium sulphate has been shown to be reduced in waters of increasing hardness and also of increasing calcium content. Water hardness is a measure of the quantities of polyvalent cations in water. Hardness generally represents the concentration of calcium (Ca\(^{2+}\)) and magnesium (Mg\(^{2+}\)) ions, because these are the most common polyvalent cations. Other ions, such as iron (Fe\(^{3+}\)) and manganese (Mn\(^{2+}\)), may also contribute to the hardness of water but are generally present in much lower concentrations. Water hardness is standardized by converting the calcium and magnesium content of the solution into calcium carbonate equivalences. As both calcium and magnesium are summed into a total hardness value, waters containing significantly different ratios of calcium and magnesium can still have comparable hardness values.
The formula used to calculate water hardness is as follows:

\[
\text{Total hardness (mg/L as CaCO}_3\text{)} = 2.497 \ [\text{Ca}^{2+}] + 4.118 \ [\text{Mg}^{2+}]
\]

Where: \([\text{Ca}^{2+}]\) is the calcium ion concentration in the solution in mg/L
\([\text{Mg}^{2+}]\) is the magnesium ion concentration in the solution in mg/L

One possible explanation as to why sodium sulphate toxicity is reduced in waters of increasing hardness is the possible formation of ion pairs between calcium and sulphate ions. In seawater, approximately 60 percent of the sulphate is ion paired to sodium, calcium and magnesium cations. However, seawater has extremely high concentrations of these cations in comparison to the amount of sulphate ions. The approximate concentrations of \text{Ca}^{2+}, \text{Mg}^{2+} and \text{Na}^+ ions in seawater is 400 mg/L and 1.272 and 10.556 g/L compared to freshwaters which typically have concentrations of these cations in amounts less than 50 mg/L. Waters of low ion content form few ion pairs as the amount of pairing is a function of the relative concentration of the ions in solution. Furthermore, ion pair modeling using PHREEQC (Parkhurst & Appelo 2001) indicated that less than 0.1 percent of the total sulphate would complex with calcium to form \text{CaSO}_4, \text{MgSO}_4 or \text{NaSO}_4^\text{-} ion pairs in waters with hardness values less than 100 mg/L as CaCO_3. Therefore, some other mechanism is influencing toxicity.

The majority of the work examining \text{Ca}^{2+} effects on ion exchange involve fish species. Studies examining toxicity reduction of metals (Markich & Jeffree 1994) and saline solutions have also found that Ca has a greater effect in reducing toxicity than Mg. Hille et al. (1975) in Markich and Jeffree (1994) showed that the binding affinity of Ca to the metal receptors at the mouth of \text{Ca}^{2+} channel on membrane surfaces exceeded that of \text{Mg}^{2+}. Metals from the aquatic environment enter the body fluids via calcium channels which disrupt the ion exchanging capacity of gill membranes (Galvez & Wood 1997). This results in a loss of ion regulation capacity of the organism leading to chronic or acute toxicity. Wendelarr Bonga et al. (1983) examined the effects of Ca and Mg on Talapia (\textit{Sarotherodon mossambicus}) gill membranes and found that Mg was not as effective at reducing osmotic influx of water across the gills. Although no information
could be found on the role of calcium in reducing the toxicity of sodium sulphate exposure, there is evidence that sodium sulphate is less toxic in waters with higher calcium contents (Davies 2001b). Ca\(^{2+}\) plays a fundamental role in the regulation of sodium between the gill membranes and the surrounding aquatic media with various studies examining ion transport in fish gill membranes (Cuthbert and Maetz (1972), Eddy (1975), Pic and Maetz (1981)) indicate that Ca\(^{2+}\) reduces the permeability of gills to sodium and hydrogen ions. As previously mentioned, the osmotic pressure exerted by a solution is a function of the concentration of solute rather than its mass (Potts & Parry 1964). As sodium sulphate has double the molar concentration of sodium compared to sulphate, the reduction of sodium sulphate toxicity observed in waters of increasing calcium content may indicate that the sodium cation may be causing a portion of the toxicity rather than the toxicity simply coming from the sulphate ion. However, as no studies could be found that examine the direct effects of Ca\(^{2+}\) on the permeability of gill membranes to sodium and sulphate ions collectively, further studies are required to delineate these interactions.

**Antagonistic effects of sulphate to other pollutants**

Sulphate can also play a role in aquatic systems in reducing the toxicity of some substances. Selenium is found in some drainage waters and can cause environmental impacts by the toxic effects and bioaccumulation of the metal. The addition of sodium sulphate has been found to ameliorate Se accumulation in aquatic macrophytes, more so than the addition of sodium chloride (Wu & Guo 2002). Furthermore, Williams et al. (1994) examined the effects of selenate on the green algae *Selenastrum capricornutum* and determined that sulphate and selenate compete for active transport across cell membranes via a common permease. Low levels of sulphate were able to significantly decrease the selenate accumulation in the algae.

Sulphate is very similar stereochemically to molybdate (MoO\(_4^{2-}\)), the most common and thermodynamically stable form of molybdenum found in oxic environments of neutral pH (Howarth et al. 1992). Consequently, sulphate can inhibit the uptake and assimilation
of molybdenum. This phenomenon has been shown in algae, bacteria and tomato plants. Sulphate has been used to reduce the amount of molybdenum uptake by sheep, which were exposed to food with a high molybdenum concentration (Howarth et al. 1992).
Chapter III

Current sulphate guideline for the protection of aquatic life

Although there are guidelines for sulphate which have been proposed in both the United States and the World Health Organization, these specifically address sulphate in drinking water rather than its effects on aquatic life. Both of these guidelines suggest maximum drinking water limits up to 250 mg/L sulphate as higher concentrations can cause a laxative effect to humans. The federal drinking water guideline in Canada is an aesthetic objective of less than 500 mg/L (Health Canada 1996). The sulphate guidelines proposed by the province of British Columbia to protect aquatic life appears to be the only guideline of its kind. The current guideline for the protection of aquatic life is 100 mg/L of dissolved sulphate, as a maximum concentration. The rationale of this guideline is based primarily on three studies, which indicated certain species were extremely sensitive to dissolved sulphate as sulphate is generally considered to be of low toxicity. The rationale is as follows (excerpt from Singleton (2000)):

i. Hughes (1973) reported 1, 2, 3, and 4 day LC50s of 2000, 1000, 500, and 250 mg/L for SO$_4^{2-}$, and LC0s (no effect) of 500, 100, 100, and 100 mg/L, respectively, for striped bass (Morone saxatilus) larvae.

ii. Unpublished data from a series of toxicity tests performed by The Pacific Environmental Science Centre (Pacific Environmental Science Centre (PESC) 1996) for BC MELP in 1996 showed that the amphipod, H. azteca, was sensitive to sulphate in soft water, but not in medium (100 mg/L as CaCO$_3$) to hard water (250 mg/L as CaCO$_3$). PESC reported 96 hour LC50s for H. azteca in soft, medium and hard water of 205, 3711, and 6787 mg/L SO$_4^{2-}$, respectively. A water quality guideline of 100 mg/L provides protection with a 2:1 safety factor in soft water, and a significantly greater safety factor in harder water more typical throughout BC.

iii. (Frahm 1975) demonstrated that a concentration of 100 mg/L SO$_4$ was toxic to the aquatic moss, Fontinalis antipyretica, a species which is known to be widely distributed throughout BC. Toxicity of SO$_4^{2-}$ to four other species of aquatic moss
ranged from 100 to >250 mg/L. There are more recent data (Beak International Incorporated & Michigan Technological University 1998) that conflicts with these earlier (Frahm 1975) data but the chosen endpoint of the newer data is in question.

iv. There is some evidence that elevated sulphate levels (average of 71 mg/L sulphate; range of 27.7 to 189 mg/L) can stimulate large sulphur bacteria growths which can cover creek beds and result in significant changes to the macroinvertebrate community. Anecdotal evidence is not used to derive water quality guidelines due to the absence of scientific defensibility of such information. But such information is worth noting to provide the impetus to stimulate the necessary future research into such observations.

Response to current guideline

The justifications used to develop the current guideline of 100 mg/L sulphate are being re-examined as a number of the studies outlined above have flaws which make them unsuitable for the use in water quality guideline development. There has been further research done on the subject of sulphate toxicity in peer reviewed journals which provide further insight into sulphate toxicity. Therefore, the guidelines will be reassessed with the new data being incorporated into a proposed guideline.

In addition, the guideline does not take into account the influence of water hardness on sulphate toxicity. An approximate halving of sulphate toxicity was observed in tests using *H. azteca* (Davies 2002a) and *D. magna* (Davies 2002b) with increases in hardness of 25 mg/L as CaCO3. The trend of reduced sulphate toxicity in waters of increasing hardness has also been observed in the aquatic moss (*F. antipyretica*) (Davies 2002d) and studies using rainbow trout (*Oncorhynchus mykiss*) (PESC, 1996). These studies, including an examination of the literature in regards to the influence of water hardness on sulphate toxicity will provide a framework in which sulphate discharges will be dependant upon the water hardness of the receiving waters.
Guideline justification evaluation

i. Hughes (1973) describes the toxicity of thirty chemicals to striped bass larvae and fingerlings. In general, fish have generally much higher tolerances to salts in comparison to most other organisms (Hart et al. 1991). For example, PESC (1996) reported 96 hour LC50 values for coho salmon and rainbow trout to be >9550 and >9750 mg/L sulphate (in water of 100 mg/L hardness (as CaCO₃)). Furthermore, Mount et al. reported a 96 hour LC50 for fathead minnow to be 5380 mg/L sulphate. Intuitively, striped bass should be even more tolerant to high levels of salts as they are principally a marine species where the ambient background sulphate concentration is approximately 2700 mg/L. The high levels of other salts found in seawater require that marine organisms (with few exceptions) to be good osmotic and ionic regulators.

The reported toxicities of some of the other chemicals tested are substantially higher in comparison to similar studies in the literature, bringing the validity of the entire study done by Hughes (1973) into question (Table 2).
Table 2. Comparison of acute toxicity of trace metals to striped bass, reported by Hughes and others, for chemicals assessed in U.S. EPA water quality criteria documents (from Beak Consultants Incorporated (1997))

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Hardness (mg/L as CaCO₃)</th>
<th>96-hr LC50 (µg/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium</td>
<td>55</td>
<td>1 100 (fingerlings)</td>
<td>(Rehwoldt et al. 1972)</td>
</tr>
<tr>
<td></td>
<td>34.5</td>
<td>1 (larvae)</td>
<td>(Hughes 1973)</td>
</tr>
<tr>
<td></td>
<td>34.5</td>
<td>1 (fingerlings)</td>
<td>(Hughes 1973)</td>
</tr>
<tr>
<td>Zinc</td>
<td>55</td>
<td>6 800 (fingerlings)</td>
<td>(Rehwoldt et al. 1972)</td>
</tr>
<tr>
<td></td>
<td>137</td>
<td>1 180 (fry)</td>
<td>(O'Rear 1972)</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>100 (larvae)</td>
<td>(Hughes 1973)</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>100 (fingerlings)</td>
<td>(Hughes 1973)</td>
</tr>
<tr>
<td>Copper</td>
<td>55</td>
<td>4 300 (fingerlings)</td>
<td>(Rehwoldt et al. 1972)</td>
</tr>
<tr>
<td></td>
<td>34.5</td>
<td>50 (larvae)</td>
<td>(Hughes 1973)</td>
</tr>
<tr>
<td></td>
<td>34.5</td>
<td>50 (fingerlings)</td>
<td>(Hughes 1973)</td>
</tr>
</tbody>
</table>

Furthermore, the lack of information regarding the methodology of the study also raises concerns about the studies scientific validity. There is no statistical analysis found in the article. The LC50s for 1, 2, 3, and 4 day bioassays reported for many of the substances do not decrease over exposure time. For example, the LC100 values reported for “Instant Sea” is 14,500 mg/L (reported as mg/L chloride) and this value is identical for 1, 2, 3 and 4 days of exposure.

This phenomenon is also seen in other the results presented in Hughes (1973) for the chemicals 2, 4-D butyl ester, Diquat and calcium hypochlorite. This trend in toxicological data would only be possible if mortality only occurred in the first 24 hours and then immediately halted. Furthermore, the 96 hour LC100 for “Instant Sea” of 14,500 mg/L was reported as mg/L chloride. This is far from the LC100 Hughes reported for chloride of 2000 mg/L after 96 hours. In addition, many of the LC50s that do decrease overtime frequently go down in a geometric stepwise process, such as the presented LC50s for sulphate which decreased by exactly half every 24 hours.
The inconsistencies of the results presented by Hughes (1973) are not consistent with the general trends seen in toxicological studies. Furthermore, the article appears in conference proceedings and shows no evidence of being peer reviewed.

This study was replicated (Davies 2002c) and the extremely low values reported in Hughes (1973) were attributed to culture water toxicity which likely exaggerated the toxicity of the substances tested. The culture water used in the original Hughes study had very low salinity and was in soft water of approximately 34.5 hardness (as CaCO$_3$). The replicated study (Davies 2002c) found that the addition of sulphate in the form of sodium sulphate actually increased the survival of the striped bass from less than 20 percent in controls which had low salinity to up to 85 percent in the 4000 mg/L sulphate exposures (this was the highest concentration tested). The phenomenon of low survival of striped bass larvae in waters of low salinity has been reported in the literature (Morgan & Rasin 1981, Winger et al. 1997); however, salinity addition in these tests was in the form of sodium chloride rather than sodium sulphate. All of the results presented in Hughes (1973) likely present a biased evaluation of all the substances tested and should not be used in the development of any water quality guideline. In contrast to the high sensitivity of striped bass to sulphate, this study indicates that sodium sulphate is beneficial rather than detrimental to striped bass larvae survival in waters of low salinity.

ii. PESC (1996) reported that the amphipod, $H. azteca$ was extremely sensitive to sulphate exposure (added as sodium sulphate) in soft but not medium or hard water. The study was repeated and the results indicated that the high sensitivity to sulphate in soft water reported by PESC (1996) was likely due to test water quality characteristics, which were not consistent with accepted exposure water formulation methodology for $H. azteca$. The ion composition of the test water in the soft water treatment was similar (but diluted by a factor of four) to that found in the experimental protocol guidelines for $Ceriodaphnia dubia$ and $D. magna$; however it is not consistent with those recommended in the current EPA guidelines (USEPA 2000). Specifically, the culture water used in the soft water treatment in the PESC experiment was
completely lacking in Cl' ions which resulted in poor survival in the control treatments. The four replications of the experiment consistently resulted in poor survival in the control test water that was used in the original study PESC study (Davies 2002a). Therefore, LC50 data for the soft water test series in the PESC (1996) study is not an accurate assessment of sulphate toxicity to *H. azteca*. Davies (2002a) demonstrated that in soft water, *H. azteca* is not as sensitive as PESC (1996) reported. Two tests done in soft water resulted in an LC50 of 491 mg/L and NOE of 453 mg/L sulphate. The NOE of 453 mg/L sulphate suggests that the LC50 calculated may be at the lower end of the tolerance range of *H. azteca*. However, further tests are needed to confirm this. The LC50s calculated in a range of water hardness treatments in standard laboratory water are as follows (Table 3):

Table 3. Reduction in toxicity of sodium sulphate in waters of increasing water hardness to the amphipod *H. azteca* (Davies 2002a).

<table>
<thead>
<tr>
<th>Water Hardness (as CaCO3)</th>
<th>LC50 (mg/L sulphate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>491</td>
</tr>
<tr>
<td>50</td>
<td>1518</td>
</tr>
<tr>
<td>75</td>
<td>1700</td>
</tr>
<tr>
<td>100</td>
<td>2971</td>
</tr>
<tr>
<td>250</td>
<td>4864</td>
</tr>
</tbody>
</table>

iii. In the current guideline, (Frahm 1975) is cited as reporting that 100 mg/L of sulphate is toxic to the aquatic moss *Fontinalis antipyretica*. However, in that study, sulphate is added in the form K2SO4 which raises concerns whether the toxicity observed was due to sulphate or the associated potassium ion. A study by Egan & Ungar (1998), examined the toxicity of K2SO4, KCl, NaCl and Na2SO4 to the terrestrial weed, *Atriplex prostrata*, and found the toxicities of the following to be K2SO4 > KCl > NaCl = Na2SO4 when exposed by comparable osmotic potentials. At the end of the 5 week test, all plants exposed to K2SO4 and 40 percent of the plants exposed to KCl were dead. However, no detrimental impacts on survival was observed in either the NaCl or Na2SO4 treatments. There is also evidence in the literature that
$K_2SO_4$ is significantly more toxic than $Na_2SO_4$ to invertebrates and fish as well (Table 4).

Table 4. Toxicity comparison of potassium sulphate in comparison to sodium sulphate to *Ceriodaphnia, D. magna* and Fathead minnow (*Pimphales promelas*) (Mount et al., 1997)

<table>
<thead>
<tr>
<th></th>
<th>LC50 of $K_2SO_4$ (mg/L)</th>
<th>LC50 of $Na_2SO_4$ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 hour <em>Ceriodaphnia</em></td>
<td>&lt;680</td>
<td>3080</td>
</tr>
<tr>
<td>48 hour <em>Daphnia magna</em></td>
<td>720</td>
<td>4580</td>
</tr>
<tr>
<td>96 hour Fathead minnow</td>
<td>680</td>
<td>7960</td>
</tr>
</tbody>
</table>

A similar study to Frahm (1975) was done; however, $Na_2SO_4$ was used rather than $K_2SO_4$. The toxic threshold of *F. antipyretica* was estimated at 800 mg/L sulphate (Davies 2002d) in water of approximately 17 mg/L hardness (as CaCO$_3$). Furthermore, this toxicity appeared to be somewhat alleviated in waters of increasing water hardness. An explanation of the apparent ameliorative effects of sodium sulphate toxicity in waters of increasing hardness may be an indication of sodium ion toxicity. The mechanism of sodium toxicity to plants occurs from sodium ions displacing calcium from the cell surface (Kinraide 1999). This allows Na$^+$ ions to cross the cell membrane to toxic levels in the cell cytoplasm. However, this can be reduced in solutions with higher Ca$^{2+}$ concentrations (Tyerman & Skerrett 1999, Reid & Smith 2000) through competition between Ca$^{2+}$ and Na$^+$ for binding sites. Although Ca$^{2+}$ is able to ameliorate the toxicity caused by high intracellular Na$^+$, it is not able to overcome the osmotic deficits associated with high salinity (Kinraide 1999). A study done by Hocking (1981) on *Typha domingensis*, an emergent macrophyte, indicated that sodium tended to be more toxic than chloride and that “sodium appears to retard plant growth through the effect on osmotic adjustment, ion uptake, enzyme activity and hormonal balance” (Hart et al. 1991). Evidence that sulphate does not cause specific ion toxicity and that sodium can cause significant amounts of toxicity to plants is supported by Stanley (1974) who studied the effects of metals and salts to the Eurasian watermilfoil (*Myriophyllum spicatum L*). The 50 percent inhibition levels of sulphate and chloride reported indicated
that Na₂SO₄ causes less toxicity than NaCl with the exception of affecting shoot length (Table 5).

Table 5. The effects of Na₂SO₄ and NaCl on the growth of Eurasian watermilfoil (Myriophyllum spicatum)

<table>
<thead>
<tr>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compound</strong></td>
</tr>
<tr>
<td>--------------------</td>
</tr>
<tr>
<td>Na₂SO₄</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
</tbody>
</table>

I₅₀RW = 50% inhibition of root dry weight; I₅₀SW = 50% inhibition of shoot dry weight; I₅₀RL = 50% inhibition of root length; I₅₀SL = 50% inhibition of shoot length (Stanley, 1974).

Analysis of the data regarding the inconsistency in the increased inhibition of shoot length by Na₂SO₄ over NaCl indicated that the mechanism of toxicity of both involved an imbalance of osmotic pressure in the plant cells. The reduced relative toxicity of NaCl compared to Na₂SO₄ was attributed to the rapid uptake kinetics of chloride ions compared to sulphate ions. It was hypothesized that the rapid uptake of NaCl allowed the plant to maintain adequate turgor pressure to allow for shoot growth while sulphate caused a negative osmotic gradient resulting in a loss of turgor pressure. This demonstrates that although both salts are acting osmotically, NaCl acts internally while Na₂SO₄ was acting externally. This suggests that sulphate toxicity may be reduced through gradual acclimation to exposure by allowing the plants to take up enough sulphate ions to equalize osmotic gradients.

BEAK International Incorporated, together with Michigan Technical University (1998), conducted a toxicity investigation on a similar species (Fontinalis neomexicana) that indicated lower sensitivity to sulphate than reported in Frahm (1975). No observable effect on chlorophyll levels was observed up to the maximum exposure concentration of 500 mg/L sulphate (added as Na₂SO₄) at a water hardness of 160 mg/L (as CaCO₃) for an exposure of 14 days. However, Ministry of Lands and Parks (MELP) scientists did not consider this a valid endpoint since aquatic mosses grow very slowly and chlorophyll
levels would likely not be affected in a relatively short experiment (Singleton 2000). A 14 day testing period may be sufficient since mosses are beginning to receive attention as bioindicators as they have been reported to demonstrate relatively high metabolic activity even in autumn and winter (Frost 1990 cited in Siebert et al. 1996). Immersion times of 1 and 10 days have been considered sufficient (Lopez et al. 1994), though longer experiments of up to 4 weeks have been conducted.

In summary, the study by Frahm (1975) which reported the toxicity threshold of *F. antipyretica* to be 100 mg/L sulphate by using potassium sulphate likely attributed the toxicity to the incorrect ion. Potassium has been reported to have a relatively high toxicity to plants, fish and invertebrates. Furthermore, the results reported in Davies (2002d) indicate that toxicity observed in *F. antipyretica* to sodium sulphate exposure is likely partially due to the associated sodium cation and cannot be fully attributed to the sulphate anion. The toxic threshold reported in Davies (2002d) in very soft water (17 mg/L as CaCO₃) was approximately 800 mg/L sulphate after 3 weeks exposure; however, this appeared to be partially alleviated in waters of increasing hardness.

iv. The anecdotal evidence that elevated sulphate levels (average of 71 mg/L sulphate; range of 27.7 to 189 mg/L) can stimulate large sulphur bacteria growths is likely due to an environmental condition associated with sulphate discharges rather than to sulphate itself. In aerobic waters, SO₄²⁻ can be taken up, reduced and assimilated into amino acids and ester compounds by vascular plants, algae and bacteria (Howarth et al. 1992). However, sulphate is rarely a limiting nutrient under aerobic conditions as such little amounts of sulphur are biologically required. Due to the excess supply of sulphur in aquatic environments, aquatic organisms do not generally take up excess sulphur beyond what is required for growth (Howarth et al. 1992). An experiment at the Experimental Lake Area (EPA) in Northwestern Ontario showed that increased dissolved sulphate concentrations did not increase sulphate uptake rates in algae (Creusius, Columbia University, unpub. Data in Howarth et al. 1992). Furthermore, Knauss and Porter (1954) in Howarth et al. (1992) observed less than a doubling of sulphate content in *Chlorella* when sulphate
concentrations were increased in test solutions from 120 to 4200 mg/L sulphate. However, sulphate can become a limiting nutrient in anaerobic environments where it can be utilized as an electron acceptor by microorganisms in the genera *Desulfovibrio* and *Desulfotomaculum* (Nriagu 1978). Sulphate is a relatively poor electron acceptor and is only utilized after all the oxygen, nitrate and ferric iron (Fe$^{3+}$) have been reduced as those electron acceptors yield more energy per glucose molecule oxidized. Under anaerobic conditions, sulphate is transformed into sulphides (H$_2$S and HS$^-$) which can react with available dissolved iron (producing pyrite) and other metals to form precipitates. Under these conditions, with available carbon and nutrient sources, large sulphur bacteria blooms can occur.

The existence of lakes with sulphate in the thousands of mg/L that do not have perpetual sulphur bacteria blooms indicate that these blooms are not due solely to the presence of sulphate. Furthermore, the observed bacteria blooms frequently disappear during heavy rains (Ian Sharpe, pers. comm. 2002). The disappearance of the bacteria mats is likely due to the washing away of the present bacteria as well as a re-oxygenation of the affected waters. This allows aerobic bacteria to recolonize as they have a competitive advantage over the facilitative anaerobes in aerobic environments. The cycle would then likely repeat in periods of high temperature which reduce oxygen solubility and again favour the production of anaerobic bacteria (sulphate is approximately 100 times more soluble in water than is oxygen at 25 °C and 1 atm (Nriagu 1978)). The production of these blooms is likely due to the anaerobic conditions in the creeks in which they exist and not to the presence of sulphate alone. Therefore, simply maintaining dissolved oxygen levels above anoxic conditions in high sulphate systems would likely stop the establishment of these bacteria blooms.

**Re-evaluation of current BC sodium sulphate guidelines**

Summary of the effects of sodium sulphate on various aquatic biota can be found in the current “Ambient water quality guidelines for Sulphate” Singleton (2000). As previously mentioned in the above sections, studies which used sulphate salts other than sodium sulphate, likely give an overly toxic evaluation for sulphate. The additional information
in regards to the ameliorative effect of water hardness and calcium levels on sodium sulphate toxicity gained from Davies (2002a, 2002b) in a wide range of water hardness treatments, and the studies done by PESC provide sufficient evidence that a guideline taking into account the local water hardness of receiving waters is justified.

**Additional data not cited in the current guideline is as follows:**

**Fish Species**

Mount et al. (1997) reported 96 hour LC50 to the Fathead minnow to be 7960 mg/L sodium sulphate (range 6800 – 10 000 mg/L) based on three replicates of the experiment. This corresponds to a sulphate concentration of 5384 mg/L sulphate (range 4597 – 6763 mg/L).

A study by Koel & Peterka (1995) examined the effects of sulphate saline waters on the reproductive success of walleye (*Stizostedion vitreum*), northern pike (*Esox licius*), yellow perch (*Perca flavescens*), white sucker (*Catostomus commersoni*) and the common carp (*Cyprinus carpio*). In general, these species exhibited a decline in survival to hatching in sulphate saline waters of TDS content between 1150 and 2400 mg/L. The proportion of sulphate in this water was approximately 600 and 1238 mg/L. However, these should be considered conservative estimates as the sulphate exposure waters had significant amounts of other potentially toxic ions such as potassium and magnesium. The authors admitted that some of their data showed higher toxicity in some cases compared to other studies in the literature. Furthermore, it was hypothesised that acclimation of the fish to the test waters before the test would have likely increased the reproductive success above what was observed.

**Invertebrates**

Mount et al. (1997) reported 24 and 48 hour LC50 values for the Cladoceran, *Ceriodaphnia dubia* to be 3590 (range 3540 – 3740) and 3080 mg/L (range 1770 – 3540) sodium sulphate respectively based on 4 replications of the experiment. This corresponds to 2428 (range 2394 – 2530) and 2083 mg/L (1197 – 2364) sulphate. The test was done in standard reconstituted water of hardness of approximately 100 mg/L (as CaCO₃) as
outlined in USEPA (1991) and is consistent with standard test procedures accepted in Canada (Environment Canada July 1990).

These data are supported by Warne and Schifko (1999) who reported comparable 48 hour LC50 values numbers for *Ceriodaphnia* of 3150 mg/L sodium sulphate (95% confidence limits 2807 – 3535). This corresponds to 2131 mg/L sulphate (95% confidence limits 1899 – 2391). This bioassay was conducted in dechlorinated, aged city tap water.

Mount et al. (1997) reported 24 and 48 hour LC50 values for *D. magna*, indicating less sensitivity to sodium sulphate in comparison to *Ceriodaphnia*. The 24 and 48 hour LC50 values reported were 6290 (range 5790 – 7070) and 4580 (range 4060 – 5360) sodium sulphate respectfully based on four replicates each of the experiment. This corresponds to sulphate exposure concentrations of 4254 (range 3916 – 4782) and 3098 mg/L (range 2746 – 3625) after the 24 and 48 hour test exposure period respectfully. This test was also executed in water of approximately 100 mg/L hardness (as CaCO₃) with an ion content following the guidelines found in USEPA (1991) and Environment Canada (July 1990). These data are comparable to an experiment by Davies (2002b) which examined the effects on sodium sulphate toxicity in waters in the hardness range of 25 – 100 mg/L (as CaCO₃) as well as the relative importance of calcium and magnesium (as components of water hardness) in reducing sodium sulphate toxicity. In waters of comparable hardness and ion content to that used in Mount et al. (1997), Davies (2002b) reported a comparable 48 hour LC50 for *D. magna* of 3244 mg/L sulphate. In treatments of increasing water hardness, the toxicity of sodium sulphate was reduced (Figure 3). Waters of comparable hardness but greater relative calcium content in both soft (Figure 4) and medium hardness waters was also less toxic (Figure 2, page 7).
Figure 3. Toxicity of sulphate to *D. magna* in waters of increasing hardness. • corresponds to LC50 values in standard waters (Davies 2002b) and ▲ to Pacific Environmental Science Centre toxicity data performed in well water PESC (1996). Dashed line shows trend only.

Figure 4. Reduction of sodium sulphate toxicity to *D. magna* in soft water of increasing calcium content. Waters have identical water hardness of 25 mg/L (as CaCO₃) (Davies 2002b). Dashed line shows trend only.
Unpublished data from a series of toxicity tests performed by The Pacific Environmental Science Centre (PESC) for BC MELP (BC Ministry of the Environment, Lands and Parks) in 1996 (unpublished data) showed *D. magna* was highly sensitive to sulphate in soft water (25 mg/L as CaCO₃), but not in medium (100 mg/L) or hard water (250 mg/L). PESC reported 48 hour LC50s for *D. magna* in soft, medium and hard water of 537, 6281, and 7442 mg/L sulphate, respectively. The LC50 value reported by PESC is likely on the conservative end of the tolerance range of *D. Magna* for 48 hour exposure as other studies have reported higher LC50 values in this hardness range. Specifically, Dowden & Bennett (1965) reported an LC50 of 740 mg/L and Davies (2002b) reported an LC50 of 957 mg/L sulphate. Therefore, the mean of 745 mg/L sulphate between these tests likely provides the best assessment of acute toxicity sodium sulphate in soft waters.

Although waters with higher Ca/Mg ratios caused significantly lower mortality to *D. magna*, Ca/Mg ratios alone do not completely explain the substantially lower toxicity that PESC reported (LC50 of 6281 mg/L sulphate) in water of 100 mg/L hardness (as CaCO₃). The well water used in the tests done by PESC had a Ca/Mg ratio of 6.7 in comparison to the high calcium water used in Test #2 (in Davies 2002b) which had a Ca/Mg molar ratio of 6.95 which resulted in an LC50 of 4541 mg/L sulphate. These differences may be due to population specific differences in the test organisms or possibly other water quality characteristics. As PESC conducted its test in well water, there may be other water quality characteristics influencing sodium sulphate toxicity in their bioassay.

The LC50s reported by PESC (1996) for *H. azteca* were found to be extremely low in soft water test conditions due to inappropriate test waters lacking in chloride ions (Davies 2002a). Sodium sulphate toxicity to this amphipod was also found to be reduced in waters of increasing water hardness (Figure 5).
Figure 5. Sulphate toxicity to the amphipod \(H. \text{azteca}\) in waters of increasing hardness (Davies 2002a). □ correspond to LC50 values from this investigation in standard waters and ▲ to PESC experimental data which was performed in well water (PESC 1996). Dashed line shows trend only.

**Aquatic Macrophytes**

A replication of Frahm (1975) indicated that sodium sulphate was much less toxic to the aquatic moss *Fontinalis antipyretica* than the 100 mg/L toxic threshold reported (Davies 2002d). The lowest observable effect (LOE) level was observed by a reduction in mean chlorophyll levels at 400 mg/L sulphate in soft water conditions of approximately 19.3 mg/L (as CaCO₃) and the moss appeared dead at exposure above 800 mg/L sulphate. In a replication of that experiment, the moss showed sensitivity to sodium sulphate exposure in soft water conditions (26 mg/L as CaCO₃) but it did not display a significant reduction in mean chlorophyll levels until 1500 mg/L sulphate, when the moss appeared dead.
However, in water of 100 mg/L hardness and up to a maximum exposure of 1500 mg/L sulphate (Figure 6), no significant reduction in chlorophyll levels were observed.

![Figure 6. Response of increasing sulphate concentration on chlorophyll a & b content of the moss *Fontinalis antipyretic* after 21 days exposure (Davies 2002d). Coefficient of variation bars are included. • and ■ correspond to soft water treatments (hardness 19.3 and 26 mg/L as CaCO₃ respectfully). ▲ correspond to medium water hardness treatment group (hardness 105 mg/L as CaCO₃).]

In response to the claim by MELP scientists claiming that the 14 day study done by Beak International & Michigan Technological University (1998) on the moss, *Fontinalis neomexicana*, was not long enough to cause a reduction in chlorophyll levels, it was found through an examination of studies using mosses as biomonitors that the exposure period was likely sufficiently long enough to promote a toxic response. Furthermore, the report by Beak International (1998) indicating no observable effect in chlorophyll levels to *F. neomexicana* up to 500 mg/L in water of 160 mg/L as CaCO₃ hardness is consistent with the results presented here and with the general observation that freshwater macrophytes are usually tolerant to salinities less than 1000 – 2000 mg/L (as NaCl) (Hart et al. 1991). However, as no observable effect was seen, an extrapolation of the effects of longer exposures is impossible to hypothesize. The increased energy requirements required to maintain ion balance in a medium of amplified ion content and how this
would affect the long term sustainability of moss populations is problematical when no effect is observed.
Chapter IV

Guideline Recommendation

The chronic toxicity tests using the aquatic moss *(F. antipyretica)* and the acute toxicity tests using *D. magna* and *H. azteca* in a wide hardness range provide data which can be used in the development of a new sulphate guideline for the protection of aquatic life. This new information regarding the reduction of sodium sulphate toxicity in waters of increasing hardness indicate that allowable discharge limits for sulphate should be partially dependent with the water hardness of the receiving waters. Therefore, three water hardness categories and allowable sulphate discharges within those categories are proposed. The three water hardness categories are <50, 50 to 100 and >100 mg/L hardness (as CaCO₃).

The current “Ambient water quality guideline for sulphate” of 100 mg/L sulphate was based on studies which after critical review and replication were ultimately deemed flawed in either their design or interpretation of results. In the current sulphate guideline, a safety factor of 2:1 largely based on a 96 hour LC50 of *H. azteca* bioassay was considered appropriate for sulphate discharges due to the steep response curve observed in waters of increasing sulphate salinity. In general, toxicity occurs once the osmoregulatory mechanisms of an organism are overpowered by the osmotic potentials created by the presence of excessive amounts of ions in the surrounding media (Goodfellow et al. 2000). The metabolic demands put on organisms in saline waters are not well quantified using acute, rather than chronic bioassays as acute tests do not provide a measure of the effects on growth and longer term effects of salinity that sub lethal chronic tests would provide. Furthermore, short term acute toxicity tests may overestimate toxicity, as many organisms are able to acclimate to waters of higher salinities by increasing ion uptake and reduce the osmotic potential between their cells and the surrounding environment which subsequently reduces osmotic stress. Therefore, bioassays examining long term chronic effects of sulphate salinity on sensitive organisms
likely provide the best tool in developing a guideline which is protective in an ecosystem context as well.

Plant species have been identified as wetland biota which is most sensitive to increases in salinity (Hart et al. 1991). Therefore, in soft water conditions, the study examining the effects of sodium sulphate exposure on the aquatic moss (*F. antipyretica*) (Davies 2002d) is likely the best study to use for developing a guideline in soft water conditions. That study indicated no-observable-effect (NOE) to mean chlorophyll concentrations of 200 mg/L sulphate is water with a hardness of 19.3 mg/L as CaCO$_3$ after 3 weeks of exposure. However, a replication of the experiment in waters of slightly higher water hardness (26 mg/L) showed NOE up to 1000 mg/L sulphate. Therefore, a guideline of 200 mg/L in waters of 50 mg/L and less would likely be protective of any potential ecosystem effects sulphate discharges may have. This is more conservative than the current sulphate guideline which uses a 2:1 safety factor which was largely based on a 96 hour LC50 of 205 mg/L sulphate for *H. azteca* done by PESC (1996). The replication of the PESC (1996) study using *H. azteca* indicated a flaw in the experimental design of that study and two new endpoints were calculated for *H. azteca* in soft water conditions. A 96 hour LC50 of 491 and a NOE of 453 mg/L over two separate tests were calculated (Davies 2002a). However, the LC50 of 491 mg/L may possibly be at the lower tolerance end for *H. azteca* for sulphate in comparison to the second test which indicated NOE up to 453 mg/L sulphate. In light of these new data, a revised guideline of 200 mg/L sulphate in waters of <50 mg/L hardness takes a more conservative safety factor than the current sulphate guideline.

In the 50 mg/L to 100 mg/L hardness range, the information which provides the most resolution of the reduction of sodium sulphate toxicity in waters of increasing hardness is provided by acute studies using *D. magna* and *H. azteca* (Davies 2002a, 2002b). These studies reported 48 and 96 hour LC50 values of 1768 and 1518 mg/L sulphate respectfully in waters with a hardness of 50 mg/L. A conservative 5:1 safety factor based on the 96 hour LC50 value obtained for *H. azteca* and 48 hour *D. magna* study should be adequate to compensate for the uncertainties inherent in using acute studies which do not
address the sub lethal metabolic effects of sulphate on organisms and extrapolating single species bioassays into an ecosystem context. Again, this is substantially more conservative than the safety factor used in the current guideline; however, with the uncertainties inherent in acute studies in assessing the potential metabolic demands placed on organisms in sulphate saline waters, the extra precaution in justified. Therefore, a maximum discharge of 300 mg/L sulphate in the hardness range of 50 – 100 mg/L as CaCO$_3$ should be protective.

In receiving waters with hardness above 100 mg/L, most species appear to be relatively tolerant to high sulphate concentrations. A sub lethal study was done using *D. magna* by PESC (1996) examining the effect of sulphate on the reproduction of *D. magna*. A 21 day test was done and NOEC was reported as 625 mg/L sulphate in water of 100 mg/L hardness. The most sensitive acute test is found in Mount et al. (1997) and Warne and Schifko (1999) for *Ceriodaphnia dubia*. An average 48 hour LC50 of 2121 mg/L sulphate (based on five replicates: four replicates from Mount et al. (1997) and one from Warne and Schifko (1999) were calculated). Maintaining a 5:1 safety factor will result in a guideline of 400 mg/L sulphate. This is roughly 65 percent the NOEC reported by PESC and should therefore provide sufficient safety to aquatic environments.

The majority of these studies were done in standard reconstituted waters which have a Ca/Mg ratio of 0.7. As outlined in previous sections, natural waters on average have a Ca/Mg ratio of roughly 2.7. The evidence that the toxicity caused by sodium sulphate is reduced in waters of increasing Ca/Mg ratios (Davies 2002b); therefore, these proposed guidelines have an additional safety factor built in to them for most receiving water environments.
Summary

Sulphate toxicity to aquatic organisms

Sulphate is commonly found in environments and frequently acts as the main sulphur source for plants and bacteria. In general, organisms have a relatively high tolerance for sulphate with some organisms, such as some fish species, able to withstand sulphate concentrations in excess of 10000 mg/L. The current “Ambient Water Quality Guidelines for Sulphate for the protection of aquatic life (Singleton 2000)” has set a discharge limit of 100 mg/L sulphate as a maximum concentration which should not be exceeded at anytime. However, the guideline appears to be overly conservative, as the principle rational in developing the guideline is based on toxicity investigations which seem to exaggerate the toxicity of sulphate. These studies investigated the effects of sulphate on stripped bass larvae (*Morone saxatilis* (Hughes 1973)), an aquatic moss (*Fontinalis antipyretica* (Frahm 1975)), and the amphipod *Hyalella azteca* (PESC 1996). These studies were replicated, and the original studies were found to exaggerate the toxicity of sulphate for the following reasons:

1. The original study using striped bass reported a 4 day LC50 of 250 mg/L sulphate (added as sodium sulphate). The replicated study (Davies 2002c) had low survival in control groups; however, survival dramatically increased in treatments with additions of sodium sulphate. The best survival was observed in the highest sulphate exposure groups of 4000 mg/L sulphate (added as sodium sulphate). Other studies have reported poor survival of stripped bass in waters with similar water chemistry used in the control treatments of original and replicated study. Therefore, the high sensitivity of striped bass larvae to sulphate as reported by Hughes (1973) was likely due to the unsuitability of exposure waters rather than toxicity to sulphate.

2. The original study using the aquatic moss *F. antipyretica* (Frahm 1975) reported an extremely low toxic threshold to sulphate of 100 mg/L. However, the original study used the salt K₂SO₄ which has been shown to be extremely toxic to fish,
plants and invertebrates due to $K^+$ toxicity rather than toxicity from the sulphate ion. Therefore, a replication of this study (Davies 2002d) used $Na_2SO_4$ and reported a toxic threshold (using chlorophyll content as an endpoint) of approximately 800 mg/L sulphate is soft waters (hardness 19.3 mg/L as CaCO$_3$). No observable effect in waters of 100 mg/L hardness (as CaCO$_3$) up to sulphate concentrations of 1500 mg/L were observed.

3. Toxicity tests using *H. azteca* done by PESC (1996) reported a 4 day LC50 of 205 mg/L sulphate (added as Na$_2$SO$_4$) in soft water conditions (25 mg/L as CaCO$_3$). However, the toxicity was reported to be greatly alleviated in waters of medium hardness (100 mg/L as CaCO$_3$) reporting a 4 day LC50 of 3711 mg/L sulphate. Multiple replications and modifications to the experimental methods of the original study established that the exposure water used in the original study caused increased toxicity in the soft water treatments due to a deficiency in chloride ions (Davies 2002a). The replicated study calculated a NOE of 453 mg/L and an 4 day LC50 of 491 mg/L sulphate (added as Na$_2$SO$_4$) and also found that sulphate toxicity was greatly reduced in waters of increasing water hardness. The LC50 reported by PESC (1996) in soft water conditions should not be considered an accurate assessment of sulphate toxicity to *H. azteca*.

The reduced toxicity of sulphate observed in a variety of organisms in waters of increasing water hardness motivated a study to investigate how modifying the principle components of water hardness (calcium and magnesium) affected sulphate toxicity (Davies 2002b). It was found that waters which had comparable hardness, but elevated calcium and magnesium ratios (Ca/Mg ratios of 0.7 to 7.0 expressed as a molar basis) had significant different 2 day LC50s using the cladoceran *D. magna*. Specifically, waters at 100 mg/L hardness (as CaCO$_3$) resulted in 2 day LC50s of 3247 and 4541 mg/L sulphate in waters with Ca/Mg ratios of 0.7 and 7.0 respectfully. As the majority of toxicity tests are done in waters with Ca/Mg ratios of 0.7 and the mean Ca/Mg ratio of waters bodies in the environment have significantly higher ratios, this suggests that laboratory toxicity
tests using standard reformulated water likely exaggerate the toxicity of sulphate over what would be observed in many natural environments.

The incorporation of the replicated studies into a newly proposed sulphate guideline indicate that in consideration to the relatively low toxicity of sulphate, the current guideline is overly conservative and proposes unnecessarily strict discharge limits. Therefore, a new guideline, taking into consideration the water hardness of receiving waters is proposed (Table 6).

Table 6. Proposed maximum allowable sulphate discharge limits for the protection of aquatic life at difference receiving water hardness levels.

<table>
<thead>
<tr>
<th>Water Hardness (as CaCO₃)</th>
<th>Maximum allowable discharge (as SO₄²⁻)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50 mg/L</td>
<td>200 mg/L</td>
</tr>
<tr>
<td>50 to 100 mg/L</td>
<td>300 mg/L</td>
</tr>
<tr>
<td>Above 100 mg/L</td>
<td>400 mg/L</td>
</tr>
</tbody>
</table>

The development of a guideline that is protective of all environments will almost always result in one that is overly conservative for many environments. In light of how the toxicity of sulphate is greatly influence by physical, chemical and biological contexts, the development of water quality objectives for sulphate is likely the most reasonable method in developing appropriate discharge limits. Therefore, this recommendation is likely best used not as a final application for a new sulphate guideline, but rather as a guide in the development of site-specific discharge limits.

**Molybdenum toxicity to rainbow trout**

The study investigating the toxicity of molybdenum was done as an earlier toxicological investigation reported that molybdenum was extremely toxic to rainbow trout (*Oncorhynchus mykiss*) under specific experimental conditions (28 day EA test, LC50 0.79 mg/L; Birge et al. 1980). This is in contradiction to other studies investigating the effects of molybdenum on salmonids in the literature. McConnell (1977) reported 96 hour LC50s in a static test of 1320 mg/L and 800 mg/L to rainbow trout of 55 mm and 20 mm respectfully. Furthermore, as part of the same study, a one year exposure test
starting with eyed eggs indicated no significant reduction in hematocrit, growth or mortality up to molybdenum exposures of 17 mg/L. The replication of Birge et al. (1980) found that molybdenum caused low toxicity to rainbow trout with two experiments failing to reach an LC50 concentration with molybdenum concentrations up to 400 and 1500 mg/L (Davies 2002e; molybdenum added as Na2MoO4).

**Research and development needs**

A basic lack of understanding of the specific mechanisms of how ions are toxic to organisms and the ameliorative effects between ions is prevalent throughout the majority of the literature about ion toxicities. This makes it difficult to gain any understanding of ion toxicity and interactions beyond what can be gained from single salt investigations.

Investigations examining the relative contributions to toxicity that different ions exert on an organism needs to be done. The evidence provided by Mount et al. (1997) that exposure to sodium sulphate and sodium chloride together cause a summed mass LC50 comparable to a single salt test needs further examination. Specifically, studies examining whether there is an additive toxicity effect of the two salts at lower concentrations rather simply when the salts are added on an equal mass basis.

There needs to be a basic change in the way ion toxicity tests are designed, analyzed and reported. Ion toxicity is a function of molar concentrations of the ions in solution, rather than the masses of the ions. Therefore, the tendency to use the masses of ions can potentially bias results and give an erroneous impression of the relative toxicity of ions. This is especially important for studies examining sulphate toxicity due to the relative high molecular weight of the sulphate ion (96.06) compared to other common ions such as Cl\(^-\) (35.45), Na\(^+\) (22.99), and K\(^+\) (39.10). Osmotic stress is caused by the presence of an ion imbalance between the organism and the environment. The total osmotic flux and direction will be a sum of all of the osmotic forces exerted by the relative concentrations of the surface of an organism and the environment. Therefore, further studies examining
the ion makeup of organisms would provide a better understanding of why certain species are more sensitive to ion toxicity caused by osmotic effects.

The use of chronic rather than acute tests to investigate the effects of ion toxicity to organisms needs to be adopted. Toxicity tests are done in order to provide information about the potential effects that a contaminant may have on ecosystems. The potential reductions in growth and reproductive success that may be caused to organisms under osmotic stress cannot be assessed with acute studies. Chronic studies would also provide insight into any adaptive response that organisms may be able to utilize under osmotic stress.

The adoption of these relatively small changes in experimental design will provide a standardized framework which will greatly aid in the understanding the mechanisms of ion toxicity and the potential ecosystem effects that elevated ion concentrations may have on the aquatic environment.
References


Davies, T. 2002a. Sulphate toxicity to the amphipod (Hyalella azteca): A 4-day lethal bioassay. Unpublished data (see appendix A).

Davies, T. 2002b. Relative importance of Calcium and Magnesium ratios in assessing toxicity of sulphate to the cladoceran (Daphnia magna). Unpublished data (see appendix B).


Davies, T. 2002d. Effects of sulphate on the growth and chlorophyll levels of Fontinalis antipyretica, a common BC aquatic moss. Unpublished data (see appendix D).

Davies, T. 2002e. The effects of molybdenum on rainbow trout (Oncorhynchus mykiss): A reproduction of Birge et al. (1980). Unpublished data (see appendix e).


Appendix A

Sulphate toxicity to the Amphipod *Hyalella azteca*
Introduction

Unpublished data from a series of toxicity tests performed by The Pacific Environmental Science Centre (PESC) for BC MELP (BC Ministry of the Environment, Lands and Parks) in 1996 (unpublished data) showed that the amphipod, *Hyalella azteca*, was sensitive to sulphate in soft water (25 mg/L as CaCO$_3$), but not in medium (100 mg/L) or hard water (250 mg/L). PESC reported 96-h LC50s for *Hyalella* in soft, medium and hard water of 205, 3711, and 6787 mg/L sulphate, respectively.

As the above LC50 value for soft water is extremely low in comparison to other tests found in the literature, I have repeated these tests to assess their validity.
**Materials and Methods**

Tests were designed to test the toxic effects of sulphate to *Hyalella* in waters of differing water hardness. All studies were of a water only static test design as described in Environment Canada (1997). The tests were run for a total of 96 hours at a controlled temperature of 23 ± 1 °C. The tests were conducted under a fully random design, with each replicate beaker being assigned a random location.

Within each hardness treatment, six different sulphate concentrations were tested comprising of 3 replicates each. The sulphate exposure levels used for each hardness block was dependant upon the water hardness of the treatment water and the results of earlier tests. Within each replicate, 10 *Hyalella* between 2-9 days old were placed in beakers containing approximately 200 mL of test solution. Within each beaker, an artificial substrate such as gauze or 500 micron Nytex screen approximately 1 x 1 cm was present to simulate cover and habitat for the organisms. A 16:8 light:dark photoperiod was used for the test at an approximate level of 750 lux.

This test was conducted a total of five times (including the duplication of PESC experiment) with differing results. As the inconsistencies in the toxicological data arise from the differences in water chemistry between the tests, the water chemistry of the PESC experimental groups are also included.

Sulphate was added as anhydrous sodium sulphate (Na$_2$SO$_4$) which was initially dissolved in a concentrated stock solution. The stock solution was then mixed with the appropriate amounts of control water to meet desired levels of sulphate exposure.

The organisms were fed 500 µL of yeast-cerophyl trout chow (YCT, US EPA, 2000) on days 0 and 2 of the test. At the end of the 96-hour test the organisms were counted and checked for survival. Organisms exhibiting any movement were classified as alive and those either immobile or missing were counted as dead.
**PESC experiment (unpublished, 1996)**

- Sulphate toxicity in water hardness of 25, 100 and 250 mg/l as CaCO₃ was tested.

The *H. azteca* used in this study came from an in-house culture maintained using on-site well water having a hardness of approximately 105 mg/l as CaCO₃. For the tests, reconstituted water using de-ionized water, well water, and well water with ion addition was used to make control and test solutions for the soft, medium and hard water treatments respectively (Table 1). The ion composition of the soft test water is similar (but diluted by a factor of four) to that found in the guidelines *Ceriodaphnia dubia* and *Daphnia Magna* (Smith et al., 1997) and is not consistent with those suggested in the current EPA guidelines for Hyalella toxicity tests (US EPA, 2000).

**Test ID**

- Sulphate toxicity in water hardness of 25, 100 and 250 mg/l as CaCO₃ was tested.

The *H. azteca* used in this study came from Aquatic Biosystems in Fort Collins, CO and were shipped overnight in water of approximately 150 mg/L (as CaCO₃) hardness. Reconstituted water was formulated using ion ratios consistent with published US EPA standard methods for determining toxic effects to freshwater invertebrates (US EPA, 2000).

Upon arrival, organisms were gradually acclimitized to the different water hardness levels by going through an approximate 30% water change every 2 hours for the first 6 hours. The organisms were then allowed to acclimate overnight and went through a final water change approximately 2 hours prior to the beginning of the test. This test was completed on August 30, 2000.
Test 2D

- Sulphate toxicity in water hardness of 25, 50 and 75 mg/l (PESC water formula) as CaCO₃ was tested.

The *H. azteca* used in this study came from Aquatic Biosystems in Fort Collins, CO and were shipped overnight shipping in water of approximately 144 mg/L (as CaCO₃) hardness. Acclimation procedures were similar to those in test 1D. This study was completed July 22, 2001.

Reconstituted water was formulated using the soft water chemistry conditions identical to the original PESC experiment. All water in this test was reconstituted water without the use of any well water. Ion concentrations were doubled and tripled respectively to create the 50 and 75 mg/L treatments (Table 1).

Test 3D

- Sulphate toxicity in water hardness of 25 mg/L (PESC water formula) as CaCO₃ while manipulating Ca:Mg ratios.

The *H. azteca* used in this study came from Aquatic Biosystems in Fort Collins, CO and were shipped overnight shipping in water of approximately 204 mg/L (as CaCO₃) hardness. Acclimation procedures were similar to those in test 1D. This study was completed August 20, 2001.

Four different water formulations were used in this test. Water hardness was maintained at 25 mg/L while Ca:Mg ratios were manipulated from a relatively high magnesium content (consistent with the PESC water formula) of 0.7 (expressed on a molar basis) to a high Calcium level of 3.9. The Ca:Mg ratio test blocks were 0.7, 1.8, 2.8 and 3.9.
Test 4D

- Sulphate toxicity in water hardness of 25 (PESC water formula), 25, 50 and 75 (EPA water chemistry) mg/l as CaCO₃ was tested.

The *H. azteca* used came from the PESC lab located in West Vancouver and is the same culture used for the PESC (1996) study. Acclimation procedures were similar to those in test 1D. The most recent water quality analysis on the well water indicated a hardness of 104 mg/L (as CaCO₃). This study was completed September 16th, 2001.

Three different control water formulations were used in this test. Reconstituted water similar to test 1D at hardness levels of 25, 50 and 75 mg/l, reconstituted water comparable to PESC (1996) at a hardness of 25 mg/l, and reconstituted water matching PESC (1996) at a hardness of 25 mg/l with the addition of 14 mg/l sodium chloride. The PESC water with sodium chloride addition was done as a control series only.

**Statistical methods**

The 50% lethal concentration (LC50) values were calculated using Maximum likelihood-Probit method as recommended in US EPA (2000). It was used in all tests with the exception of the 50 mg/L hardness block found in test 4D. The results of test 4D, 50 mg/L hardness test block used Log-logit interpolation method due to the lower survival found in the control series. In all tests where an LC50 was calculated, Shapiro-Wilk’s test indicated a normal distribution.

The control survival was >90% in all tests where an LC50 was calculated with the exception of test 4D in the 50 mg/L hardness (as CaCO₃) treatment. Although >80% survival in the control series is a prerequisite for test validity (Environment Canada, 1997), control survival was close to this at 77%. Furthermore, survival increased to >90% in the lowest sulphate exposure level (500 mg/L). Therefore, Log-logit interpolation was done on this test series to calculate an LC50.
**Results**

*PESC (1996) & Test 1D*

PESC (1996) reported 96-h LC50s for *Hyalella* in soft, medium and hard water of 205, 3711, and 6787 mg/L sulphate, respectively. The replicated experiment (Test 1D) obtained comparable results with 96-hour LC50s for medium and hard water (nominal hardness values of 123 and 257 mg/L as CaCO₃) of 2971 and 4864 mg/L sulphate respectively (Table 1). However, there was large discrepancy in the results for the soft water test series. At a water hardness of 30.5 mg/L (nominal value) as CaCO₃, no observable effect (NOE) was found up to 453 mg/L sulphate. This was the highest concentration tested. These tests were conducted in similar experimental conditions to each other with the exception of water ion composition (Table 1). Therefore, the test was repeated (see test 2D) using exact water chemistry formulations to the original PESC (1996) experiment. The reference toxicant conducted was considered valid as all conditions for reference toxicity validity were satisfied.

*Test 2D*

Test 2D survival was extremely poor in all the test groups and therefore an LC50 could not be calculated. Survival in control concentrations in the 25 mg/L hardness treatment was 47%, and all sulphate exposure concentrations had survival close to or at zero. The 50 mg/L hardness block had better survival in the controls (83%), however, all sulphate exposures (lowest 500 mg/L sulphate) had zero survival. The 75 mg/L hardness block had 57% survival and the lowest sulphate exposure level (500 mg/L) had 23% survival. All other sulphate exposure levels had zero survival.

A reference toxicant series was performed using CuSO₄ at a hardness of 100 mg/L (as CaCO₃) to assess organism viability. Survival was good (90%) in the control series, however, all exposure levels (range 0 to 1000 mg/L) resulted in zero survival.
Test 3D

Test 3D showed poor survival in all test groups. Survival in controls was <50% in all instances (Table 2) and survival in all sulphate exposure test groups was <10% with the majority being zero.

Test 4D

Test 4D calculated a 96-hour LC50 of 491, 1518 and 1700 mg/L sulphate in water with hardness of 25, 50 and 75 respectfully. Furthermore, control survival was 97%, 77% and 93% (Table 2) using the reconstituted water formula identical to test 1D and that found in the EPA guidelines (US EPA, 2000). Although survival in the 50 mg/L controls was below 80%, survival in the lowest sulphate concentration (500 mg/L) in that treatment series was 90%. This suggests that the low survival in the control series was likely due to random effects rather than toxicity to the test medium. Therefore, although survival in this series was below the control survival acceptance for this test (<80%) (Environment Canada, 1997) an LC50 was still calculated.

The treatment using the water chemistry similar to PESC (1996) again gave poor results. An LC50 could not be calculated, as survival in the controls was only 27%. In the lowest sulphate concentration (50 mg/L) survival was 10%, and all other sulphate exposure levels (range 0 to 4000 mg/L) had zero survival.

The treatment using water similar to PESC (1996) control water with 14 mg/L sodium chloride (8.4 mg/L Cl) addition had a survival of 97% (Table 2) indicating that the original PESC test water may be harmful to Hyalella due to a lack of chloride ions.

The LC50 results from these experiments summarizing the reduction of sulphate toxicity with increasing water hardness are summarised in figure 1.
**Discussion**

Data from the four *Hyalella* tests conducted, indicate that the high sensitivity to sulphate in soft water as reported by PESC (1996) was likely due to the water quality characteristics used in the test rather than toxicity to sulphate. The four replications of the experiment consistently resulted in poor survival in the test water that was used in the original study. Therefore, LC50 data for the soft water test series in the PESC (1996) study is not an accurate assessment of sulphate toxicity to *Hyalella azteca*.

The lack of survival found in Davies (2D & 3D) using water chemistry identical to the PESC (1996) test is supported by another study (Smith et al., 1997) which also found low survival (range 55 – 70%) in reformulated water control groups that had water chemistry similar to PESC (1996) (Table 2). Although they were not able to determine whether the low survival was due to a lack of chloride ions, they did observe an increase in survival (>80%) with the addition of 62.5 mg/L KCl that was being used as the reference toxicant. This suggested *Hyalella* required additional potassium and/or chloride ions. The large increase in survival found in test 4D with the addition of 14 mg/L NaCl (8.4 mg/L Cl⁻) suggests that it is a lack of chloride ions which is affecting survival. As chloride was added as NaCl, there is the possibility that the increase in survival is due not to the addition of chloride but rather the addition of sodium, or both. However, this is unlikely as sulphate was being added as sodium sulphate (Na₃SO₄) which added sufficient sodium to the test solutions. Therefore, if sodium was adding the necessary ion, then the lowest sulphate exposure series which provided 16.2 mg/L Na⁺ (from 50 mg/L Na₂SO₄) should have had increased survival over that of the controls. This was not observed.

The observation of the necessity of the chloride ion is contradicted by a study examining the minimum ion requirements of *Hyalella azteca* (Borgmann, 1996). The tests done by Borgman indicated that chloride was not an essential ion required for *Hyalella* survival and that sulphate can be substituted for one another when formulating test water. This is in obvious contradiction to the results reported by Smith et al., (1997) and those presented here.
Another major difference between the test water that may have affected toxicity is the difference in calcium:magnesium (Ca:Mg) ratios between the two test waters. As Ca and Mg contribute different amounts to water hardness (Calcium almost twice as much CaCO₃ equivalence as Mg) it is possible to maintain a constant hardness with a large change in the Ca:Mg content of the test medium. Tests investigating metal toxicity have found that copper toxicity is reduced both by waters with increasing water hardness and waters which contain relatively higher Ca:Mg ratios (Welsh, 2000). However, experiment 3D investigated this possibility over a range of ratios (0.7 – 3.9) and control survival was still extremely low (<50%) in all test groups. Therefore, it appears unlikely that the difference in Ca:Mg ratio increased survival in the test water between these tests.

The high LC50's calculated by PESC (1996) in medium and hard water (3711 and 6787 respectively) is comparable with test 1D (LC50 of 2971 and 4864). The latest analysis on the well water indicated high chloride ion levels (108 mg/L) in the well water which is significantly higher than the amount of chloride found in the medium hard water in test 1D which was 33.6 mg/L. As the well water used by PESC is high in chloride, the soft water, which was made using deionized water may have been contaminated by well water either through rinsing of the mixing buckets or the experimental glassware. Furthermore in test 4D, improved survival was seen when only 8.4 mg/L Cl⁻ was added to the soft water group and no further investigations were done to see what the minimum chloride ion requirements are. Therefore, the amount of chloride supplied through well water contamination may have been much less than the 8.4 mg/L Cl⁻ but may have been sufficient to improve survival.

Although the LC50 values found in the medium and hard water tests done by PESC and in test 1D were similar, the slight increase in toxicity in 1D may have been due to a lower chloride content. However, there are also a number of other ions present and other miscellaneous water chemistry differences in well water not found in reconstituted water. For example, the well water had a Ca:Mg ratio of the PESC well water was approximately 6.5 while the Ca:Mg ratio of the successful reformulated water was 3.3. An unpublished study by Davies (2002) examining the relative contributions of calcium
and magnesium ions as components of water hardness in reducing the toxicity of sulphate to *Daphnia magna* indicated that waters with higher calcium contents offer a greater protective effect than waters with comparable water hardness and lower Ca:Mg ratios. Finally, the differences between the values may simply be due to the normal variation seen between toxicity tests. It will require further investigations to delineate these interactions.

**Acknowledgements**

Thanks are given to the general staff, and in particular Janet Pickard, at BC Research Inc. for guidance, use of lab space, equipment and materials. BC Ministry of Environment (Smithers), through the Pacific Environmental Science Centre generously donated water analysis on the test solutions. Furthermore, Endako Mines, Highland Valley Copper, Inmet Mining Corp., Placer Dome Inc. and Taseko Mines provided financial assistance for this project. Finally, I would like to thank my thesis advisor, Dr. Ken Hall for his support.
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Davies, T. 2002. Relative importance of Calcium and Magnesium ratios in assessing toxicity of sulphate to the Cladoceran (*Daphnia magna*). Unpublished data.


Pacific Environmental Science Centre (PESC). 1996. Analysis of Laboratory Bioassays of Sulphate (Unpublished data).


<table>
<thead>
<tr>
<th>Nominal Hardness</th>
<th>4-day LC50 (mg/L SO₄)</th>
<th>Dilution water</th>
<th>Equivalent ions added</th>
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<tr>
<td></td>
<td></td>
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<td>NaHCO₃ (mg/l)</td>
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<tr>
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<td>NA</td>
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<tr>
<td>75²</td>
<td>1700</td>
<td>De-ionized</td>
<td>75.8</td>
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1. Corresponds to identical ion ratios in test solutions in PESC (1996)
2. Corresponds to identical ion ratios consistent with current EPA protocols
Table 2. Control water survival of *Hyalella azteca* in test water of different ion composition

<table>
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<tr>
<th>Nominal Hardness</th>
<th>Control Survival (percent)</th>
<th>NaCl (mg/l)</th>
<th>NaHCO₃ (mg/l)</th>
<th>CaSO₄·2H₂O (mg/l)</th>
<th>MgSO₄ (mg/l)</th>
<th>KCl (mg/l)</th>
<th>CaCl₂·2H₂O (mg/l)</th>
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<td>Test 2D</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>83³</td>
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<td>3</td>
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<td>57</td>
<td>-</td>
<td>90</td>
<td>57</td>
<td>57</td>
<td>4.5</td>
<td>-</td>
</tr>
<tr>
<td>Test 3D (Ca:Mg ratio test)</td>
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<td></td>
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<td>26.8</td>
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<td>19</td>
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<tr>
<td>25¹</td>
<td>97</td>
<td>13.8</td>
<td>30</td>
<td>19</td>
<td>19</td>
<td>1.5</td>
<td>-</td>
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<td>25²</td>
<td>97</td>
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<td>25.3</td>
<td>17</td>
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<td>77⁴</td>
<td>-</td>
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<td>93</td>
<td>-</td>
<td>75.8</td>
<td>49.9</td>
<td>23.7</td>
<td>3.2</td>
<td>52.3</td>
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</tbody>
</table>

1. Corresponds to identical ion ratios in test solutions in PESC
2. Corresponds to identical ion ratios in test solutions between Test 1D is consistent with current EPA protocols
3. Although survival was good in the control series, all sulphate exposure levels (minimum 500 mg/L) had zero survival
4. Although survival was low in the control series, survival in the lowest sulphate exposure level (500 mg/L) was 90% suggesting that the low survival was due to random survival difficulties rather than toxicity to the test medium.
Figure 1. Summary of 96-hour LC50 values for *Hyalella azteca* with changing water hardness. Dashed line shows trend only.
Appendix B

Relative importance of Calcium and Magnesium ratios in assessing toxicity of sulphate to the Cladoceran *Daphnia magna*
Introduction

The freshwater Cladoceran, *Daphnia magna*, is commonly used as an indicator bioassay organism due to its relative high sensitivity to pollutants (Leblanc & Surprenant 1984) and their wide distribution in the northern hemisphere.

Unpublished data from a series of toxicity tests performed by The Pacific Environmental Science Centre (PESC) for BC MELP (BC Ministry of the Environment, Lands and Parks) in 1996 (unpublished data) showed *D. magna* was highly sensitive to sulphate in soft water (25 mg/L as CaCO₃), but not in medium (100 mg/L) or hard water (250 mg/L). PESC reported 48-h LC50s for *Daphnia* in soft, medium and hard water of 537, 6281, and 7442 mg/L sulphate, respectively. From the literature review, it was found that the LC50 value calculated for soft water is extremely low in comparison to other tests in the literature investigating sodium sulphate toxicity. For example, Dowden & Bennett (1965) reported an LC50 of 740 mg/L sulphate for *D. magna* after 48 hours of exposure. Furthermore, other published work reported substantially lower LC50 values in medium hardness than those reported by PESC (1996). Specifically, Mount et al. (1997) calculated a 48 hour LC50 for *D. magna*, based on four tests to be 3098 mg/L sulphate (range 2746 – 3625) in medium hard reconstituted water (hardness 80 – 100 mg/L as CaCO₃). This is well below the 6281 mg/L sulphate LC50 reported by PESC in a culture water with a comparable hardness of 100 mg/L (as CaCO₃).

Although significant reduction in sulphate toxicity with increasing water hardness has been well established throughout the literature with a variety of different organisms, the discrepancies between these tests indicate another factor, rather than simply total water hardness (expressed as CaCO₃ equivalents) is influencing the toxicity of sodium sulphate. The water characteristics between the two tests is a potential source for the discrepancies between the LC50s calculated by PESC and Mount et al. (1997). Although the water hardness between the two tests was similar, they had substantially different calcium/magnesium ratios (Ca/Mg), with the PESC test having a substantially higher Ca content than Mount et al. (1997). Various studies investigating toxicity reduction of metals in waters of increasing water hardness have found that it is the Ca, rather than the
Mg, component of water hardness that plays a much greater role in the reduction of toxicity (Gundersen et al. 1994, Alsop & Wood 1999, Welsh et al. 2000). These studies demonstrated that competition between Ca and metal ions for binding sites on gill membranes reduced toxicity by ameliorating ion loss from gills caused by metals binding to gill membranes. In general, studies of both fish and invertebrates have shown that permeability of gill membranes to ions is reduced by Ca stabilizing cell membranes (Penttinen et al. 1998). Therefore, the increased Ca content of the PESC experimental water may be the source of the lower toxicity of sodium sulphate than that reported by Mount et al. (1997).

Therefore, this study aims to verify the high sensitivity of *D. magna* to sodium sulphate exposure in soft water reported by PESC and to investigate the reduction of sodium sulphate toxicity in standard reconstituted waters in the soft to medium hardness range (25 – 100 mg/L as CaCO₃). Furthermore, to manipulate the Ca/Mg ratios of standard culture waters will be manipulated, while maintaining a constant water hardness to investigate their relative importance in influencing sodium sulphate toxicity.

**Materials and Methods**

**General methods of all tests**

General procedures for *Daphnia magna* testing and culturing were based on the documents, "Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna* (Environment Canada July 1990)" and "Acute Lethality Test using *Daphnia* spp. (Environment Canada 1990a and May 1996 amendments)." The tests were conducted in a constant temperature controlled room which was maintained at 20±1 °C. A 16-h light/8-h dark photoperiod and a light intensity of <800 Lux were maintained during the test period.

Within each hardness experimental block, three replicates were used for each sulphate concentration (added as Na₂SO₄). Within each replicate, 10 *Daphnia* neonates less than 24 hours old, obtained from 2-5 week old females were used. The replicates were 300 ml
Plexiglas beakers containing approximately 200 mL of test solution. No artificial substrate was used, and the daphnids were not fed for the duration of the 48 hour test.

At the beginning and the end of the test, dissolved oxygen, temperature and pH were measured in each test concentration including the controls. All measurements were within test acceptability criteria. Furthermore, representative sodium sulphate and hardness samples were taken and reconfirmed by independent lab analysis. Values were within 15 percent of nominal concentrations; therefore, nominal concentrations were used for data analysis.

At the end of the test, the number of surviving daphnids in each vessel was recorded. Death was defined as the lack of all movement and heartbeat, as observed using a dissecting microscope.

Tests 2D and 3D manipulate Ca/Mg ratios while maintaining an equivalent water hardness between test groups. The formula used to calculate water hardness from first principles is as follows:

$$\text{Total hardness (mg/L as CaCO}_3) = 2.497 \times [\text{Ca}] + 4.118 \times [\text{Mg}]$$

Where:
- $[\text{Ca}]$ is the calcium ion concentration in the solution in mg/L
- $[\text{Mg}]$ is the magnesium ion concentration in the solution in mg/L

Ca/Mg ratios were calculated and expressed on a molar basis with respective concentration in the solution being divided by its molecular weight.

**PESC experiment (unpublished, 1996)**

- Sulphate toxicity in water hardness of 25, 100 and 250 mg/L as CaCO$_3$ was tested.

The *D. magna* used in this study came from an in-house stock, cultured in on-site well water which has a hardness of approximately 105 mg/l as CaCO$_3$. For the tests, reconstituted de-ionized water, well water, and well water with ion addition were used to make control and test solutions for the soft, medium and hard water test blocks respectively (Table 1). The ion composition of the soft test water is consistent with those
suggested in the current guidelines for conducting *D. magna* toxicity tests (Environment Canada 1990a and May 1996 amendments). No vitamin B12 or selenium was added to the culture water (Graham Van Aggelin 2001).

This Investigation

All organisms used in the following studies came from an in-house culture maintained at BC Research Inc. The culture water had a water hardness of approximately 100 mg/L (as CaCO₃) with the ion makeup of the solution being consistent with moderately hard reconstituted water (MHRW) as outlined in "Acute Lethality Test using *Daphnia* spp. (Environment Canada 1990a and May 1996 amendments)." Regardless of water hardness, all water was supplemented with 2 μg/L selenium and vitamin B12.

Test 1D

- Sulphate toxicity in water hardness of 25, 50 and 75 mg/L as CaCO₃ was tested.

MHRW water was diluted with de-ionized water to formulate the desired hardness levels.

Test 2D

- Sulphate toxicity in constant water hardness of 105 mg/L (as CaCO₃) with Calcium/Magnesium ratio's 0.7, 3.8 and 6.9 (expressed as molar ratios)

MHRW as described in Environment Canada (July 1990a) has a Ca/Mg ratio of 0.7. A new water formula was developed using the basic water hardness equation found above to increase the relative amounts of Ca relative to Mg while maintaining a constant water hardness of 105 mg/L (as CaCO₃). The following chemicals were added to 20 L of de-ionized water to achieve the desired ratios (Table 1). Equal amounts of both were mixed to create the water with a Ca/Mg of 3.8.
Table 1. Ion composition of reformulated water used to maintain constant water hardness while manipulating Ca/Mg ratios

<table>
<thead>
<tr>
<th>Salts added</th>
<th>Amounts (mg/L)</th>
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<tbody>
<tr>
<td>CaSO₄</td>
<td>75</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>59</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>120</td>
</tr>
<tr>
<td>KCl</td>
<td>5</td>
</tr>
<tr>
<td><strong>Calculated hardness</strong> (mg/L as CaCO₃)</td>
<td><strong>105.7</strong></td>
</tr>
<tr>
<td><strong>Ca²⁺/Mg²⁺ ratio</strong></td>
<td><strong>0.695</strong></td>
</tr>
</tbody>
</table>

**Test 3D**

- Sulphate toxicity in constant water hardness of 25 mg/L (as CaCO₃) with Calcium/Magnesium ratio's 0.7 and 6.9 (expressed as molar ratios)

Water formulations were similar to test 2D with the exception that one-quarter the amounts of ions were added.

**Statistical methods**

All 50 percent lethal concentration (LC50) values were calculated using the software “Toxcalc 5.0.” Methods used to calculate LC50s were Maximum-likelihood Probit and Logit methods and Linear interpolation, depending on the distribution of the data.

**Quality Assurance/Quality Control**

A reference toxicant (analytical grade zinc sulphate) was used to assess the relative sensitivity of daphnids and the precision of data produced during these tests (Environment Canada 1990a and May 1996 amendments). The procedures and conditions used were identical to those in standard *Daphnia magna* 48-hr LC50 bioassays. All reference toxicant tests were within the acceptable range (2 standard deviations) of variation for the culture.

The brood organisms met the following health criteria: there was less than 25 percent mortality in the parental organisms in the seven days preceding the test; the time to first
brood was less than 12 days and the females delivered greater than 15 neonates per brood.

The tests were considered valid since the control mortality was less than 10 percent and not more than 10 percent of the control organisms exhibited stressed behaviour (i.e., immobility).

Results

Test 1D

The 48-hour LC50s for the 25, 50 and 75 hardness blocks (mg/L as CaCO$_3$) were calculated at 957, 1768 and 3155 mg/L sulphate respectively (Figure 1).

![Figure 1. Reduction in toxicity of sodium sulphate in standard waters of increasing water hardness.](image)

Test 2D

The 48-hour LC50s for the 0.7, 3.8 and 6.9 Ca/Mg blocks were 3247, 3842 and 4541 mg/L sulphate respectively (Figure 2). There was a significant difference (p<0.05) between the 0.7 Ca/Mg ratio group when compared to the 3.8 and 6.9 Ca/Mg ratio groups. The difference between the 6.9 and the 3.8 Ca/Mg groups was not significant as the confidence intervals overlapped.
Figure 2. Reduction in toxicity of sodium sulphate in waters of increasing calcium content. Waters have identical water hardness of 100 mg/L (as CaCO$_3$).

**Test 3D**

The 48-hour LC50s for the 0.7 and 6.9 Ca/Mg blocks were 1303 and 2424 mg/L sulphate respectively (Figure 3). However, both these values were beyond the highest sulphate concentration tested (1300 mg/L and 1700 mg/L in the 0.7 and 6.9 Ca/Mg blocks respectively); therefore they are not statistically valid. However, the LC50 calculated for the 6.9 Ca/Mg of 2424 mg/L sulphate is substantially higher than both the LC50 calculated in the 0.7 Ca/Mg block and in Test 1D of 957 mg/L sulphate (which had the same 0.7 Ca/Mg ratio), suggesting that Ca also plays a role in reducing toxicity in soft water conditions as well.
Figure 3. Reduction in toxicity of sodium sulphate in waters of increasing calcium content. Waters have identical water hardness of 25 mg/L (as CaCO₃). Dashed line shows trend only.

**Discussion**

*Test 1D*

The results in Test 1D indicated that toxicity from sodium sulphate is reduced in waters of increasing hardness. This trend has been observed in many other taxa, including species of fish, plants and other invertebrates.

The LC50 value reported by PESC (537 mg/L sulphate) is nearly half the LC50 of 957 mg/L sulphate calculated here. This may have been due to the vitamin B12 and/or selenium addition to the culture water in this test or may have been from experiment or organism variation. As the test conditions were similar, an average of these two values is used in Figure 4.
Figure 4. Summary of data of the response of *D. magna* in waters of increasing hardness. • and ▲ correspond to LC50 values from this investigation in standard waters and PESC experimental data which was performed in well water. Dashed line shows trend only.

**Tests 2D and 3D, Changing molar ratios**

The LC50 value of 3247 mg/L sulphate calculated in standard laboratory water at 100 mg/L hardness in Test 2D is comparable to the LC50 of 3098 mg/L sulphate reported by Mount et al. (1997). Although waters with higher Ca/Mg ratios caused significantly lower mortality To *D. magna*, Ca/Mg ratios alone do not completely explain the substantially lower toxicity that PESC reported (LC50 of 6281 mg/L sulphate) in water of 100 mg/L hardness (as CaCO₃). The well water used in the tests done by PESC had a Ca/Mg ratio of 6.7 in comparison to the high calcium water used in Test #2 which had a molar ratio of 6.95 which resulted in an LC50 of 4541 mg/L sulphate. These differences may be due to population specific differences in the test organisms or possibly other water quality characteristics. As PESC conducted its test in well water, there may be other water quality characteristics influencing sodium sulphate toxicity.

Both Test 2D and 3D indicate that Ca has a greater effect than Mg on reducing the toxicity of sodium sulphate. The majority of the work examining Ca effects on ion exchange involve fish species. Studies examining toxicity reduction of metals and saline
solutions have also found that Ca has a greater effect in reducing toxicity than Mg. For example, Wendelarr Bonga et al. (1983) examined the effects of Ca and Mg on Talapia (Sarotherodon mossambicus) gill membranes and found that Mg was not as effective at reducing osmotic influx of water across the gills. Calcium may play a similar role in D. magna when subjected to the high saline conditions of the test medium examined here. Furthermore, Ca plays a fundamental role in the regulation of sodium between the gill membranes and the surrounding aquatic media. Various studies examining ion transport in fish gill membranes (Cuthbert & Maetz 1972, Eddy 1975, Pic & Maetz 1981) indicate that Ca reduces the permeability of gills to sodium and hydrogen ions. Sodium sulphate is 33 percent sodium by mass; however, two sodium ions are associated with every sulphate ion in anhydrous sodium sulphate. Therefore, sodium has double the molar concentration compared to sulphate in sodium sulphate solutions. The osmotic pressure exerted by a solution is a function of the concentration of solute particles rather than the mass of the particles (Potts & Parry 1964). Therefore, the reduction of sodium sulphate toxicity observed in waters of increasing calcium content may indicate that the sodium cation may be causing a portion of the toxicity rather than the toxicity simply coming from the sulphate ion. However, as no studies could be found that examine the direct effects of Ca on the permeability of gill membranes to sodium and sulphate ions collectively, further studies are required to delineate these interactions.

These findings have ramifications on the management of effluent discharges with elevated concentrations of sodium and sulphate as most natural waters have higher Ca/Mg ratios than the standard laboratory water used in the majority of toxicity investigations. The U.S. Geological Survey National Stream Water-Quality Monitoring Network database has information regarding the Ca and Mg ratios of natural waters (Welsh et al. 2000). Of the 660 water quality stations included in the database, the median Ca/Mg ratio was 2.05, and only 10 of the stations has ratios below 0.7. The substantially higher LC50 values reported by PESC (Figure 4) suggests that other water quality characteristics of natural waters may reduce the toxicity of sodium sulphate even further. Therefore, any guideline developed using toxicological data on sodium sulphate which utilized standard laboratory waters likely overestimates toxicity from what would occur in an environmental context.
Conclusions

The toxicity of sodium sulphate to *D. magna* is substantially reduced in water of increasing hardness. Furthermore, the calcium rather than the magnesium component of water hardness appears to play a more important role as was observed in reduced mortality of *D. magna* in waters of increasing calcium content. Specifically, approximately 50 percent more sodium sulphate was required to achieve LC50 in waters with a Ca/Mg ratio of 7.0 compared to waters of high magnesium content with a Ca/Mg ratio of 0.7. As many natural waters have Ca/Mg ratios substantially above 0.7, this may explain a portion of the reduced toxicity seen in these waters beyond what would be expected from data from toxicity tests done in standard lab water. Furthermore, the toxicity of sodium sulphate appears to be further reduced by other water quality characteristics beyond simply water hardness and calcium content. This is suggested by the elevated LC50s reported by PESC (1996) which were conducted in ground water. However, further tests are needed in order to delineate these interactions.

Acknowledgements

Thanks are given to the general staff, and in particular Janet Pickard, at BC Research Inc. for guidance, use of lab space, equipment and materials. BC Ministry of Environment (Smithers), through the Pacific Environmental Science Centre generously donated water analysis on the test solutions. Endako Mines, Highland Valley Copper, Inmet Mining Corp., Placer Dome Inc. and Taseko Mines provided financial assistance for this project. Finally, I would like to thank my thesis advisor, Dr. Ken Hall for his support.
References
Appendix C

The Effects of Sulphate on Striped Bass (*Morone saxatilus*) Larvae Survival
A Reproduction of Hughes (1973)
**Introduction**

Striped bass (*Morone saxatilus*) is an anadromous fish species native to the Atlantic Coast that was introduced from New Jersey to San Francisco Bay on the Pacific Coast in 1879. Like other anadromous species, there are landlocked populations which live and spawn entirely in freshwater; however, no land locked populations reside in British Columbia. Striped bass are most abundant in Pacific waters around the San Francisco Bay area but have been found from northern Baja, California to Barkley Sound, BC (Eschmeyer & Herald 1983). Striped bass spawn in fresh water and eggs hatch from 29 to 80 hours after fertilization, depending on the water temperature. At hatching, larvae have an average size of 3.1 mm and typically begin feeding about five days later (California Department of Fish and Game 2002).

The majority of the toxicity testing involving striped bass has been specifically aimed at determining ideal aquaculture methodology (Hughes 1971, Hughes 1973, Mazik et al. 1991, Reardon & Harrel 1994, Grizzle 1995) and investigating the effects of salinity on early life stages of striped bass (Morgan & Rasin 1981, Dwyer et al. 1992, Winger & Lasier 1994). Salinity impact studies have been done to examine the potential population impacts from seawater intrusion into striped bass spawning habitats as eggs, larvae and juveniles are more sensitive to high salinity unlike adults in anadromous populations which carry out the majority of their adult life in estuaries and coastal bays (Eschmeyer & Herald 1983). However, the larvae and juveniles do show some resistance to saline waters and actually have better survival rates in waters with salinities of 3 g/L compared to freshwater (Reardon & Harrel 1994) as the addition of salt has been shown to reduce the physiological responses associated with the stresses of capture and transport of striped bass (Harrel 1992). As it can be difficult to differentiate between the toxic effects of trace elements and salinity in waste waters, striped bass are now gaining attention as possible bioassay organisms to differentiate between these effects (Dwyer et al. 1992).
This investigation repeats the study done by (Hughes 1973) which reported 1, 2, 3 and 4 day (96 hour) median lethal concentrations (LC50s) of 2000, 1000, 500, and 250 mg/L for sulphate, and LC0s (no effect) of 500, 100, 100, and 100 mg/L, respectively, for striped bass larvae. These values are much lower than what would intuitively be suggested by the reported beneficial effects of low salinity mixtures to larvae striped bass survival. However, the high sensitivity reported may indicate that sulphate is highly toxic to striped bass under specific water quality conditions. Furthermore, this may indicate that species with a certain life history or strategy could be at risk to sulphate toxicity under certain environmental conditions as those mimicked Hughes (1973).

The British Columbia Ambient Water Quality Guidelines for Sulphate for the Protection of Aquatic Life (Singleton 2000) cites Hughes (1973) as one of the principle rationales for the current guideline of 100 mg/L sulphate. In order to ensure that guidelines are based on reproducible and defensible scientific data, this study repeats Hughes (1973) bioassays to assess its validity for the use in the BC sulphate water quality guidelines. Furthermore, as the toxicities of inorganics (such as sulphate) and salinity have been shown to be reduced in waters of increasing water hardness (Dwyer et al. 1992), this study incorporates the influence of water hardness n sulphate toxicity.

Review of “Acute Toxicity of Thirty Chemicals to Striped Bass (Morone saxatilis)” (Hughes 1973)

Hughes describes the toxicity of thirty chemicals to striped bass larvae and fingerlings. The toxicities reported are substantially lower than those reported by others regarding the sensitivity of striped bass to many of these substances (Table 1).
Table 1. Comparison of acute toxicity of trace metals for striped bass, reported by Hughes and others, for chemicals assessed in U.S. EPA water quality criteria documents (from Beak Consultants Incorporated (1997)).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Hardness (mg/L as CaCO$_3$)</th>
<th>96-hr LC50 (μg/L)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium</td>
<td>55</td>
<td>1 100 (fingerlings)</td>
<td>(Rehwoldt et al. 1972)</td>
</tr>
<tr>
<td></td>
<td>34.5</td>
<td>1 (larvae)</td>
<td>(Hughes 1973)</td>
</tr>
<tr>
<td></td>
<td>34.5</td>
<td>1 (fingerlings)</td>
<td>(Hughes 1973)</td>
</tr>
<tr>
<td>Zinc</td>
<td>55</td>
<td>6 800 (fingerlings)</td>
<td>(Rehwoldt et al. 1972)</td>
</tr>
<tr>
<td></td>
<td>137</td>
<td>1 180 (fry)</td>
<td>(O'Rear 1972)</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>100 (larvae)</td>
<td>(Hughes 1973)</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>100 (fingerlings)</td>
<td>(Hughes 1973)</td>
</tr>
<tr>
<td>Copper</td>
<td>55</td>
<td>4 300 (fingerlings)</td>
<td>(Rehwoldt et al. 1972)</td>
</tr>
<tr>
<td></td>
<td>34.5</td>
<td>50 (larvae)</td>
<td>(Hughes 1973)</td>
</tr>
<tr>
<td></td>
<td>34.5</td>
<td>50 (fingerlings)</td>
<td>(Hughes 1973)</td>
</tr>
</tbody>
</table>

There is no statistics methodology found in the article; however, 1, 2, 3 and 4 day LC50 values are reported in the Hughes article. Furthermore, the LC50 values reported for many of the substances do not decrease over exposure time. For example, the LC100 values reported for “Instant sea” is 14,500 mg/L (reported as mg/L chloride) are identical for 1, 2, 3 and 4 days of exposure.

This phenomenon is also seen in the results presented for the chemicals 2, 4-D butyl ester, Diquat and calcium hypochlorite. This trend in toxicological data would only be possible if mortality only occurred in the first 24 hours and then immediately halted. Furthermore, “Instant Sea” is 85 percent (by mass) sodium chloride. This is far from the LC100 Hughes reported for chloride of 2000 mg/L after 96 hours. In addition, many of the LC50s that do decrease overtime frequently go down in a geometric stepwise process, such as the presented LC50 values for sulphate which decreased by exactly half every 24 hours.

The inconsistencies of the results presented by Hughes (1973) are not consistent with the general trends seen in toxicological studies. The article appears in conference proceedings and shows no evidence of being peer reviewed. Therefore, the quality of the results are suspect and the experiment needs to be replicated to verify the results presented.
**Methodology**

**Water Chemistry**

The ion content in Hughes (1973) contained significant amounts of sulphate; and, as this test was specifically examining sulphate effects, a slightly different water chemistry was used in this investigation (Table 2). The hard water (250 mg/L as CaCO₃) and medium water (100 mg/L as CaCO₃) treatments would have contained 198 and 79 mg/L sulphate respectfully using the Hughes (1973) reconstituted water ion formulation. The former was approaching the 250 mg/L sulphate, 96 hour LC50 concentration reported in Hughes (1973). Furthermore, as this test was specifically aimed at looking at sulphate toxicity, it seemed prudent to remove sulphate from the control water and replace the associated anion of calcium and magnesium with chloride. All other ion ratios were comparable to that of Hughes (1973) as the toxicity of inorganics have been shown to be influenced by different ion ratios in the exposure water (Mount et al. 1997, Davies 2002).

<table>
<thead>
<tr>
<th>Davies, 2001 Quantities (mg/L)</th>
<th>Hughes 1973 Quantities (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂·2H₂O</td>
<td>85</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>75</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>137.5</td>
</tr>
<tr>
<td>KCl</td>
<td>0.6</td>
</tr>
<tr>
<td>Hardness</td>
<td>95</td>
</tr>
</tbody>
</table>

|                                |                               |
|                                | CaSO₄·2H₂O                    |
|                                | MgSO₄·7H₂O                    |
|                                | NaHCO₃                       |
|                                | KCl                           |
|                                | 35                            |
|                                | 35                            |
|                                | 55                            |
|                                | 2                             |
|                                | 34.5                          |

**Experimental methods**

Two static renewal larvae tests were conducted on striped bass larvae from May 20th to 24th and May 25th to 29th, 2001. The larvae were supplied by Professional Aquaculture Services located in Chico, CA and were approximately 6 to 8 days old on arrival. The larvae arrived in water of 20 °C with a hardness of approximately 144 mg/L as CaCO₃ and conductivity of 255 μS/cm. There was considerable trouble in receiving larvae that would survive the shipping process. In the two tests reported here, survival during shipping was below 50 percent.
Upon arrival, surviving larvae were removed from the shipping water and transferred to the reconstituted de-ionized control water of 100 mg/L as CaCO$_3$. As three different hardness treatments were to be tested (25, 100 and 250 mg/L as CaCO$_3$), the larvae were acclimated with fractional water changes to the different hardness levels over a period of one day for the first test and two days for the second.

Ten larvae were placed in 300 ml Pyrex beaker test vessels in a climate controlled room at 21 °C ± 1 °C and with 16:8 hour light/dark cycle exposed to between 500 to 1000 Lux. Each control group and sulphate exposure concentration had four replicates each. Fish were fed 500 µL of yeast-cerophyl trout chow (YCT, (U.S. Environmental Protection Agency 2000)) one hour prior to the initiation of the test and on day two of the test.

Sulphate was added as sodium sulphate anhydrous (Na$_2$SO$_4$) which was initially dissolved in a concentrated stock solution. The stock solution was then mixed with control water to meet the desired sulphate exposure levels (0, 250, 500, 750, 1000, 2000 and 4000 mg/L SO$_4$) in each hardness treatment. Sulphate exposures were reconfirmed by ion chromatography analysis and exposures tested were all within 10 percent nominal concentration levels; therefore, nominal concentrations are reported here. Due to poor survival during transport, some higher concentrations of sulphate exposure were omitted during the first experiment.

At the end of the 96-hour test, the organisms were counted and checked for survival. Organisms exhibiting any movement were classified as alive and those immobile were recorded as dead.

**Results**

Low survival in all control and low sulphate exposures were seen in all three hardness treatments. Control survival was less than 35 percent in all water hardness treatments and half of them had zero survival (Figures 1 though 3).
Survival tended to increase in all hardness treatments with increasing sulphate exposure. Survival in the second test was better in the majority of treatments compared to the first test.

**Figure 1.** Comparison of survival of larval striped bass in soft water (hardness 25 mg/L as CaCO₃) after 96 hours to increasing sulphate exposure. ▲ and ■ correspond to test #1 and test #2 respectfully, error bars are ±1 SD.

**Figure 2.** Comparison of survival of larval striped bass in medium water (hardness 100 mg/L as CaCO₃) after 96 hours to increasing sulphate exposure. ▲ and ■ correspond to test #1 and test #2 respectfully, error bars are ±1 SD.
Discussion:

The extremely low survival observed in the control water and low sulphate exposures in all water hardness treatments suggest that the water quality characteristics were unsuitable for larval striped bass survival. Therefore, no indication of reduction of toxicity caused by sodium sulphate exposure in waters of increasing hardness could be gained from this study. However, the substantial improvement to survival with increasing sulphate exposure indicates that the addition of sodium sulphate was somehow beneficial and suggests that the ionic composition of the control water was at least partially responsible for the low survival.

The water chemistry outlined in Hughes (1973) has an extremely low salinity having approximate Na\(^+\) and Cl\(^-\) concentration of 15 and 1 mg/L respectfully and, like this experiment, operated at a temperature of 21.1 °C. A previous bioassay (Morgan & Rasin 1981) investigated the effects of temperature and salinity on striped bass larvae survival. Their results indicated temperatures above 20 °C were detrimental to larvae survival and increasing salinity improved survival (Table 3). Optimal calculated temperatures and
salinity for survival was 18°C and 10 parts per thousand (‰) salinity (the highest salinity tested).

**Table 3.** Effects of salinity and temperature combinations on survival of striped bass larvae.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Salinity (‰)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>5.0</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>65.0 ± 4.8</td>
<td>68.5 ± 3.8</td>
<td>74.1 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>76.1 ± 3.5</td>
<td>80.4 ± 3.4</td>
<td>84.2 ± 5.1</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>68.6 ± 2.8</td>
<td>71.9 ± 1.8</td>
<td>75.2 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>34.8 ± 4.1</td>
<td>46.0 ± 2.6</td>
<td>47.1 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>13.3 ± 2.4</td>
<td>10.9 ± 1.4</td>
<td>12.5 ± 2.5</td>
<td></td>
</tr>
</tbody>
</table>

*Values are means and standard deviations from Morgan & Rasin (1981)*

Optimal larval survival temperature corresponds very closely to the mean temperature of peak spawning of striped bass in nature (Morgan & Rasin 1981). Therefore, the poor survival seen in Hughes (1973) and this investigation may have been in part due to high water temperatures and low salinity. However, the experiment by Morgan & Rasin was done with newly hatched larvae which may have been more sensitive to temperature than the 6-9 day old larvae used in Hughes (1973) and this experiment.

Other studies have reported poor survival in waters with low salinity (Lai et al. 1977, Geiger & Parker 1985). Also, Winger and Lasier (1994) reported extremely poor survival in freshwaters and also observed substantially increased survival in waters of moderate salinity exposure (added as seawater up to 18 ‰ when mortality of 5 day post larvae begins to occur (Table 4).
Table 4. Percent survival of striped bass larvae to increasing levels of salinity exposure (Winger & Lasier 1994). Anions exposure from seawater included.

<table>
<thead>
<tr>
<th>Salinity (%)</th>
<th>48h post hatch larvae survival (%)</th>
<th>5 day post hatch larvae survival (%)</th>
<th>Na⁺ (mg/L)</th>
<th>Cl⁻ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>6</td>
<td>80</td>
<td>88</td>
<td>1837</td>
<td>3302</td>
</tr>
<tr>
<td>12</td>
<td>64</td>
<td>98</td>
<td>3673</td>
<td>6605</td>
</tr>
<tr>
<td>18</td>
<td>25</td>
<td>84</td>
<td>5510</td>
<td>9907</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>0</td>
<td>7346</td>
<td>13210</td>
</tr>
</tbody>
</table>

The increased survival observed with the addition of seawater is comparable to that seen in this experiment when converted to equivalent sodium ion addition (Figure 4). Although the associated anion (either Cl⁻ or SO₄²⁻) may be responsible to the increase in survival, the trend is not as consistent when chloride and sulphate exposure (converted to molarity) is compared (Figure 5). However, as sodium and chloride make up 85% (by weight) of the ion content of seawater, the higher survival of the larvae exposed to seawater may have also been influenced by other ions.

Although there appears to be a decline in survival in the highest Na⁺ concentration for this test (Figure 4), the percent survival between the two test is not significantly different from each other. Furthermore, as there is not a observation point above this level, the decline in survival may not be an indication of the upper tolerance of Na⁺ exposure, but rather an outlying data point. However, further studies would be required to assess the upper tolerance of sodium sulphate to larval stripped bass survival.
Figure 4. Effects of Na\(^+\) exposure to survival of larval striped bass in test #2-hard water treatment after 96 to that seen in (Winger & Lasier 1994) after 120 hours. • and ■ correspond to test #2-hardwater treatment and (Winger & Lasier 1994) respectfully, error bars are ±1 SD.

Figure 5. Effects of anion exposure to survival of larval striped bass in test #2-hard water treatment after 96 to that seen in (Winger & Lasier 1994) after 120 hours. • and ■ correspond to test #2-hardwater treatment and (Winger & Lasier 1994) respectfully, error bars are ±1 SD.
Conclusions

The poor survival in waters of low salinity at all water hardness treatments in this experiment makes it impossible to assess whether waters hardness influences the toxicity of sodium sulphate to striped bass larvae. Further studies will be required to assess this.

The results in this study and those reported by Winger & Lasier (1994) and Morgan & Rasin (1981) indicate that striped bass survival is adversely affected in waters in temperatures above 20 °C and of low salinity. The test temperature of 21 °C and low salinity test water of Hughes (1973) and this test suggests that culture water toxicity is the source of the extremely high toxicity of sulphate and other substances reported by Hughes (1973). The low survival was likely due to a deficiency in Na\(^+\) ions and may have been exacerbated by high water temperatures. Therefore, all the results presented in Hughes (1973) are suspect and likely present a biased evaluation of all the substances tested and should not be used in the development of any water quality guideline. In contrast to the high sensitivity of striped bass to sulphate, this study indicates that sodium sulphate is beneficial rather than detrimental to striped bass larvae survival in waters of low salinity.
References:


California Department of Fish and Game. 2002. Web Site: http://www.dfg.ca.gov/


Hughes, J.S. 1973. Acute Toxicity of Thirty Chemicals to Striped Bass (Morone saxatilis). pp. 399 Proceedings of 53rd Annual Conference Western Association of State Game and Fish Commissioners, Salt Lake City, Utah.


Appendix D

Effects of sulphate on the growth and chlorophyll levels of *Fontinalis antipyretica*, a common BC aquatic moss
Introduction

Mosses have been used in various biomonitoring investigations in several types of pollution in surface waters (Mersch & Reichard 1998) and have frequently been used as bioindicators as their lack of well-developed cuticula and vascular tissue make them sensitive to environmental pollutants (Raeymaekers & Glime 1986). Their appeal is based on laboratory and field investigations that have shown exchange kinetics in mosses to be very rapid and predominantly based on biosorptive processes (Mersh & Reichard 1998). Furthermore, although the majority of the research on moss toxicity is based upon trace metal studies, the rapid uptake kinetics found in mosses have shown to provide reliable responses over relatively short exposure periods (Mersh & Reichard 1998). Specifically, immersion times of 1 and 10 days have been considered sufficient (Lopez et al. 1994), though longer experiments of up to 4 weeks have been conducted.

This investigation looks at three characteristics to assess the toxicity of sulphate on *F. antipyretica*. Dry weight, shoot growth and chlorophyll levels are compared to control levels in the 21-day experiment. Lopez et al. (1994) indicated that a period of between 7 to 15 days should be sufficient to obtain a response from *F. antipyretica*, although longer exposure times were encouraged. Furthermore, reduction in chlorophyll levels has been observed in various moss species including *F. antipyretica* in response to organic pollution (Martinez-Abaigar et al. 1993) and heavy metal (Bruns et al. 1997) exposure. As the stability of a wide range of metabolic processes is expressed via the growth response (Sidhu & Brown 1996), these measures should provide data assessing sub lethal effects of sulphate exposure to *F. antipyretica*.

The aquatic moss, *F. antipyretica* was selected as it is one of the most widely distributed species in Fontinalaceae (Biehle et al. 1998) and has wide distribution in BC (Warrington, 1994). A previous study claimed that a one week exposure to 100 mg/L sulphate (added as K₂SO₄) was toxic to this aquatic moss (Frahm 1975). In contradiction, BEAK International Incorporated, together with Michigan Technical University (1998), conducted a toxicity investigation on a similar species (*Fontinalis neomexicana*) that indicated much less sensitivity to sulphate than reported in Frahm (1975). No observable
effect on chlorophyll levels was observed up to the maximum exposure concentration of 500 mg/L sulphate (added as Na$_2$SO$_4$) at a water hardness of 160 mg/L (as CaCO$_3$) for an exposure of 14 days. However, Ministry of Lands and Parks (MELP) scientists did not consider this a valid endpoint since aquatic mosses grow very slowly and chlorophyll levels would likely not be affected in a relatively short experiment (Singleton 2000). A 14 day testing period may be sufficient, however, since mosses are beginning to receive attention as bioindicators as they have been reported to demonstrate relatively high metabolic activity even in autumn and winter (Frost 1990 cited in Siebert et al., 1996)

**Site and moss characteristics**

*F. antipyretica* is easily identifiable from other mosses found in BC with its sporophyte (reproductive structure) closely associated with the gametophyte of the plant. It can be very large and grows in dense clumps with stems growing from 50 cm (Siebert et al. 1996) to 90 cm (Biehle et al. 1998) in length and a diameter of 0.2 – 0.5 mm. The leaves on the stems are arranged in a “3-winged” form. The plant attaches itself to hard substrate with its rhizoids and is able to propagate both vegetatively and sexually although the latter is rare under natural conditions (Siebert et al. 1996)

Moss was collected from two field sites. One close to the campus of the University of British Columbia (UBC) (site #1), the other in a slough of the Miami River in the town of Harrison, BC (site #2). Identification was done using Schofield (1992) and reconfirmed by Wilf Schofield, Professor Emeritus of the Botany department at UBC. The moss collected at site #2 differed taxonomically from specimen collected at site #1. Specimens from site #2 displayed more branching and appeared to have a more “woody” main stem. This may be attributed to the different flow rates between the two sites. Biehle et al. (1998) reported that specimens from areas with higher flow velocities tended to have significantly more strengthening tissue and different branching angles of the leaves.
Site #1

The moss was located in a shallow, slow moving stream in Pacific Spirit Park in approximately 20 – 30 centimeters deep. The water had a brownish colour and substantial suspended solids were present. There was substantial overhanging canopy with little or no direct sunlight being exposed to the area. The water collected had a hardness of 19.3 mg/L as CaCO₃.

Site #2

The moss was found in a slow moving portion of a slough of the Miami River in Harrison Hot Springs in approximately 45 to 60 centimeters of water. The water was clear with little suspended solids and had direct sunlight exposure. The water collected had a hardness of 41.6 mg/L as CaCO₃.

Materials and Methods

Experiment #1

Moss was collected from the field sites in 20 L plastic buckets. The moss was kept submerged in site water during transport from the field site to the test lab at BC Research Inc. The water collected from the field sites was put through a glass fiber filter to remove organisms and suspended solids that may be detrimental to moss growth and its survival over the test duration. This water was used as exposure water. A concentrated sulphate stock solution was added to control water in varying amounts to get the desired sulphate exposure levels of 0, 200, 400, 600, 800, 1000 and 1500 mg/L. The Pacific Ecology Science Centre reconfirmed sulphate concentrations and sulphate exposure levels were all within ten percent of nominal concentrations. Therefore, nominal concentrations were used in analysis. Sulphate was added in the form of anhydrous sodium sulphate (Na₂SO₄). All exposure concentrations were formulated at the beginning of the test. Water, which was to be used for water changes, was put in opaque 1 L plastic bottles and stored in a 8°C cold room until the morning of the water change when it was warmed to approximately 15°C, the temperature of the test lab. Moss was collected the day prior to
the start of the test and the moss was left to sit overnight in the cold room in collection buckets.

**Experiment #2**

As the moss collected from site #1 displayed increased sensitivity to sulphate exposure during the first experiment, a second experiment was designed to investigate whether there was a water-hardness based response to sulphate toxicity in *F. antipyretica* as found in other organisms. Fresh samples were to be collected for this experiment; however, the region had been undergoing extremely dry conditions and the site where moss was previously collected had substantially reduced water levels and moss populations. As there was moss remaining that appeared to be in a healthy condition remaining from the previous experiment that had been stored with an open lid in the test room since the execution of the first experiment (approximately 5 weeks) and no other moss was available, this moss was used for the 2nd experiment.

Well water was used as control water and was collected from the Pacific Ecology Science Centre (PESC) located in West Vancouver. The water collected had a hardness of approximately 104 mg/L (as CaCO₃) and was used undiluted for the medium hardness test block. The other test block used well water diluted 3:1 with deionized water to bring the water hardness down to 25 mg/L. The well water was not filtered and no additives such as plant nutrients were supplemented.

**General Procedures**

Both studies were of a static-renewal aqueous test design with water changes and shoot measurement occurring on days 0, 5, 10, 15 and 21. The tests were run for a total of 21 days at a controlled temperature of 15 ± 1°C. The tests were conducted under a randomized block design with each replicate flask being assigned a random location within each block. Blocks were assigned by site water chemistry and were used for ease of setup rather than any expected difference between test locations. Furthermore, flasks
were given a new random position after each water change and block position was rotated.

Individual gametophores (referred to here as shoots) were taken from the moss clumps and 2 cm apical segments were excised and temporarily stored in control water. After enough shoots were collected, they were randomly assigned to test concentrations within each test block. Test vessels were 250 ml Erlenmeyer flasks filled to 125 ml and left uncovered for the duration of the test to ensure sufficient gas exchange. Humidity in the room was 100 percent and evaporation was not significant during the course of the experiment.

Ten 2 cm shoots were placed in each flask and allowed to float freely. There were 4 replicates for each test concentration. Flasks were positioned on a shaker table and were lightly gyrated to ensure sufficient nutrient and gas exchange from the shoots, medium and atmosphere. Light was provided by four full spectrum florescent light bulbs supplying 1500 – 2000 Lux. The lights were left on continuously for the duration of the experiment.

Length and Dry weight measurements
After experimental takedown on day 21, shoots that were not used for chlorophyll analysis were oven dried at 80 °C for 24 hours and individually weighed. Length measurements collected on days 5, 10 and 15 were not used in analysis.

Chlorophyll a and b calculations and extraction procedures
At experimental takedown, all shoots were measured, air dried and stored in 30 ml glass bottles and placed in a –20 °C freezer for approximately six weeks. The shoots were thawed and 2 were taken from each replicate set and cut to standard 1-cm lengths. These were placed in 10 ml glass tubes with 5 ml dimethyl sulphoxide (DMSO) and sealed. The tubes were placed in a 60 °C oven for 12 hours, after which the shoot tips were removed and placed in a drying oven for 24 hours at 80 °C.
The absorbance was measured at wavelengths of 648.2 and 664.9 nm on a Spectronic Unicam UV-300 spectrophotometer in a 1 cm cell to calculate chlorophyll levels. The equations used to calculate chlorophyll concentrations are as follows (Barnes et al. 1992).

\[
\begin{align*}
C_a &= (14.85A_{664.9} - 5.14A_{648.2}) \times \frac{V}{DW} \\
C_b &= (25.48A_{648.2} - 7.36A_{664.9}) \times \frac{V}{DW}
\end{align*}
\]

Where:
- \(C_a\) is chlorophyll a concentration (mg chl/mg moss)
- \(C_b\) is chlorophyll b concentration (mg chl/mg moss)
- \(A_{648.2}\) and \(A_{664.9}\) are the absorptive measurements at 648.2 and 664.9 nm.
- \(V\) is the volume of DMSO solvent in mL.
- \(DW\) is the dry weight of the moss tip in mg.

**Statistical Methodology**

Statistical analysis of experimental data was done using the software package “Toxcalc 5.0”. One tailed analysis of variance tests were done with a significant probability level of less than five percent. With the exception of the total length data for experiment #1, site #2, Shapiro-Wilk’s Test indicated a normal distribution and Bartlett’s Test indicated equal variances of the data.

**Results**

**Experiment #1**

**Shoot length (figure 1)**

The moss shoots collected at site #1 had minimal branching throughout the experiment and displayed low variance in their response to sulphate exposure. Moss shoots displayed a significant reduction in final length in response to increasing sulphate exposure. The lowest observable effect (LOE, \(P<0.05\)) was at the 400 mg/L sulphate exposure level.
The moss shoots collected at site #2 showed significant branching throughout the experiment. Variance seen in total measured length in this group was high; therefore, shoot length was likely not an accurate measure of overall growth of the shoots.

![Graph showing the response of increasing sulphate concentration on moss shoot growth after 21 days exposure. Coefficient of variation bars are included. ■ correspond to group of moss collected at site #1 with hardness of 19.3 mg/L as CaCO₃ and ▲ with dashed line correspond to group of moss collected at site #2 with a hardness of 41.6 mg/L as CaCO₃.](image)

**Figure 1.** Response of increasing sulphate concentration on moss shoot growth after 21 days exposure. Coefficient of variation bars are included. ■ correspond to group of moss collected at site #1 with hardness of 19.3 mg/L as CaCO₃ and ▲ with dashed line correspond to group of moss collected at site #2 with a hardness of 41.6 mg/L as CaCO₃.

**Dry Weight (figure 2)**

Variance in dry weight measurements was approximately 20 percent in both test groups. Moss shoots collected from site #1 displayed a relatively constant and significant reduction in final dry weight in response to increasing sulphate exposure. The LOE (P<0.05) was at the 600 mg/L sulphate exposure level.

The moss shoots collected at site #2 did not show a significant reduction in final dry weight in all sulphate exposure treatments up to 1500 mg/L sulphate. However, the trend seen from 800 mg/L to 1500 mg/L suggests the beginnings of a downward trend indicating that 1500 mg/L sulphate exposure may be the upper limit of sulphate tolerance.
Figure 2. Response of increasing sulphate concentration on moss final dry weight after 21 days exposure. Coefficient of variation bars are included. ■ correspond to group of moss collected at site #1 with hardness of 19.3 mg/L as CaCO₃ and ▲ with dashed line correspond to group of moss collected at site #2 with a hardness of 41.6 mg/L as CaCO₃.

Chlorophyll levels (figure 3)

The shoots from site #1 had the highest chlorophyll content compared to shoots from site #2. The shoots in the control series from site #1 had an average chlorophyll content of 6.9 mg Chl a & b / g DW, and showed a continual decline with increasing sulphate exposure. The LOE level was 400 mg/L sulphate. Above 800 mg/L sulphate, when chlorophyll levels decreased below 3 mg Chl a & b / g DW the shoots from site #1 showed obvious signs of distress by beginning to turn brown. This can be quantitatively seen in the drop in chlorophyll levels from 400 mg/L of 5.2 mg Chl a & b / g DW in comparison to those levels in the 600 mg/L exposure group of 2.3 mg Chl a & b / g DW.
Figure 3. Response of increasing sulphate concentration on chlorophyll a & b content after 21 days exposure. Coefficient of variation bars are included. ■ correspond to group of moss collected at site #1 with hardness of 19.3 mg/L as CaCO$_3$ and ▲ with dashed line correspond to group of moss collected at site #2 with a hardness of 41.6 mg/L as CaCO$_3$.

The shoots in the control series from site #2 had a much lower chlorophyll content of 4.1 mg Chl a & b / g DW than compared to the moss shoots from site #1. This may have been due to the morphological differences between the two populations where the moss collected from site #2 seemed to have much woodier stems than those from site #1. A significant decline in chlorophyll was seen in the moss from site #2 at the 400 mg/L sulphate exposure level (LOE, P<0.05). However, increasing sulphate exposure beyond this level did not cause a significant reduction in chlorophyll levels with no significant difference between exposures 400 mg/L through 1000 mg/L. Moreover, the 1500 mg/L exposure treatment did not have significantly lower chlorophyll levels than the control treatment.

Most importantly, the highest sulphate exposure did not result in plant death as it did in the shoots collected from site #1 suggesting that the moss or water chemistry characteristics from site #2 resulted in less overall sensitivity to sulphate exposure.
Discussion of experiment #1

The were differences between the mosses collected at site #1 and site #2 with respect to final length, dry weight and chlorophyll levels. However, as the experiment used moss and water specific to each site it is difficult to determine whether the differences are due to site water characteristics, the particular responses from distinct moss populations or a combination thereof. Therefore, the only conclusion that can be realistically gained from this experiment is that the moss at site #1 would be more at risk due to sodium sulphate exposure compared to the moss found site #2.

Experiment #2

Shoot Length (figure 4)

As shoots were used that were collected from site #1, minimal branching occurred in both exposure groups. The soft water treatment (26 mg/L as CaCO₃) showed a significant reduction in growth (LOE, P<0.05) compared to control groups at 1000 mg/L sulphate. The medium water treatment (105 mg/L as CaCO₃) displayed a significant reduction in shoot growth (LOE, P<0.05) at 1500 mg/L sulphate.

The moss in the soft water treatment had overall slightly better growth than that in the medium water treatment. However, both groups had less growth in the control groups compared to the control treatment in experiment #1, site #1 with average length being 2.40 cm and 2.26 cm in the soft and medium water treatments respectfully, compared to 2.58 cm in the experiment #1, site #1 controls. The shoots in the control treatments in experiment #2 exhibited on average approximately 31 and 55 percent less growth in the soft water and medium water treatments respectfully than experiment #1, site #1 controls.
**Figure 4.** Response of increasing sulphate concentration on moss shoot length after 21 days exposure. Coefficient of variation bars are included. ■ correspond to group of moss in soft water treatment (hardness 26 mg/L as CaCO₃) and ▲ correspond to medium water treatment group (hardness 105 mg/L as CaCO₃). Dashed Line with ● correspond to moss treatment group from site#1, experiment #1 (hardness 19.3 mg/L as CaCO₃).

**Dry Weight (figure 5)**

The soft water treatment (25 mg/L as CaCO₃) showed a significant reduction in final dry weight (LOE, P<0.05) compared to control groups at 1500 mg/L sulphate. The medium water treatment (100 mg/L as CaCO₃) displayed a significant reduction (LOE, P<0.05) in shoot growth at 400 mg/L sulphate.
Figure 5. Response of increasing sulphate concentration on moss final dry weight growth after 21 days exposure. Coefficient of variation bars are included. ■ correspond to group of moss in soft water treatment (hardness 26 mg/L as CaCO$_3$) and ▲ correspond to medium water treatment group (hardness 105 mg/L as CaCO$_3$). Dashed Line with ● correspond to moss treatment group from site#1, experiment #1 (hardness 19.3 mg/L as CaCO$_3$).

Chlorophyll levels (figure 6)

Both soft and medium water treatments showed a comparable, yet limited, response to increasing sulphate exposure up until the highest exposure sulphate treatment of 1500 mg/L. Although a statistically significant reduction in chlorophyll levels was seen in the soft water treatments at 600 mg/L (LOE, P<0.05), this response may have been an outlier as no significant response was seen at the 800 mg/L treatment which exhibit higher chlorophyll levels than the 200 mg/L exposure treatment. However, chlorophyll levels plummeted in the 1500 mg/L treatment where the shoots appeared dead and browned.

No significant reduction in chlorophyll levels was seen in the medium water group and no mortality was observed. A comparison in chlorophyll between the soft and medium water treatments at 1500 mg/L sulphate can be seen in figure 7.
Figure 6. Response of increasing sulphate concentration on chlorophyll a & b content after 21 days exposure. Coefficient of variation bars are included. ■ correspond to group of moss in soft water treatment (hardness 26 mg/L as CaCO₃) and ▲ correspond to medium water treatment group (hardness 105 mg/L as CaCO₃). Dashed Line with ● correspond to moss treatment group from site#1, experiment #1 (hardness 19.3 mg/L as CaCO₃).

Discussion of experiment #2

The dry weight and shoot length data from this experiment provide little insight into the effects of sulphate exposure as a consistent trend was hard to discern from the data. However, the chlorophyll analysis provided evidence that sulphate seemed to be less toxic in water with increasing water hardness (figure 7). A response in chlorophyll content can be seen in *F. antipyretica* due to stress caused by increasing sulphate exposure and possibly provides a better measure of plant health than growth (figure 6).
Figure 7. Comparison of chlorophyll absorbance scan of soft water and medium water treatments in experiment #2 at 1500 mg/L sulphate. The lower line corresponds to the soft water treatment.

The dry weight measurements in hard water suggest that the shoots in that treatment exhibited the most growth (Figure 5); however, this is contradicted from the total length data where it displayed the least growth overall up to 600 mg/L sulphate exposure (figure 4). This may be due to higher ion content of the medium water, which had a hardness of 105 mg/L as CaCO₃ while the soft water had a hardness of 26 mg/L. In waters of increasing ion concentration, many non-halophytes increase ion uptake to compensate for the adverse effects caused by the osmoregulatory imbalance (Hart et al. 1991). Although the differences in weights in the controls were relatively large (1.83 and 2.59 mg in soft and medium water respectfully) the difference of less than 0.8 mg may simply have been due to the moss taking up ions from solution to help it maintain osmotic balance in the harder water. Unfortunately, no analysis of the plant tissues was done so this is simply speculation. Also, the shoots were not rinsed with de-ionized water prior to drying and weighing. This may have also added weight to the dried shoots.
Comparisons between tests

As previously mentioned, the moss collected at site #2 and used in experiment #1 cannot be reasonably compared to the other moss treatment blocks due to the differences in water chemistry and the potential for specific ecotype sulphate tolerance.

The lower total length and chlorophyll levels in the control groups in experiment #2 compared to the controls of experiment #1, site #1 suggest that water characteristics and/or plant health may have influenced the results in experiment #2. However, the general trend in chlorophyll (figure 6) levels suggest that *F. antipyretica* is less sensitive to sulphate in water of increasing hardness. Furthermore, the reduced toxicity observed in experiment #1 in comparison to experiment #1, site #1 may have been influenced by factors other than simply water hardness.

Water hardness is a measure of the quantities of polyvalent cations in water. Hardness generally represents the concentration of calcium (Ca\(^{2+}\)) and magnesium (Mg\(^{2+}\)) ions, because these are the most common polyvalent cations. Other ions, such as iron (Fe\(^{2+}\)) and manganese (Mn\(^{2+}\)), may also contribute to the hardness of water, but are generally present in much lower concentrations. Water hardness is standardized by converting the calcium and magnesium content of the solution into calcium carbonate equivalences. As both calcium and magnesium are summed into a total hardness value, waters containing significantly different ratios of calcium and magnesium can still have comparable hardness values. The calcium and magnesium ratios between the two tests were considerably different. The waters used in experiment #1 and #2 had calcium/magnesium ratios (Ca/Mg) of 2.4 and 6.7 (based on a molar basis) respectfully. Various studies investigating the reduction of toxicity observed in fish to metals in waters of increasing water hardness have found that it is the Ca, rather than the Mg, component of water hardness that plays a much greater role in the reduction of toxicity (Gundersoen et al. 1994, Alsop & Wood 1999, Welsh et al. 2000). Furthermore, Davies (2002) reported that the toxicity caused by sodium sulphate exposure to *D. magna* was significantly reduced in waters with increased Ca/Mg ratios. However, no information could be found indicating that calcium plays a greater role than magnesium in reducing
the toxicity of metals or sodium sulphate to bryophytes specifically. Therefore, further studies are required to assess this hypothesis.

**Discussion**

The toxic threshold of sulphate to *F. antipyretica* of 100 mg/L sulphate as reported by Frahm (1975) is in stark contradiction to the results presented here. The exaggerated toxicity reported by Frahm is likely from the associated cation rather than sulphate toxicity. Frahm added sulphate as potassium sulphate (K$_2$SO$_4$) compared to sodium sulphate (Na$_2$SO$_4$) which was used in this experiment. There is evidence in the literature that K$_2$SO$_4$ is significantly more toxic than Na$_2$SO$_4$ to invertebrates and fish (Table 1). Unfortunately, there is limited evidence of toxic responses of K$_2$SO$_4$ to aquatic macrophytes.

**Table 1.** Comparison of the toxicity of potassium sulphate and sodium sulphate to *Ceriodaphnia, Daphnia magna* and Fathead minnow (*Pimephales pronelas*) (Mount et al., 1997)

<table>
<thead>
<tr>
<th></th>
<th>LC50 of K$_2$SO$_4$ (mg/L)</th>
<th>LC50 of Na$_2$SO$_4$ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48-hour <em>Ceriodaphnia</em></td>
<td>&lt;680</td>
<td>3080</td>
</tr>
<tr>
<td>48-hour <em>Dapnia magna</em></td>
<td>720</td>
<td>4580</td>
</tr>
<tr>
<td>96-hour Fathead minnow</td>
<td>680</td>
<td>7960</td>
</tr>
</tbody>
</table>

However, a study by Egan & Ungar (1998), examined the toxicity of K$_2$SO$_4$, KCl, NaCl and Na$_2$SO$_4$ to the terrestrial weed, *Atriplex prostrata* and found the toxicities of the following to be K$_2$SO$_4$ > KCl > NaCl = Na$_2$SO$_4$ when exposed by comparable osmotic potentials. At the end of the 5 week test, all plants exposed to K$_2$SO$_4$ and 40 percent of the plants exposed to KCl were dead. However, no detrimental impacts on survival was observed in either the NaCl or Na$_2$SO$_4$ treatments. This is consistent with the trend observed in the toxicity to invertebrates and fish where individual ion toxicity for *Ceriodaphnia, D. magna* and Fathead minnows is K$^+$ > Cl$^-$ > SO$_4^{2-}$ (Mount *et al.*, 1997).

An explanation of the apparent ameliorate effects of increasing water hardness to sulphate toxicity may also be due to the toxicity of the associated cation. Sodium must
also be considered a potential source of toxicity as sulphate was added in the form of sodium sulphate. Sodium sulphate contains 32.3 percent sodium by mass; therefore, the highest concentrations of sulphate exposure (1500 mg/L) also resulted in an exposure to 718 mg/L sodium. Furthermore, the osmotic pressure exerted on a cell is a function of the concentration rather than the mass of the solute particles (Potts & Parry 1964). The molarity of the 1500 mg/L sulphate exposure was 15.6 mM of sulphate and 31.2 mM of sodium. Therefore, the higher molarity of sodium compared to sulphate indicated that the osmotic stress is also being placed on the plant from the associated sodium ion.

Studies done on salinity exposure (salts added as NaCl) to aquatic macrophytes show that reduced plant growth or death can occur due to both specific ion toxicity and/or a reduction in the ability of plants to extract water or necessary nutrients from the surrounding media due to osmotic gradients (Hart et al. 1991). The mechanism of sodium toxicity to plants occurs from sodium ions displacing calcium from the cell surface (Kinraide 1999). This allows Na\(^+\) ions to cross the cell membrane to toxic levels in the cell cytoplasm. However, this can be reduced in solutions with higher Ca\(^{2+}\) concentrations (Reid & Smith 2000; Tyerman & Skerrett 1999) through competition between Ca\(^{2+}\) and Na\(^+\) for binding sites. However, although Ca\(^{2+}\) is able to ameliorate the toxicity caused by high intracellular Na\(^+\), it is not able to overcome the osmotic deficits associated with high salinity (Kinraide 1999). A study done by Hocking (1981) on *Typha domingensis*, an emergent macrophyte, indicated that sodium tended to be more toxic than chloride and that “sodium appears to retard plant growth through the effect on osmotic adjustment, ion uptake, enzyme activity and hormonal balance” (Hart et al. 1991). Evidence that sulphate does not cause specific ion toxicity is supported by Stanley (1974) who studied the effects of metals and salts to the Eurasian Watermilfoil (*Myriophyllum spicatum* L.). The 50 percent inhibition levels of sulphate and chloride reported indicated that Na\(_2\)SO\(_4\) has lower toxicity than NaCl with the exception of affecting shoot length (table 2).
Table 2. The effects of Na$_2$SO$_4$ and NaCl on the growth of Eurasian Watermilfoil (Myriophyllum spicatum)

<table>
<thead>
<tr>
<th>Compound</th>
<th>$I_{50}$RW</th>
<th>$I_{50}$SW</th>
<th>$I_{50}$RL</th>
<th>$I_{50}$SL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$SO$_4$</td>
<td>10 228</td>
<td>9 376</td>
<td>10 370</td>
<td>4 120</td>
</tr>
<tr>
<td>NaCl</td>
<td>8 183</td>
<td>5 962</td>
<td>8 008</td>
<td>7 423</td>
</tr>
</tbody>
</table>

$I_{50}$RW = 50% inhibition of root dry weight; $I_{50}$SW = 50% inhibition of shoot dry weight; $I_{50}$RL = 50% inhibition of root length; $I_{50}$SL = 50% inhibition of shoot length (Stanley, 1974).

Analysis of the data regarding the inconsistency in the increased inhibition of shoot length by Na$_2$SO$_4$ over NaCl indicated that the mechanism of toxicity of both involved an imbalance of osmotic pressure in the plant cells. The reduced relative toxicity of NaCl compared to Na$_2$SO$_4$ was attributed to the rapid uptake kinetics of chloride ions compared to sulphate ions. It was hypothesized that the rapid uptake of NaCl allowed the plant to maintain adequate turgor pressure to allow for shoot growth while sulphate caused an negative osmotic gradient resulting in a loss of turgor pressure indicating that although both salts are acting osmotically, NaCl acts internally while Na$_2$SO$_4$ was acting externally. This suggests that sulphate toxicity may be reduced through gradual acclimation to exposure by allowing the plants to take up enough sulphate ions to equalize osmotic gradients.

Another potential mechanism of reduction of sulphate toxicity with increasing water hardness in the formation of ion pairs between calcium and sulphate. However, calcium concentration was 0.9 mM/L and sulphate levels were 15.6 mM/L in the medium water at 1500 mg/L sulphate exposure; therefore, even if all the calcium ion paired to the sulphate ions a reduction in toxicity would be unlikely. Furthermore, modeling using PHREEQC (Parkhurst & Appelo, 2001) indicate that less than 0.1 percent of the total sulphate would complex with calcium to form CaSO$_4$ ion pairs.

The report by Beak International (1998) indicating no observable effect in chlorophyll levels to F. noemexicana up to 500 mg/L in water of 160 mg/L as CaCO$_3$ hardness is consistent with the results presented here and with the general observation that freshwater
macrophytes are usually tolerant to salinities less than 1000 – 2000 mg/L (Hart et al. 1991). However, as no observable effect was seen, an extrapolation of the effects of longer exposures is impossible to hypothesize. The increased energy requirements required to maintain ion balance in a medium of amplified ion content and how this would affect the long term sustainability of moss populations is problematical when no effect is observed.

The measurement of chlorophyll levels appears to be an acceptable method in assessing sublethal bryophyte health. Exposure to osmotic stress from Na₂SO₄ showed a significant reduction in chlorophyll levels in *F. antipyretica* in very soft water but tended to be alleviated in waters of increasing water hardness (figure 6).

**Conclusion**

An assessment of the toxicity of sulphate to *F. antipyretica* is confounded by the presence of sodium ions even though sodium is considered one of the least toxic cations associated with sulphate. The mode of toxicity from sulphate is due to the creation of an unsustainable osmotic imbalance between the plant and its surrounding environment. The presence of confounding ions influence that balance and interact with each other through the creation and nullifying of osmotic gradients and cellular ion excess or deficits. Therefore, attributing toxicity of sodium sulphate directly to sulphate likely paints an overly simplistic and inaccurate picture of sulphate ion toxicity to *F. antipyretica* and other plant species.

**Acknowledgements**

Thanks are given to the general staff, and in particular Janet Pickard, at BC Research Inc. for guidance, use of lab space, equipment and materials. BC Ministry of Environment (Smithers), through the Pacific Environmental Science Centre generously donated water analysis on the test solutions. Endako Mines, Highland Valley Copper, Inmet Mining Corp., Placer Dome Inc. and Taseko Mines provided financial assistance for this project. I would like to thank my thesis advisor, Dr. Ken Hall and Dr. Wilf Schofield for their support.
References


Davies, T. 2002. Relative importance of Calcium and Magnesium ratios in assessing toxicity of sulphate to the Cladoceran (*Daphnia magna*). Unpublished data


Appendix E

The Effects of Molybdenum on Rainbow Trout (*Oncorhynchus mykiss*)

A Reproduction of Birge et al. (1980)
Introduction

The purpose of this study was to replicate an earlier study done on the toxic effects of molybdenum on rainbow trout (*Oncorhyncuss mykiss*) conducted by Birge et al. (1980). The results from that study calculated a 29-day LC50 to newly fertilized eggs to be 0.79 mg/L. A number of studies have reported substantially lower toxicity of molybdenum than that reported by Birge et al. (1980). Pickard et al., (1998) and McDevitt et al. (1999) both reported that no effects on growth and survival were observed at concentrations averaging 30 mg/L and up to 49 mg/L at maximum. McConnell (1977) reported 96 hour LC50s in a static test of 1320 mg/L and 800 mg/L to rainbow trout of 55 mm and 20 mm respectfully. Furthermore, as part of the same study, a one year exposure test starting with eyed eggs indicated no significant reduction in hematocrit, growth or mortality up to molybdenum exposures of 17 mg/L.

These values are comparable to the reported toxicity of molybdenum to other fish species. Easterday and Miller (1963) reported a 96 hour LC50 to the bluegill (*Lepomis macrochirus*) of 1320 mg/L and Peterson (1974) reported that the LC50s for bluegill, rainbow trout, fathead minnow, and channel catfish ranged from 2500 to greater than 10000 mg/L molybdenum (added as sodium molybdate) (in McConnell (1977)).

However, test species, temperature, test apparatus, and dilution water may affect molybdenum toxicity causing large discrepancies between LC50s. Therefore, efforts were made to duplicate the Birge et al., 1980 study to determine whether molybdenum is highly toxic under those specific test conditions or whether the results from Birge et al. 1980 are artefacts as the other conflicting studies suggest. Furthermore, a study using the standard methodology outlined in “Biological Test Method: Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout), July 1998” was done to determine molybdenum toxicity under laboratory conditions using accepted test protocols.
Methodology

Test #1

A static renewal Embryo/Alevin (EA) test was conducted on rainbow trout (*Oncorhynchus mykiss*) beginning Feb 9, 2000 and ending March 11, 2000. An EA test was chosen both to replicate the Birge et al. (1980) study as well as to observe the effects of molybdenum on multiple phases of development. Prior to egg fertilization, sperm was checked for viability under a microscope.

Approximately 100 newly fertilized eggs were placed in each aerated 600 ml Pyrex beaker test vessel. Four replicates were conducted for target exposures 0 mg/L, 50 mg/L and 100 mg/L, while three replicates were used for the other exposures of 0.5 mg/L, 1.0 mg/L, 10 mg/L, 200 mg/L, and 400 mg/L. Test water was reconstituted from de-ionized water. Salts were added to duplicate the hardness and ion content of the test water found in the Birge et al., 1980 experiment (Table 1). Water hardness ranged from 93 to 113 mg/L CaCO₃, and pH varied from 7.4 to 8.0. Aeration was used to maintain dissolved oxygen in a range of 6.5 to 10.6 mg/L. The test was conducted in an environmentally controlled room that maintained the temperature between 11.9°C and 13.4°C. Batches of 180 litres of reconstituted water were made every six days. The water was then transferred to 21-litre plastic pails, and molybdenum, as sodium molybdate (Na₂MoO₄), was added to bring the test water to the required concentrations. After the molybdenum addition, water was allowed to sit a minimum of 12 hours before use to ensure complete mixing of the molybdenum in the test water.
Table 1. Ion content of test waters used in the Birge et al. (1980) and this study.

<table>
<thead>
<tr>
<th></th>
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</tr>
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<tbody>
<tr>
<td>Target Hardness as CaCO₃</td>
<td>100 mg/L</td>
<td>100 mg/L</td>
</tr>
<tr>
<td>Hardness, as mg/L CaCO₃</td>
<td>101.6 ± 4.4</td>
<td>104.7 ± 5.7</td>
</tr>
<tr>
<td>pH</td>
<td>7.7 ± 0.01</td>
<td>7.8 ± 0.2</td>
</tr>
<tr>
<td>Conductivity, µmhos/cm</td>
<td>176.0 ± 1.0</td>
<td>377.5 ± 5.1</td>
</tr>
<tr>
<td>Ca</td>
<td>27.1</td>
<td>27.1</td>
</tr>
<tr>
<td>Mg</td>
<td>7.4</td>
<td>7.4</td>
</tr>
<tr>
<td>Na</td>
<td>27.4</td>
<td>27.4</td>
</tr>
<tr>
<td>K</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Cl</td>
<td>52.3</td>
<td>47.9</td>
</tr>
<tr>
<td>HCO₃</td>
<td>72.6</td>
<td>72.6</td>
</tr>
<tr>
<td>SO₄</td>
<td>29.2</td>
<td>29.2</td>
</tr>
</tbody>
</table>

Eggs were exposed to the various concentrations of molybdenum within one hour of embryo fertilization and continued for 7 days post-hatch (50% hatch in control vessels). This resulted in a test exposure period of 32 days. A 14-light/10-dark photoperiod was imposed with water renewal times occurring at approximate 12-hour intervals for the duration of the 32-day test.

Test populations were examined twice daily to tabulate lethality and teratogenesis. At takedown, alevins exhibiting obvious developmental defects were counted as non-viable.

Two replicates were lost during the experiment in the nominal concentrations of 10.0 and 400 mg/L. One 10.0 mg/L vessel replicate was accidentally dropped, and the majority of the fish were lost. Although the bulk of the survivors did survive through the remainder of the test, it was difficult to calculate reasonable proportions for mortalities prior to the accident. Therefore, this replicate was discarded from statistical analysis.

Near the end of the test, 68 embryos and alevins died within 3 ½ days at the 400 mg/L exposure level. Afflicted embryos turned a milky white and then ruptured, depositing a sticky white substance on the bottom of the test vessel. Embryos and alevins got caught in the substance and seemed to contract the infection and either die as a result, undergone mechanical damage from struggling in the substance, or die from a possible loss of O₂ absorption area. As mortality appeared to be caused by other than molybdenum
exposure, this replicate was discarded from the data set. This problem was also present in some of the other replicates. However, removing the majority of the white substance during water changes seemed to control the problem and keep mortality to a minimum.

**Test #2**

Another static renewal EA test was conducted on rainbow trout (*Oncorhynchus mykiss*) beginning Oct 30\textsuperscript{th}, 2002 and ending Nov 30\textsuperscript{th}, 2002. The general procedures and specific water chemistry of the test can be found in "Biological Test Method: Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout), July 1998."

The tests were conducted in a constant temperature controlled room which was maintained at 14±1 °C. Dissolved oxygen and pH concentrations varied in the test vessels between 8.3 to 10.1 mg/L and 7.7 to 8.0 respectfully for the duration of the test. The test was done in darkness except during water changes; however, light levels were <500 Lux. Soft, reconstituted dilution water was used, as recommended in the guidelines and it had a hardness of approximately 48 mg/L (as CaCO\textsubscript{3}). The test had seven molybdenum (added as Na\textsubscript{2}MoO\textsubscript{4}) exposure levels (0, 100, 250, 500, 750, 1000, 1500 mg/L), each with four replicates each. Within each replicate, 60 newly fertilized eggs were placed after water hardening in exposure water. The test apparatus were the redesigned 4 litre static renewal incubation unit found in the Environment Canada (July 1998) guidelines.

Water changes and mortality tabulations were three times per week. Appropriate amounts of molybdenum was dissolved in exposure water at least 2 hours prior to each water change. During changes, deaths were recorded and carcasses were removed.

**Statistical Analysis**

Statistic analysis was done using Toxcalc 5.0. Data sets for both tests had a normal distribution (Shapiro Wilk’s and Bartlett’s Test). Endpoints (LC20 and LC50) were calculated using the Probit Method.
Quality Assurance / Quality Control

The relative sensitivity of the group of embryos used was assessed by a reference toxicant test. The reference toxicant test (ZnSO₄ ·7H₂O) was done according to BC Research Laboratory procedures so as to compare the results of these tests to previous reference toxicant tests. Reference toxicant tests for both investigations were within the acceptable range of two standard deviations compared to previous tests done by BC Research Inc.

The tests were considered valid as all conditions for reference toxicity validity were satisfied. Molybdenum exposure levels were confirmed by independent lab analysis, and exposure concentrations were found to be within 10 percent of nominal concentrations. Therefore, nominal concentrations have been used for reporting and statistical analysis. Water quality analysis was provided by BC Ministry of Environment (Smithers), through the Pacific Environmental Science Centre.

Results

Test #1

There was no significant difference in survivorship between the control populations up to the 200 mg/L molybdenum concentration (Table 3). However, survivorship in the 400 mg/L test vessels was significantly lower than that found in the control groups.

A 32-day LC50 value was estimated to be 645.27 mg/L molybdenum (Table 3); however, as this value is beyond the data set, it is not statistically valid.

Obvious sub lethal effects were relatively rare in the fish present. A total of two spinal deformities were observed in the 25 mg/L replicate set. These were counted as non-viable during analysis. However, in higher concentrations, some fish did not completely exit their egg cases at hatching. This resulted in a loss of 7 fish from the 400 mg/L group, one from the 200 mg/L group, and 3 from the 50 mg/L group. These were also counted as non-viable.
Test #2

As in Test #1, an LC50 could not be calculated as 50 percent mortality did not occur in any of the replicates up to 1500 mg/L molybdenum. The LOEC was 1000 mg/L which showed a slight decline in survival compared to controls. Mortality was even greater in the 1500 mg/L molybdenum exposure; however, mean survival at this exposure level was still fairly good at 82.5 percent compared to controls which had a mean survival of 94.6 percent. Therefore, the highest mortality statistic that could be gained, that was bound by the molybdenum exposure levels, was an LC20 of 1424.8 mg/L molybdenum. An LC50 was projected out of the data set at 11977.1 mg/L molybdenum. However, as this is outside the data set, as in Test #1, this value is also not statistically valid.

Table 3. Summary of toxicological results of this study describing the toxicity of molybdenum to rainbow trout (Oncorhynchus mykiss)

<table>
<thead>
<tr>
<th>Calculated Effect Levels</th>
<th>mg/L molybdenum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test #1</strong></td>
<td></td>
</tr>
<tr>
<td>NOEC</td>
<td>200</td>
</tr>
<tr>
<td>LOEC</td>
<td>400</td>
</tr>
<tr>
<td>32-day LC15</td>
<td>365.4</td>
</tr>
<tr>
<td>Projected 32-day LC50</td>
<td>645.27</td>
</tr>
<tr>
<td><strong>Test #2</strong></td>
<td></td>
</tr>
<tr>
<td>NOEC</td>
<td>750</td>
</tr>
<tr>
<td>LOEC</td>
<td>1000</td>
</tr>
<tr>
<td>32-day LC20</td>
<td>1424.8</td>
</tr>
<tr>
<td>Projected 32-day LC50</td>
<td>11977.1</td>
</tr>
</tbody>
</table>

Discussion

Test #1 indicates that the test conditions used in Birge et al. (1980) do not cause extreme toxicity of sodium molybdate to rainbow trout. A notable difference between the original Birge et al. study and test #1 is the differences in conductivity between the two (Table 1). Calculations using conductivity factors (APHA, 1980) of ions the used in the reconstituted waters in both this investigation and Birge et al. (1980) indicate that the conductivity of both solutions should be approximately 300 μS/cm. The elevated
conductivity found in Test #1 may have been due to the conductivity of the hydrogen and hydroxyl ions present in the reconstituted water which was not used in the 300 μS/cm calculation. It is unknown how the conductivity of 176.0 μS/cm reported in the Birge et al. (1980) study is possible with the ion concentration that were reported.

The better survival observed in Test #2 in comparison to Test #1 may have simply been due to better methodology. The high EA density in Test #1 may have resulted in increased mortality due to lower dissolved oxygen concentrations or higher concentrations of metabolic wastes around the organisms which may have had an adverse effect on survival. As an exposure above 400 mg/L was not done in Test #1, a confirmation that molybdenum was toxic at molybdenum levels at, and above 400 mg/L was not available.

The results obtained in Test #2 indicate that molybdenum is not toxic to developing rainbow trout at concentrations below 750 mg/L. This is consistent with other results that have indicated that salmonids are not affected by molybdenum concentrations up to 1000 mg/L (Hamilton & Buhl 1990; McConnell 1977).

Both tests indicate that rainbow trout are not as highly sensitive to molybdenum as was reported by Birge et al. (1980). The cause of the high toxicity reported by Birge et al. (1980) is unknown; however, the two tests reported here suggest that the results reported in Birge et al. (1980) are artifacts and are not an accurate description of the toxicity of molybdenum to rainbow trout.

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References:


