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Sulphate toxicity to the aquatic moss, *Fontinalis antipyretica*

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Abstract

The aquatic moss, *Fontinalis antipyretica* was exposed to elevated sulphate concentrations for 21-days. Gametophores were sectioned to 2 cm lengths and exposed to sulphate concentrations up to 1500 mg/l, in waters of different water hardness. Significant reductions in shoot length, dry weight, and chlorophyll *a* and *b* concentrations (per gram dry weight) were observed in soft water (19 mg/l as $CaCO_3$); however, effects were significantly reduced in waters of increasing hardness (up to 105 mg/l as $CaCO_3$). The substantial reduction of sulphate toxicity in waters of increasing hardness suggests water chemistry plays a significant role in affecting sulphate toxicity and should be considered when setting sulphate discharge limits.

Keywords: Bryophytes; Sulphur pollution effects; Chlorophyll concentration; Water hardness

1. Introduction

Sulphate is ubiquitous in freshwater environments and is a common sulphur source for plants and bacteria. Sulphate is usually found in low concentrations in most freshwater systems, although high concentrations can be found in areas where sulphate-containing ores or anthropogenic activities exist. In the province of British Columbia (BC), Canada, most lakes and rivers have mean sulphate concentrations between 2 and 30 mg/l; however, some saline lakes in the interior of BC have high natural sulphate levels in the thousands of mg/l (Singleton, 2000).

The amount of sulphate discharged from anthropogenic sources into aquatic systems can be significant. Indeed, anthropogenic sources of sulphate can account for 20 to over 90 percent of the sulphur found in some surface waters (Nriagu, 1978). Major anthropogenic sources of sulphate include industrial waste waters, mine wastes, smelting, the burning of fossil fuels, agricultural runoff, and domestic sewage. High sulphate concentrations are of particular concern to the mining industry, as sulphate is often a major contaminant of mine water and can be the dominant contributor to salinity of mine water discharges (Bowell, 2000). In response to concerns that elevated sulphate concentrations from anthropogenic activities may cause detrimental effects on aquatic ecosystems, the provincial government of BC developed ambient-water quality guidelines for sulphate for the protection of freshwater aquatic life.

A focal study used in the development of the BC water quality guideline for sulphate is a study by Frahm (1975) that investigated the effect of elevated sulphate concentrations on the aquatic moss, *Fontinalis antipyretica*. This aquatic moss has a wide distribution in BC (Warrington, 1994) and is well represented in the literature as a biomonitoring organism (Bleuel et al., 2005). Mosses have been used in various biomonitoring investigations of several types of pollution in surface waters (Mersch and Reichard, 1998; Fernández et al., 2006). They have frequently been used as bioindicators, given their wide geographical distribution and lack of well-developed cuticula and vascular tissue that make them sensitive to environmental pollutants (Raeymaekers and Glime, 1986; Aceto et al., 2002). Their appeal as monitoring species is based on laboratory and

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field investigations that have shown exchange kinetics in mosses to be very rapid and predominantly based on biosorptive processes (Martins et al., 2004).

Frahm (1975) reported a one-week exposure to 100 mg/l sulphate (added as K_2SO_4) to be toxic to *F. antipyretica*, with observed toxicity attributed to the sulphate ion. Consequently, the study was used to justify a sulphate discharge limit of 100 mg/l sulphate, to not be exceeded at any time, and an "Alert level" of 50 mg/l, which when exceeded, triggers a requirement to monitor aquatic moss populations on an occasional basis (Singleton, 2000). In contrast, Beak International Incorporated and Michigan Technological University (1998) conducted a toxicity investigation using a similar species (*F. neomexicana*), reporting no observable effect on chlorophyll levels at exposures up to the maximum concentration of 500 mg/l sulphate (added as Na_2SO_4, at a water hardness of 160 mg/l $CaCO_3$ over an exposure of 14 days). However, Singleton (2000) did not consider this a valid endpoint, given aquatic mosses grow very slowly and chlorophyll levels would likely not be affected in a relatively short experiment. However, a significant reduction in chlorophyll levels has been observed in various moss species including *F. antipyretica* within one week in response to organic pollution (Martinez-Abaigar et al., 1993) and within 80 h from UV-B exposure (Núñez-Olivera et al., 2005). Given that a wide range of metabolic processes are expressed via the growth response (Sidhu and Brown, 1996), using chlorophyll levels as a proxy of plant health should provide an indication of the possible sub-lethal effects of sulphate exposure.

Results of other investigations of sulphate toxicity suggest that the large discrepancy between results reported by Frahm (1975) and Beak International Incorporated and Michigan Technological University (1998) may be a misattribution of the source of toxicity observed by Frahm (1975) to the sulphate ion rather than the potassium ion. Mount et al. (1997) reported the relative ion toxicity to the daphnids, *Ceriodaphnia dubia* and *Daphnia magna*, and fathead minnows (*Pimephales promelas*) to be potassium (K^+) > bicarbonate (HCO_3^-) ≈ magnesium (Mg^{2+}) > chloride (Cl^-) > sulphate (SO_4^{2+}). In addition, Pillard et al. (2000) reported relative ion toxicity to the marine mysid (*Americamysis bahia*) to potassium (K^+) > bicarbonate (HCO_3^-) > calcium (Ca^{2+}) > magnesium (Mg^{2+}) > bromide (Br^-) > sulphate (SO_4^{2-}).

Increased water hardness has been shown to reduce the toxicity of some substances to aquatic organisms. This relationship is well established for certain metals (Jayaraj et al., 1992; Gundersen et al., 1994; Galvez and Wood, 1997; Perschbacher and Wurts, 1999; Hansen et al., 2002). For example, cadmium and zinc have been shown to be less toxic to rainbow (*Oncorhynchus mykiss*) and bull trout (*Salvelinus confluentus*) in waters of increasing hardness (Hansen et al., 2002); however, this trend has not been observed in *F. antipyretica* (Martins et al., 2004). There is little explicit evidence that sulphate toxicity is reduced in waters of increased hardness, and none exists for *F. antipyretica*. Reduced toxicity from sulphate, however, has been observed for the amphipod *Hyalella azteca* and the freshwater cladoceran *Daphnia magna* in waters of increased water hardness (Davies, unpublished data).

The objective of this study was to investigate the effects of increased sulphate concentrations on the growth and chlorophyll levels of *F. antipyretica* in waters of different hardness levels over a 21-day exposure period. The results of this study should help clarify conflicting results as they pertain to aquatic mosses considered in the current BC water quality guideline for sulphate for the protection of aquatic life. Also, results can be used for the development of a new guideline or for site-specific water quality objectives.

2. Materials and methods

2.1. Moss collection site and moss characteristics

F. antipyretica is easily identifiable from other mosses found in BC because its sporophyte (reproductive structure) lacks a distinct stalk and emerges among its leaves. It can be very large and grows in dense clumps, with stems growing between 50 cm (Siebert et al., 1996) and 90 cm (Biehle et al., 1998) in length and a diameter of 0.2–0.5 mm. Leaves on stems are arranged in a "3-winged" form and lack a costa (rib). 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 near the University of British Columbia (UBC) campus at Vancouver, BC. Identification was confirmed by Wilf Schofield, Professor Emeritus of the Botany department at UBC. The moss was located in a shallow, slow-moving stream approximately 20–30 cm deep. The water exhibited a brownish colour and substantial suspended sediment, and a hardness of 19 mg/l as $CaCO_3$. There was substantial overhanging canopy with little or no direct sunlight. Moss was collected from the field site in 20 l plastic buckets and kept submerged in ambient stream water during transport from the field site to the test lab.

2.2. Test design

Two separate tests were conducted: the first to assess toxicity of sulphate (as Na_2SO_4); and the second to assess the effects of water hardness on sulphate toxicity. The first test used stream water obtained from the *F. antipyretica* collection site as the test medium (hardness 19 mg/l as $CaCO_3$). Stream water was passed through a glass–fiber filter to remove organisms and suspended solids that could potentially affect moss growth and survival. The second test used undiluted well-water and well-water diluted 3:1 with de-ionized water (105 mg/l and 26 mg/l hardness) to simulate medium and soft-water hardness respectively. Well water was not filtered and no additives such as plant nutrients were supplemented.

A concentrated sulphate stock solution was made using anhydrous sodium sulphate (Na_2SO_4) added to test waters in various amounts to achieve the desired sulphate concentrations of 200, 400, 600, 800, 1000 and 1500 mg/l. Sulphate concentrations were confirmed through ion chromatography for anions (ICA) by the Pacific Environmental Sciences Centre located in North Vancouver, BC. Exposure concentrations were determined to be within 10 percent of nominal concentrations; therefore, nominal concentrations were used for statistical analysis. All exposure concentrations were formulated at the beginning of each test and stored at 8 °C in opaque 1 l plastic bottles. Moss was collected the day prior to the start of the first test and stored overnight at 15 °C in collection buckets.

The second experiment was designed to investigate whether there was a hardness-based response to sulphate toxicity in *F. antipyretica*. Fresh moss samples could not be collected for this experiment due to extremely dry conditions at the moss collection site. Therefore, moss collected for the first test also was used for the second experiment.

Both studies used a static-renewal aqueous tests design, with water changes occurring every five days. Tests were run for a total of 21-days at a controlled temperature of 15 ± 1 °C. 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. 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% and evaporation was not significant during the course of the experiment. Four replicates of approximately 10 shoots were placed in each a flask at each test concentration and allowed to float freely. Flasks were randomly assigned placement after each weekly water change. Light was provided by four full-spectrum halogen light bulbs supplying 1500–2000 Lux measured at the level of the exposure flasks. Lights were left on continuously and flasks were gently gyrated on a shaker table to ensure sufficient nutrient and gas exchange for the duration of the experiments.

2.3. End-point measurements

At the end of the exposure period, the lengths of all shoots were measured, air-dried for 2 h and stored in 30 ml glass bottles in a −20 °C freezer for approximately six weeks. Shoots that were not used for chlorophyll analysis were oven-dried at 80 °C for 24 h and individually weighed.

Chlorophyll was extracted with dimethyl sulphoxide (DMSO), using methods adapted from Barnes et al. (1992). Two shoots were randomly selected from each replicate set for chlorophyll analysis and cut to standard 1 cm lengths. These were placed in 10 ml glass tubes with 5 ml DMSO. The tubes were sealed and placed in a 60 °C oven for 12 h. Shoot tips then were removed and placed in a drying oven for 24 h at 80 °C and individually weighed. Absorbance was measured at wavelengths of 648.2 and 664.9 nm on a spectrophotometer in a 1 cm cell. Equations used to calculate chlorophyll *a* and *b* concentrations are found in Barnes et al. (1992).

All statistical analysis was done using the statistical programming language "*R*" version 2.1.1 (Ihaka and Gentleman, 1996). Data were analyzed using linear mixed-effects models with beakers nested within treatments. All tests were two-tailed with comparisons made between treatments compared to lower concentration treatment groups ($p < 0.05$). Data normality and heterogeneity of variance were confirmed by residual analysis.

3. Results

3.1. Shoot length

All moss shoots displayed a reduction in final shoot length with increasing sulphate concentration (Fig. 1). Shoots in ambient stream control water had the greatest increase in length during the exposure period and showed a relatively linear reduction in mean final length with increasing sulphate concentration. Shoots in soft and medium-hardness waters grew less than those in ambient stream water up to 600 mg/l sulphate. Increasing sulphate concentrations had the greatest impact on shoot length in ambient stream water; soft-water treatments showed a lesser response; and the medium-hardness treatment did not show a significant reduction in shoot length until the highest sulphate exposure of 1500 mg/l sulphate.

3.2. Dry weight

Moss shoots in the ambient stream treatment displayed a relatively linear reduction in final dry weight in response to increasing sulphate concentrations, with the first significant difference detected at 600 mg/l sulphate (Fig. 2). The soft-water treatment did not show a significant reduction in dry weight relative to the control until 1500 mg/l sulphate. Shoots in the medium-hardness treatment had the highest dry weight of all groups and showed a significant reduction in growth relative to the control group at 400 mg/l sulphate. However, dry weights did not decline significantly up to 1500 mg/l sulphate relative to the 600 mg/l sulphate ($p < 0.05$) exposure group. Mean dry weight was highest in the medium-hardness treatment, with a final mean weight of 2.1 mg, relative to the ambient-water and soft-water treatments of 1.3 and 1.6 mg respectively.

3.3. Chlorophyll levels

Shoots in all hardness treatments showed a reduction in chlorophyll *a* and *b* content (on a dry weight basis) with increasing sulphate exposure (Fig. 3). Shoots grown in stream water had significantly higher chlorophyll *a* and *b*

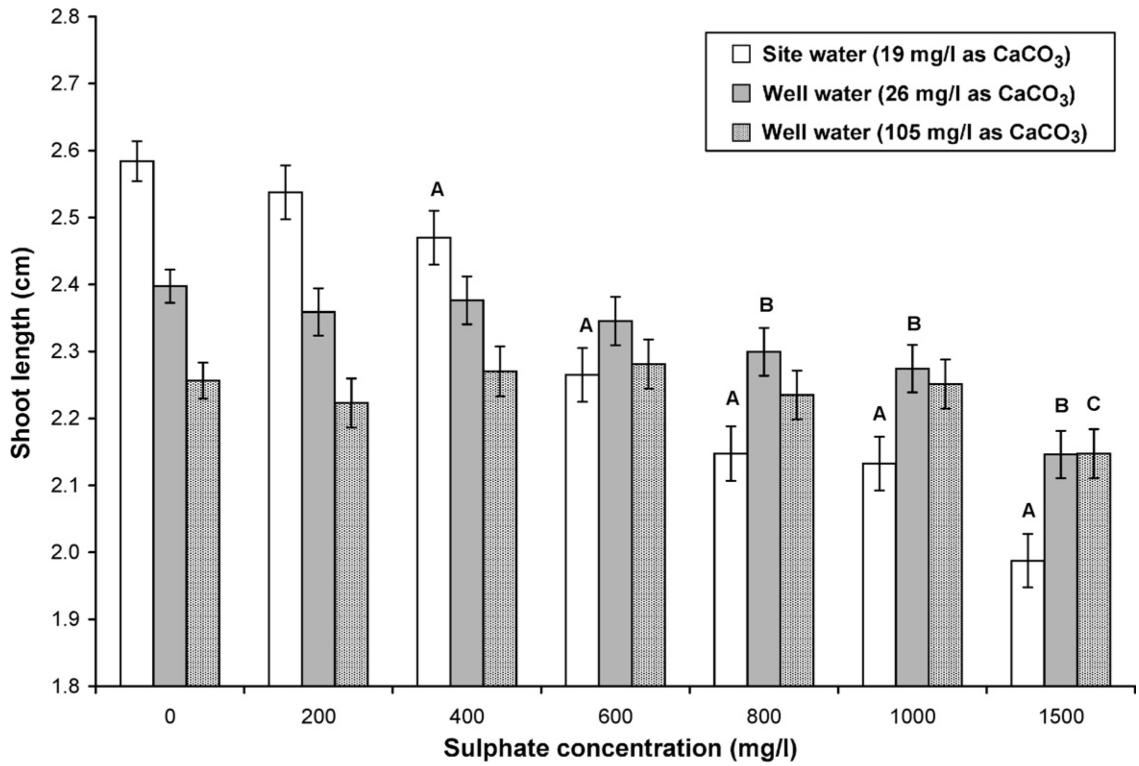

Fig. 1. Response to increasing sulphate concentration on final shoot length ($n \approx 10$ for each replicate (4) at each sulphate concentration) of *Fontinalis antipyretica* after 21-days exposure. Data are means ± standard error. Letters correspond to treatments which are significantly different from controls ($p < 0.05$). Shoot length at the beginning of test was 2 cm.

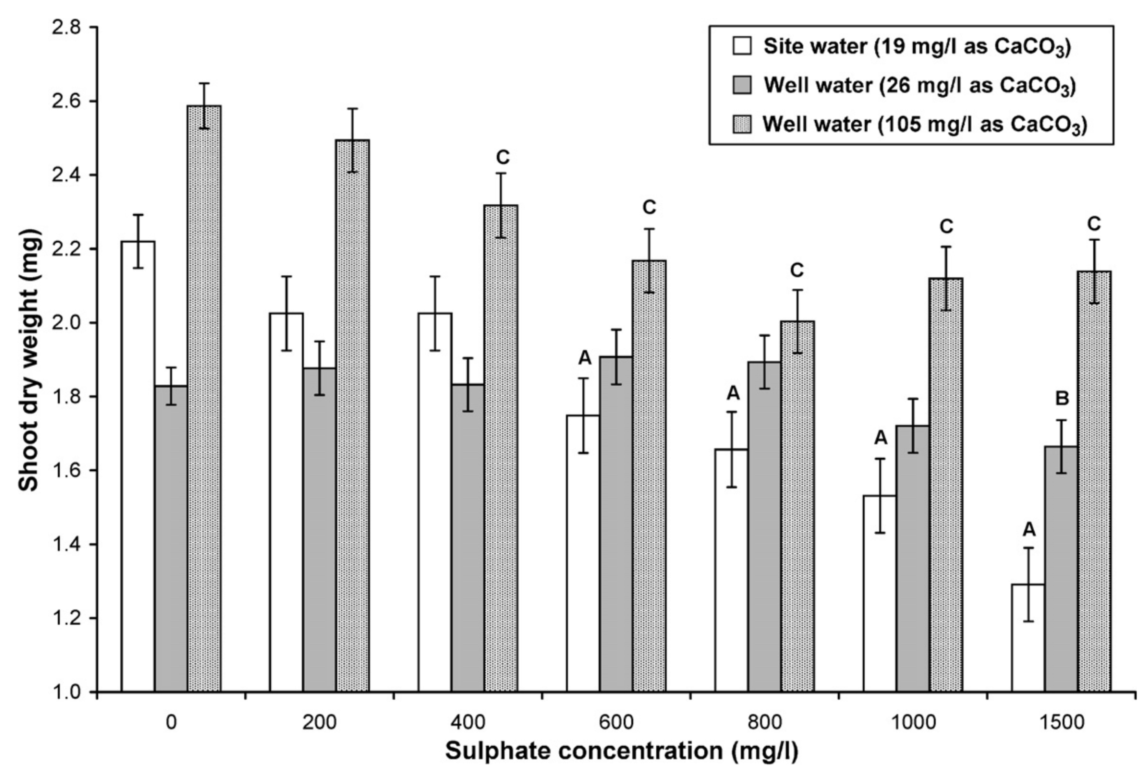

Fig. 2. Response to increasing sulphate concentration on final shoot dry weight ($n \approx 8$ for each replicate (4) at each sulphate concentration) of *Fontinalis antipyretica* after 21-days exposure. Data are means ± standard error. Letters correspond to treatments which are significantly different from controls ($p < 0.05$).

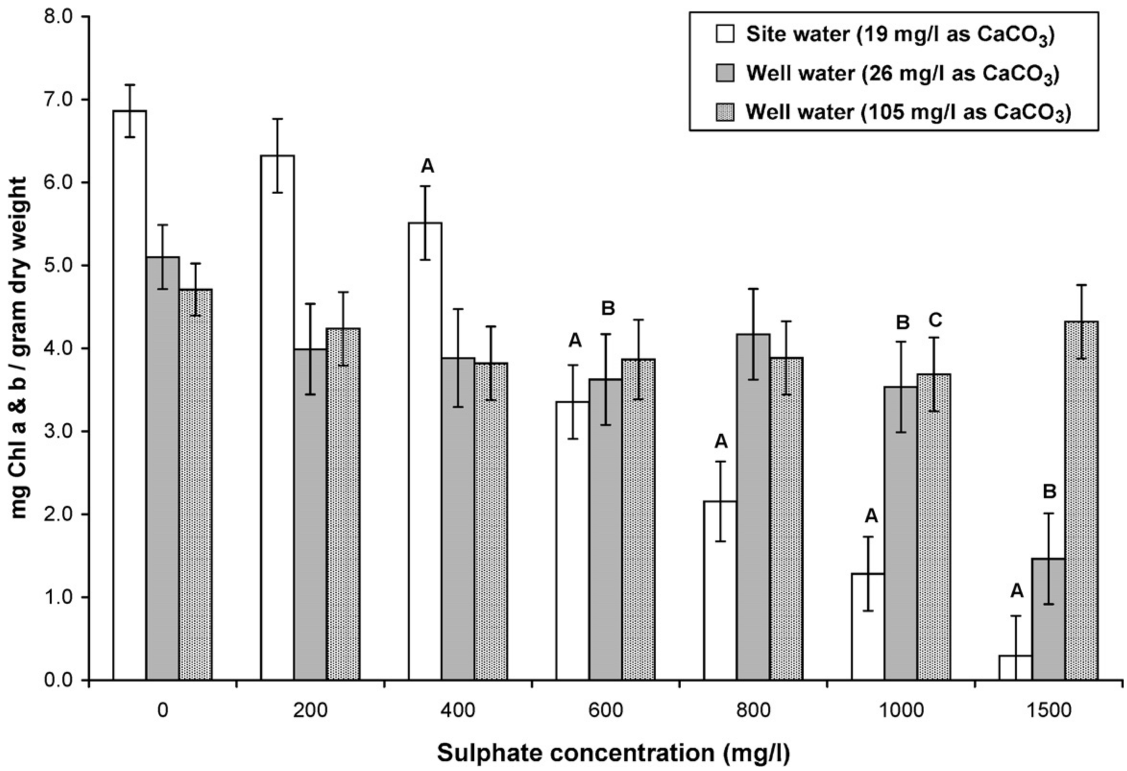

Fig. 3. Response to increasing sulphate concentration on the chlorophyll *a* and *b* content (per gram dry weight; ($n = 3$–4 per sulphate concentration) of *Fontinalis antipyretica* after 21-days exposure. Data are means ± standard error. Letters correspond to treatments which are significantly different from controls ($p < 0.05$).

concentrations per gram dry weight (chl *a* and *b*/g DW) in the control groups in comparison to both well-water control groups (Fig. 3). Shoots in the stream-water control had a mean chlorophyll content of 6.9 mg chl *a* and *b*/g DW and showed a continual decline with increasing sulphate exposure; a significant decline was first detected at 400 mg/l sulphate. Above 800 mg/l sulphate, chlorophyll levels were below 3 mg chl *a* and *b*/g DW and shoots began to show obvious signs of stress by beginning to turn brown. Both soft- and medium-water treatments showed a comparable, yet limited, response to increasing sulphate exposure, up to the highest exposure sulphate treatment of 1500 mg/l. Although a statistically significant reduction in chlorophyll levels was observed at 600 mg/l sulphate relative to the control treatment in the soft well-water treatments ($p < 0.05$), no significant difference was observed between the 200 mg/l sulphate treatment and all other treatments until 1500 mg/l sulphate ($p < 0.05$), in which shoots appeared dead and brown. In the medium-hardness group, a significant reduction in chlorophyll levels was observed at 1000 mg/l sulphate, but not at 1500 mg/l sulphate.

4. Discussion

Reduction in shoot length and chlorophyll content provides evidence that there is an inverse relationship between sensitivity of *F. antipyretica* to elevated sulphate concentrations and the hardness of the exposure water. The effect of increasing sulphate exposure on dry weight is difficult to interpret because a consistent trend is lacking. Dry weight measurements from the medium-hardness water treatment suggest that these shoots exhibited the most growth (Fig. 2); however, this differs from the total length results where the medium-hardness treatment exhibited the least overall growth up to 600 mg/l sulphate exposure. This may possibly be explained due to the higher ion content of the medium-hardness water, which had a hardness of 105 mg/l as $CaCO_3$ in comparison to the soft water of 26 mg/l as $CaCO_3$. In waters of increasing ion concentration, many non-halophytes increase ion uptake to compensate for the adverse effects caused by an osmoregulatory imbalance (Hart et al., 1991). Although differences in weights in controls were relatively large (1.83 versus 2.59 mg in soft and medium-hardness water respectively), the difference (<0.8 mg) may be a result of greater ion uptake by the moss in the more ion rich harder water in order to maintain osmotic balance. This is speculative as no analysis of the plant tissues was done.

Alternatively, different forms and concentrations of nitrogen have been shown to increase shoot mass by encouraging branching and the development of wider leaves without an associated increase in shoot length in the aquatic moss *Vesicularia dubyana* (Alghamdi, 2003). As no nutrients were added to the diluted well-water treatments, the non-diluted well water used for the medium-hardness test medium had a fourfold concentration of nitrate. This may have inadvertently influenced the level of branching resulting in greater dry weight without an associated increase in shoot length. Anecdotally, no obvious difference in branching was observed among water hardness test groups; however, this was not formally tested.

Chlorophyll analysis provided evidence that sulphate is less toxic in water of increasing water hardness. Reduction of chlorophyll content in *F. antipyretica* suggests that chlorophyll may be a better metric of plant health in response to elevated sulphate exposure than either shoot length or dry weight. The lower total length and chlorophyll levels in the control groups of the well water relative to stream water suggest that water characteristics and/or plant health may have influenced results. Regardless, the general trend in chlorophyll concentrations per mg DW suggests that *F. antipyretica* is less sensitive to sulphate in water of increased hardness. However, differences between the stream and soft-water groups may be exaggerated due to the much higher calcium content of the soft well water.

Water hardness is a measure of the quantity 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 and manganese, may also contribute to the hardness of water, but are generally present in much lower concentrations. Converting the calcium and magnesium content of the solution into calcium carbonate equivalents standardizes water hardness. 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. Steam water and well water had calcium/magnesium ratios (Ca/Mg) of 2.4 and 6.7 (based on a molar basis) respectively. Various studies investigating the reduction of metal toxicity in waters of increasing water hardness have demonstrated that it is the calcium rather than the magnesium component of hardness that plays a greater role in the reduction of metal toxicity in fish (Gundersen et al., 1994; Alsop and Wood, 1999; Welsh et al., 2000). 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. Further studies are required to assess this hypothesis.

Another potential explanation for reduced sulphate toxicity with increasing water hardness is the formation of ion pairs between calcium and sulphate. However, calcium concentrations were 0.9 mm/l and sulphate levels were 15.6 mm/l in the medium water at 1500 mg/l sulphate exposure; therefore, a significant reduction in toxicity would be unlikely even if all the calcium paired to sulphate ions. Furthermore, modeling using the program PHREEQC (Parkhurst and Appelo, 2001) indicated that less than 0.1 percent of the total sulphate would complex with calcium to form $CaSO_4$ ion pairs.

The toxic threshold of sulphate to *F. antipyretica* of 100 mg/l sulphate as reported by Frahm (1975) was not replicated in the results presented here. The enhanced toxicity reported by Frahm likely resulted from adding sulphate as K_2SO_4. Egan and Ungar (1998) examined the toxicity of K_2SO_4, KCl, NaCl and Na_2SO_4 to the terrestrial weed, *Atriplex prostrata* and found the toxicities of the following to be $K_2SO_4 > KCl > NaCl = Na_2SO_4$ when exposed to comparable osmotic potentials. At the end of the five-week test, all plants exposed to K_2SO_4 and 40 percent of the plants exposed to KCl were dead. However, no detrimental impacts on survival were observed in either the NaCl or Na_2SO_4 treatments. This is consistent with the trend observed in the toxicity to invertebrates and fish where individual ion toxicity for *Ceriodaphnia*, *Daphnia magna* and Fathead minnows (*Pimephales pronelas*) is $K^+ > Cl^- > SO_4^{2-}$ (Mount et al., 1997).

The report by Beak International Incorporated and Michigan Technological University (1998) indicating no observable effect in chlorophyll levels to *F. noemexicana* up to 500 mg/l sulphate 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 of salinities below 1000–2000 mg/l (Hart et al., 1991). However, as no observable effect was seen, an extrapolation of the effects of longer exposures cannot be made. The increased energy requirements to maintain ion balance in a medium of amplified ion content, and how this would affect the long-term sustainability of moss populations is problematic when no effect is observed.

The assessment of sulphate toxicity to *F. antipyretica* is confounded by the presence of sodium ions even though sodium is considered one of the least toxic cations. The mode of toxicity from sulphate relates 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 by creating or nullifying osmotic gradients. Therefore, attribution of sodium sulphate toxicity solely to the sulphate ion may give a simplistic assessment of sulphate ion toxicity to *F. antipyretica* and other plant species.

Future experimental design needs to control for the influence of different concentrations of plant nutrients, particularly nitrate, in the different hardness treatments. In this study, well water was diluted with de-ionized water to simulate exposure waters of different hardness. This may have influenced results by having different concentrations of nitrate and phosphorus in the different hardness treatments. Better methods would be to either create exposure waters using reconstituted de-ionized water or add calcium and magnesium to soft well water to make the medium-hardness treatment. These two methods would hold water quality variables constant and potentially clarify the confounding results of this study.

This study indicates that *F. antipyretica* is much more tolerant to sulphate than is indicated in the current BC water quality guideline for sulphate for the protection of freshwater aquatic life. The toxicity reported by Frahm (1975) was likely from the associated potassium ion, rather than the sulphate ion, and should not have been used in a sulphate water quality guideline. Chlorophyll levels and shoot length were useful measures in assessing the effects of sulphate on *F. antipyretica*, in contrast to shoot weight, which appeared to be confounded by other factors.

Chlorophyll concentration is a relatively simple measure to obtain and appears to be a good indicator of moss health in response and should be considered as an indicator for monitoring aquatic moss health to elevated sulphate concentrations. The substantial reduction of sulphate toxicity in waters of increasing hardness suggests that development and application of site-specific water quality objectives, rather than a single provincial guideline, would be more appropriate when establishing sulphate discharge limits.

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