# Effects of Sulfate Addition and Soil Physico-Chemical Characteristics on the Abundance of Fe(III)-Reducing Bacteria in Subalpine Wetland Soils

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## ABSTRACT

Observed elevations in dissolved organic carbon (DOC) concentrations in surface waters worldwide signal a declining capacity of soils to stabilize soil organic matter (SOM), a function important to managing drinking water quality as well as maintaining a large terrestrial carbon (C) pool integral to global carbon balance. In wetlands, anaerobic respiration via microbial reduction of terminal electron acceptors including iron (Fe) and sulfate ($SO_4^{2-}$) is a major driver of Fe cycling, which is key to preservation and decomposition processes in SOM cycling. Given Fe-S coupled cycling and previously demonstrated fallibility of a long-standing thermodynamic hierarchy used to predict the sequence of terminal electron accepting processes, effects of changing the relative availability of S on Fe-reducing populations are unclear. This study elucidates the effects of observed increases in $SO_4^{2-}$ deposition levels in subalpine wetlands of Colorado's Fraser Experimental Forest on the abundance of soil Fe(III)-reducing bacteria (IRB). IRB in a slope and a depressional wetland were enumerated using the most probable number (MPN) method before and after intact soil cores were injected with artificial $SO_4^{2-}$-enriched porewater. $SO_4^{2-}$ addition significantly increased IRB abundance in most study soils, implying that $SO_4^{2-}$ deposition can be a contributing factor to rising DOC levels in watersheds. Dry bulk density was found to have a significant positive effect on IRB abundance that was enhanced upon $SO_4^{2-}$ addition and N content only became relevant to IRB abundance after $SO_4^{2-}$ addition. Complexities in Fe-S cycling linkages combined with physico-chemical heterogeneity of soils pose challenges to determining the extent to which $SO_4^{2-}$ addition affects biotic Fe(III) reduction in subalpine wetlands.

## KEYWORDS

Iron-sulfur coupled cycling, dissolved organic carbon, soil organic matter, subalpine watersheds, wetland biogeochemistry

## INTRODUCTION

Exports of dissolved organic carbon (DOC), defined as organic compounds in water that can pass through a 0.45-μm filter, from land to surface waters of glaciated landscapes have dramatically risen around the world over the past decade (Roulet and Moore 2006; Freeman et al. 2004; Evans et al. 2006; Monteith et al. 2007; de Wit et al. 2007; Clark et al. 2010; Knorr 2013). High concentrations of DOC can lead to the formation of carcinogenic disinfection by-products and elevate costs during drinking water treatment processes (Roulet and Moore 2006; Chowdhury et al. 2009). DOC concentrations in watersheds depend on the stabilization of soil organic matter, or SOM, which consists of material produced by biota that returns to the soil and undergoes decomposition. SOM promotes the productivity and long-term sustainability of soils by storing organic and inorganic nutrients in forms available to plants (Bot and Benites 2005). With global soil organic carbon (C) stocks at approximately 1,500 billion metric tonnes, exceeding the amount of carbon stored in phytomass and the atmosphere (Scharlemann et al. 2014), SOM stabilization plays an important role in the capacity of soils for C sequestration. Not only do increasing DOC concentrations pose public health risks, but they also indicate a shift in the global carbon balance via the associated decrease in the terrestrial soil carbon pool (Qafoku 2015).

Mountain snow packs serve as drinking water supply and storage for over one-sixth of the global population (Barnett et al. 2005). The subalpine forests of the Rocky Mountains in Colorado provide municipal, agricultural, and industrial services to a population of over 25 million (Schneider 2017). Local and regional sources of air pollution such as coal-fired power plants in southwestern Wyoming (Mast et al. 2001) and northwestern Colorado (Turk and Campbell 1997) have generated hot spots of atmospheric sulfate ($SO_4^{2-}$) deposition, increasing concentrations of $SO_4^{2-}$ from north to south throughout the Rocky Mountains. Relatively high $SO_4^{2-}$ deposition levels of 8.00 – 10.0 kg/ha were found along the Front Range, an area adjacent to a large urban corridor (Nanus et al. 2003). Increasing stringency on emissions standards had decreased atmospheric $SO_4^{2-}$ deposition in recent years, shifting microbial dominance from $SO_4^{2-}$-reducing bacteria (SRB) to IRB and thereby increasing DOC concentrations in the watershed (Evans et al. 2006; Clark et al. 2010; Knorr 2013). However, the new federal administration's efforts to roll back environmental regulations such as the Environmental Protection Agency's

$CO_2$ standards and the Department of the Interior's coal leasing moratorium on federal lands generate prospects for a recovery of United States coal production and consumption (Trump 2017). National coal consumption is expected to rise by up to 16% relative to the Obama policy baseline in 2025 and by 37 percent in 2030, indicating an associated likelihood of increasing $SO_4^{2-}$ deposition rates in the next few years (Houser et al. 2017).

As C is transported from stable soil reservoirs into waterways, its bioavailability for microbial metabolism increases (Schneider 2017). Wetlands functioning as links between terrestrial and aquatic ecosystems promote high levels of microbial activity as they contain large amounts of organic matter, 45 to 50% of which consist of C (Bano et al. 1997, Zweifel 1999). A key component of C cycling within wetlands is anaerobic respiration, or C mineralization via the reduction of terminal electron acceptors (TEAs) such as $SO_4^{2-}$, iron (Fe), and nitrate ($NO_3^-$) (Kayranli et al. 2010). In particular, the role of microbial metabolism in Fe cycling is a major driver of soil Fe reductive dissolution. Fe-associated organic carbon is present in 2,900-6,800 times the amount of atmospheric carbon (Lalonde et al. 2012), an indication of the importance of Fe cycling to preservation and decomposition processes in SOM cycling. Co-precipitation and direct chelation comprise most of the associations interlinking the biogeochemical cycles of solid Fe phases and organic carbon and promote the preservation of organic carbon in sediments (Lalonde et al. 2012). Dissimilatory Fe-reducing bacteria (DIRB or simply IRB) also couple the oxidation of organic matter to the reduction of ferric iron, or Fe(III), in the process of anaerobic respiration. IRB use Fe(III) as a TEA to oxidize organic compounds to $CO_2$ or other oxidized C metabolites (Myers and Nealson 1990). Most characterized IRB can oxidize organic substrates using alternative TEAs such as nitrate, fumarate, and elemental sulfur (S) in the absence of Fe(III) (Lovley et al. 1997), potentially affecting dynamics such as resource competition within microbial communities. The effects of these alternative electron acceptors to Fe on SOM stabilization and release may be significant.

A long-standing thermodynamic-based hierarchy has been used to predict the dominance of microbial respiratory pathways based on the net energetic yields from different terminal electron accepting processes (Lovley 1987). In deference to this simplified model, often referred to as the "redox tower" or "redox ladder," $SO_4^{2-}$ reduction observed in high-Fe and low-$SO_4^{2-}$ environments has been attributed to the presence of highly crystalline Fe oxides that are less biologically available and hence allow $SO_4^{2-}$ reduction to be more energetically favorable. Under

these conditions, sulfidization of Fe(III)-minerals is a dominant pathway in Fe reduction (Hansel et al. 2015). Yet inverse redox zonations have been observed in which biotic $SO_4^{2-}$ reduction preceded or occurred simultaneously with ferrihydrite reduction despite a higher net energetic yield from the latter terminal electron accepting process. This indicates the fallibility of the "redox ladder" in predicting microbial metabolic pathways and geochemical zonation (Chapelle and Lovley 1992). Evidence in the literature indicates interlinkages between Fe and S cycling in terrestrial and marine sediments (Koretsky et al. 2003; Holmkvist et al. 2011; Akob et al. 2008; Komlos et al. 2008; Pester et al. 2012; Osorio et al. 2013; Flynn et al. 2014; Kwon et al. 2014). Because ferrihydrite is a dominant product of Fe oxidation in the roots of terrestrial and wetland plants (Emerson et al. 1999), unexpectedly high $SO_4^{2-}$-reducing activity in low-$SO_4^{2-}$ wetland systems can potentially be attributed to Fe oxide-based S recycling (Pester et al. 2012). Given the fallibility of this model and evidence of interlinkages between Fe and S biogeochemistry as well as the importance of Fe in SOM stabilization, the response of IRB to $SO_4^{2-}$ addition is not well understood and merits investigation.

This study investigates the effects of $SO_4^{2-}$ addition on the abundance of IRB in subalpine wetland soils of the Fraser Experimental Forest in Colorado. Because environmental parameters characterize the ecological traits of whole microbial communities, contributing directly to ecosystem and food web dynamics, physico-chemical soil properties may also play roles in affecting the response of IRB populations to $SO_4^{2-}$ addition (Wallenstein and Hall 2012). IRB in the study soils were enumerated using the most probable number (MPN) method before and after $SO_4^{2-}$ addition and soil properties were characterized using standard soil analysis techniques. By improving current understanding about a biological mechanism through which $SO_4^{2-}$ addition by facilities such as coal-burning power plants affects environmental health and the global carbon balance, this study can aid efforts to inform policymakers and industry about the impacts of $SO_4^{2-}$ deposition.

# METHODS

## Study site

Established in 1937 as part of a national network of outdoor research laboratories and maintained by the Rocky Mountain Research Station, the Fraser Experimental Forest in Colorado is a 9,308-hectare representative site for studies on timber harvest impact on alpine and subalpine ecosystems of the central Rocky Mountains (39°50' N, 105°54' W). The forest is integral to managing water quality in the snowmelt-dominated Fraser River watershed that serves as drinking water storage and supply to Denver, located approximately 112 kilometers southeast from the site. Elevations in the forest range between 2,680 – 3,900 m; the overall climate is humid with frigid, long winters and cool, short summers. Annual precipitation averages 737 mm, approximately two-thirds of which falls as snow from October to May ("Fraser Experimental Forest - Site Description" n.d.).

## IRB abundance

Soil cores were collected from a slope and a depressional wetland located at subalpine elevations. The slope wetland is groundwater-fed with fast-flowing water and a permanent hydroperiod and was sampled in June of 2015. In the snowmelt-fed depressional wetland two different sites were sampled: the transition site with a seasonal hydroperiod and the center site with a permanent hydroperiod in June and September of 2015, respectively. Once collected in the field, the soil cores were capped and sealed to maintain anoxic conditions and stored at approximately 4°C prior to analysis and experiments. Shallow (13.5 to 30 cm) and deep (55 to 72 cm) portions of each of the mineral soil profiles were treated as separate samples to investigate the effect of soil depth on the response variables.

Intact soil cores were used in flow-through reactor experiments to determine potential $SO_4^{2-}$ reduction rates, Fe(II) export rates (lower-bound estimates of potential Fe(III) reduction rates), and dissolved organic carbon export rates (Schneider 2017). A synthetic porewater medium containing $SO_4^{2-}$ was injected through the input channel to the intact soil cores at increasing concentrations (0.05 to 0.75 mM) over the course of 25 days.

For biological characterization of each soil sample, I used the most probable number (MPN) method (Woomer 1994), a procedure for estimating the population density of viable microorganisms in a test sample, to enumerate all six soil samples for IRB both before and after the flow-through experiments (Schneider 2017). The MPN method applies probability theory to the numbers of observed positive growth responses to a standard dilution series of inoculated microtiter plate wells.

To prepare for the enumeration of IRB, I first synthesized ferrihydrite ($Fe_5HO_8 \cdot 4H_2O$) to act as the terminal electron acceptor for the IRB by creating a ferric chloride solution, titrating it, and washing the produced Fe oxides following the protocol of (add reference). Ferrihydrite was used as it is a dominant product of Fe oxidation in the rhizosphere of terrestrial and wetland plants (Emerson et al. 1999; Hansel et al. 2001). First, I mixed 9.585 g of ferric chloride ($FeCl_6 \cdot H_2O$) into 575 ml of deionized water and titrated it with 0.4N NaOH until a pH of 7.2 was reached. The Fe oxides were allowed to settle overnight, after which the supernatant was decanted and the Fe oxides re-suspended with deionized water. I then centrifuged the ferrihydrite at 6,000 rpm for 15 minutes four times, each time decanting the supernatant and resuspending with deionized water. Next, I oven-dried the ferrihydrite for 24 hours at 105°C before grinding it in with an agate mortar and pestle.

Soil slurries were made for each sample by suspending approximately 1 g of soil in 10 ml of sterile saline solution (NaCl, 2%; $MgCl_2$, 0.3%) in a blender and mixing for 1.5 minutes at high speed (Pallud and Van Cappellen 2006). I also analyzed the water content of each sample by oven-drying approximately 1 g of soil for 24 hours at 105°C, calculating the mass of the water in each sample, and replicating this procedure three times to obtain an average.

To prepare the microtiter plates for incubation, working under sterile conditions, I prepared a basal freshwater growth medium for IRB with the following composition in g/L: NaCl (1.0), $MgCl_2 \cdot 6H_2O$ (0.4), $CaCl_2 \cdot 2H_2O$ (0.15), $KH_2PO_4$ (0.2), KCl (0.5), and $NH_4Cl$ (0.25) (Dubinsky et al. 2010). On the day of incubation, I added sodium acetate (10 mmol/L) to act as the electron donor in the microbial metabolic processes and ferrihydrite (30 mmol/L) to act as the terminal electron acceptor. The medium was adjusted to a pH of 5.0 with 1 M HCl and autoclaved at 121°C for 30 minutes. I also added a selenite-tungstate solution (Widdel and Bak 1992) and vitamin B12 solution (Pfennig and Trüper 1992) under sterile conditions before

dispensing the medium into 96-well microtiter plates. Soil slurries were serially diluted in the plates to a dilution of $10^{-8}$ with eight replicates per dilution. Inoculated and negative-control plates were incubated anaerobically in sealed bags inside a glove box (1 to 3.5% $H_2$, 99 to 96.5% $N_2$) at 25 ˚C for 4 and 6 weeks. To visualize positive growth of the IRB at the end of incubation, I added a ferrozine reagent (0.1% w/w in 50 mM HEPES buffer) that turned wells a purple color when complexed with of Fe(II).

I also inoculated microtiter plates using a growth medium specifically for SRB and completed serial dilutions of the samples using the same processes (Pallud and Van Cappellen 2006). Inoculated and negative-control plates were incubated anaerobically in sealed bags inside a glove box (1 to 3.5% $H_2$, 99 to 96.5% $N_2$) at 25˚C for 6 weeks. Because I found no evidence of growth of SRB, I did not proceed to analyze SRB growth. The lack of observed SRB growth may be attributable to suboptimal incubation pH conditions; pH of the SRB growth medium had been adjusted to fall between 6.8 and 7.0 using 1 M HCl. While an optimal pH level for SRB growth has been reported to be 7.0 (Al Zuhair et al. 2008), SRB in wetlands have also been found to grow at pH levels between 4.0 and 4.6 (Koschorreck 2008). Possibly, the incubation pH used in this study was too high for SRB in the subalpine wetlands to adapt and proliferate.

MPN estimates were derived from Table 4 of Appendix 2 in the United States Food and Drug Administration's Bacteriological Analytical Manual (Blodgett 2010).

**Soil physico-chemical characteristics**

Physical, chemical, and biological characteristics of the shallow and deep portions of the mineral soil profile for each sample were analyzed using standard soil analysis techniques. $pH_{water,}$ $pH_{KCl}$, clay:sand ratios, porosity, dry bulk density, organic matter content, S content, Fe content, organic carbon content (DOC concentration), nitrogen (N) content, and molar C:N ratio were for all samples.

Active acidity and exchangeable acidity, characterized by $pH_{water}$ and $pH_{KCl}$, respectively, were measured by air drying another portion of each soil for 48 hours at 105 °C, mixing a portion of the sediments with deionized water and another portion with 1 M KCl on stir plates. pH for both mixtures was determined using a Denver Instrument Model 215 pH meter. I analyzed the soil texture, represented by clay:sand ratio, of each sample by taking hydrometer

readings from 0.5 to 1,440 minutes for each sample to separate silt, clay, and sand; oven drying a portion of each soil for 48 hours at 105 °C to sieve for sand fraction calculations; and using a soil triangle to identify soil texture ("Soil Texture Calculator" n.d.). Porosity and dry bulk density were calculated using results of the water content analysis for each sample. A portion of each sample was also sent to the University of Massachusetts Amherst Soil and Plant Nutrient Testing Lab ("Soil and Plant Nutrient Testing Laboratory" n.d.) to analyze for organic matter content, S content, and Fe content. Total C, assumed here to be organic C as soils contained no carbonates, and N content were analyzed via dry combustion on a LECO TruSpec Micro CN analyzer (Leco Corp., St. Joseph, MI, USA).

**Statistical analyses**

Statistical analyses were conducted using version 3.3.1 of the R programming language at a significance threshold of 0.05 (R Core Team 2016; Graves et al. 2015). A t-test was also conducted to determine whether conducting two separate flow-through reactor experiments on the same samples significantly affected MPN estimates of IRB. Because this effect was significant ($p \leq 0.001$), I chose to examine data retrieved from both experiments (hereafter referred to as Experiment 1 and Experiment 2) separately for all subsequent analyses. Statistically significant differences for IRB cell densities across soils (slope wetland, center site of depressional wetland, or transition site of depressional wetland), depth within the mineral soil profile (shallow or deep), and $SO_4^{2-}$ addition were determined through a multi-factorial analysis of variance (ANOVA) followed by a post-hoc Tukey Honestly Significant Difference (HSD) test. The roles of soil physico-chemical characteristics ($pH_{water,}$ $pH_{KCl}$, clay-to-sand ratio, porosity, dry bulk density, S content, Fe content, organic matter content, organic C content, N content, and molar C:N ratio) interacting with $SO_4^{2-}$ addition in driving IRB cell densities were determined using an analysis of covariance (ANCOVA).

# RESULTS

## Soil physico-chemical characteristics

Soil physico-chemical properties varied by wetland type, site, and soil depth (Table 1). In general, the slope wetland had lower clay:sand ratio, porosity, Fe content, S content, organic matter content, carbon content, and molar C:N ratio as well as higher dry bulk density than the depressional wetland.

All study soils were acidic. The soil active acidity, or $pH_{water}$, of the six samples ranged from 4.11 to 5.60 while the soil exchangeable acidity, or $pH_{KCl}$, ranged from 3.33 to 4.92. The shallow depth of the slope wetland had the lowest active acidity and lowest exchangeable acidity of all soils. The shallow depth of the transition site of the depressional wetland had the highest active acidity while the transition site overall had the highest exchangeable acidities.

The deep section of the transition site of the depressional wetland had the highest clay:sand ratio (21.9%) of all soils and exhibited the greatest range in soil texture by depth (5.96 to 21.9%). The deep slope wetland had the lowest ratio (0.141%). Porosities and dry bulk densities were similar for both the center and transition sites of the depressional wetland, ranging from 0.77 to 0.83 and 0.31 to 0.42 g $cm^{-3}$, respectively. In general, soil from the slope wetland had lower porosity and higher dry bulk density than that of the depressional wetland.

The shallow depth of the depressional wetland center site contained the highest Fe concentration (3.18 μmol $g^{-1}$) while the slope wetland had the lowest Fe content (0.53 μmol $g^{-1}$) at the same depth. Similarly, the slope wetland had the lowest S content (0.82 and 0.16 μmol $g^{-1}$ in shallow and deep zones, respectively) while the shallow zone of the center site of the depressional wetland had the highest (1.48 μmol $g^{-1}$). Shallow depths in both the center and transition sites of the depressional wetland had the highest organic matter content (30.5% and 42.8%, respectively) while the slope wetland contained the lowest levels (12.9% and 1.08% in shallow and deep zones, respectively). The slope wetland contained approximately 10 to 18 times proportionally less carbon than the depressional wetland regardless of site or soil depth. Additionally, the slope wetland contained 12 to 37 times less N than the depressional wetland. Molar C:N ratios ranged from 9.2 to 19.5 across all soils with the deep zone of the slope wetland

**Table 1. Physical and chemical characteristics of the study soils by wetland type, site, and depth.**

| Soil Properties | Slope Wetland Shallow | Slope Wetland Deep | Depressional Wetland Center Shallow | Depressional Wetland Center Deep | Depressional Wetland Transition Shallow | Depressional Wetland Transition Deep |
|---|---|---|---|---|---|---|
| $pH_{water}$ | 5.60 | 4.86 | 4.81 | 5.05 | 4.11 | 4.60 |
| $pH_{KCl}$ | 4.92 | 3.60 | 4.08 | 4.08 | 3.59 | 3.33 |
| Clay (%) | 36.6 | 11.6 | 37.3 | 43.0 | 58.6 | 62.2 |
| Sand content (%) | 4.1 | 82.3 | 4.4 | 12.9 | 9.8 | 2.8 |
| Clay:Sand ratio (%) | 8.88 | 0.141 | 8.43 | 3.33 | 5.96 | 21.9 |
| Porosity | 0.59 | 0.39 | 0.77 | 0.83 | 0.77 | 0.79 |
| Dry bulk density (g $cm^{-3}$) | 0.83 | 1.6 | 0.36 | 0.31 | 0.31 | 0.42 |
| S content ($\mu$mol $g^{-1}$) | 0.82 | 0.16 | 1.48 | 0.91 | 0.97 | 0.36 |
| Fe content ($\mu$mol $g^{-1}$) | 0.53 | 0.68 | 3.18 | 1.8 | 2.86 | 0.86 |
| Organic matter content (%) | 12.9 | 1.08 | 30.5 | 18.3 | 42.8 | 13.9 |
| Organic C content (%) | 1.6 | 0.09 | 10.9 | 17.3 | 18.6 | 14.1 |
| N content (%) | 0.08 | 0.02 | 0.92 | 0.88 | 1.37 | 0.75 |
| Molar C:N ratio | 18.6 | 9.2 | 11.8 | 19.5 | 13.6 | 18.7 |

having the smallest ratio and the deep zone of the center site of the depressional wetland having the largest.

**IRB abundance**

IRB abundance varied with wetland type, site, and depth before and after $SO_4^{2-}$ addition in both experiments conducted (Figure 1). Prior to $SO_4^{2-}$ addition in Experiment 1, IRB abundance in the deep soil of the slope wetland was found to be similar to that of both center and transition sites in the depressional wetland (2.73, 1.55, and $1.46 \times 10^2$ IRB $g^{-1}$ dry soil, respectively; Figure 1a). These levels of IRB abundance were the lowest among all study soils. On average, shallow soils contained 350 times more IRB than deep soils with the shallow soil from the slope wetland having the greatest IRB abundance ($1.93 \times 10^5$ IRB $g^{-1}$ dry soil). IRB abundance in the center and transition sites of the depressional wetland were not found to be significantly different from each other. The trend of decreasing IRB abundance with depth within the depressional wetland was reversed upon $SO_4^{2-}$ addition. Deep soils from both center and transition sites had similar IRB population sizes (3.85 and $1.42 \times 10^7$ IRB $g^{-1}$ dry soil, respectively), the largest IRB populations among all soils; they had on average 600 times more IRB than shallow soils within the same wetland ($1.95 \times 10^3$ and $8.60 \times 10^4$ IRB $g^{-1}$ dry soil, respectively). Between the shallow soils of those two sites, however, the transition site had approximately 44 times more IRB than the center site soil. $SO_4^{2-}$ addition did not significantly affect IRB abundance in the shallow part of the center site of the depressional wetland. No trend of IRB abundance with depth was observed for the slope wetland.

Similar to as observed in Experiment 1, IRB abundance in the deep soil of the slope wetland prior to $SO_4^{2-}$ addition in Experiment 2 was found to be similar to that of both center and transition sites in the depressional wetland (2.73, 1.55, and $1.46 \times 10^2$ IRB $g^{-1}$ dry soil, respectively; Figure 1b). Unlike in Experiment 1, IRB abundance within the depressional wetland did not decrease with depth after $SO_4^{2-}$ addition. IRB abundance was highest in the shallow soil of the center site ($1.77 \times 10^6$ IRB $g^{-1}$ dry soil) and was approximately 12,000 times greater than the IRB abundance found in the deep soil of the transition site ($1.52 \times 10^2$ IRB $g^{-1}$ dry soil). $SO_4^{2-}$ addition did not affect IRB abundance in the deep parts of both the slope wetland and the transition site of the depressional wetland. The shallow and deep parts of the

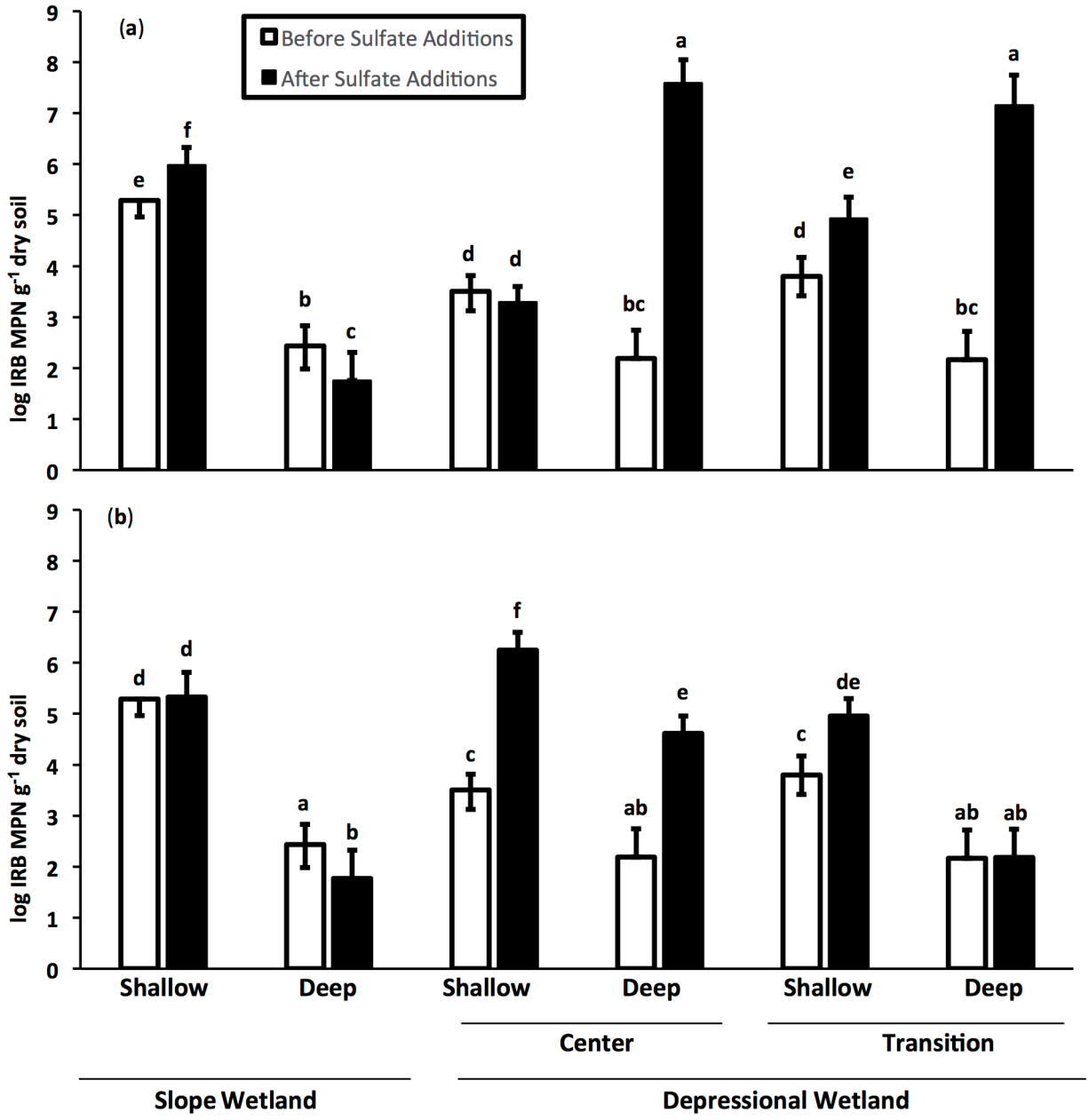


**Figure 1. Most probable number estimates of IRB in intact soil cores collected from the six study soils noted on the x-axis before and after sulfate addition.** Figures 1a and 1b refer to results from two separately conducted sulfate addition experiments referred to as Experiments 1 and 2, respectively. Letters above bars indicate significant pairwise differences for all possible pairs when different within each experiment. Error bars indicate standard error.

depressional wetland's center site experienced the largest IRB population growth upon $SO_4^{2-}$ addition in comparison to the other soils; IRB abundance increased to approximately 560 times the original abundance in the shallow soil prior to $SO_4^{2-}$ addition ($3.18 \times 10^3$ IRB $g^{-1}$ dry soil) and increased to approximately 270 times the original abundance in the deep soil ($1.55 \times 10^2$ IRB $g^{-1}$ dry soil). In both experiments, IRB abundance significantly decreased after $SO_4^{2-}$ addition only in the deep soil of the slope wetland.

**Interacting effects of $SO_4^{2-}$ addition and soil physico-chemical characteristics on IRB abundance**

The effects of soil physico-chemical properties on the abundance of IRB in response to $SO_4^{2-}$ addition varied across Experiments 1 and 2 (Table 2). $pH_{water}$, which represents the active acidity of the soils, did not significantly affect the abundance of IRB in Experiment 1. Upon $SO_4^{2-}$ addition to the systems, however, increasing $pH_{water}$ leads to a significant decrease in the abundance of IRB. In Experiment 2, $pH_{water}$ had a positive significant effect on the abundance of IRB and $SO_4^{2-}$ addition did not significantly change this relationship. In Experiment 1, clay:sand ratio had a significant negative effect on the abundance of IRB, an effect dampened by the addition of $SO_4^{2-}$; in Experiment 2, the proportion of clay-sized to sand-sized particles did not significantly affect the abundance of IRB before or after $SO_4^{2-}$ addition. Similarly, in Experiment 1, porosity significantly decreased the abundance of the original IRB, an effect dampened by $SO_4^{2-}$ addition; in Experiment 2, porosity did not significantly affect the abundance of IRB before or after the treatment. Experiment 1 showed that the S content of soils had no role in controlling the abundance of IRB regardless of whether $SO_4^{2-}$ had been added to the system or not; Experiment 2, on the other hand, indicated that S content had a significant positive effect on the abundance of IRB prior to treatment and had a greater effect upon $SO_4^{2-}$ addition. Similar results were found for the impacts of Fe content and organic matter content. In Experiment 1, organic C content had a significant negative effect on the abundance of IRB prior to $SO_4^{2-}$ addition; this effect was dampened once externally sourced $SO_4^{2-}$ was introduced to the system. In Experiment 2, organic C content did not have a role in controlling the abundance of IRB until $SO_4^{2-}$ was added, whereupon organic C content had a significant positive effect on growth. A significant positive effect of molar C:N ratio on IRB abundance was strengthened by $SO_4^{2-}$ addition in

**Table 2. ANCOVA tables for the soil physico-chemical properties of the six study soils and the response variable, IRB abundance, interacting with sulfate addition.** The two tables from right to left represent ANCOVA analyses conducted separately for Experiments 1 and 2, respectively.[1,2] "Main effect"[a] refers to the effect of the physical or chemical soil property on the respective response variable. "Interaction effect"[b] refers to the effect of the soil physical or chemical property on the observed change in the response variable to incubation with sulfate additions for 25 days. NS represents a non-significant effect at a significance threshold of 0.05.

| Soil properties | MPN IRB[1] | | | | MPN IRB[2] | | | |
|---|---|---|---|---|---|---|---|---|
| | Main effect[a] | | Interaction effect[b] | | Main effect[a] | | Interaction effect[b] | |
| | Effect | p-value | Effect | p-value | Effect | p-value | Effect | p-value |
| $pH_{water}$ | NS | > 0.05 | – | 0.014 | + | ≤ 0.001 | NS | > 0.05 |
| $pH_{KCl}$ | + | ≤ 0.001 | NS | > 0.05 | + | ≤ 0.001 | NS | > 0.05 |
| Clay:Sand ratio | – | 0.002 | + | ≤ 0.001 | NS | > 0.05 | NS | > 0.05 |
| Porosity | – | ≤ 0.001 | + | ≤ 0.001 | NS | > 0.05 | NS | > 0.05 |
| Dry bulk density | – | ≤ 0.001 | – | ≤ 0.001 | – | ≤ 0.001 | – | ≤ 0.001 |
| S content | NS | > 0.05 | NS | > 0.05 | + | ≤ 0.001 | + | ≤ 0.001 |
| Fe content | NS | > 0.05 | NS | > 0.05 | + | ≤ 0.001 | + | ≤ 0.001 |
| Organic matter content | NS | > 0.05 | NS | > 0.05 | + | ≤ 0.001 | + | ≤ 0.001 |
| Organic C content | – | 0.014 | + | ≤ 0.001 | NS | > 0.05 | + | 0.007 |
| N content | NS | > 0.05 | + | 0.023 | NS | > 0.05 | + | 0.004 |
| Molar C:N ratio | + | ≤ 0.001 | + | ≤ 0.001 | NS | > 0.05 | NS | > 0.05 |

Experiment 1; in the other experiment, molar C:N ratio did not have a significant role in controlling the abundance of IRB at all.

The effects of $pH_{KCl}$, dry bulk density, and N content on pre-treatment and post-treatment IRB population densities were consistent across both experiments. In both experiments, pHKCl, or exchangeable acidity, significantly increased the abundance of IRB prior to $SO_4^{2-}$ addition. $SO_4^{2-}$ addition did not change this positive effect. Dry bulk density also increased the abundance of IRB prior to treatment, an effect strengthened by the addition of $SO_4^{2-}$ to the system. N content did not have a statistically significant effect on IRB abundance until $SO_4^{2-}$ was added to the system. Upon $SO_4^{2-}$ addition, N content had significant positive effects.

## DISCUSSION

### Variations in response of IRB abundance to $SO_4^{2-}$ addition

In situ pore water $SO_4^{2-}$ concentrations in the subalpine wetland study sites were relatively low, ranging between 0.005 and 0.09 mM. Differences in these concentrations between the slope and depressional wetlands, however, may have played a role in the differences in MPN estimates of IRB communities before and after $SO_4^{2-}$ addition. The slope wetland had the lowest in situ S concentration at 0.005 mM while the center and transition sites of the depressional wetland had concentrations of 0.02 mM and 0.09 mM, respectively. Stimulation of $SO_4^{2-}$-reducing activity via $SO_4^{2-}$ addition may have indirectly stimulated IRB population growth by increasing levels of acetate, a favored C substrate for IRB (Lovley and Phillips 1986). SRB produce acetate as its primary fermentation intermediate during the decomposition of complex organic matter. Acetate, a primary electron donor for Fe(III) reduction, is readily consumed in sediments in which Fe(III) reduction is the predominant terminal electron accepting process (Cord-Ruwisch et al. 1998; Lovley and Phillips 1986; Lovley and Phillips 1988). Higher in situ $SO_4^{2-}$ concentrations in the depressional wetland indicate a higher likelihood of $SO_4^{2-}$-reducing microbes being initially present or present in larger quantities; this may serve as a possible explanation for the significant increase in IRB community sizes upon $SO_4^{2-}$ addition. The IRB populations in the deep sections of the depressional wetland in both the center and transition sites grew to be the largest in magnitude across all IRB communities.

The seasonal hydroperiod of the depressional wetland may also have played a role in the strong growth response of the IRB communities present. Ferrihydrite, used in this study as a substrate for microbial oxidation in the IRB growth medium, is highly reactive and is the most bioavailable phase of Fe(III) oxide (Hansel et al. 2015). $SO_4^{2-}$ can impact Fe(III) oxide nucleation and precipitation processes as well as affect Fe(III) oxide formation under acid drainage conditions, which commonly arise from the exposure and subsequent oxidation of Fe sulfides (Majzlan and Myneni 2005). During the relatively dry summer and fall seasons, increased exposure of the depressional wetland surface soil to atmospheric oxidation promotes regeneration of Fe(III) and possibly $SO_4^{2-}$, recycling terminal electron acceptors available for microbial metabolism and thereby enhancing abundance.

**Roles of dry bulk density and N content in response of IRB abundance to $SO_4^{2-}$ addition**

$SO_4^{2-}$ addition strengthened the significant negative effect of dry bulk density on IRB abundance, indicating that soil physical structure has a significant role in IRB growth response to increased levels of $SO_4^{2-}$. This finding is consistent with literature on the effects of dry bulk density on microorganisms; lower dry bulk density presumably provides more favorable soil physical conditions and more organic substrates provided by plant roots (Li et al. 2002). Soils with higher bulk density have also less pore space for microorganisms, limiting the size of their populations (Zabinski and Gannon 1997). The more optimal soil conditions provided by lower dry bulk density may have accelerated biotic $SO_4^{2-}$ reduction rates upon $SO_4^{2-}$ addition, increasing acetate production and providing more organic substrate for IRB to metabolize.

Only upon $SO_4^{2-}$ addition did N content have significant positive effects on IRB abundance, particularly in soils with relatively high N content. The only soil in which $SO_4^{2-}$ addition had a significant negative effect on IRB abundance also had the lowest N content out of all soils sampled. Possibly, the increase of biotic $SO_4^{2-}$ reducing activity and indirect stimulation of IRB growth due to increased acetate production upon $SO_4^{2-}$ reduction also increased competition for limiting resources among the microbial community. N availability limits the growth of biomass in terrestrial environments (Bell et al. 2013; Wang and Bakken 1997). With increased pressure on the N pool in the soil as a result of increasing Fe(III)- and $SO_4^{2-}$-reducing microbial activity, N content becomes a significant determinant of IRB growth after $SO_4^{2-}$

addition. That N content only becomes relevant to IRB abundance under $SO_4^{2-}$ addition implies that $SO_4^{2-}$ deposition may affect microbial community structure in subalpine wetland systems by increasing resource competition.

**Limitations and future directions**

Although the scope of this study was limited to subalpine wetland ecosystems in the Fraser Experimental Forest, this study provides insights into the complex nature of how $SO_4^{2-}$ addition may affect the biotic component of Fe cycling in terms of IRB abundance. Future research in this area can entail conducting more experiments of adding $SO_4^{2-}$ to Fe-dominated soil environments in order to shed light on inconsistencies in analyses between the two separately run experiments discussed in this study. SRB can also be enumerated in order to provide a more comprehensive portrait of changes in the microbial community composition given the perturbation of $SO_4^{2-}$ addition.

**Broader implications**

This study not only found that $SO_4^{2-}$ addition has significant effects on the growth of IRB but also indicated the existence of interacting effects of $SO_4^{2-}$ addition and soil physico-chemical characteristics on IRB abundance. Complexities in the Fe-S cycling linkages combined with the heterogeneity of soil microoenvironments pose challenges to determining the extent to which $SO_4^{2-}$ addition affects IRB abundance and thus biotic Fe(III) reduction in subalpine wetlands. The generally positive effects of $SO_4^{2-}$ addition on biotic Fe(III) reduction found in this study have implications for increasing DOC exports from wetland soils into watersheds. Increasing levels of Fe(III) reduction in wetlands have been shown to enhance photooxidation of organic matter, which promotes the loss of organic matter from the water column through aggregation and sedimentation (Kritzberg and Ekström 2012).

An enhanced understanding of the interlinkages between Fe and S biogeochemistry also has applications beyond drinking water quality management. Use of Fe(III)-reducing microorganisms in bioremediating contaminated subsurface environments and in harvesting electricity from aquatic sediments and waste organic matter is being explored (Lovley et al.

2004); understanding how the introduction of $SO_4^{2-}$ to these systems may impact the ability of IRB to serve these functions. In addition to its impacts on biotic Fe cycling and water quality by association, $SO_4^{2-}$ deposition may impact other parts of the subalpine wetland system. Soil-plant interactions are, for instance, affected by $SO_4^{2-}$ addition in wetland ecosystems (Pezeshki and DeLaune 2012). $SO_4^{2-}$ adsorption on mineral surfaces is also important, particularly in $SO_4^{2-}$-enriched acidic soils and areas impacted by acid mine drainage. In such environments, $SO_4^{2-}$ forms a template for the formation of schwertmannite, a highly reactive material that adsorbs to toxic elements and can enhance or inhibit adsorption of metals, radionuclides, anions, and organic molecules on mineral surfaces (Zhu et al. 2013). The addition of $SO_4^{2-}$ to a subalpine wetland system can have a multitude of effects on soil microbiology and characteristics that alter the soil's mineralogy and biota.

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## REFERENCES

Akob, D. M., H. J. Mills, T. M. Gihring, L. Kerkhof, J. W. Stucki, A. S. Anastacio, K.-J. Chin, K. Kusel, A. V. Palumbo, D. B. Watson, and J. E. Kostka. 2008. Functional Diversity and Electron Donor Dependence of Microbial Populations Capable of U(VI) Reduction in Radionuclide-Contaminated Subsurface Sediments. Applied and Environmental Microbiology 74:3159–3170.

Al Zuhair, S., M. H. El-Naas, and H. Al Hassani. 2008. Sulfate inhibition effect on sulfate reducing bacteria. Journal of Biochemical Technology 1:39–44.

Bano, N., M. A. Moran, and R. E. Hodson. 1997. Bacterial utilization of dissolved humic substances from a freshwater swamp. Aquatic Microbial Ecology 12:233–238.

Barnett, T. P., J. C. Adam, and D. P. Lettenmaier. 2005. Potential impacts of a warming climate on water availability in snow-dominated regions. Nature 438:303–309.

Blodgett, R. 2010. Appendix 2: Most Probable Number Determination from Serial Dilutions. Page Bacteriological Analytical Manual. U.S. Food and Drug Administration.

Bot, A., and J. Benites. 2005. The importance of soil organic matter: Key to drought-resistant soil and sustained food production. Food and Agriculture Organization of the United Nations, Rome.

Chapelle, F. H., and D. R. Lovley. 1992. Competitive exclusion of sulfate reduction by Fe(III)-reducing bacteria: a mechanism for producing discrete zones of high-iron ground water. Ground Water 30:29–36.

Chowdhury, S., P. Champagne, and P. J. McLellan. 2009. Models for predicting disinfection byproduct (DBP) formation in drinking waters: A chronological review. Science of The Total Environment 407:4189–4206.

Clark, J. M., S. H. Bottrell, C. D. Evans, D. T. Monteith, R. Bartlett, R. Rose, R. J. Newton, and P. J. Chapman. 2010. The importance of the relationship between scale and process in understanding long-term DOC dynamics. Science of The Total Environment 408:2768–2775.

Cord-Ruwisch, R., D. R. Lovley, and B. Schink. 1998. Growth of Geobacter sulfurreducens with Acetate in Syntrophic Cooperation with Hydrogen-Oxidizing Anaerobic Partners. Applied and Environmental Microbiology 64:2232–2236.

Emerson, D., J. V. Weiss, and J. P. Megonigal. 1999. Iron-oxidizing bacteria are associated with ferric hydroxide precipitates (Fe-plaque) on the roots of wetland plants. Applied and Environmental Microbiology 65:2758–2761.

Evans, C. D., P. J. Chapman, J. M. Clark, D. T. Monteith, and M. S. Cresser. 2006. Alternative explanations for rising dissolved organic carbon export from organic soils. Global Change Biology 12:2044–2053.

Flynn, T. M., E. J. O'Loughlin, B. Mishra, T. J. DiChristina, and K. M. Kemner. 2014. Sulfur-mediated electron shuttling during bacterial iron reduction. Science 344:1039.

Fraser Experimental Forest - Site Description. (n.d.). https://www.fs.usda.gov/main/fraser/about/site-description.

Freeman, C., N. Fenner, N. J. Ostle, H. Kang, D. J. Dowrick, B. Reynolds, M. A. Lock, D. Sleep, S. Hughes, and J. Hudson. 2004. Export of dissolved organic carbon from peatlands under elevated carbon dioxide levels. Nature 430:195–198.

Graves, S., H.-P. Piepho, and L. Selzer. 2015. multcompView: Visualizations of Paired Comparisons.

Hansel, C. M., S. Benner, B. W. Wielinga, and S. E. Fendorf. 2001. Evolution of Fe(II) mineralization induced by dissimilatory iron-reducing bacteria.

Hansel, C. M., C. J. Lentini, Y. Tang, D. T. Johnston, S. D. Wankel, and P. M. Jardine. 2015. Dominance of sulfur-fueled iron oxide reduction in low-sulfate freshwater sediments. The ISME Journal 9:2400–2412.

Holmkvist, L., T. G. Ferdelman, and B. B. Jørgensen. 2011. A cryptic sulfur cycle driven by iron in the methane zone of marine sediment (Aarhus Bay, Denmark). Geochimica et Cosmochimica Acta 75:3581–3599.

Houser, T., J. Bordoff, and P. Marsters. 2017. Can Coal Make a Comeback? Page 55. Columbia University Center on Global Energy Policy, New York, New York, USA.

Kayranli, B., M. Scholz, A. Mustafa, and Å. Hedmark. 2010. Carbon Storage and Fluxes within Freshwater Wetlands: a Critical Review. Wetlands 30:111–124.

Knorr, K.-H. 2013. DOC-dynamics in a small headwater catchment as driven by redox fluctuations and hydrological flow paths – are DOC exports mediated by iron reduction/oxidation cycles? Biogeosciences 10:891–904.

Komlos, J., H. S. Moon, and P. R. Jaffé. 2008. Effect of Sulfate on the Simultaneous Bioreduction of Iron and Uranium All rights reserved. No part of this periodical may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without permission in writing from the publisher. Journal of Environmental Quality 37:2058–2062.

Koretsky, C. M., C. M. Moore, K. L. Lowe, C. Meile, T. J. DiChristina, and P. Van Cappellen. 2003. Seasonal oscillation of microbial iron and sulfate reduction in saltmarsh sediments (Sapelo Island, GA, USA). Biogeochemistry 64:179–203.

Koschorreck, M. 2008. Microbial sulphate reduction at a low pH. FEMS Microbiology Ecology 64:329–342.

Kritzberg, E. S., and S. M. Ekström. 2012. Increasing iron concentrations in surface waters—a factor behind brownification? Biogeosciences 9:1465–1478.

Kwon, M. J., M. I. Boyanov, D. A. Antonopoulos, J. M. Brulc, E. R. Johnston, K. A. Skinner, K. M. Kemner, and E. J. O'Loughlin. 2014. Effects of dissimilatory sulfate reduction on FeIII (hydr)oxide reduction and microbial community development. Geochimica et Cosmochimica Acta 129:177–190.

Lalonde, K., A. Mucci, A. Ouellet, and Y. Gélinas. 2012. Preservation of organic matter in sediments promoted by iron. Nature 483:198–200.

Li, C. H., B. L. Ma, and T. Q. Zhang. 2002. Soil bulk density effects on soil microbial populations and enzyme activities during the growth of maize (Zea mays L.) planted in large pots under field exposure. Canadian journal of soil science 82:147–154.

Lovley, D. R. 1987. Organic matter mineralization with the reduction of ferric iron: a review. Geomicrobiology Journal 5:375–399.

Lovley, D. R., J. D. Coates, D. A. Saffarini, and D. J. Lonergan. 1997. Dissimilatory iron reduction. Pages 187–215 *in* G. Winkelmann and C. J. Carrano, editors. Iron and related transition metals in microbial metabolism. Harwood Academic Publishers, Chur, Switzerland.

Lovley, D. R., D. E. Holmes, and K. P. Nevin. 2004. Dissimilatory Fe(III) and Mn(IV) Reduction. Pages 219–286 Advances in Microbial Physiology. Elsevier.

Lovley, D. R., and E. J. P. Phillips. 1986. Organic Matter Mineralization with Reduction of Ferric Iron in Anaerobic Sediments. Applied and Environmental Microbiology 51:683–689.

Lovley, D. R., and E. J. P. Phillips. 1988. Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Applied and Environmental Microbiology 54:1472–1480.

Mast, A. M., J. T. Turk, G. P. Ingersoll, D. W. Clow, and C. L. Kester. 2001. Use of stable sulfur isotopes to identify sources of sulfate in Rocky Mountain snowpacks 35:3303–3313.

Monteith, D. T., J. L. Stoddard, C. D. Evans, H. A. de Wit, M. Forsius, T. Hogasen, A. Wilander, B. L. Skjelkvale, D. S. Jeffries, J. Vuorenmaa, B. Keller, J. Kopacek, and J. Vesely. 2007.

Dissolved organic carbon trends resulting from changes in atmospheric deposition chemistry. Nature 450:537–540.

Myers, C. R., and K. H. Nealson. 1990. Respiration-linked proton translocation coupled to anaerobic reduction of manganese (IV) and iron (III) in Shewanella putrefaciens MR-1. Journal of Bacteriology 172:6232–6238.

Nanus, L., D. H. Campbell, G. P. Ingersoll, D. W. Clow, and M. Alisa Mast. 2003. Atmospheric deposition maps for the Rocky Mountains. Atmospheric Environment 37:4881–4892.

Osorio, H., S. Mangold, Y. Denis, I. Ñancucheo, M. Esparza, D. B. Johnson, V. Bonnefoy, M. Dopson, and D. S. Holmes. 2013. Anaerobic Sulfur Metabolism Coupled to Dissimilatory Iron Reduction in the Extremophile Acidithiobacillus ferrooxidans. Applied and Environmental Microbiology 79:2172–2181.

Pallud, C., and P. Van Cappellen. 2006. Kinetics of microbial sulfate reduction in estuarine sediments. Geochimica et Cosmochimica Acta 70:1148–1162.

Pester, M., K.-H. Knorr, M. Friedrich, M. Wagner, and A. Loy. 2012. Sulfate-reducing microorganisms in wetlands – fameless actors in carbon cycling and climate change. Frontiers in Microbiology 3:72.

Qafoku, N. P. 2015. Chapter Two - Climate-Change Effects on Soils: Accelerated Weathering, Soil Carbon, and Elemental Cycling. Pages 111–172 *in* Donald L. Sparks, editor. Advances in Agronomy. Academic Press.

R Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Roulet, N., and T. R. Moore. 2006. Browning the Waters. Nature 444:283–284.

Scharlemann, J. P., E. V. Tanner, R. Hiederer, and V. Kapos. 2014. Global soil carbon: understanding and managing the largest terrestrial carbon pool. Carbon Management 5:81–91.

Schneider, L. M. 2017, May. Iron and carbon cycling in subalpine wetland soils: interactions with increased temperature and altered sulfate concentrations. University of California, Berkeley, Berkeley, California.

Soil and Plant Nutrient Testing Laboratory. (n.d.). . https://ag.umass.edu/services/soil-plant-nutrient-testing-laboratory.

Soil Texture Calculator. (n.d.). https://www.nrcs.usda.gov/wps/portal/nrcs/detail/soils/survey/?cid=nrcs142p2_054167.

Trump, D. J. 2017, March 28. Presidential Executive Order on Promoting Energy Independence and Economic Growth.

Turk, J. T., and D. H. Campbell. 1997. Are Aquatic Resources of the Mt. Zirkel Wilderness Area in Colorado Affected by Acid Deposition and what Will Emissions Reductions at the Local Power Plants Do? US Department of the Interior, US Geological Survey.

Wallenstein, M. D., and E. K. Hall. 2012. A trait-based framework for predicting when and where microbial adaptation to climate change will affect ecosystem functioning. Biogeochemistry 109:35–47.

de Wit, H. A., J. Mulder, A. Hindar, and L. Hole. 2007. Long-Term Increase in Dissolved Organic Carbon in Streamwaters in Norway Is Response to Reduced Acid Deposition. Environmental Science & Technology 41:7706–7713.

Zabinski, C. A., and J. E. Gannon. 1997. Effects of recreational impacts on soil microbial communities. Environmental Management 21:233–238.

Zweifel, U. L. 1999. Factors Controlling Accumulation of Labile Dissolved Organic Carbon in the Gulf of Riga. Estuarine, Coastal and Shelf Science 48:357–370.