An investigation into the influence of microbes on the cycling of sulfur in a net neutral oxidation reservoir in Sudbury

by

Darla Jane Bennett

A thesis submitted in conformity with the requirements
for the degree of Master of Science

SGES
McMaster University

Abstract

The generation of acid and H_2S associated with microbial cycling of intermediate sulfur species, (sulfur oxidation intermediates; SOIs), is a global mining industry management issue. Both the role of bacteria in SOI transformations as well as comprehensive understanding of the SOIs that can occur within mining wastewaters, are poorly constrained. Key impediments to the industry's ability to improve wastewater sulfur management have been securing a better understanding of the specific sulfur oxidation intermediate (SOI) species that occur in wastewaters, as well as microbial transformations of these sulfur species.

One of the significant prohibitions on the advancement of knowledge in the area of SOI transformations in mine waste waters has been the lack of analytical methods for these species as well as lack of understanding of the controls on these transformations. A significant step forward was established through the development of robust analytical methods using derivatization and HPLC analysis to characterize sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$), sulfide (ΣH_2S) as well as elemental sulfur (S^0). These methods enabled assessment of these sulfur compounds in >60 seasonally and spatially varying wastewater samples collected from Sept 2014 to May 2016. Results identified SOIs were present in all wastewater samples and there were seasonal variations in both concentrations and occurrence of specific SOIs. The mass balance analysis of bulk water samples show that the total sulfur concentration varies seasonally in the system. Higher total sulfur occurred during spring and summer (8.4-13.1 mM) with lower (5.3-10.8 mM) total sulfur observed during the fall and winter sampling campaigns. Further, the proportion of the total sulfur pool associated with sulfate, indicative of complete oxidation of sulfur, were highest during spring and summer (75-100%) with a decreasing

trend through fall (60-75%) and lowest in the winter under ice (10-20%); suggesting temperature may be an important ecological control on sulfur redox biogeochemistry. Corresponding to the observed decreasing seasonal sulfate trend, an increasing trend in the proportion of unanalyzed sulfur species (e.g. $S_4O_6^{2-}$, $S^{2-}_{n+1,}$ $S_nO_6^{2-}$) was also observed, increasing from 0-25% (spring, summer) to 80-90% under ice. Further, elemental sulfur (S^0), which emerged as an important part of the sulfur cycle in these waters, ranged in proportional abundance from 25-99% of the analyzed sulfur species. Elemental sulfur increased during the fall and winter (75-99%), compared to 25-65% during the spring and summer.

Enrichment of sulfur oxidizing microbes (SOM) was conducted to determine whether SOM's were present in endemic waters, and if so, what were the controls on these microbes in terms of cycling SOI's and producing protons. Enrichment experiments were successful from all >60 water samples collected indicating the presence of these bacteria throughout the system over seasonal scales. These SOM catalyzed sulfur transformations consistent with the seasonal SOI characterization results which indicates that SOM are likely important players in sulfur cycling within mine wastewaters. Consumption of thiosulfate was limited to SOM enrichments from waters which were 10 °C or warmer (i.e. spring/summer) and generated sulfate and unanalyzed SOIs in lower and higher proportions respectively than those observed in summer field samples. Consistent with winter field results evidencing lower concentrations of sulfur and sulfate occurrence, winter SOM enrichments only partially consumed thiosulfate and cycled sulfur through different reactions compared to those catalyzed by warmer SoM enrichments.

Analysis of SOI and endemic microbial communities provide a key assessment link in mine environmental management. The new methods that were developed enable more accurate determination of SOI in mining wastewaters. Assessment of SOI within mining waste waters demonstrate that simple H_2S/ SO_4^{2-} measurements will not comprehensively represent sulfur reactions and therefore accurately predict water quality outcomes that occur. Similarly, microbial sulfur metabolism was shown to be possible throughout space and time, but with differing seasonal implications for S cycling in these waters. The inclusion of SOI and SOM understanding into mine wastewater biogeochemical sulfur models will provide prophylactic rather than reactive management strategies.

Acknowledgments

I would like to thank Lesley Warren for her tremendous support and encouragement in all that I needed to manage in these last two years. Her understanding and unwavering commitment to my success as a student allowed me to reach at this point.

I would like to thank Tara Colenbrander-Nelson for her support, both personally and in the lab. Her help with every aspect of the work that we do is invaluable. Her insight and guidance allowed me to work more efficiently and successfully.

Thanks to all members of the McMaster Warren lab group. An amazing group of people that are always willing to lend a hand.

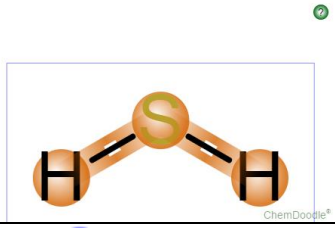
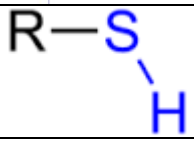
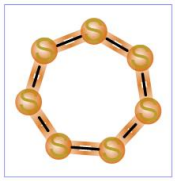
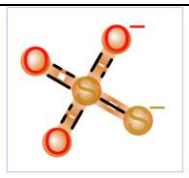
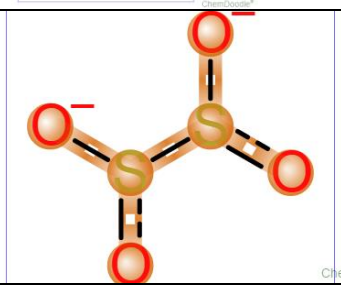
Table of Contents

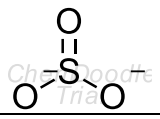
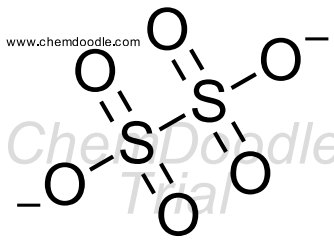
List of Figures

List of Tables:

List of equations:

Table 1: Sulfur species in mine wastewaters with chemical formula structure and common name

Oxidation number	Name	Formula	Molecular structure
-2/-1	Hydrogen sulfide/metal sulfides	H_2S Ie; Fe_2S	
-2	Thiols a	R-SH	
0	Sulfur	S^0 S_8	
2	Thiosulfate	$S_2O_3^{2-}$	
3	Dithionite	$S_2O_4^{2-}$	

4	Sulfite	SO_3^{2-}	
5	Dithionate	S_2O_6	

1 Outline of the project and mining and developments in remediation of pyrite oxidation throughout time.

Sulfuric acid, historically known as vitriol, has been a topic of interest and study since the 16th century (Lottermoser, 2007). Diego Delgado conducted the first recorded experiment on acid mine drainage (AMD) affected waters in 1556 in Spain (Rickard, 2015; Lottermoser, 2007). He documented the waters effect on iron (both present in the water and added by Delgado) and sediment which was present in the river; he also placed a living frog into the water and recorded the frogs' rapid demise (Rickard, 2015; Lottermoser, 2007). The water he conducted these experiments on was Rio Tinto, a river which flows through an area that has been mined for thousands of years (Rio Tinto, 2016). Delgado was actually preforming a series of experiments which tested the geochemical characteristics of AMD.

Mining and environmental issues associated with mining have existed for thousands of years. As the waste associated with mining is very high volume, the first practices of waste control focused on minimizing costs and space (Bell and Bullock, 1996; Bell and Donnelly, 2006; Freihammer, 2016; Ledin, 1996; Lottermoser, 2007). Up until the turn of the 20th century, many mines simply released their waste into the nearest water body or heaped piles of waste rock in the nearest convenient area (Lottermoser, 2007). As mining practices evolved and grew throughout the industrial times, so did the industries knowledge of their impact on the environment. Increased mining and knowledge led to increased governmental regulations which governed these

companies (Bell and Bullock, 1996; Bell and Donnelly, 2006; Freihammer, 2016; Ledin, 1996; Lottermoser, 2007). This increase in mining practices and necessity of waste management brought about increased research on mining waste, in specific, the sulfide minerals which, as a result of mining practices, are exposed to conditions which compromise their stability and generate acidity (Bell and Bullock, 1996; Bell and Donnelly, 2006; Freihammer, 2016; Ledin, 1996; Lottermoser, 2007).

Prior to ~1920 AMD was thought to be driven purely by chemical kinetics. The thought being that reduced sulfur species are stable under anoxic or microaerobic conditions and when they are brought to the surface and exposed to air, they undergo oxidation reactions which bring about AMD conditions (Loew, 1894; Lackey, 1938; Lackey, 1939). Convention of the times was to air seal mines when production was completed in an attempt to stop this process from occurring (Loew, 1894; Lottermoser, 2007; Lackey, 1938; Lackey, 1939). In 1925 studies of AMD sites began to identify that although these areas were fairly devoid of life, there was a significant microbal community which existed in these waters and studies were undertaken in an attempt to constrain which microbes were present (Lackey, 1938; Lackey, 1939). In the late 1930's some of these studies were complete and identified that, although life was significantly hampered by increased acidity, at low acidities microbes and other microscopic life forms were present (Lackey, 1938; Lackey, 1939). It was identified also that these microbes were highly diverse, specific to AMD waters and presenting in novel ways (Colmer and Hinkle, 1947; Lackey, 1938; Lackey, 1939). *The hypothesis resulting from some of these findings was that these microbes represented a path forward, that they were in fact the precipitators of change, the change being a return to more neutral life*

sustaining waters (Colmer and Hinkle, 1947; Lackey, 1938; Lackey, 1939). In spite of this hypothesis and these findings, microbes were still not thought to play a role in AMD in any regard—they only represented hope for remediation (Colmer and Hinkle, 1947; Lackey, 1938; Lackey, 1939). It wasn't until the 1940's that the role microbes play in AMD formation was investigated (Colmer and Hinkle, 1947). At this time experiments were done which tested AMD water under variable aerobic conditions and with and without the presence of microbes (Colmer and Hinkle, 1947). These experiments showed that without microbes AMD did not readily form (Colmer and Hinkle, 1947). This knowledge was fundamental to understanding and dealing with mine waste and also began to shift the focus from macroscopic and chemical processes to the fundamental role microscopic life forms play in nearly every aspect of life on this earth.

Although mine waste treatment had improved since the turn of the century, understanding exactly what was causing the rapid formation of acid from mine tailings allowed research to begin on what could be done to prevent this process from occurring. There was an increased number of remediation and treatment strategies which were developed and initiated during the time period of 1940-1990 (Akcil and Koldas, 2006; Bell and Bullock, 1996; Bell and Donnelly, 2006; Colmer and Hinkle, 1947; Gazea, Adam and Kontopoulos, 1996; Gray, 1997; Johnson and Hallberg, 2005; Ledin, 1996; Lottermoser, 2007). Increased knowledge developed as a result of the increasing capacity to identify microbes and their functions. This was coupled with an increased ability to model systems to determine what changes were likely to occur and an increased ability to measure systems due to advances in analytical instruments. The ability to model systems lead to increasing ability to predict how systems would change

over time and what was necessary to treat these systems (Reardon and Beckie, 1987; Singer and Stumm, 1970). All of this research lead to a more “systems” approach when dealing with mine waste as opposed to a “simple” chemical treatment (Lottermoser, 2007). The way mine waste systems were thought about and treated underwent a revolution based on scientific exploration and advances in techniques; mainly instrumental and sequencing (Akcil and Koldas, 2006; Gazea, Adam and Kontopoulos, 1996; Ledin, 1996; Neculita, Zagury and Bussière, 2007). These advances sparked a plethora of research into AMD, the microbes which function in these systems and the chemistry and geochemistry of these systems (Akcil and Koldas, 2006; Baker and Banfield, 2003; Barton, Mandl and Loy, 2010; Bell and Donnelly, 2006; Gazea, Adam and Kontopoulos, 1996).

The focus of all of this research however remained on the remediation and treatment of waste which was generated by AMD microbes and sulfide reactions. Although this research and the resulting remediation methods brought increased environmental and public safety, remediation still has not reached the point where we can successfully remediate mine waste. After many years of research perhaps the time has come to incorporate another line of inquiry into remediation efforts. This new line of research can now focus on prophylactic methods of mine waste treatment. Prophylactics are the course of action which usually develops after much is known about particular system of interest—as is the case with mine waste today. Prophylactics for mining waste are possible and were initiated first with modelling programs (Reardon and Beckie, 1987; Singer and Stumm, 1970). Mine waste modelling programs however are unable to factor in all of the complexities of mine wastewater. These complexities

are mainly a result of microbes and their interactions with other microbes, chemical species (ie: C, S and Fe) and physicochemical conditions and changes which are present in these waters. New remediation strategies need to take into consideration all that is currently known and incorporate new research. This new research is based on advances in sequencing capabilities and analytical instrumentation and methodology.

In the case of mining waste--we now know that it is not just microbial and O_2 driven oxidation of pyrite to sulfate which are the perpetrators of problems in mine wastewater. Intermediate sulfur reactions (SOI) can occur within these waters and, just as AMD is the result of microbial action, this sulfur cycling is heavily biologically catalyzed. Just as historically we had missed the fact that microbes are the drivers for AMD reactions, it is the case now that we are missing some of the more complex sulfur reactions that are occurring and potentially contributing to sulfur cycling issues in these waters.

The SOI reactions, which are occurring in these waters, remain poorly constrained beyond a general understanding. As mentioned previously—modelling apps, such as PHREEQC or PHREEQC-I are unable to accurately predict which sulfur redox reactions will occur and under which conditions (Wwwbrr.cr.usgs.gov, 2016). So there is, at this time, an industry wide need to understand thiosalt behavior.

Innovative science can help identify some of the missing links and could enable mines to more effectively monitor, manage and control thiosalts.

A key component of starting to fill in these blanks is better characterization of the S redox species in conjunction with microbial community characterization within mine waters themselves.

1.1 The global sulfur cycle and its main players.

Carbon, at high pressure and temperature may be a girl's best friend; but sulfur with its impressive redox and bonding capacities, is humankind's best friend. Sulfur is the tenth most abundant element on earth (Judge, 2009), sixth when considering microbial biomass (Poole, 2012). Sulfur is present in the air, earth, and water and is a component of many economically viable ores (Appendix 1) (Vaughan and Craig, 1978). Not only are sulfurous compounds environmentally ubiquitous, sulfur is one of the elements essential to all life; whether it be due to the metabolic processes of microbes or the necessity of S components in eukaryotic cells (Falkowski, 2015). The three most abundant forms of sulfur are elemental sulfur, sulfate, and sulfide (with oxidation states of 0, +6 and -2 respectively) followed by the intermediate redox state forms sulfite, dithionate, thiosulfate and polythionites (+4, +3, +5/-1 and 0/+5 respectively) (Poole, 2012). (Benn, Mareschal and Condie, 2006; Canfield, Rosing and Bjerrum, 2006; Poole, 2012).

The sulfur cycle seen in Figure 2 is diverse and complex, but the main components of the cycle are oxidation and reduction of the various sulfur species—either biotically or abiotically (Table 2: Important biotic and abiotic S reactions (Rickard & Luther, 2007 & Figure 2). Considering that just the reduction of sulfate coupled with the oxidation of organic matter in anaerobic environments produces ~2.7 $\times 10^9$ tons of H_2S annually *(*Finster*,* 2008) then estimating that a very small fraction (~1-2%) of this sulfide is permanently (on human time scales) sequestered as pyrite,

the remaining sulfide (~98-99%) must be oxidized either chemically or biologically by various microbes (Finster, 2008). These microbes must couple this oxidation with the reduction of oxygen, nitrate, metal oxides and other electron acceptors (Figure 1) to cycle these components, all of which are substantial fractions of our atmosphere and biosphere. Following this out, it is evident that microbial S metabolisms play a fundamental role in the biogeochemical cycles on earth (Finster, 2008).

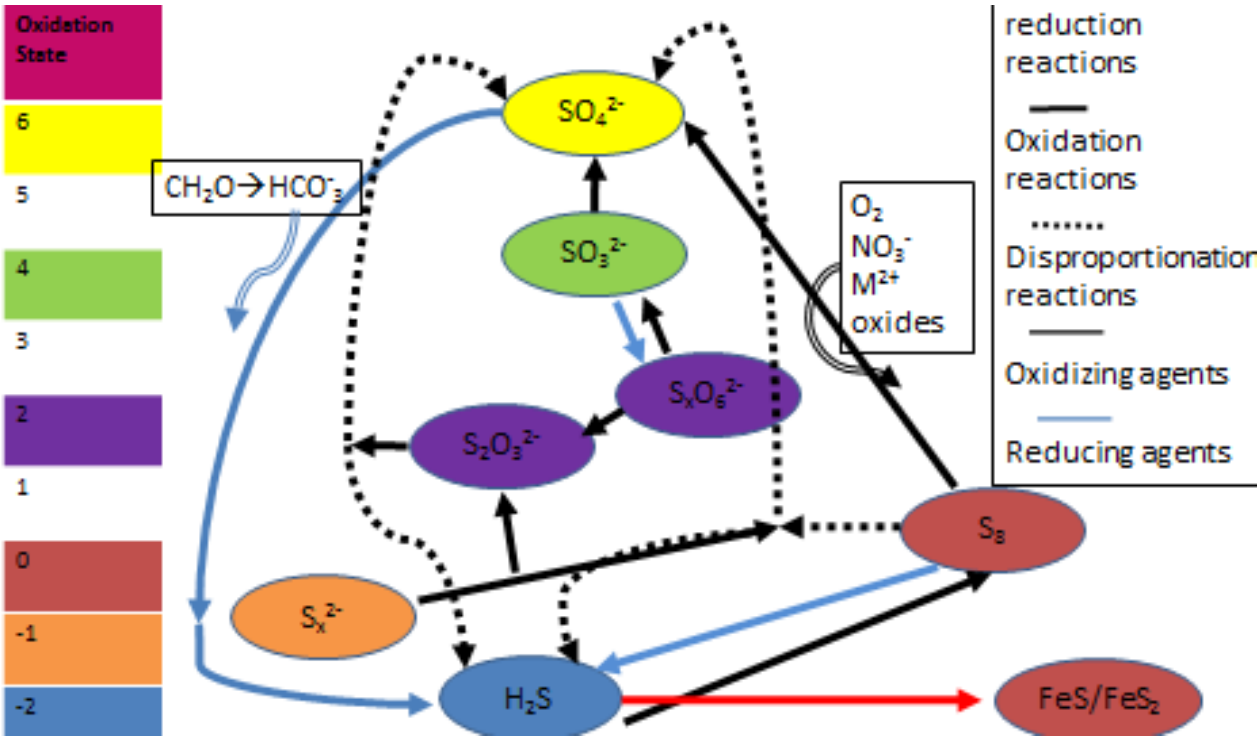


Figure 1: The sulfur cycle showing oxidation and reduction with various electron acceptors and donators. Adapted from (Zopfi, Ferdelman and Fossing, 2004)

As mentioned, sulfide, sulfate and elemental sulfur are the main sulfurous components on earth and as such they are a substantial part of the redox cycles of sulfur. They are also the “apparent” part of the sulfur cycle and are relatively easily and well-studied (Finster, 2008). The mechanisms of sulfate and sulfur reduction by

microbes such as eubacteria and archaea have been well documented since the 1800's (Brunton and Macfadyen, 1889; Loew, 1894). Sulfide and sulfur oxidation reactions by phototrophic and chemolithotrophic bacteria are likewise well documented since the 1800's ((Brune, 1989; Friedrich et al., 2001; Ghosh and Dam, 2009; Gottschalk, 1986; Tang, Baskaran and Nemati, 2009; Winogradsky, 1890).

Following the main species of the sulfur cycle--H_2S is oxidized to sulfate or elemental sulfur by sulfur oxidizing bacteria or chemically under oxygenated/nitrogen rich environments (Kuenen, Robertson And Van Gemerden, 1985). Elemental sulfur and sulfate can then be reduced to sulfide by sulfur-reducing bacteria (Kuenen, Robertson And Van Gemerden, 1985). In most cases, the microbial oxidation of sulfide coupled with the reduction of carbon dioxide/oxygen/nitrate produces organic molecules/water/nitrogen gas. These two complimentary actions—sulfur oxidizing microbes and sulfur reducing microbes make up a substantial part of the sulfur cycle (Kuenen, Robertson And Van Gemerden, 1985). They are likewise dependent on one another for survival (Baker and Banfield, 2003)

Intermediate sulfur species are likely to play an important role for these cycles and can be termed the invisible S cycle. This "invisibility" is due to the fact that it is much more difficult to detect and understand these processes in environmental systems (Bernier, 2008; Justice et al., 2014; Tang, Baskaran and Nemati, 2009). Current issues are an incomplete understanding of microbial interactions, the metastability of the intermediate species and therefore difficulties in accurate analyses and the lack of knowledge of the metabolic

processes of the microbes involved (Kamyshny et al., 2004; Kuenen, Robertson And Van Gemerden, 1985; Montoya et al., 2015; Poole, 2012).

The cycling of intermediate sulfur compounds is likely heavily influenced by microbial disproportionation reactions. Unlike reductions and oxidations, disproportionation reactions are not as well defined, and mention of this type of microbial metabolism was not present until later in the 20th century (Finster, 2008; Tuttle and Jannasch, 2015). Many microbes can disproportionate sulfur intermediates but do not solely live off this type of metabolism because it has lower energy yields (Figure 2 and Figure 6).

microbes which utilize sulfur species for their metabolic functions	**sulfite**	**thiosulfate**
organism		
sulfate reducing microbes		
D. curvatus	/	D
D. hydrogenophilus	/	D
D. postgatei	/	/
D. autotrophicum	/	/
D. phenolicum	/	/
D. propionicus	/	D
D. multivorans	/	D
D. orientus	/	/
D. desulfuricans CSN	G	D
D. gigas	/	
D. sapovorans	/	
D. sulfodismutans	G	G
D. vulgaris Strain NTA 3 and Bra02	G	G
where G=growth, D=disproportionation with or without growth		

(Table adapted from Kramer and Cypionka,1989)

Table 2: Microbes known to be capable of intermediate sulfur species disproportionation

As mentioned above, the energy gained from disproportionation is less than reduction or oxidation of sulfur compounds. *However,* it serves two important functions. Disproportionation allows an organism to use sulfur intermediates, which are often a product of incomplete oxidation/reduction; and it should be more favorable if substrates are limited (Rabus, Hansen and Widdel, 2006; Rickard, 2012; Tang, Baskaran and Nemati, 2009; Tuttle and Jannasch, 2015). Disproportionation can lead to the cycling of substrates where the reduced product can subsequently be re-oxidized back to the intermediate used for disproportionation, perpetuating a sulfur cycle amongst intermediate species (Figure 3 black circle) (Hubbard et al., 2014; Podgorsek and Imhoff, 1999). In these ways microbial disproportionation allows for adaptation by increasing the range of energy yielding reactions and by increasing the opportunity for mutualism or symbiosis with other S respiring microbes (Podgorsek and Imhoff, 1999). Disproportionation reactions are of particular interest in that they have the ability to be a proton neutral, consuming or generating process in varying degrees depending on the specific reactions occurring and the species being cycled (Equation 14, Equation 15, Equation 16; pages 45-46) Because of their ability to consume protons and operate at a proton neutral level, disproportionating bacteria may play a key role in mediating more acid generating processes in natural water systems (Figure 3).

Disproportionation in red	Oxidations and reductions in black
Red stars=H^+ production	Blue stars= H^+ consumption

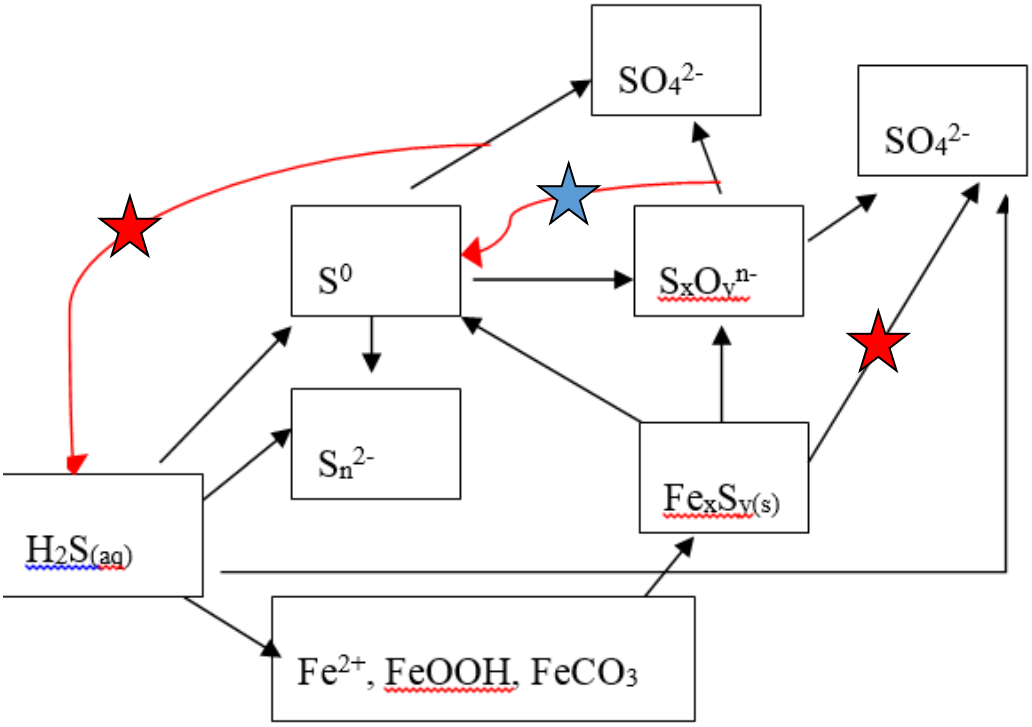


Figure 2: Possible disproportionation reactions of sulfur intermediates

1.2 Mine sulfur cycle

The mining of metal ores produces significant waste, as generally the target material is present in rocks in the lower percent to ppm range (Johnson, 2014). The waste can be classified in two general streams—waste rock or gangue, in mining terms, and tailings (Johnson, 2014). The waste rock is separated into large piles and stored on site as they generally have less tendency toward acid mine drainage (AMD) formation than tailings do (Lengke, Davis and Bucknam, 2010; Johnson, 2014). Their lower tendency to reactivity is due to the surface area of the rock compared to the tailings, which have been ground very fine to allow for metal separation. Most metals which are mined contemporarily are sulfide minerals; metal sulfides are often found together and so with the target material comes other ancillary metal sulfides which are not mined for (Johnson, 2014; Nancucheo and Johnson, 2011). Typically the undesirable sulfide present in the highest concentration is pyrite (FeS_2) (Johnson, 2014; Nancucheo and Johnson, 2011). Pyrite contains reduced sulfur and it is therefore unstable under surface ambient conditions. Whereas in waste rock the sulfides are not as exposed to direct oxygen and water, the tailings are already in an aqueous stream and are very susceptible to oxidation due to their high surface area to volume ratio. Pyrite exposed to water and oxygen has the tendency towards the following reaction:

Equation 1: $\mathbf{8\ FeS_2 + 30\ O_2 + 18\ H_2O \rightarrow Fe_8O_8(SO_4)(OH)_6 + 15\ SO_4^{2-} + 30\ H^+}$

(Johnson, 2014; Nancucheo and Johnson, 2011)

The kinetics of this reaction are quite slow however. The rate of this reaction (k) (moles/m^2-s) = $10^{-8.10}$ *[O_2] $^{0.5}$ /[H^+] $^{0.11}$(Moses et al., 1987; Williamson and Rimstidt,

1994). At ambient oxygen concentrations this results in a pyrite oxidation rate of $1.8*e^{-5}$ moles/m^2*day. This reaction does result in the generation of a large quantity of protons as it proceeds however. The generation of acid allows for the growth of *Thiobacillus ferrooxidans. T. ferrooxidans* catalyzes the following reaction increasing in rate as colonies grow:

Equation 2: $\mathbf{4Fe^{2+}_{(aq)} + O_2 + 4H^+ \rightarrow 4Fe^{3+}_{(aq)} + 4H_2O}$

(Johnson, 2014; Nancucheo and Johnson, 2011).

And herein lies the key to the development of many problems with mine waste. As mentioned earlier, the oxidation of pyrite with only oxygen is slow, however the oxidation of pyrite using Fe^{3+} is more rapid. The rate of this reaction (k) (moles/m^2 -s) = $10^{-8.58\,*}$ $[Fe^{3+}]^{\,0.3}$ / $[Fe^{2+}]^{\,0.47}$ *$[H^+]^{\,0.32}$(Moses et al., 1987; Williamson and Rimstidt, 1994). This results in a pyrite oxidation rate of $5.8*e^{-3}$ moles/m^2*day. In one week the difference in pyrite oxidation is 11 moles/m^2*week (abiotically) compared to 3508 moles/m^2*week with bacterial catalysis. The reason for this lies in the production of Fe^{3+} ions from these microbes, their proximity to the material—they often bind to the surface, low pH which inhibits chemical oxidation mechanisms, and their use of enzymes to facilitate the reaction.

Equation 3: $\mathbf{S^{2-}/S^0 + 4Fe^{3+} + 3H_20 \rightarrow H_2SO_3 + 4Fe^{2+} + 4H^+}$

(Evangelou, 1995)

Equation 4: $\mathbf{FeS_2 + 14\ Fe^{3+} + 8\ H_2O \rightarrow 15\ Fe^{2+} + 2\ SO_4^{2-} + 16\ H^+}$

(LIZAMA and SUZUKI, 1989)

The ferric iron produced is an efficient oxidizer and reacts with pyrite to create even more acidity and a sustaining reaction loop.

This is the situation for mines which are acid generating. However the majority of contemporary mines are not acid generating. Their sulfur chemical cycling follows different paths and may be under the influence of different microbes. What those microbes are, how they function, and how they influence their surroundings is not well known, studied, or understood. What is known is some general chemistry about these systems. General chemistry however is based on ideal conditions and is not an accurate reflection of these systems but it does give us a launching pad to start our research.

In net neutral systems the oxidation of pyrite and other sulfide minerals occurs via chemical oxidation and microbial action. The complete oxidation of the end member sulfides (H_2S or FeS_2 with oxidation states of -2 and -1 respectively) to sulfate (SO_4^{2-} oxidation state 6+) requires the transfer of 7 to 8 electrons. The energy required for this complete oxidation makes it unlikely to occur in one step. The oxidation of reduced sulfur species goes through a number of intermediate species, which are termed sulfur oxidation intermediates (SOI) (figure 1 & table 3). SOI's are also produced during mining processes such as flotation extraction and creating fine particles from the ore (Johnson, 2014). What SOI's are present at any given time and which are stable is dependent on a number of factors which include pH of the solution, available electron donors and acceptors, the presence of microbes which utilize these compounds, and

other physicochemical characteristics of the water system (see figure 3). These SOI's pose a significant risk to water bodies as reactions involving them can delete oxygen in the water body as well as generate acid, albeit on slower time scales than *T. Acid.* (Johnson, 2014).

SOI's are important sulfur species in mine waters as they can be precursors to more serious issues with water quality, dependent upon how they are cycled. Equations 4 through 6 show some disproportionation examples. As one can see in some cases protons are consumed or produced and in some hydroxides are consumed or produced. Which of these reactions occur and how many occur at any given time is dependent upon: the microbes, which species are available for redox coupling and what the water characteristics are. Therefore to understand mine wastewater SOI cycling in neutral conditions many parameters need to be studied and taken into consideration.

Equation 5: $S_2O_3^{2-} + OH^- \leftrightarrow HS^- + SO_3^{2-} + 1/2O_2$

(Amend, Edwards and Lyons, 2004)

Equation 6: $4SO_3^{2-} + H^+ \rightarrow HS^- + 3SO_4^{2-}$

(Schulz and Zabel, 2006)

Equation 7: $S^0 + 4H_2O \rightarrow SO_4^{2-} + 4H^+$

(Schulz and Zabel, 2006)

Equation 8: $S_nO_6^{2-} + S_2O_3^{2-} \leftrightarrow S_{n+1}O_6^{2-} + SO_3^{2-}$

(Evangelou, 1995)

In addition to microbial action and chemical characteristics, SOI's are also controlled by the presence of other SOI's in solution. Therefore there is no reliable way to predict what is present, what reactions are happening and likely to occur and how these nutrients are being utilized by microbes unless we sample a particular site and take all of these parameters into consideration. Equation 7 predicts likely behavior of SOI's in solution. This reaction is likely to move to the right at pH<7 and to the left at pH>7. Equation 7 again gives us a starting point in which to determine which species are more likely to be stable at a given pH however, as with many other chemical predictors—the presence of "life" changes the outcome. Additionally the precipitation of sulfur also effects the concentration of SOI species in mine waters by increasing the relative oxidation state of soluble sulfur species (Nordstrom, 2000).

As mentioned, pH values affect the speciation of SOI's in water—at pH values under 7 thiosulfate is sensitive to acid decomposition and sulfite is more susceptible to oxidation (equation 9). This leads to an accumulation of elemental sulfur and polythionates in waters, excluding microbial cycling (Druschel, Hamers and Banfield, 2003; Kamyshny et al., 2004; Licht and Davis, 1997; Meyer, 1977; Nordstrom, 2000). Temperature also effects speciation, where higher temperatures lead to an increased amount of polysulfides and increased chain length (Kamyshny et al., 2008). At pH values greater than 7, thiosulfate is more stable and very resistant to oxidation and will therefore persist in aqueous solution (Nordstrom, 2000).

Equation 9: $5S_2O_3^{2-}+18H^+ \rightarrow S_8+2SO_3^{2-}+9H_2O$

(Konhauser, 2007)

In section 1 it was shown that microbes have a significant impact on the global sulfur cycle in acidic and neutral conditions. This role is explored more in depth in section 7 and 8 however suffice it to say that microbes are also likely to play a significant role in the cycling of sulfur in neutral mine environments, just as they have been shown to influence mine waters which are acidic. Microbes are known to utilize SOI's in oxidation as well as disproportionation reactions (please see section 7 and 8) however how these electron sources/sinks are cycled, supplied and the reactions which dominate in certain conditions, are not known. As shown above, the conditions of the water system, the concentrations of other sulfur species, and microbial catalysis, can all have a significant effect on which sulfur species dominate, which reactions are favored, and which reactions microbes can use to gain energy. Greater understanding of these important controls on sulfur dynamics will be very useful in predicting and modelling mine wastewater systems over time.

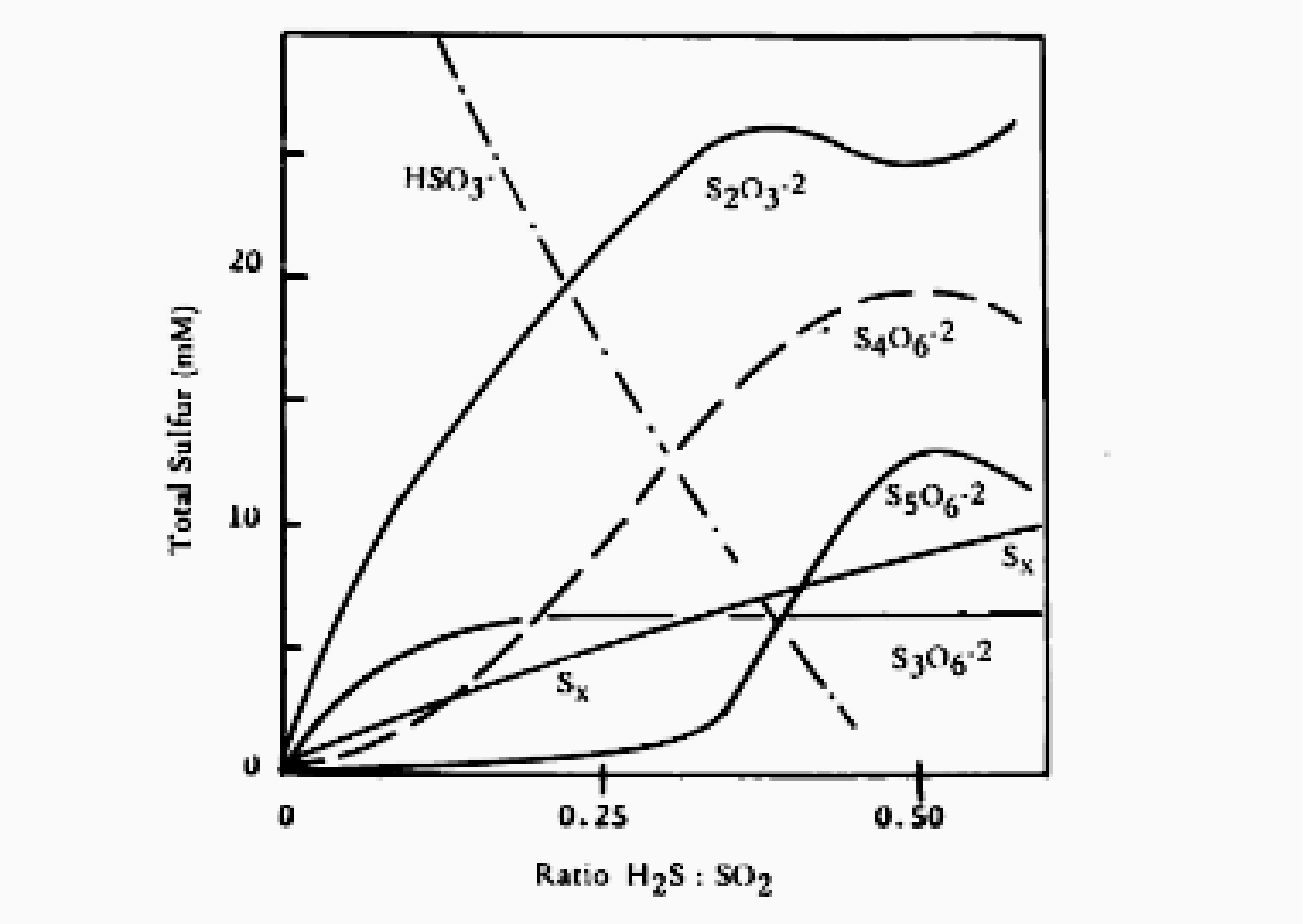


Figure 3: SOI as a function of sulfur species in wackenroder's solution (Reproduced with permission from Meyer, 1977).

1.3 Abiotic reactions involving solid and aqueous sulfur species in neutral mine wastewaters

Intermediate sulfur compounds are abundant in aqueous environments and have important and poorly characterized roles in the sulfur cycle—especially in mine wastewaters. Below are a few of the reactions which form important sulfur intermediates without the aid of microbes. This demonstrates that, even abiotically, there is an available supply of these species for microbes in neutral environments.

In oxygen rich waters sulfide produced will be oxidized to sulfate—the stable S phase. In anoxic waters iron hydroxides and oxyhydroxides can oxidize sulfide to sulfur. This process happens via sorption processes; the kinetics of this reaction are dependent on the surface area to volume ratio (Rickard, 2012).

Equation 10: $Fe^{3}OH + HS^{-} \rightarrow Fe^{3}S^{-} + H_2$

(Rickard, 2012)

Equation 11: $Fe^{3}S^{-} \rightarrow Fe^{2}S^{*}$

(Rickard, 2012)

Equation 12: $Fe^{2}S^{*} + H_2O \rightarrow Fe^{2}OH_2^{+} + S^{*-}$

(Rickard, 2012)

Equation 13: $Fe^{3}OH + S^{*-} \rightarrow S_8 + Fe$

(Rickard, 2012)

Manganese hydroxides/oxyhydroxides can also oxidize sulfides—more effectively than its iron counterparts due to the reaction (note 2 FeOOH for 1 MnO_2) (Rickard, 2012):

Equation 14: $MnO_2 + HS^- + 3H^+ \rightarrow Mn^{2+} + S + 2H_2O$

(Rickard, 2012)

Equation 15: $2FeOOH + HS^- + 5H^+ \rightarrow 2Fe^{2+} + S + 2H_2O$

(Rickard, 2012)

Thiols are formed via reactions between organic matter and aqueous sulfate and are dependent upon the sulfate concentration in solution (Bernier, 2008).

There are more allotropes of sulfur than any other element with the exception of carbon (Greenwood and Earnshaw, 1984). Elemental sulfur forms rings of various numbers of S, with S_8 rings being the most common. It also reacts with sulfide in solution to produce polysulfides (Greenwood and Earnshaw, 1984).

Thiosulfate is formed via reaction between HSO_3 and HS^- (Equation 16) and in aqueous solution can be oxidized by reductants such as chlorite; it will also react with

ferric iron, free protons or organic matter to produce polythionates, sulfite, and organically bound thiosulfate respectively (Liebensteiner et al., 2014; Rickard & Luther, 2007).

Thiosulfate formation:

Equation 16: $4\ HSO_3 + 2\ HS^- \rightarrow 3\ S_20_3^{2-} + 3\ H_20$.

(Liebensteiner et al., 2014)

Polythionates are various S chain length molecules in which the chain length is a function of the following equilibrium (Equation 17) (Druschel, Hamers and Banfield, 2003)

Equation 17: $S_xO_6 + S_2O_3^{2-} \rightarrow 7\ S_{x+1}O_6 + SO_3^{2-}$

(Druschel, Hamers and Banfield, 2003)

As mentioned, polythionates are typically found in chain lengths ranging from 3→6 (Druschel, Hamers and Banfield, 2003). The abundances of each are a function of the following reaction(s) (Equation 18):

Equation 18: $2\ S_4O_6^{2-} \leftrightarrow S_3O_6^{2-} + S_5O_6^{2-}$

(Druschel, Hamers and Banfield, 2003)

Tetrathionate in particular reacts with sulfite to produce thiosulfate and trithionate, with sulfide to form elemental sulfur and thiosulfate, and with water to produce thiosulfate, elemental sulfur and sulfate (Podgorsek and Imhoff, 1999).

key sulfur oxidation reactions relevant to mine waters
Abiotic
$H_2S+0.5\ O_2 \rightarrow S^0+H_2O$
$H_2S+2Fe(OH)_{3(s)}+6H^+ \rightarrow 2\ Fe^{2+}+S^0+6\ H_2O$
$H_2S+O_2 \rightarrow 0.5\ S_2O_3^{2-}+0.5\ H_2O+H^+$
$FeS_2+6\ Fe^{3+}+3\ H_2O \rightarrow 7Fe^{2+}+S_2O_3^{2-}+6H+$
$H_2S+1.5O_2 \rightarrow 2\ Fe^{2+}+SO_3^{2-}+2\ H^+$
$S^0+\ H_2SO_3 <\rightarrow S_2O_3^{2-}+2\ H^+$
$2\ S_2O_3^{2}+2Fe^{3+} \rightarrow 2\ FeS_2O_3^{+} \rightarrow 2Fe^{2+}+\ S_4O_6^{2-}$
$S_4O_6^{2}+3Fe^{3+}+2.75\ O_2+4.5\ H_2O \rightarrow 4SO_4^{2-}+9H^+ +3Fe^{3+}$
$SO_3^{2-}+0.5O_2 \rightarrow\ SO_4^{2-}$
$Fe^{3+}+\ HSO_3^- \rightarrow SO_3^- +Fe^{2+}+H^+$
Biotic
$H_2S+0.5\ O_2 \rightarrow\ S^0+H_2O$
$S_2O_3^{2-}\ +2\ H_2O \rightarrow\ S^0+\ H_2SO_3+2OH^-$
$2S_2O_3^{2-}\ +\ 0.5\ O_2\ +\ 2H^+ \rightarrow\ S_4O_6^{2-}\ +H_2O$
$S^0+\ H_2O\ +O_2 \rightarrow\ H^+ +\ HSO_3^-$
$4Fe^{3+}+S^0+3\ H_2O \rightarrow H_2SO_3\ +\ 4\ Fe^{2+}+4\ H^+$
$S_4O_6^{2-}\ +\ 14Fe^{3+}+10\ H_2O \rightarrow 4SO_4^{2-}+14\ Fe^{2+}+20\ H^+$
$2S_4O_6^{2-}+\ 2\ H_2O \rightarrow 2S_2O_3^{2-}+\ 2\ S^0+\ 2\ SO_4^{2-}+4\ H^+$
$H_2SO_3+\ 0.5\ O_2\ \rightarrow SO_4^{2-}+2\ H^+$
$H_2SO_3\ +\ 2Fe^{3+}+\ H_2O\ \rightarrow 4\ H^+ +\ 2Fe^{2+}+\ SO_4^{2-}$

Table 3: Important biotic and abiotic S reactions

1.4 Possible microbial sulfur/sulfide oxidizing and disproportionating metabolisms and reactions in neutral mine wastewaters

Microbes utilize compounds in the environment according to thermodynamic principles, which vary for particular environmental conditions such as pH, temperature, electron donor and acceptor concentrations, and reaction kinetics—microbes will oxidize/reduce species which will yield the greatest energy (Figure 5) (Friedrich and Finster, 2014). The following description mentions S reducers and acknowledges their importance in S cycling however this section focuses on the products of S oxidation and disproportionation as these reactions are the likely and relevant reactions in net neutral mine waste (Barton, 1995; Rabus, hansen and Widdel, 2006; Tuttle and Jannasch, 2015)

redox couples	E'0 (V)
CO_2/glucose	-0.43
2H+/H_2	-0.42
CO_2/methanol	-0.38
NAD+/NADH	-0.32
CO_2/acetate	-0.29
elemental sulfur/hydrogen sulfide	-0.29
sulfate/hydrogen sulfide	-0.22
pyruvate/lactate	-0.18
tetrathionate/thiosulfate	0.02
Fe3+/Fe2+ (pH 7)	0.1
NO_3-/NO_2-	0.45
NO_3-/1/2N_2-	0.75
Fe3+/Fe2+ (pH 2)	0.77
1/2 O_2/H_2O	0.81

(adapted from Koofers, 2015)

Figure 4: Redox energy for some specific redox couples related to mine waste nutrients and cycles

Sulfur respiring microbes are a diverse group (Table 4) some of the most prominent are the colourless sulfur bacteria which are found in a wide range of environmental conditions.

Colourless sulfur microbes:

Colourless sulfur microbes are a large and diverse group of gram negative microbes, which include the genus *thiobacilli* and *thiobacterium* (Kuenen, Robertson And Van Gemerden, 1985). Of these the *thiobacilli* are the most prominent. They can function as heterotrophs or autotrophs (Table 3) and can utilize both O_2 and nitrate as electron acceptors (Kuenen, Robertson And Van Gemerden, 1985; Rickard, 2013). They are found growing in all concentrations of O_2 and can utilize substrates such as

tetrathionate, thiosulfate, and thiocyanate in anaerobic conditions and elemental sulfur and sulfide in aerobic conditions (Kuenen, Robertson And Van Gemerden, 1985; Rickard, 2013).

	metabolic definition used to describe colourless sulfur microbes			
	energy source		carbon source	
	reduced sulfur compounds	organic compounds	CO2	organic compounds
obligate autotroph	+	-	+	-
facultative autotroph	+	+	+	+
chemolithotroph	+	+	-	+

(adapted from Kuenen, Robertson And Van Gemerden, 1985)

Table 4: metabolic categories of colourless sulfur bacteria

Table I. Metabolic Types Found among Colorless Sulfur Bacteria

Metabolic definition	Genera	Representative species	Respiratory type	Special requirement
Obligate chemolithotroph	*Thiobacillus, Thiomicrospira*	*H. neapolitanus*	O_2	
		Thiobacillusferrooxidans	0_2	Acidophilic
		Thiobacillus denitrificans	$0_2/NO^{3-}$	
		Sulfurimonas denitrificans	O_2/NO^{3-}	Microaerophilic
Facultative chemolithotroph	*Thiobacillus, Sulfolobus, Ther.nothri.x, Faracoccus, Thiosphaera, Beggiatoa*	*Thiobacillus intermedius*	0_2	
		Beggiatoa	o_2	Microaerophilic
		Thiobacillus acidophilus	0_2	Acidophilic
		Sulfolobus acidocaldarius	0_2	Acidophilic, thermophilic
		Thiobacilus versutus	$O_2/N0^{3-}$	Denitrifies heterotrophically
		Thiosphaera pantotropha	O_2/NO^{3-}	
Chemolithoheterotroph	*Thiobacillus, Pseudomonas*	*Thiobacillus perometabolis*	0_2	
Heterotroph (oxidizes S_2 but does not appear to gain energy)	*Beggiatoa, Pseudomonas*	*Beggiatoa*	$0_2/S^0$	

(adapted from Kuenen, Robertson And Van Gemerden, 1985)

Table 5: colourless sulfur microbe respiration

Within the broad category of “colourless sulfur bacteria” there are subdivisions:

Obligate Chemolithoautotrophs (S oxidizers/ S reducers/disproportionators):

Obligate Chemolithoautotrophs can only grow autotrophically; they fix CO_2 via the Calvin cycle and oxidize inorganic substrates such as sulfides, and thiosulfate to produce produce sulfate, and tetrathionates (Kuenen, Robertson And Van Gemerden, 1985; Lengeler, Drews and Schlegel, 1999; Rickard, 2013).

Facultative Chemolithotrophs (S oxidizers/ S reducers/ disproportionators):

Facultative Chemolithotrophs are able to grow heterotrophically, autotrophically and mixotrophically. They are extremely adaptive and have been shown in culture to be capable of adjusting their metabolism to suit the substrate available in time frames as

short as four hours (Kuenen, Robertson And Van Gemerden, 1985; Rickard, 2013). In regard to their S respiring capacities, they oxidize reduced S compounds to produce sulfate, sulfite, and elemental sulfur. They couple this with the reduction of CO_2 or utilize other small organic molecules (Kuenen, Robertson And Van Gemerden, 1985; Lengeler, Drews and Schlegel, 1999; Rickard, 2013).

Chemolithoheterotrophs (S oxidizers/S reducers/disproportionators):

Chemolithoheterotrophs that oxidize/disproportionate use reduced sulfur compounds coupled with the oxidation of organic carbon compounds for energy. They cannot fix CO_2 and thus exist in environments which have abundant organic matter and where CO_2 fixation would not be energetically viable (Kuenen, Robertson And Van Gemerden, 1985; Rickard, 2013). The end products of these metabolisms are CO_2, sulfate and sulfur—some microbes store sulfur in their cells to utilize when sulfide is in short supply (Kuenen, Robertson And Van Gemerden, 1985; Lengeler, Drews and Schlegel, 1999; Rickard, 2013).

Denitrifying Sulfur Bacteria (S oxidizers/disproportionators):

In microaerobic or anaerobic conditions these microbes utilize nitrate as an electron acceptor and oxidize reduced sulfur species such as H_2S, S^0, $S_2O_3^{2-}$ (Flere And Zhang, 1998; Kuenen, Robertson And Van Gemerden, 1985). The end product of these microbes metabolisms are sulfate and nitrogen gas (Kuenen, Robertson And Van Gemerden, 1985; Lengeler, Drews and Schlegel, 1999; Rickard, 2013).

Coloured sulfur bacteria:

Phototrophic Sulfur Bacteria (S oxidizers/disproportionators):

There are many various phototrophic bacteria operating within the sulfur cycle.

Cyanobacteria are one of the oldest known. Cyanobacteria can utilize water as an electron donor to produce oxygen. Other species such as green and purple bacteria grow in anaerobic conditions and do not produce oxygen. They utilize reduced sulfur compounds (sulfide, elemental sulfur, thiosulfate, and sulfite), hydrogen or organic material as electron donors to produce sulfur, tetrathionate, thiosulfate or sulfate (Equation 19), water, and organic matter (Kuenen, Robertson And Van Gemerden, 1985; Lengeler, Drews and Schlegel, 1999; Podgorsek and Imhoff, 1999; Rickard, 2013).

Equation 19: $S_4O_6^{2-} + S^* \rightarrow + 2S_20_3^{2-} + S^0$

(Rickard, 2013)

Obligate Phototrophs (S oxidizers):

Obligate phototrophs require sulfide to grow—they need sulfide for both assimilatory processes (building R-SH molecules) and as an electron donor for photosynthesis, they produce R-SH, sulfur and sulfate (Kuenen, Robertson And Van Gemerden, 1985; Lengeler, Drews and Schlegel, 1999; Rickard, 2013).

Facultative Photolithotrophic Bacteria (S oxidizers):

Facultative Photolithotrophic Bacteria can grow mixotrophically. Experimental evidence has shown that they can grow on sulfide and carbon dioxide, sulfide and acetate and sometimes sulfide and organic molecules, the outcome of this metabolism is sulfur and sulfate (Kuenen, Robertson And Van Gemerden, 1985; Lengeler, Drews and Schlegel, 1999; Rickard, 2013). These microbes make up the aqueous sulfur cycle, some reduce, some oxidize, some disproportionate, however, it is the cycling of S compounds among these microbes which form the basis of the sulfur cycle currently on earth.

1.5 Sulfur disproportionation

Sulfur disproportionation was first discovered in the late 1970's during experiments on sulfur reducing microbe *Desulfobulbus propionicus* in which the media was enriched with intermediate sulfur species instead of sulfate although growth was quite slow (Finster, 2008; Hardisty et al., 2013; Licht and Davis, 1997. Given that it is likely that environmental sulfur reducing microbes would be exposed to temporally varying degrees of O_2 concentrations, it was hypothesized that disproportionation may be a significant part of these microbes metabolic capabilities, and a significant part of the sulfur cycle and further studies were initiated on disproportionation (Finster, 2008; Hardisty et al., 2013; Licht and Davis, 1997). After experimental evidence of microbial disproportionation was revealed, in situ experiments where undertaken in which tracers were added to marine sediments; these experiments revealed that disproportionation of intermediate sulfur species was indeed an important part of the sulfur cycle (Finster, 2008; Hardisty et al., 2013; Licht and Davis, 1997).

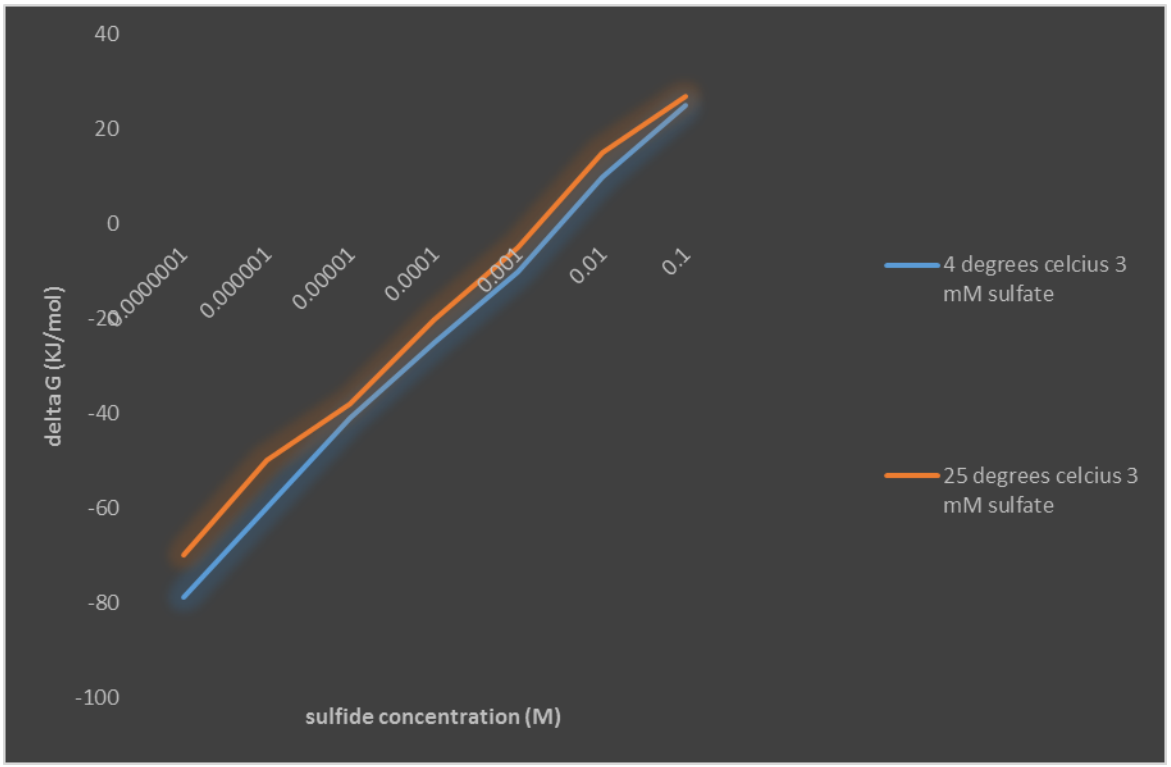
Disproportionation, or dismutation is comparable to the fermentation of compounds such as glucose and is sometimes termed inorganic fermentation (Finster, 2008). The disproportionation of sulfur species is a microbial and chemical process. In fresh neutral waters, at moderate temperatures, it is mainly a function of the metabolic processes of chemolithotrophic microbes (Finster, 2008; Hardisty et al., 2013; Licht and Davis, 1997). The general process utilizes compounds such as elemental sulfur, thiosulfate, and sulfite which serve as the electron donor and acceptor to produce two products—one more oxidized and one more reduced as compared to the start materials (Finster, 2008; Hardisty et al., 2013; Licht and Davis, 1997).

Sulfur disproportionation is often not the most energetically favorable (Table 6) reaction however, energy yields increase as a function of increases in pH and decreases in concentrations of the products (Figure 5) (Finster, 2008; Hardisty et al., 2013; Licht and Davis, 1997). For instance: the disproportionation of elemental sulfur; the activity of any element is equal to 1 and so the free energy of the reaction is a function of the concentration of the products and the pH of solution (Finster, 2008; Hardisty et al., 2013; Licht and Davis, 1997). Considering that most of the products are metastable and that they are substrates for other metabolic reactions, they are removed rapidly from solution, allowing for the reaction to be more favorable than it would otherwise be (Finster, 2008; Hardisty et al., 2013; Licht and Davis, 1997).

Gibbs Energies of Sulfur Disproportionalion Reactions	
(25°C, **0.1** MPa)	ΔG, kJ mol-^{1}
$4S^0 + 4H_20 \rightarrow 3HS + SO_4^{2-} + 2H^+$	+202
$4SO_3^{2-} + 2H^+ \rightarrow H_2S + 3SO_4^{2-}$	-315
$S_2O_3^{2-} + H_20 \rightarrow H_2S + SO_4^{2-}$	+17.7
$4S^0 + 3H_20 \rightarrow 2S_2O_3^{2-} + H_2S + 2H^+$	+244.6

(adapted from Rickard, 2013)

Table 6: Free energy of sulfur disproportionation

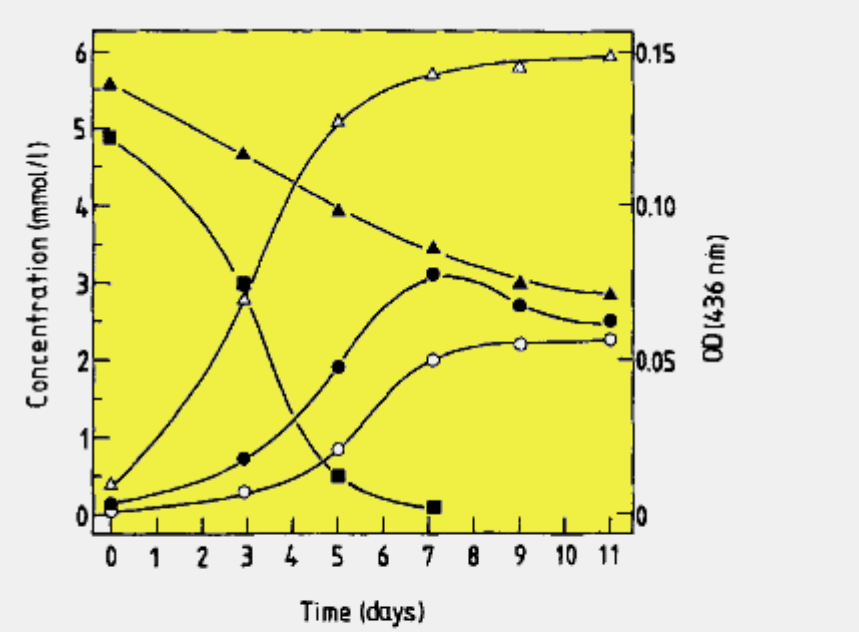


(adapted from Finster, 2008)

Figure 5: Free energy as a function of sulfide concentration and temperature

Chemical disproportionation of sulfur also allows this metabolism to have a leg up in environmental conditions. Chemical dismutation occurs naturally to form hydrogen sulfide and intermediate sulfur oxo anions; microbes can then disproportionate the sulfur oxo anions to sulfide and sulfate, sulfur reducing microbes can then reproduce elemental sulfur or sulfide which can react to form further sulfur intermediates (Finster, 2008; Friedrich and Finster, 2014; Hardisty et al., 2013; Licht and Davis, 1997). These biotic and abiotic micro cycles are another way these ingenious microbes can gain the most energy out of their environment with the least work and certainty that reactants will be in ample supply (Finster, 2008; Hardisty et al., 2013; Licht and Davis, 1997).

There are microbes that disproportionate sulfur and sulfur intermediates for growth such as *Desulfobulbus, Desulfofustis,* and *desulfocapsa* and there are those which can disproportionate for growth or not such as the bacteria *Desulfovibrio* (Table 5). The latter microbes reduce sulfur compounds such as sulfur, thiosulfate, dithionate, and to a lesser extent tetrathionate for growth (Finster, 2008; Hardisty et al., 2013; Licht and Davis, 1997).



Simultaneous formation of sulfate from sulfite and oxidation of ethanol by *Desulfovibrio sulfodismutans*. Symbols used are: △, optical density (436 nm); concentrations of ●, sulfate; ○, sulfide; ■, sulfite; ▲, ethanol

(Kramer and Cypionka, 1989)

Figure 6: changes in product and reactant concentration via disproportionation by Desulfovibrio reproduced with permission from H. Cypionka

In regard to the particular mechanisms of disproportionation, most microbes appear to utilize similar pathways (Finster, 2008; Hardisty et al., 2013; Licht and Davis, 1997). The microbe accomplishes oxidation via AMP and APS kinase which adds the sulfite group to AMP with the loss of hydrogen to create elemental hydrogen and APS. The sulfite group of APS pulls the O of

the terminal phosphate of AMP closer to it and weakens/lengthens the P-O bond. This bond lengthening is due to the shared electrons with the P in the terminal phosphate group of AMP. With the enzyme ATP sulfurylase and pyrophosphate the sulfite group is oxidized to sulfate with the generation of 1 ATP molecule (Kramer et al., 1989). Sulfite is reduced via six hydrogens and thiosulfate reductase (Rickard, 2013).

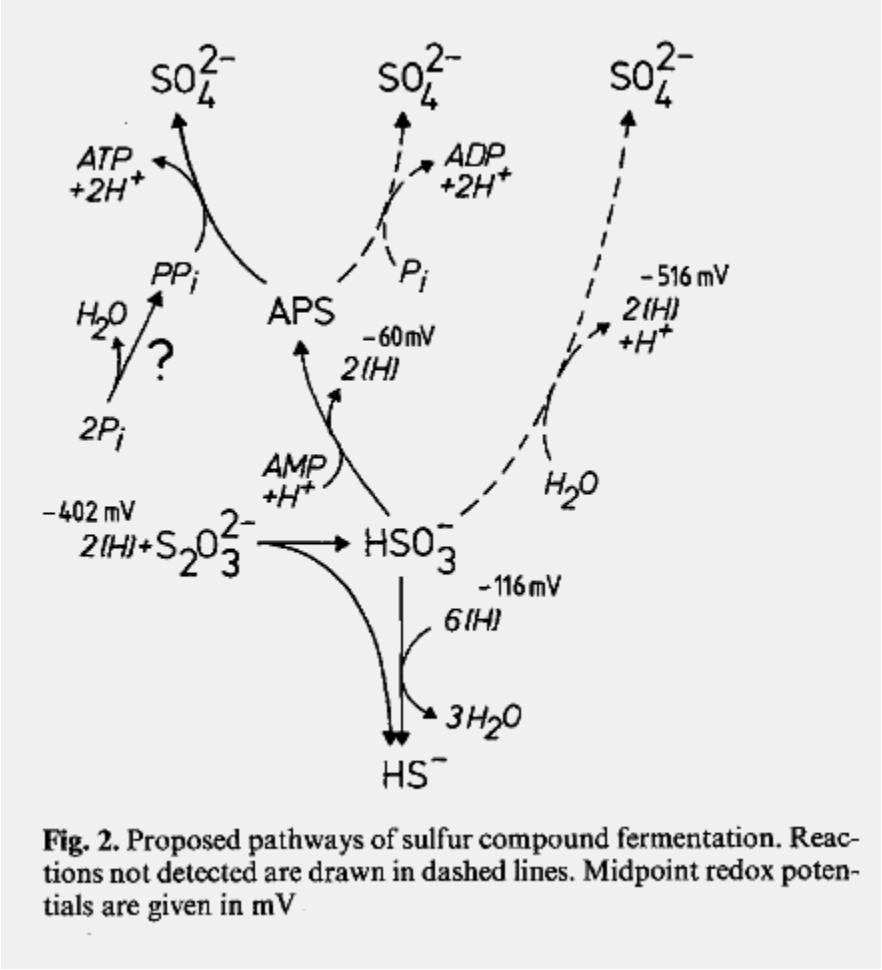


Fig. 2. Proposed pathways of sulfur compound fermentation. Reactions not detected are drawn in dashed lines. Midpoint redox potentials are given in mV

Figure 7: Pathways common in sulfur disproportionation. Reproduced with permission from Hardisty. (Hardisty et al., 2013)

Disproportionations have the ability to be a proton neutral process, consume or generate protons in varying degrees depending on the reactions occurring and the species being cycled (Equation 20, 21 & 22) (Finster, 2008; Friedrich and Finster, 2014; Hardisty et al., 2013; Licht and Davis, 1997). Because of their ability to consume protons and operate at a proton neutral level, disproportionators may play a key role in mediating more acid generating processes in natural water systems. In industrial wastewaters proton generating processes may be operating and being concealed by disproportionation reactions. It is unknown how long, or under what conditions these reactions mediate proton generation. Understanding how disproportionators are cycling intermediate sulfur species, which microbes participate in these reactions, and the conditions under which these cycles begin to change may change how wastewaters are understood and monitored.

Equation 20: $S_2O_3^{2-} + H_2O \rightarrow H_2S^- + SO_4^{2-}$

(Konhauser, 2007)

Equation 21: $4S_0 + 4H_2O \rightarrow 3H_2S^- + SO_4^{2-} + 2H^+$

(Konhauser, 2007)

Equation 22: $4\ SO_3^{2-} + 2H^+ \rightarrow H_2S^- + 3SO_4^{2-}$

(Konhauser, 2007)

1.6 Methods of detection—SOI's

Sulfur intermediates are metastable and so great care must be taken in the sampling procedure to ensure that representative samples are obtained—or analysis could be done on site via ion chromatography or some other portable field instrument. SOI's can be detected in the range of nmol→µmol for HPLC and ion chromatography. XANES and similar methods can validate the species present in solution but cannot provide concentrations. [2]

The following section details the analysis of certain SOI and some pros and cons to each method.

1.6.1 Sulfide characteristics:

Hydrogen sulfide is a weak acid and breaks down to sulfide and protons in water (Rickard, 2013). Hydrogen sulfide is unstable in oxic environments, and will react with cations rapidly in anoxic environments where it is stable. Hydrogen sulfide can be both a reducing and oxidizing agent in solution (Kamyshny et al., 2004; Lengeler, Drews and Schlegel, 1999; Rickard, 2013). It can behave as an oxidizing agent because its highest occupied molecular orbital (HOMO) has energies of ~10 eV which is of a similar value, or slightly less stable value than the lowest unoccupied molecular orbital (LUMO) of metal cations in solution (Rickard, 2013). Sulfide minerals are very abundant and include pyrite, chalcopyrite, and cinnabar to name a few. These minerals are oxidized abiotically and biotically to produce a wide range of intermediate sulfur species (Figure 1) (Barton, 1995; Barton, Mandl and Loy, 2010; Kuenen, Robertson And Van Gemerden, 1985).

1.6.2 Sulfide analysis methods:

Sulfide can be detected via the methylene blue reaction in concentrations ranging from 0-1000 µg/L (CLINE, 1969; Kamyshny et al., 2011). This reaction was first recorded in the late 1800's and

has served as an easy and reliable sulfide detection method ever since (Figure 8) (Fischer, 1883). The reaction requires the solution to be acidified and also requires ferric iron as an oxidizing agent. Ferric chloride oxidizes N,N-dimethyl-p-phenylenediamine which reacts with sulfide in solution to produce the coloured complex methylene blue. The sensitivity of this reaction is a change of 5 µg/L per every 0.01 AU (HACH, 2016). This reaction has many advantages first and foremost that it may be done in the field and therefore can most closely ensure that sulfide

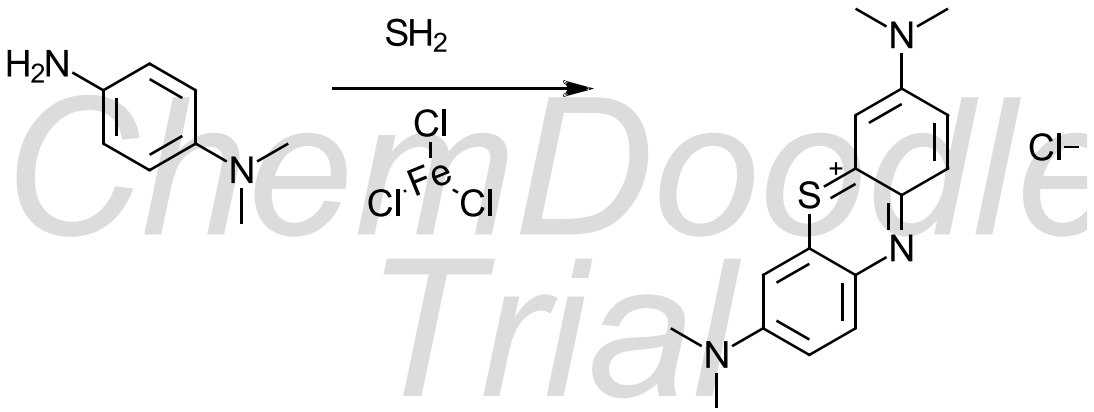


Figure 8: sulfide reaction to form coloured complex (methylene blue)

Concentration remains consistent with the sampling environment. However there are some flaws with this reaction—it is unspecific with regard to hydrogen sulfide and can measure any acid labile sulfide including polythionates and transition metal sulfides among others (Kamyshny et al., 2004). One of the most difficult disadvantages to overcome when measuring sulfide by this method is the interferences. This is especially notable in mine waters which contain many different chemical compounds, not only from the ore itself but also from the chemical extraction steps. Interferences include barium, turbidity, and most notable SOI species and other reducing agents. Thiosulfate, sulfite

and other reducing agents can decrease the intensity of the colour produced by the reaction (HACH, 2016).

Sulfide can also be analyzed by ion chromatography by oxidizing the sample with an oxidizing agent such as H_2O_2 to produce sulfate (Ubuka et al., 2001). The upper detection limit at 30 µg/L creates a small detection range especially for sulfide rich systems as error would be more likely due to significant dilution (Radford-Knoery and Cutter, 1993). Also the sampling procedures are much more involved and would be very time consuming for multiple sample locations or any experiment where a large number of samples are required (Radford-Knoery and Cutter, 1993). In addition to these issues, since all of the sulfur that is susceptible to oxidation will be oxidized to sulfate the remainder of the SOI's are not able to be analyzed and so many samples would need to e taken to measure each separate sulfur species (Radford-Knoery and Cutter, 1993; Ubuka et al., 2001). This would also create such a large concentration of sulfate that reading it via this method would be highly impractical. In some areas this would be entirely practical, however mine wastewaters with their higher concentrations of sulfur species would not be suitable for ion chromatography analysis.

Sulfide can also be analyzed via Gas chromatography (GC)-FPD (flame photmetric detector) (Cutter and Oatts, 1987; Radford-Knoery and Cutter, 1993; Ubuka et al., 2001). The FPD is ideal for sulfur analysis as it creates a reduced sulfur species via the flame and has high specificity for reduced sulfur species (Cutter and Oatts, 1987; Radford-Knoery and Cutter, 1993; Ubuka et al., 2001). This sulfur species can then be analyzed via its emission spectra (HiQ, 2016). The range for this method is 0-140 pmol/L sulfide and requires relatively simple preparation steps (Cutter and Oatts, 1987; Radford-Knoery and Cutter, 1993; Ubuka et al., 2001). Analysis via GC-FPD is simple, can be done with a field GC and has precise and accurate results. However, it does require that you have easy access to a GC and this particular detector (FPD) or a very reliable way to store the samples without sulfide loss until the analysis is able to be accomplished. Another drawback is the relatively

narrow detection range which, for mine waters, may require additional diluting and the potential for loss of sulfide that goes along with that.

1.6.3 Polysulfide characteristics:

Polysulfides ($S^{2-} + nS \rightarrow S_{n+1}$) have formal oxidation numbers ranging from -1 and +6 and form from reactions between sulfur and sulfides in solution (Rickard, 2012; Kamyshny et al., 2004; Kuenen, Robertson And Van Gemerden, 1985; Licht and Davis, 1997; Schippers and Sand, 1999). They are also intermediate compounds in the oxidation of hydrogen sulfide in neutral to basic environments (Rickard, 2012; Kamyshny et al., 2004). They are found in oxic and anoxic environments and are thought to be significant players in the sulfur cycle due to their metastability and thus high reactivity (Rickard, 2012; Kamyshny et al., 2004; Kuenen, Robertson And Van Gemerden, 1985; Licht and Davis, 1997; Schippers and Sand, 1999).

1.6.4 Polysulfide detection methods:

Inorganic polysulfides can be detected by derivatization with methyl triflate with subsequent separation on an HPLC (Kamyshny et al., 2006; Kamyshny et al., 2008). The process is similar to the derivatization methods required for sulfite thiosulfate and sulfide analysis on the HPLC which is described in chapter 2. It can detect and separate sulfur chains between 4 and 7 in length (Kamyshny et al., 2006; Kamyshny et al., 2008).The detection range for this method is between 15−70 nM (Kamyshny et al., 2006; Kamyshny et al., 2008). Many other detection methods such as IC or electrophoresis which can be used with other SOI's are not possible for polysulfides due to their reactivity (O'Reilly et al., 2001).

1.6.5 Polythionate characteristics

Polythionates ($S_{n+2}O_6^{2-}$) which are also known as polysulfane disulfonic acids or polysulfane disulfonates, are intermediates in pyrite formation (Druschel, Hamers and Banfield, 2003; Rickard,

2012). They are sulfur chain molecules of various lengths which are terminated at both ends by sulfite groups (Druschel, Hamers and Banfield, 2003; Kamyshny et al., 2004: Rickard, 2012). Polythionates are ubiquitous and play important roles in sulfur redox reactions in aqueous environments, and microbial sulfur metabolisms (Druschel, Hamers and Banfield, 2003; Kamyshny et al., 2004: Rickard, 2012). The chain length of these molecules can be up to 22 S, but typically they exist in lengths 3□6 (Bernier, 2008).

Polysulphane monosulfonic acids (S_x0_3 where x >2) are relatively newly recognized and poorly defined players in the aqueous S cycle. They are assumed to be intermediates in reactions involving thiosulfate and polythionate and their single sulfite group makes them highly reactive and thus important molecules in aqueous sulfur reactions (Bernier, 2008).

1.6.6 Polythionate detection methods

Polythionates are as equally difficult to analyze as polysulfides due to their similar chemical characteristics and reactivity (Miura and Kawaoi, 2000; Miura and Watanabe, 2001; O'Reilly et al., 2001). IC and electrophoresis are methods to characterize polythionates, with IC having good success at separating polysulfides and maintaining their speciation throughout separation (Miura and Kawaoi, 2000; Miura and Watanabe, 2001; O'Reilly et al., 2001). Currently IC is the only analytically robust method for detecting polythionates in solution.

1.6.7 Thiosulfate characteristics

Thiosulfate is an intermediate sulfur species which occurs naturally and biochemically. It is stable only under basic and neutral conditions as it undergoes acid decomposition to sulfite and elemental sulfur (Antoine.frostburg.edu, 2016). This is an important sulfur species in mining as it is produced and used in disproportionation reactions which may produce acid (Jørgensen, 1990). It may also give us an idea as to what microbes are active in solution—this is discussed in more detail in the previous sections 3, 4 &5.

1.6.8 Thiosulfate detection methods

Thiosulfate is an important component in many different systems and therefore methods to separate and detect it have been studied and tested in greater detail. The main analytical technique used for thiosulfate qualification and quantification is via HPLC due to its ability to separate various sulfur compounds that are present in solution (see wackenroders solution equation section 3) and the relatively easy derivatization reaction to produce a fluorescent species (CLINE, 1969; Hurse and Abeydeera, 2002; Keller-Lehmann et al., 2016; Lin Ling, Dewaele and Baeyens, 1990; Newton, Dorian and Fahey, 1981; O'Reilly et al., 2001; Rethmeier et al., 1997; Sardi et al., 2013; Zopfi, Ferdelman and Fossing, 2004). Other advantages to HPLC analysis are that the fluorescence reaction is specific to sulfur compounds and creates a very bright signal on a very dim background as none of the other components of the system fluoresce in the same wavelength (Rethmeier et al., 1997; Sardi et al., 2013; Zopfi, Ferdelman and Fossing, 2004. The elution of uncomplicated sulfur species can be tuned to elute without any interference from one another, and all are capable of very linear responses between 0 and ~2 mmol species (CLINE, 1969; Hurse and Abeydeera, 2002; Keller-Lehmann et al., 2016; Lin Ling, Dewaele and Baeyens, 1990; Newton, Dorian and Fahey, 1981; O'Reilly et al., 2001; Rethmeier et al., 1997; Sardi et al., 2013; Weir, Butler and Haddad, 1994; Zopfi, Ferdelman and Fossing, 2004). Thiosulfate may also be detected electrochemically via the oxidation of silver via the anion thiosulfate up to 5 ppb (Cheng, Jandik and Avdalovic, 2005). The disadvantage to this method is that only sulfide and thiosulfate may be detected (Cheng, Jandik and Avdalovic, 2005). Ion chromatography is another way to analyze thiosulfate, recoveries are ~ 90% for this sulfur species (Miura and Kawaoi, 2000; Jørgensen, 1990; Siriraks, Kingston and Riviello, 1990). This method has several advantages in that it can analyze many of the sulfur species which are in solution, albeit via different methodologies. For thiosulfate detection a cation exchange column would be used; this column could also separate polythionates, polysulfides, sulfide, tetrathionate and sulfate—sulfur would require HPLC analysis (Miura and Kawaoi, 2000; Jørgensen, 1990; Rethmeier

et al., 1997; Sardi et al., 2013; Siriraks, Kingston and Riviello, 1990; Weir, Butler and Haddad, 1994; Zopfi, Ferdelman and Fossing, 2004).

1.6.9 Sulfite characteristics

Sulfite is a weak base that readily oxidizes to sulfate in solution. It is produced in mine water by disproportionation reactions and polythionate propagation see figure 8 and reactions 9 and 11 thiosulfate oxidation and polythionate production respectively. Reactions involving sulfite can consume protons or produce protons on the way to sulfate, dependent upon the mechanism (figure 8). Reactions involving sulfite are both biotical and abiotically driven in mine waste.

1.6.10 Sulfite detection methods:

Sulfite has similar detection methods to thiosulfate, however there are many more references regarding fluorescent derivatizations due to the presence of sulfites in food and beverages and the impact it may have on humans.

1.6.11 Sulfur characteristics

Sulfur is a third row element and is in the group termed chalcogens; these elements have 6 valence electrons and sulfur is unique in having 5 empty d orbitals (Greenwood and Earnshaw, 1984; www2.chemistry.msu.edu, 2015). Sulfur has an extensive redox capacity which is due to its relatively small atomic radius, few electron shells, and empty d shell which can be used for bonding (Greenwood and Earnshaw, 1984; www2.chemistry.msu.edu, 2015). The only elements that sulfur does not form stable bonds with are the noble gases (Greenwood and Earnshaw, 1984).

Elemental sulfur is very stable and highly insoluble; it can be oxidized to an intermediate oxidation state or it can react with sulfides to form polysulfides—these reactions are both biotic and

abiotically driven (Barton, 1995; Barton, Mandl and Loy, 2010; Kuenen, Robertson And Van Gemerden, 1985).

1.6.12 Sulfur detection methods

Elemental sulfur is difficult to analyze as there are many forms of it that can be in solution in both soluble and insoluble states. Typically to analyze sulfur one must extract the sulfur in solution to an organic liquid and conduct the analysis on that solution. This presents some difficulties as many field test methods are not amendable to reading organic liquids.

In the field and in the lab Gas Chromatography is an elemental sulfur analysis method. An organic extraction is done on the aqueous sample between 1-3x, depending on the type of sulfur in the system and that organic sample is run on a GC with an electron capture detector, flame photometric detector or mass spectrometry detector (Chen, Joly and Belzile, 1997; Richard, Vick and Junk, 1977; Shearer, Poole and Nowalk, 1993; Yin et al., 2014). The detection limits are in the µM-mM range and R^2 values are in the upper .90's to 1.0 (Chen, Joly and Belzile, 1997; Richard, Vick and Junk, 1977; Shearer, Poole and Nowalk, 1993; Yin et al., 2014). These analysis methods have been long developed and are a reliable way to detect elemental sulfur in solution. However, this is a destructive analysis method and it requires specialized detectors.

A HPLC instrument may also be used to detect sulfur via UV/Vis detectors at ~ λ of 254 nm (Alberta environment and parks, 2015; Buchanan et al., 1993; Hurse and Abeydeera, 2002; Kamyshny et al., 2008; Nageswari, 201; Newton, Dorian and Fahey, 1981; O'Reilly et al., 2001; Rethmeier et al., 1997; Yin et al., 2014; Zopfi, Ferdelman and Fossing, 2004). The analysis occurs after extraction with an organic solvent 1-3 x dependent upon the type of elemental sulfur in solution. Chloroform is the extractant of choice as it has higher extraction efficiencies as compared to dichloromethane, acetone, and methanol (Alberta environment and parks, 2015). HPLC analysis has

the advantage of being able to detect a number of SOI's on each run which minimizes analysis time and solvent use. Also as UV/vis detectors are fairly ubiquitous, this method is more transferable to labs of many different specializations.

1.7 Difficulties transferring methods and knowledge to mine water analysis

There are a number of papers and studies which look at the analysis of sulfur species in solution, the problem is that they are looking at relatively uncomplicated samples. Lakes, rivers and other fresh water bodies have significantly less sulfur and therefore less sulfur speciation. They also have fewer sulfur active microbes in general, as there is less of it to cycle. Ocean water analysis is complicated by the high ionic strength of the solution, however ocean water is fairly well characterized and there are known ways of overcoming the ionic strength issues. With mine water the components of the solution are variable with: time, amount of ore processed, type of ore processed, temperature, pH, among others and so the methods used for analysis can be difficult to pin down and difficult to transfer between different mines.

1.8 Future directions:

Given the importance of sulfur intermediates on the global sulfur cycle, and our relatively poor knowledge of these intermediates, the microbes which cycle them, and the relationships which foster this part of the sulfur cycle, it seems apparent that more experimental and field analyses are needed. These studies should emphasis collection methods, storage procedures, in field analyses, and analytical methods and sources of error. A greater understanding of these cycles and the microbes which play a part in them will allow much more sophisticated monitoring techniques and potentially predictive tools for contemporary industrial practices.

Microbial disproportionation and the microbes which play a role in these systems and cycles may be very important in the development of monitoring and remediation strategies of industrial wastewaters and therefore a comprehensive knowledge of these microbes is necessary.

1.9 Research objectives:

Given the current state of mining practices and knowledge, the timing is ideal to start to explore microbial –SOI connections in mine waters. Much is known about AMD and its key players, however limited understanding of the factors which may influence mine wastewater sulfur cycling and potential for AMD development exists. In particular knowledge of the specific SOIs that can occur as well as the role SOM can play in the cycling of sulfur will improve the ability to forecast mine wastewater changes over time.

Thus, the key questions for this research are:

(1) What are the key SOI species, and

(2) How do endemic SOM affect sulfur cycling of SOI over seasonal scales in a net neutral mine waters as well as within its inputs?

The hypotheses that will be tested in this research are:

(1) Diverse SOI occur within these mine wastewaters.

(2) SOM are present in these waters; they are capable of acid generation.

(3) Seasonal patterns in SOI and SOM sulfur cycling will occur.

Research goals for this project can be broken into two separate and cohesive parts— (1) characterization of SOI species present seasonally and spatially within a mine wastewater system, and (2) experimental assessment of SOI cycling via endemic microbes present in disparate geochemical and temporal conditions within a mine wastewater system. To fulfill the goals necessary to test the hypothesis several areas needed to be covered.

The first was based on method development. Knowledge regarding methods to analyze sulfur species in water is poor. This is especially true for mine wastewaters in general, which possess a significant amount of other chemical species which may affect/interfere with the accurate analysis of sulfur species. Due to this lack of knowledge, the first objective of this research was to set up a comprehensive method to accurately quantify and qualify distinct sulfur species in mine wastewater. This will be discussed in further detail in Chapter 2.

In Chapter 3 the application of these methods to mine wastewater samples will be discussed and analyzed.

Chapter 4 summarizes the results of enrichment experiments that assessed the role of sulfur oxidizing microbes (SOM) on sulfur cycling within the same waters characterized for SOI. Research findings from Marshall et al. (in preparation) were used to identify linkages between field characteristics and observed SOI and microbial SOI cycling.

2 SOI analysis methods and development

There are a limited number of scientific publications regarding the analysis of sulfur species in aqueous samples (Böttcher and Thamdrup, 2001; Chen, Joly and Belzile, 1997; Cutter and Oatts, 1987; Hurse and Abeydeera, 2002; Kamyshny et al., 2006). The reasons for this are multifold. Sulfur species with its 6 valance states and redox diversity is difficult to pin down and even more difficult to track its original state and the states that it may have passed through on the way to the current one. Aside from that, as mentioned previously, sulfur is not an easily analyzed species. Spectrophotometers can only measure chemical species, which have significant absorbance in the UV/VIS spectrum. Many significant sulfur species do not absorb light in this spectrum and are unable to be reacted to create a complex, which does absorb in this spectrum. Nuclear magnetic resonance NMR can only identify a few of the sulfur species common in mine wastewaters and it is not a quantitative method of analysis. The other analytical instruments could be run through regarding their limitations, however suffice it to say that no other analytical instrument, which the exception of Ion Chromatography(IC) and High Performance Liquid Chromatography (HPLC) can provide qualitative and quantitative measurements for sulfur species such as those seen in mine wastewaters. Figure 9 outlines the sampling steps.

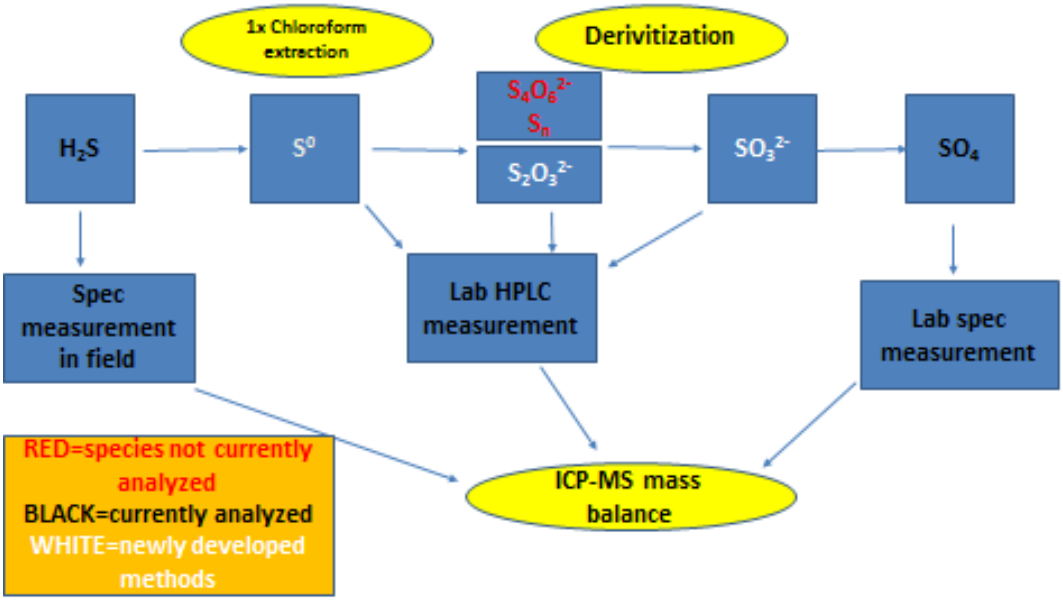


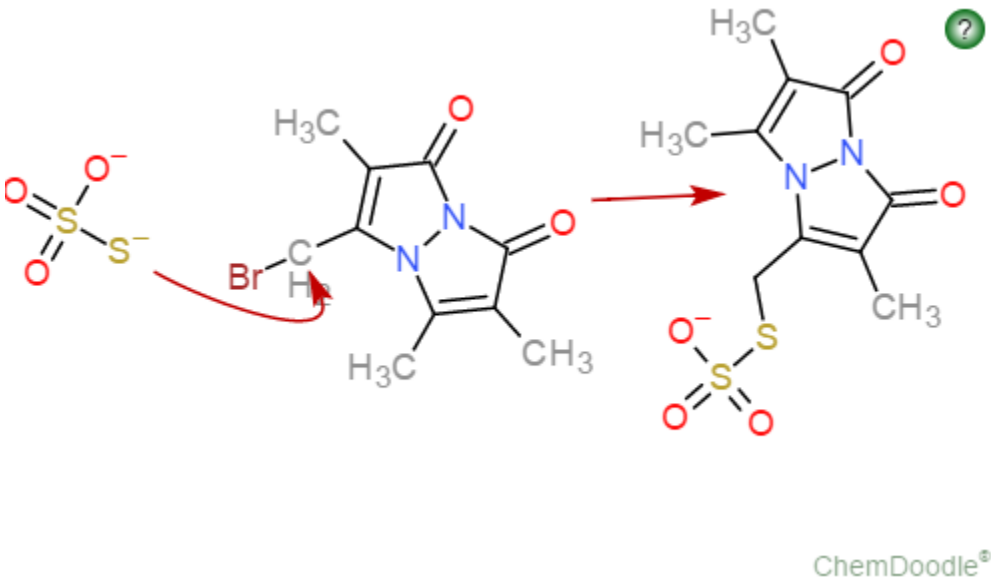
Figure 9: Preparation and analytical steps for sulfur species potentially present in mine wastewaters (black: methods available prior to this thesis; red: species that remain unanalyzable; white: methods developed in this research for specific species.

Although no current analysis of sulfur species in mine wastewaters was able to be uncovered via academic searches, there are a few papers which have analyzed sulfur oxidation intermediates (SOI) in fresh, salt and wastewaters. These papers all used either a method developed on a HPLC or a combination of methods developed on both a HPLC and an IC. Notable among these papers are (Hurse and Abeydeera, 2002; Kamyshny et al., 2011; Keller-Lehmann et al., 2016; Rethmeier et al., 1997; Zopfi, Ferdelman and Fossing, 2004). The method I developed to analyze the SOI in mine wastewaters was based on Rethmeier et al's paper with modifications for column type and differences in water composition.

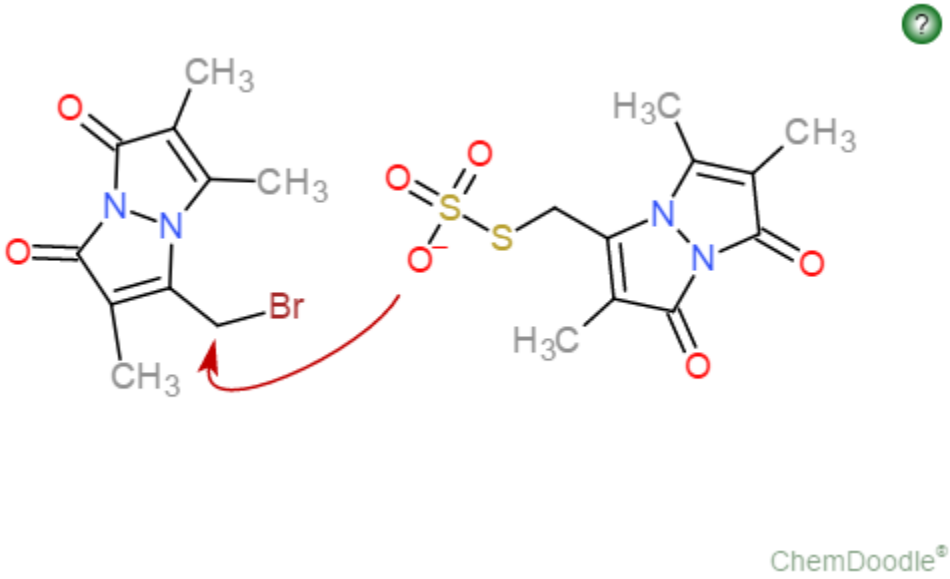
As mentioned previously, many sulfur species are unable to be detected via typical spectrophotometric methods as they do not absorb in the UV or visible spectrum.

There is a reaction however which creates a compound which fluoresces from chemical species which have the ability to undergo nucleophilic substitution reactions. In this reaction an electron rich “nucleophile” attacks an electron deficient “electrophile”. A leaving group is produced and a new chemical species is formed. In the case of thiosulfate the nucleophile is the negative sulfur on thiosulfate and the electrophile is the carbon species bonded with bromine. For the reaction below thiosulfate will be used as an example.

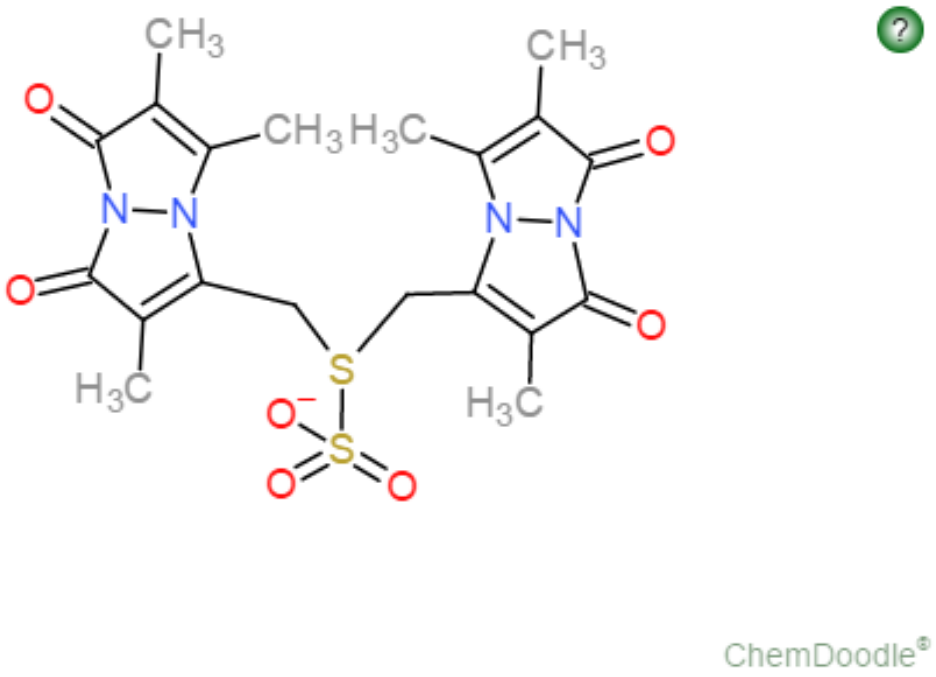
Step 1: the nucleophile attacks the electrophile creating an intermediate species



Step 2: another equivalent of MBB present in solution is attacked once again by a nucleophile.



Step 3: A fluorescent product is formed from the alkylation of the sulfur species



This reaction is termed the substrate nucleophilic substitution reaction in which an intensely fluorescent thioether is formed from an intermediate product--the thiolate and a nearly nonfluorescent bromoalkane by the incorporation of pi bonding (Lin Ling, Dewaele and Baeyens, 1990; Sardi et al., 2013).

Elemental sulfur is unaffected by this reaction and is extracted after derivatization via a one time chloroform extraction.

Before going into the specifics of the method development, there are a number of reasons why this reaction is suitable and highly effective for the detection and quantification of thiosalts in mine

wastewater. The first is sensitivity; the sulfide dibimane species (SdB) is highly fluorescent against the dark background of the reagents, none of which are aromatic or have significant delocalized electrons, this allows the detection of nanomolar concentrations of product—dependent upon instrumentation noise. The small size and uncharged nature of MBB are also advantageous due to the fact that there are no extreme steric demands. The steric freedom increases access for reaction ease and thoroughness and the absence of ionizable or charged groups makes the reagent nearly indifferent to the electrostatic surroundings (Lin Ling, Dewaele and Baeyens, 1990; Montoya et al., 201; Sardi et al., 2013). The reaction happens rapidly and is selective to sulfides. The reaction requires very low amounts of the solvents and the solvents used and relatively non-hazardous to human, aquatic or plant life. Because the SdB reaction is so rapid, the reaction can be stopped to avoid any contaminants from reacting and increasing or blocking the signal.

The disadvantages are that there are a number of solvents which need to be pre prepared and used in each reaction and this is time consuming and renders the reaction susceptible to increased amounts of human error.

The method development was a straightforward activity and relied on the method laid out by Rethmeier et al., 1997 with adjustments due to the differences in column polarity and solution chemistry and components.

Rethmeier et al.'s mobile phase consisted of .25% acetic acid (A) and 100% methanol (B). Their protocol was as follows: 0-7 mins 88% A, 12% B, 7-15 mins 12-30% linear gradient B, 15-19 mins 30% isocratic B, 19-23 mins 30-50% linear gradient B, 23-30 mins 50-100 linear gradient B, 30-33 mins 100% isocratic B, 33-33.1 min 100-12% linear gradient B, 33.1-36 mins 12% isocratic B

Calibrations were done using standards of sodium thiosulfate ranging from 0 to 1.5 mM, sodium sulfite from 0-1 mM, 0-32 mM for sulfur and sodium sulfide 0-1.5 mM. The retention times for Retmeier et al were 2.38 min for sulfite, 5.08 min for thiosulfate and 24.55 min for sulfide.

The column used for this research was significantly more hydrophobic (appendix 4) and so performed quite differently. The mobile phase needed to be adjusted so that the hydrophobic methanol phase was of increased prominence. The mobile phase was adjusted eventually to 70/30 acetic acid (.25%) and methanol (100%) respectively. This produced good sharp peaks for both thiosulfate and sulfite without the need to incorporate a gradient. The flow rate was adjusted from 1.0 mL/min to 1.5 mL/min until 6 mins where it drops to 0.85 mL/min. This adjustment was necessary due to tailing from the sulfide peak.

For elemental sulfur, a successful run at a flow rate of 1 mL/min and a mobile phase of 50 methanol and 50 water was elucidated.

Standard curves were done using standards of sodium thiosulfate ranging from 0 to 1.5 mM, sodium sulfite from 0-1.7 mM, 0-32 mM for sulfur and sodium sulfide 0-1.5 mM. The chemicals used were all ACS grade and obtained from Fischer Scientific. Standards were prepared by creating a stock solution in deionized water (DI)—for thiosulfate and sulfite the standards were prepared in 100 mM concentrations by back weighing the appropriate amounts of sodium thiosulfate and sodium sulfite and then filling the volumetric flask with degassed DI. A series of 6-7 dilutions were made from the stock to create a standard curve. For sulfide a standard solution was prepared in concentrated NaOH by back weighing the appropriate amount into a volumetric flask and then filling to the line. The concentrated NaOH was to preserve the sulfite concentration in the water prior to analysis. 6-7 dilutions were made from that standard to construct the standard curve. Elemental sulfur was prepared by back weighing the appropriate amount into a volumetric flask and then diluting to the line

with chloroform. The chloroform was the only solvent attempted which dissolved the sulfur in sufficient amounts.

2.1 Final method and reaction steps

50 µL aqueous samples were added to a previously prepared derivatization mixture which contained 50µL acetonitrile, 50µL of 50mM HEPES/ 5mM EDTA (pH 8) buffer and 10µL of 48mM monobromobimane in acetonitrile, based on the method by Rethmeier et al., 1997. This mixture underwent a derivatization reaction in the dark for 30 minutes, after which time the reaction was stopped with 100µL of 101mM methanesulfonic acid. The derivitization reaction is very rapid and is stopped after 30 minutes to avoid reaction of any possible contaminants in the system.

The derivitized samples were then stored at -20 °C until analysis.

The derivatization reaction creates a fluorescent species from $S_2O_3^{2-}$, SO_3^{2-}, any elemental sulfur in these samples is unaffected by this reaction.

To extract elemental sulfur a 210 µL aliquot of chloroform was added to the derivitized sample and extracted 1x. The sulfur partitions into the chloroform which was separated from the aqueous phase and was stored at -20 °C until analysis.

The $S_2O_3^{2-}$ and SO_3^{2-} fluorescent species was read on a Shimadzu LC-20AD prominence HPLC instrument equipped with a fluorescence detector. The wavelength of excitation was 387 nm, the wavelength of emission was 478 nm. The mobile phase was 70/30 acet/MeOH, the flow rate was 1.5 mL/min until 6 mins at which point it drops to 0.85 mL/min. The oven temperature was 35°C and was held stable throughout the run. The column used was the Alltima HP C18 (5µm x 150mm x 4.6mm) reverse phase column. The sulfide peak elutes at 2.3 minutes and thiosulfate elutes at 3.5 minutes.

Both peaks are sharp and well defined. Concentrations were determined by comparing peak area against a calibration curve of peak area to analyte concentration.

Elemental sulfur was analyzed on the same HPLC instrument with a UV/Vis detector. The wavelength of detection was 254 nm. The mobile phase was 50:50 MeOH:H_2O. The flow rate was 1 mL/min. and the oven was off throughout the run. The column used was the Alltima HP C18 (5µm x 150mm x 4.6mm) reverse phase column. Elemental sulfur elutes at 2 minutes with slight tailing. Concentrations were determined by comparing peak area against a calibration curve of peak area to analyte concentration.

All standard curves used have an R^2 value of .9 or greater (please see appendix 3).

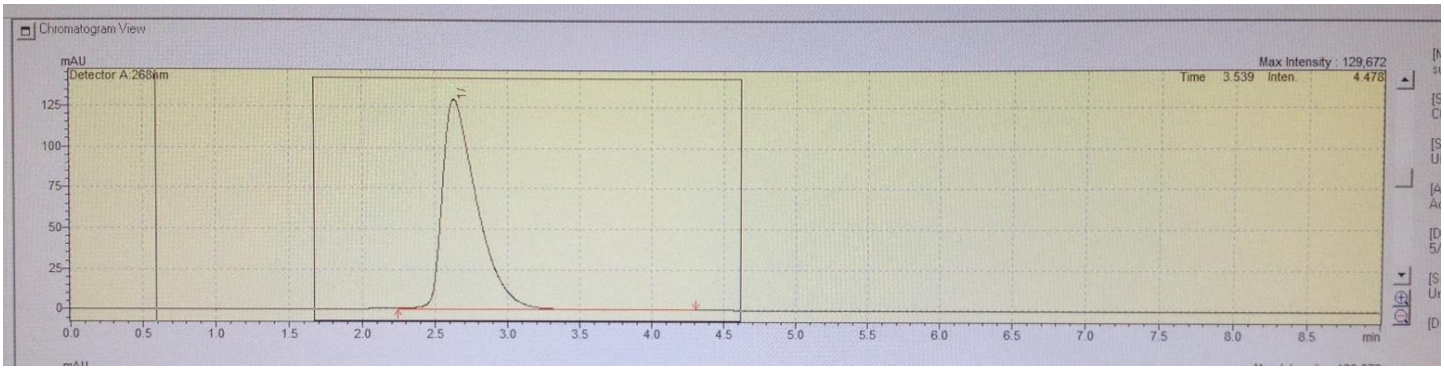


Figure 10: Chromatogram of a sulfur peak

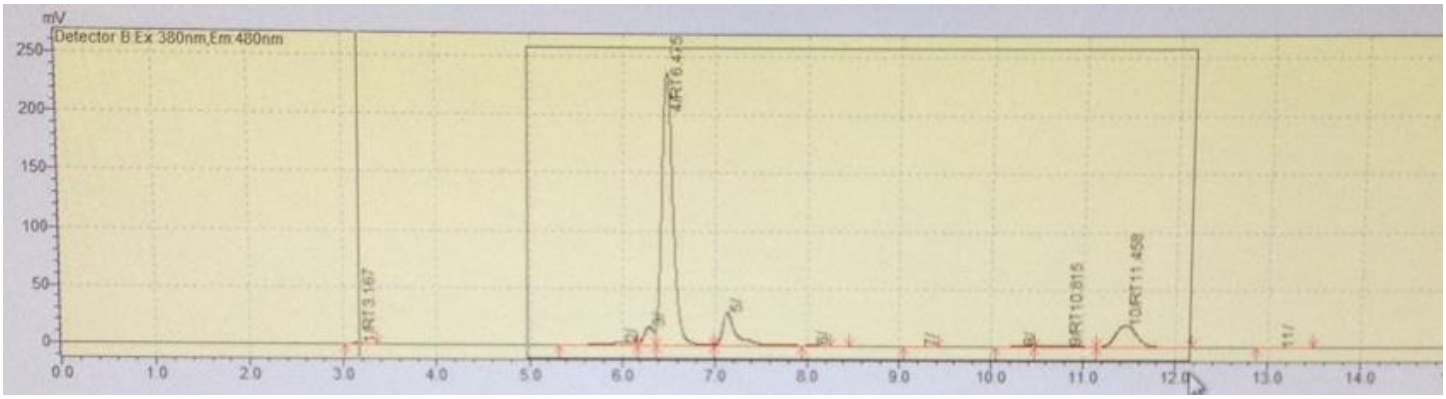
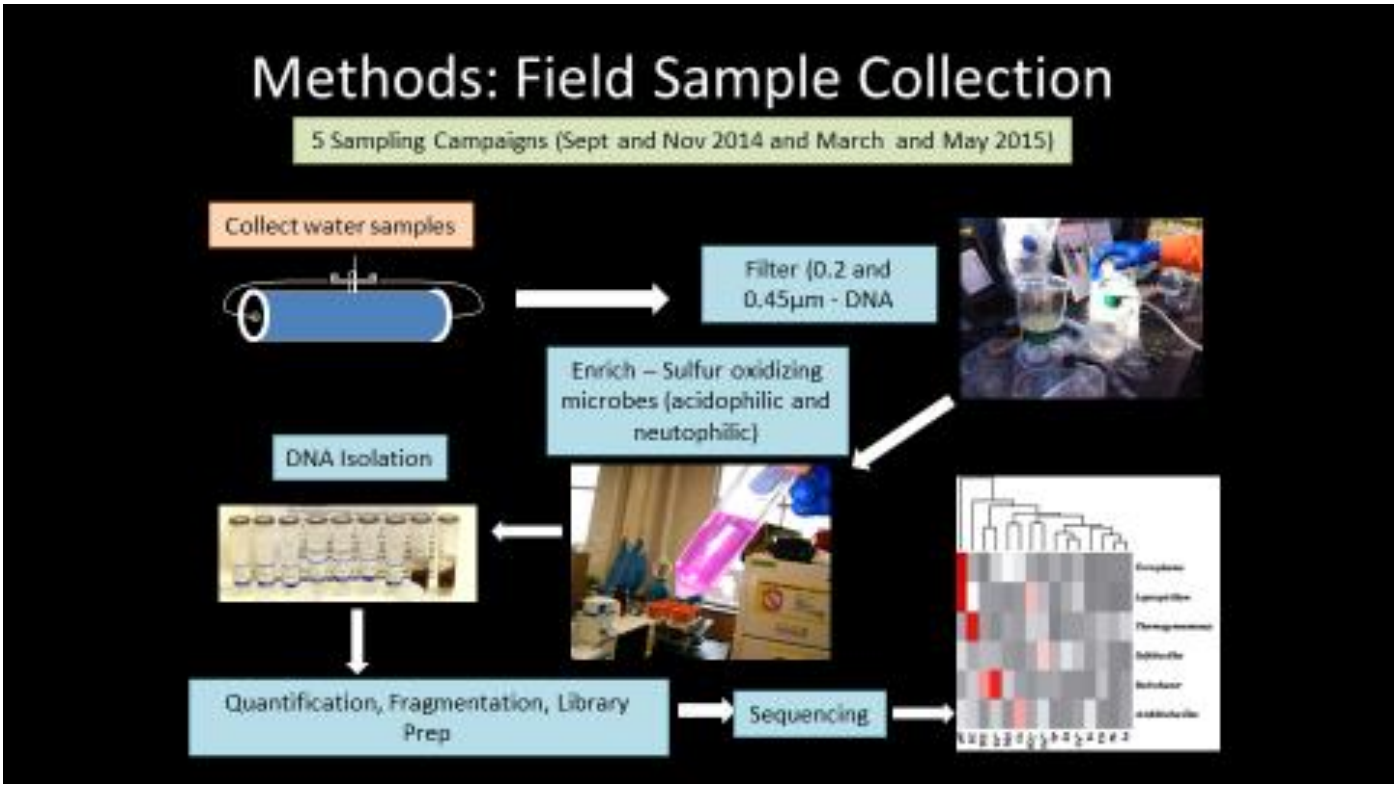


Figure 11: Chromatogram of sulfide (3.1 mins), thiosulfate (6.4 mins), and sulfide (11.4 mins)

3 Method application to mine wastewater samples

The site of this research is the Glencore Sudbury Integrated Nickel Operations (INO) wastewater system near Sudbury, Ontario (add map showing location to Fig 13 as panel insert). The oxidation reservoir is currently ~neutral and receives a variety of inputs. The inputs are of variable compositions and chemical characteristics—including waste rock discharge, tailings deposit runoff, and inputs from three separate mines. These mines and mills are processing different ores to extract different metals and range from basic pH to acidic pH with variable dissolved oxygen, suspended solids, conductivity, temperature, Fe, dissolved organic carbon DOC, SO_4, and N concentrations (data reported in Marshall et al. in prep).

The objectives of this laboratory based thesis are part of a larger project incorporating field characterization along with laboratory assessment and molecular microbiology. As part of that larger project samples were provided for analyses here. Briefly, during sampling campaigns to ensure robust samples were obtained and transported from the sample site—the derivatization cocktail was prepared in advance of field sampling trips and as the samples were obtained from the site, 50 µL of mine water sample was added to 110 µL derivatization mixture which contained 50 µL of 50 mM HEPES buffer, 50 µL acetonitrile, and 10 µL monobromobimane (48 mM). The reaction was stopped after 30 minutes using 100 µL methanesulfonic acid (65 mM). Samples were frozen at -20 °C upon return from the field until analysis.



Tara Colenbrander Nelson, 2015

Figure 12: Field collection steps for SOI samples, microbial enrichments and bulk mine water for water chemical analyses. Adapted with permission from Tara Colenbrander-Nelson.

Figure 13: Map of the Glencore/Sudbury INO site

Table 7: Sample season and sample site names

Season	Site name as listed on figure 13	Site name
Fall 2014	Waste rock runoff	Waste rock 1 Waste rock 2
	Tailings deposit	Strathcona Tails
	Oxidation reservoir	Oxidation reservoir Epilimnion Oxidation reservoir Hypolimnion
	Follows the same route as waste rock runoff	Mine 1 Mine 2 Mine 3
Winter 2014	Waste rock runoff	Waste rock 1 Waste rock 2
	Tailings deposit	Strathcona Tails
	Oxidation reservoir	Oxidation reservoir Epilimnion Oxidation reservoir Hypolimnion
	Follows the same route as waste rock runoff	Mine 1 Mine 2 Mine 3
Spring 2015	Waste rock runoff	Waste rock 1 Waste rock 2
	Tailings deposit	Strathcona Tails
	Oxidation reservoir	Oxidation reservoir Epilimnion Oxidation reservoir Hypolimnion
		Mine 1

	Follows the same route as waste rock runoff	Mine 2 Mine 3
Summer 2015	Waste rock runoff	Waste rock 1 Waste rock 2
	Tailings deposit	Strathcona Tails
	Oxidation reservoir	Oxidation reservoir Epilimnion Oxidation reservoir Hypolimnion
	Follows the same route as waste rock runoff	Mine 1 Mine 2 Mine 3
Fall 2015	Waste rock runoff	Waste rock 1 Waste rock 2
	Tailings deposit	Strathcona Tails
	Oxidation reservoir	Oxidation reservoir Epilimnion Oxidation reservoir Hypolimnion
	Follows the same route as waste rock runoff	Mine 1 Mine 2 Mine 3
Winter 2016	Waste rock runoff	Waste rock 1 Waste rock 2
	Tailings deposit	Strathcona Tails
	Oxidation reservoir	Oxidation reservoir Epilimnion Oxidation reservoir Hypolimnion

	Follows the same route as waste rock runoff	Mine 1 Mine 2 Mine 3
Spring 2016	Waste rock runoff	Waste rock 1 Waste rock 2
	Tailings deposit	Strathcona Tails
	Oxidation reservoir	Oxidation reservoir Epilimnion Oxidation reservoir Hypolimnion
	Follows the same route as waste rock runoff	Mine 1 Mine 2 Mine 3

Figure 13 shows a map of the Sudbury INO Onaping Craig water system which was the water system used for this study. Arrows indicate the water's movement and stars indicate the sampling sites. This water system includes waste from three separate mines which vary in their operational schedule and metal mined. This system is unique in that it allows for the setup of exeriments which can compare differences in mine influences on the endemic microbes and their respective inflence on the cycling of sulfur in this water system.

3.1 Site characteristics

Briefly, > 60 samples were collected over the period ranging from September 2014- May 2016 ensuring seasonal assessment for SOI characterization and enrichment experimentation. For these >60 samples, temperaure ranged from 0.4°C to 25.55°C, pH ranged from 2.75 to 11.69, dissolved oxygen (DO) percent saturation ranged from 0 to >100, and total organic carbon (TOC, mg/L) ranged from 0 to 17.4 Table 6 (Marshall et al in preparation).

Table 8: seasonal field data reproduced from Marshall et al. (in progress) showing seasonal variations in temperature, pH, dissolved oxygen concentration and total organic carbon.

site	season	temp. (°C)	pH	DO%	TOC (mg/L)
ox res epi	sept	16.37	6.92	27.8	3.1
ox res epi	nov	8.17	6.63	55.7	3.1
ox res epi	march	0.4	8.32	13.3	5.4
ox res epi	aug	18.47	6.86	53.6	6.2
ox res hypo	sept	15.89	7.66	4.9	3.3
ox res hypo	nov	8.13	7.23	51.4	5.5
ox res hypo	march	3.23	8.76	0.2	4.0
ox res hypo	aug	16.23	7.19	0	3.2
mine dewatering	sept	13.17	7.01	97.6	10.6
mine dewatering	nov	7.28	6.66	100.4	17.4
mine dewatering	may	15.83	5.16	97.5	6.4
mine 1	nov	6.29	3.56	ND	3.3
mine 1	may	15.38	2.75	94	0.8
mine 1	aug	21.07	2.97	100.1	0.9
mine 2	March	17.3	4.68	107.7	1.6
mine 2	May	15.6	3.91	99.9	1.6
mine 2	August	22.07	5.43	104.1	3.2
mine 3	May	13.71	6.23	95	0.0
mine 3	August	19.81	6.52	90.8	3.7
mine 3	Novemb	9.28	6.57	96.6	2.3
waste rock 1	sept	15.27	3.47	61.3	6.2

Figures 14 through 41 details the sulfur species in the oxidation reservoir and its inputs over seasonal time scales. Note that the total sulfur concentrations (analyses conducted by Land and Water, CSIRO Sydney, NSW, Australia) includes all sulfur species in the waters with the exception of elemental sulfur which is filtered out of the sample when preparation for the ICP-AES is conducted. All sulfur species on the left of the pie charts should equate to the right. Any inequalities are due to species which are present in the waters which were not analyzed specifically, ie unknown SOI species. Throughout all sites sampled, the winter season shows the greatest disparity in the sulfur balance indicating the largest pool of unaccounted for S. The portion of unanalyzed sulfur species in the winter sampling season ranges from 80%-90% (Figures 14 through 27). In contrast, for all other sampling seasons across this unaccounted for SOI pool is negligible as measured concentrations of specific SOI analyzed here equalled 90% or greater of the total S concentration.

3.1.1 Importance of unanalyzed SOI's in mine wastewater S budget

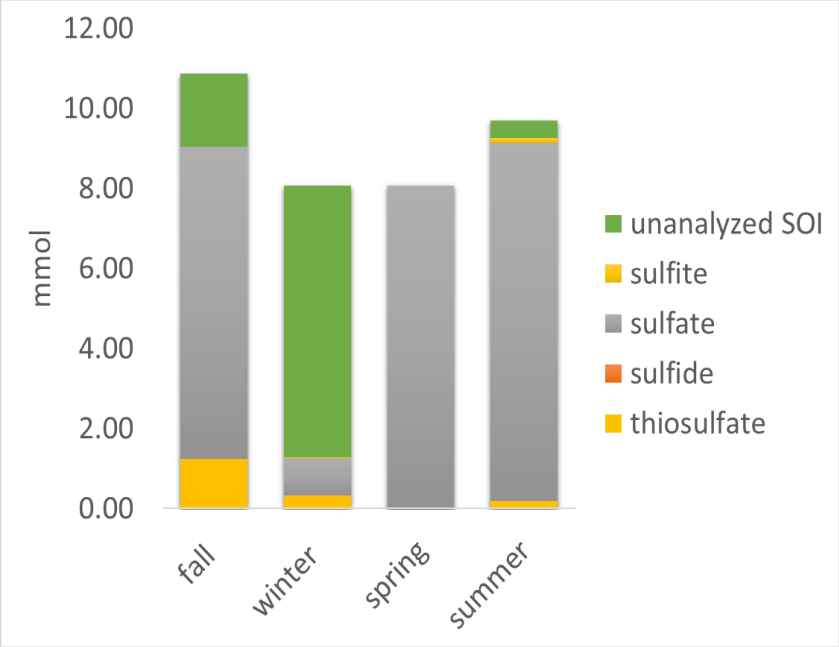


Figure 14: Waste Rock 1 Fall, Winter 2014 SOI balance & Spring, Summer 2015 SOI balance

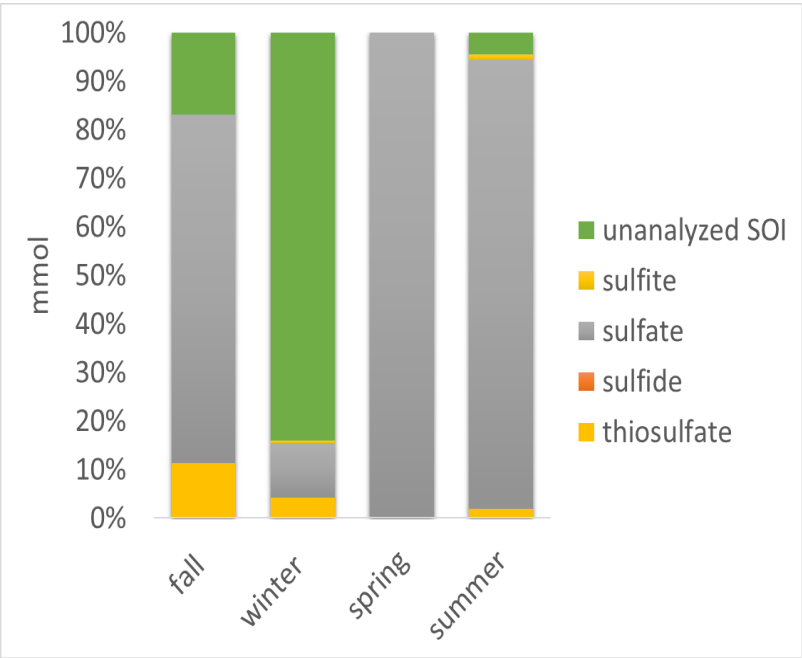


Figure 15: Waste Rock 1 Fall, Winter 2014 SOI balance & Spring, Summer 2015 SOI balance (%)

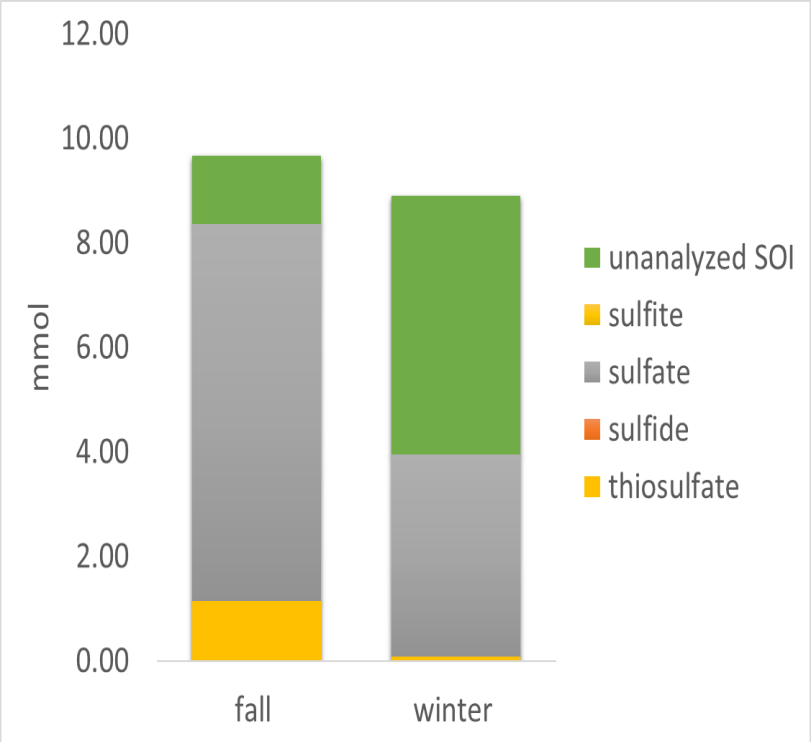


Figure 16: Waste Rock 2 Fall 2014 SOI balance

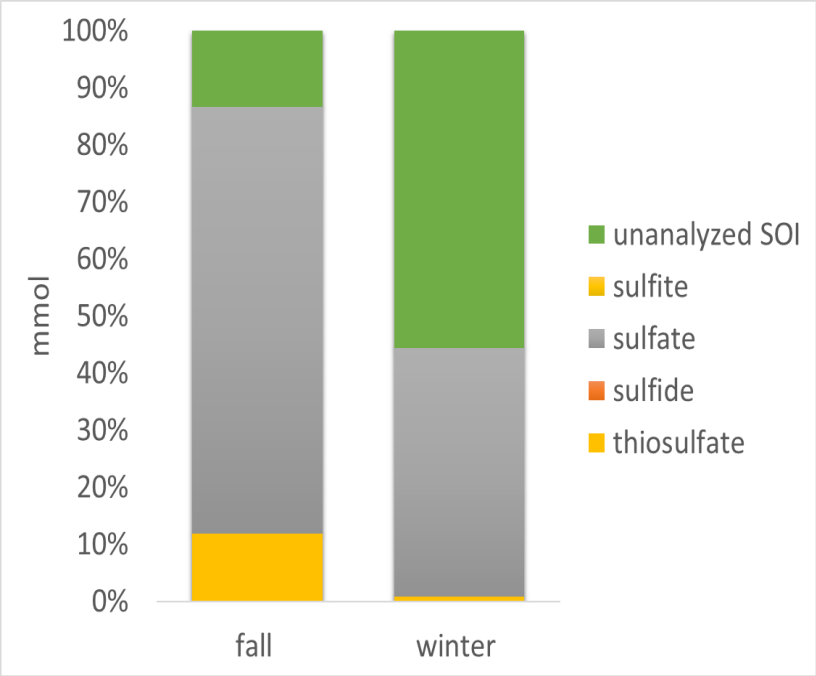


Figure 17: Waste Rock 2 Fall 2014 SOI balance (%)

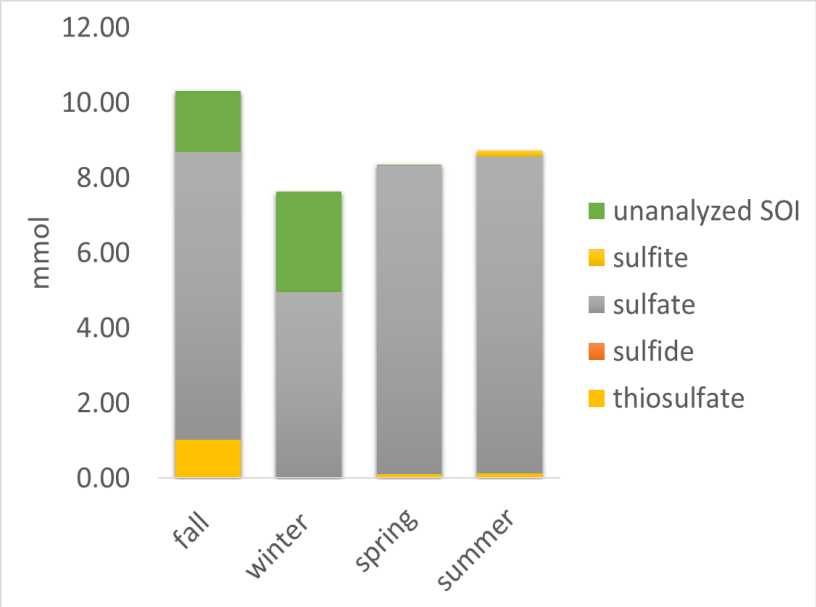


Figure 18: Oxidation Reservoir epilimnion Fall and winter 2014, spring and Summer 2015

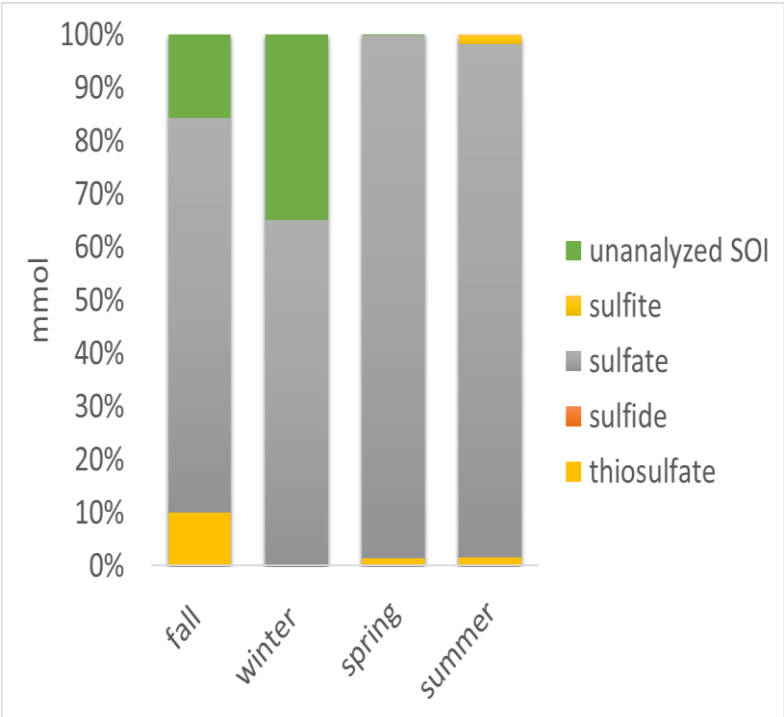


Figure 19: Oxidation Reservoir epilimnion Fall and winter 2014, spring and Summer 2015 (%)

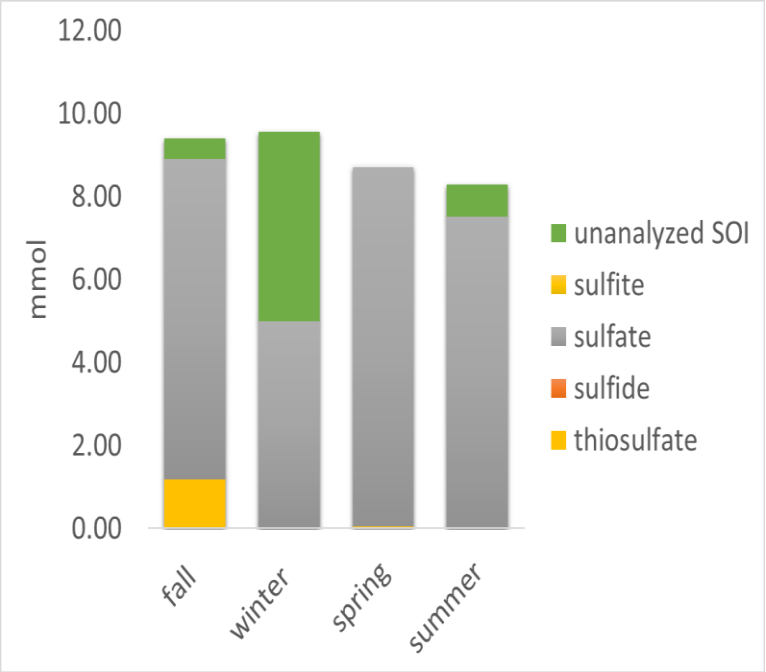


Figure 20: Oxidation Reservoir hypolimnion Fall and winter 2014, Spring, Summer 2015

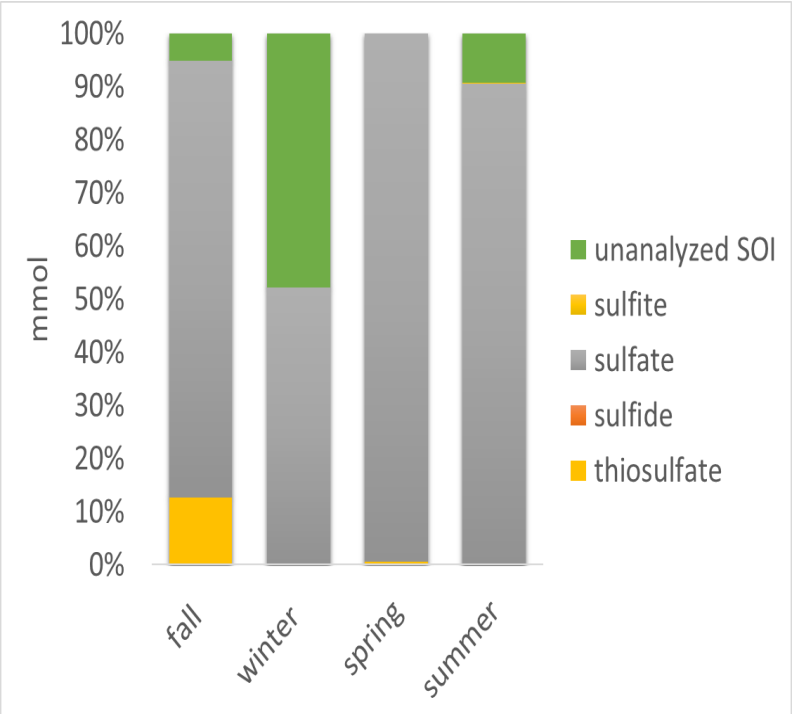


Figure 21: Oxidation Reservoir hypolimnion Fall and winter 2014, Spring, Summer 2015 (%)

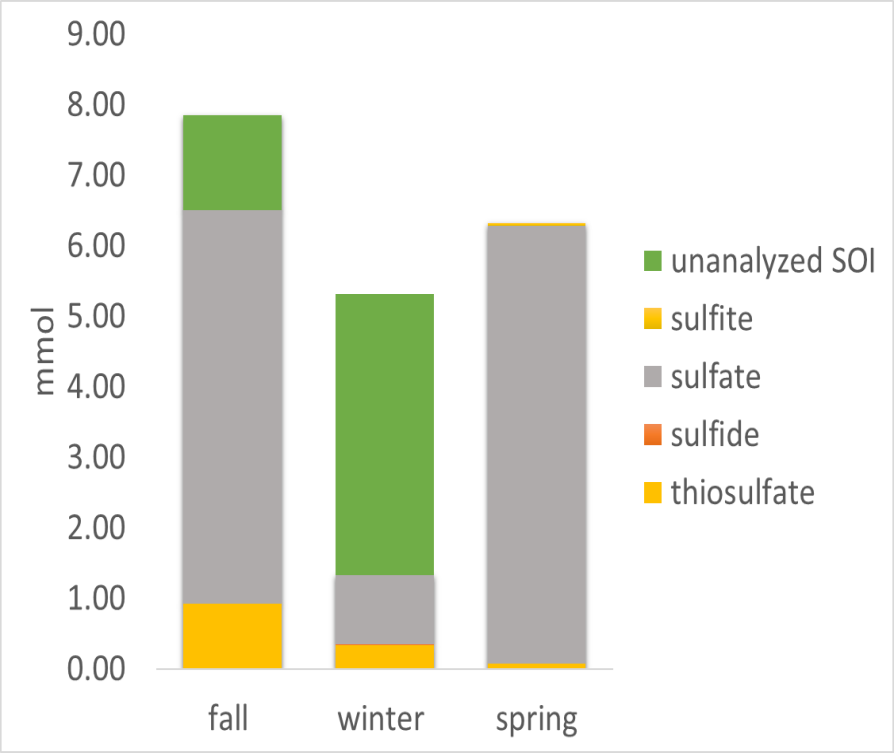


Figure 22: Strathcona tails Fall and winter 2014, Spring 2015

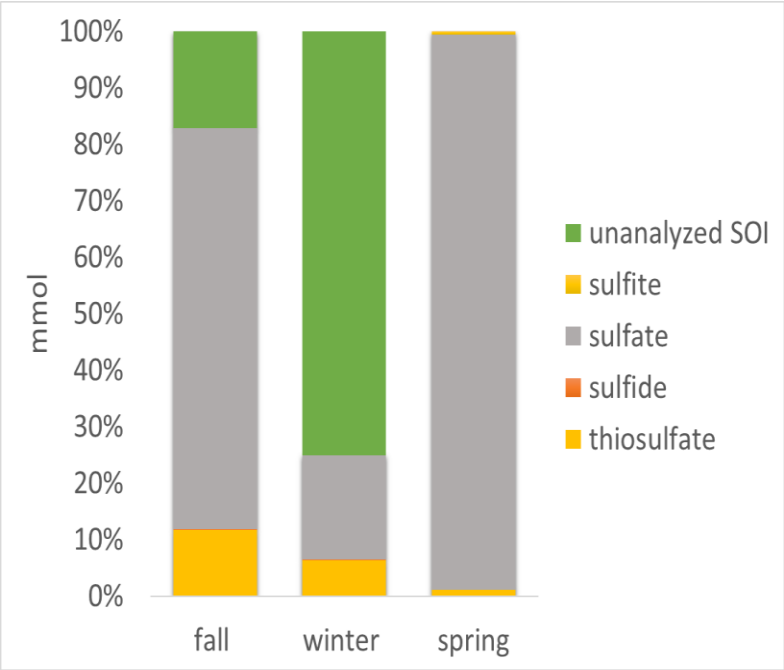


Figure 23: Strathcona tails Fall and winter 2014, Spring 2015 (%)

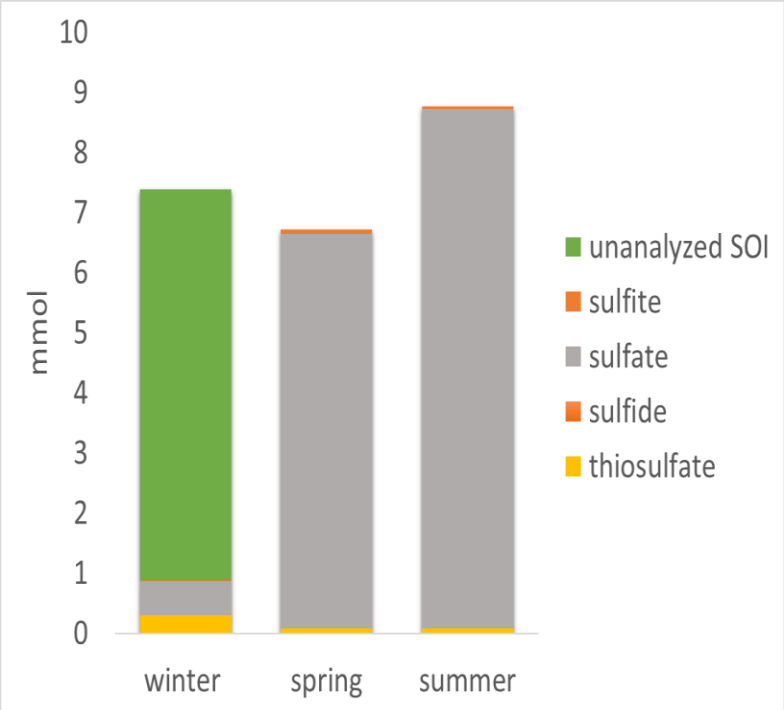


Figure 24: Mine 1 winter 2014, spring, summer 2015

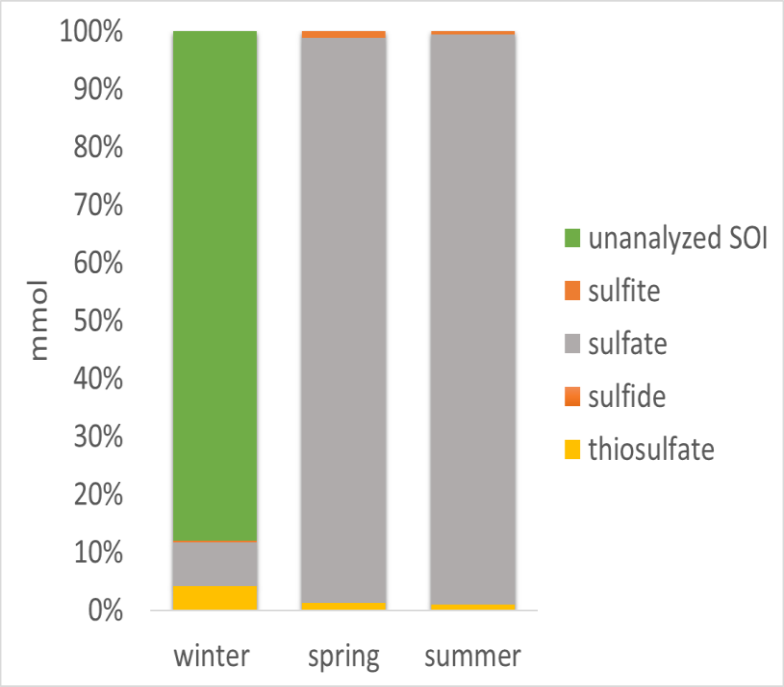


Figure 25: Mine 1 winter 2014, spring, summer 2015

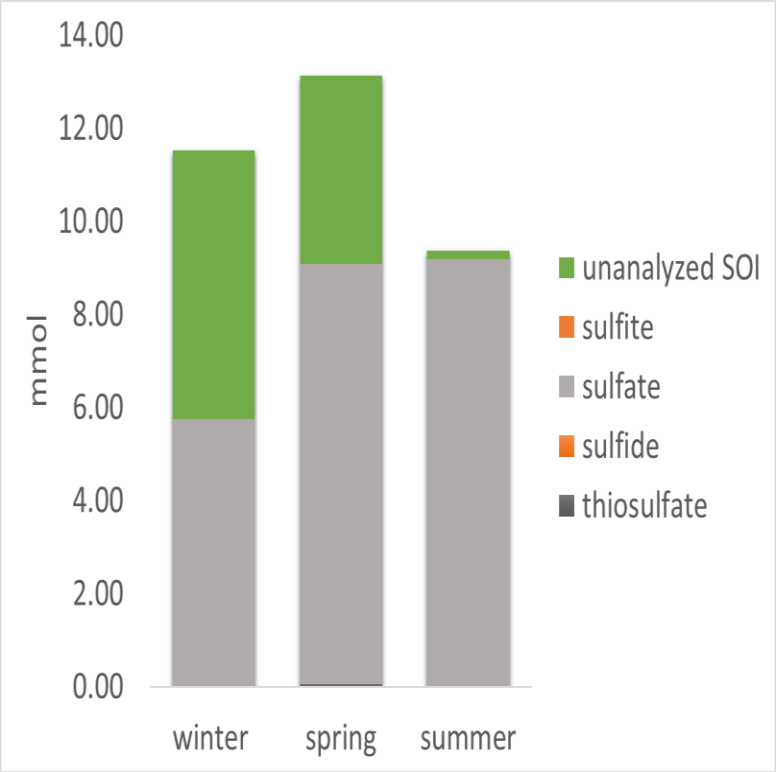


Figure 26: Mine 2 Winter 2014, Spring, Summer 2015

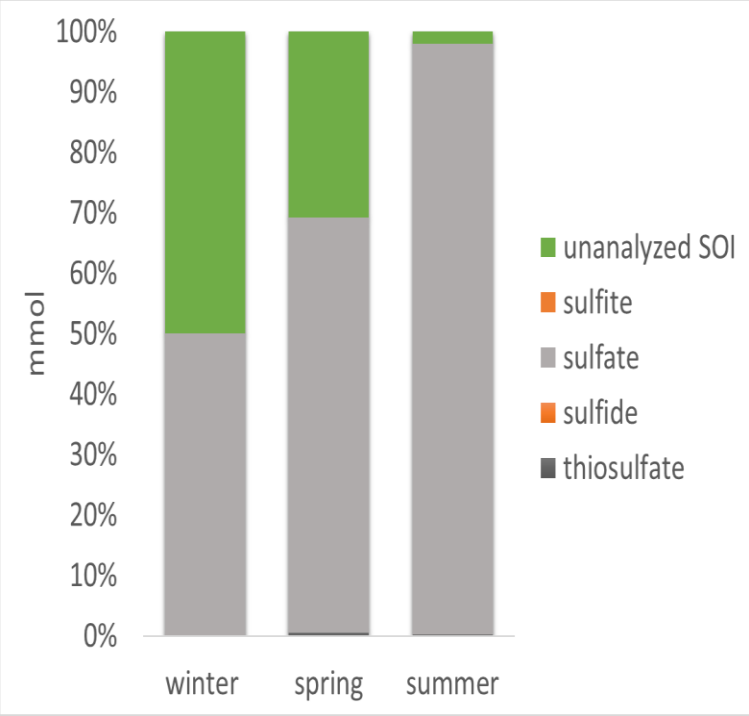


Figure 27: Mine 2 Winter 2014, Spring, Summer 2015 (%)

Another important finding was the proportion of elemental sulfur in these waters. Figures 28 through 41 detail the amount of elemental sulfur in comparison to other sulfur species in these waters. In some cases the amount of elemental sulfur is in great excess of the other sulfur species (60-90%) and is likely to play an important role in microbial cycling in these waters. In general the elemental sulfur tends to peak in the winter and decline in the warmer months. Potentially due to the fact that polysulfide formation is more likely in warmer waters (Kamyshny et al., 2008).

The occurrence, significant in the winter months of unanalyzed sulfur species such as polysulfides/polythionates and tetrathionate (Figures 14 through 27) , and the high elemental sulfur load (25-99%) results (Figs 28 through 41) identify the need for their assessment within mine wastewater sulfur budget determination. This will require further method development specifically for polysulfides/polythionates and tetrathionate and some further refining of the method for elemental sulfur analysis in these waters.

3.1.2 Importance of elemental S (S0) in mine wastewater S budget

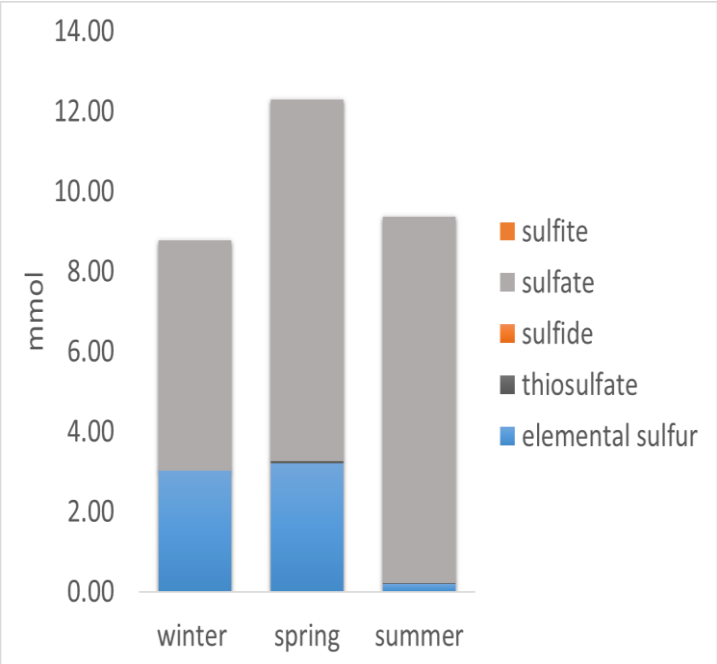


Figure 28: Mine 2 Winter 2014, Spring, Summer 2015

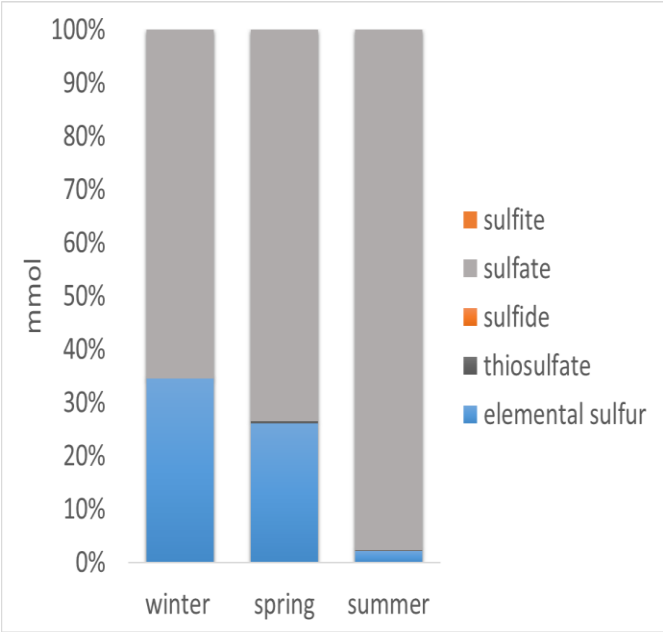


Figure 29: Mine 2 Winter 2014, Spring, Summer 2015 (%)

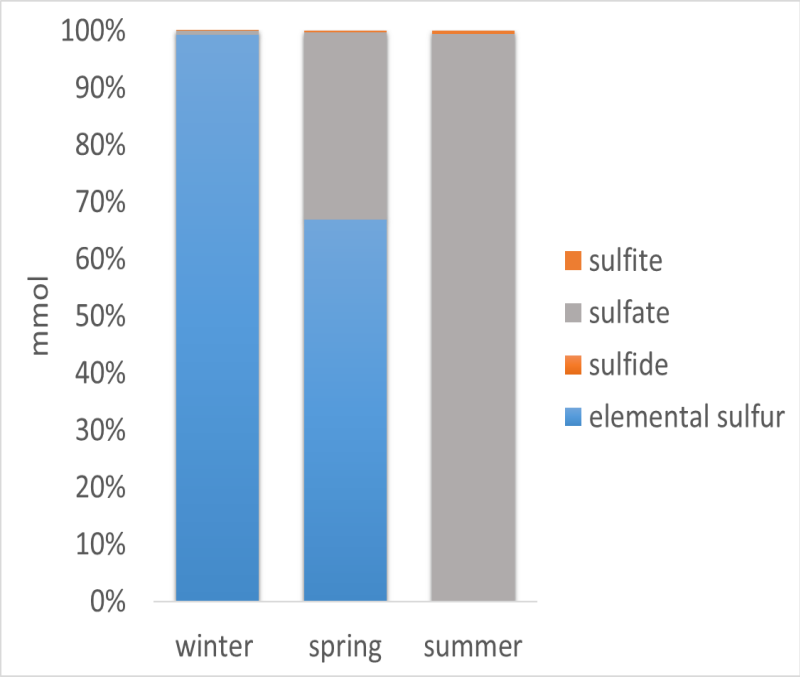


Figure 30 : Mine 1 Winter 2014, Spring, Summer 2015

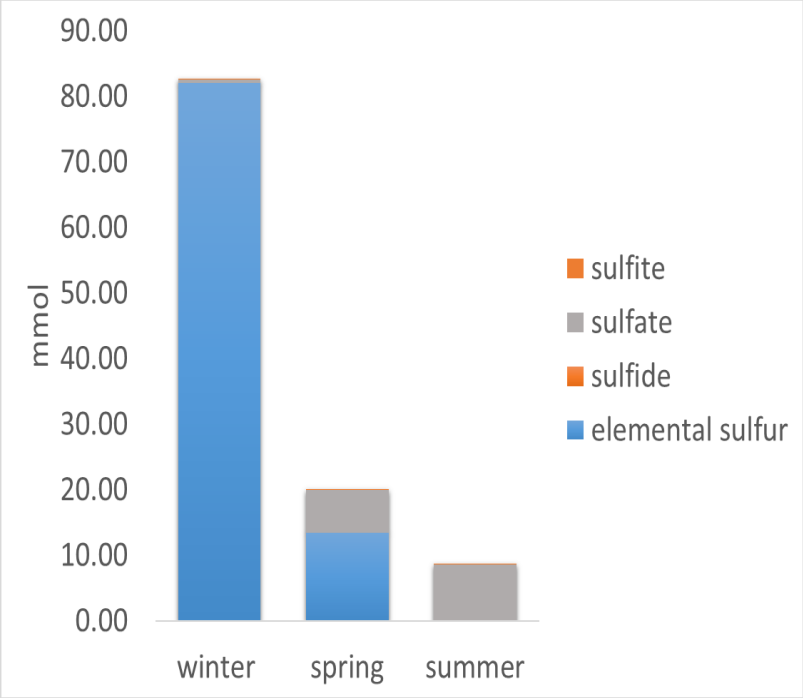


Figure 31: Mine 1 Winter 2014, Spring, Summer 2015

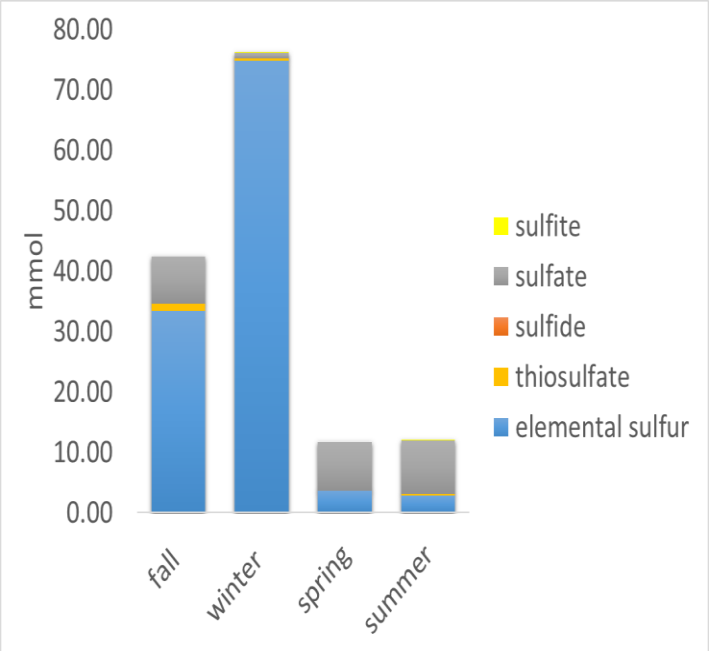


Figure 32: Waste rock 1 Fall and Winter 2014, Spring, Summer 2015

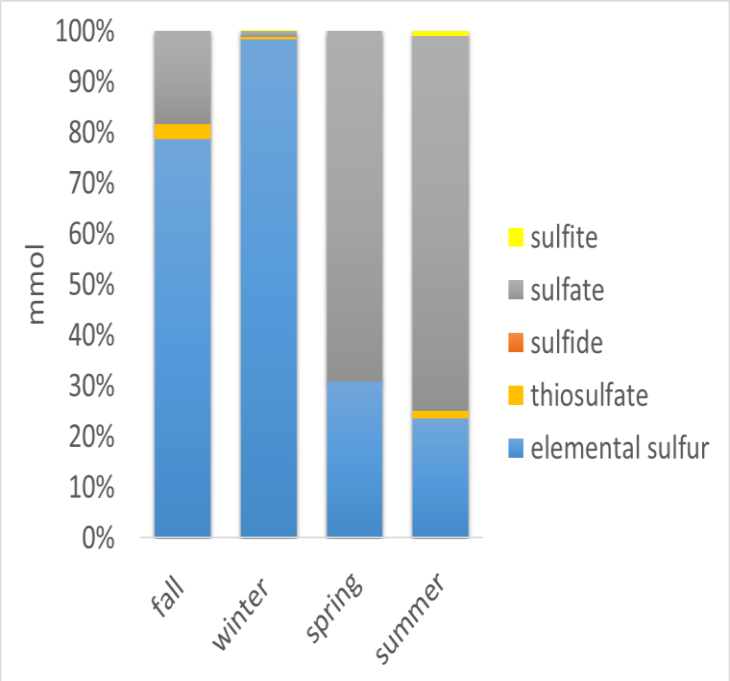


Figure 33: Waste rock 1 Fall and Winter 2014, Spring, Summer 2015

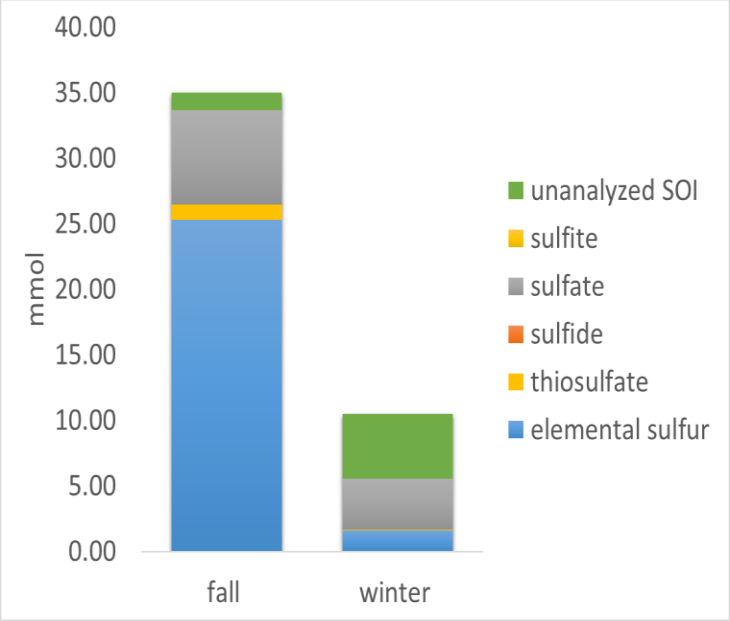


Figure 34: Waste rock 2 Fall 2014, winter 2015

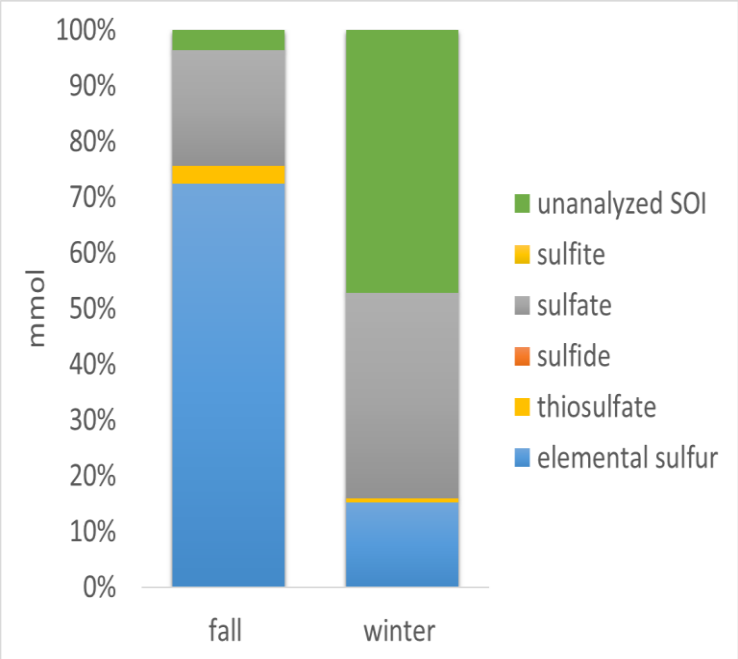


Figure 35: Waste rock 2 Fall 2014, winter 2015 (%)

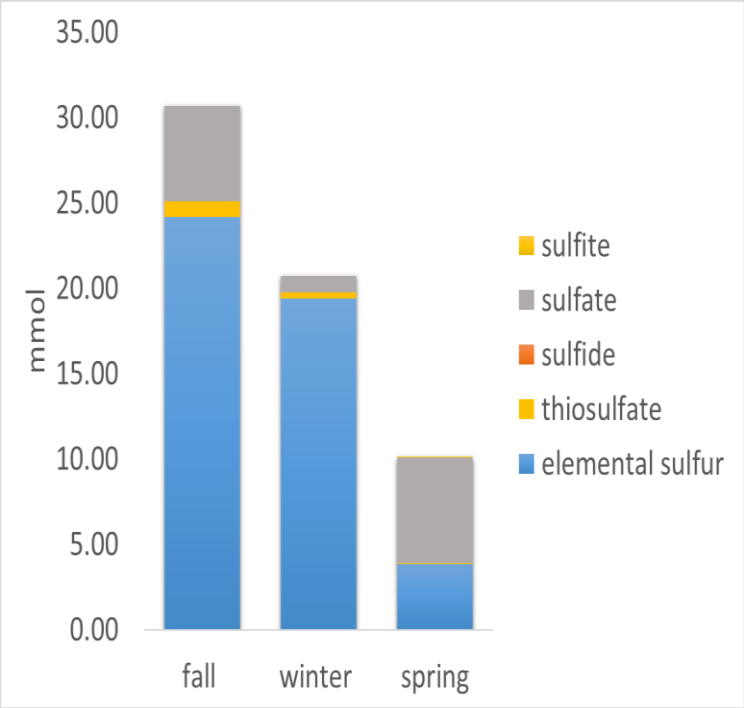


Figure 36: Strathcona tails Fall & Winter 2014, Spring, Summer 2015

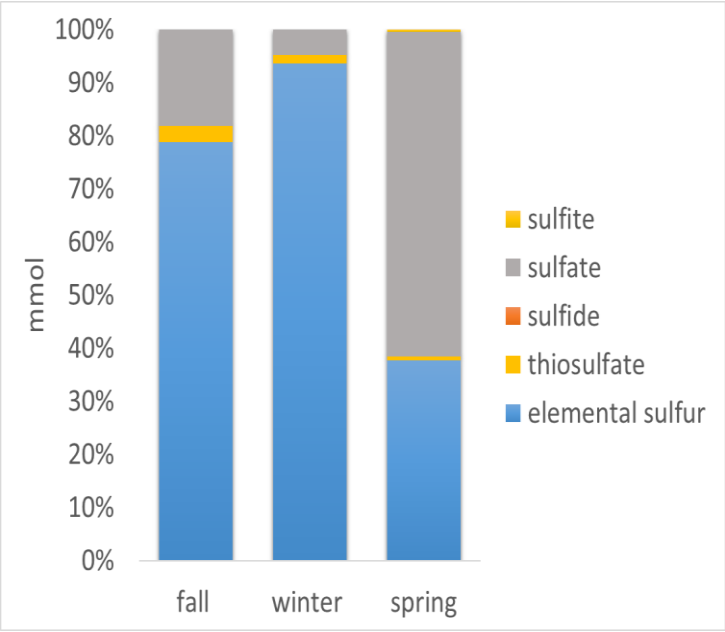


Figure 37: Strathcona tails Fall & Winter 2014, Spring, Summer 2015 (%)

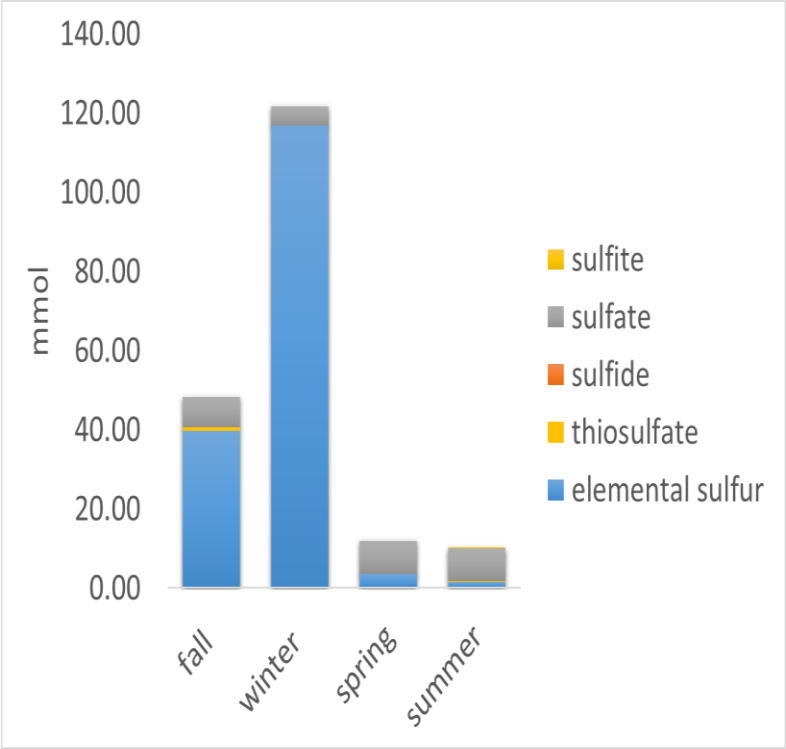


Figure 38: Oxidation Reservoir Epilimnion Fall & Winter 2014, Spring, Summer 2015

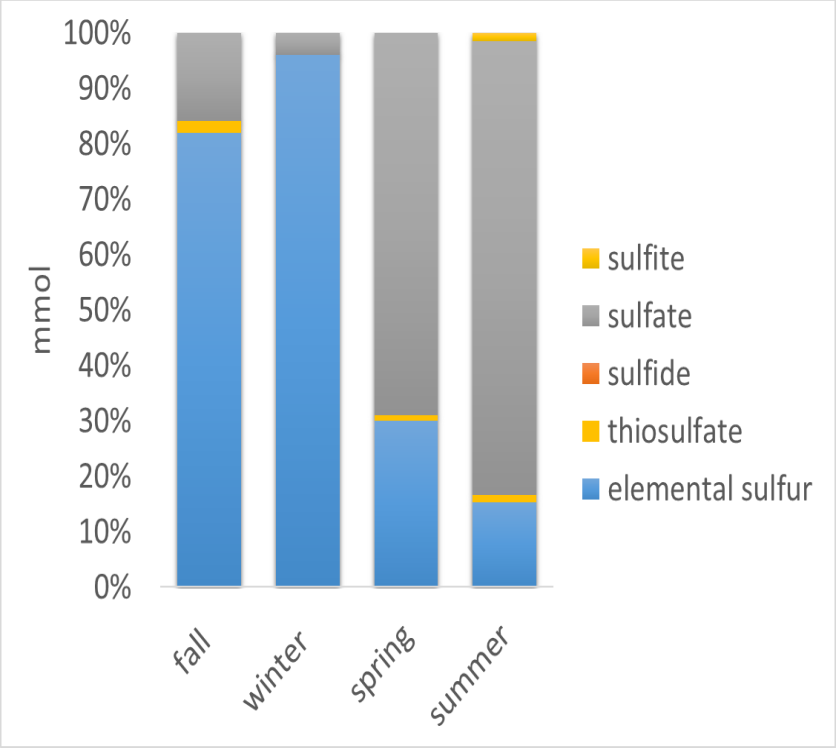


Figure 39: Oxidation Reservoir Epilimnion Fall & Winter 2014, Spring, Summer 2015(%)

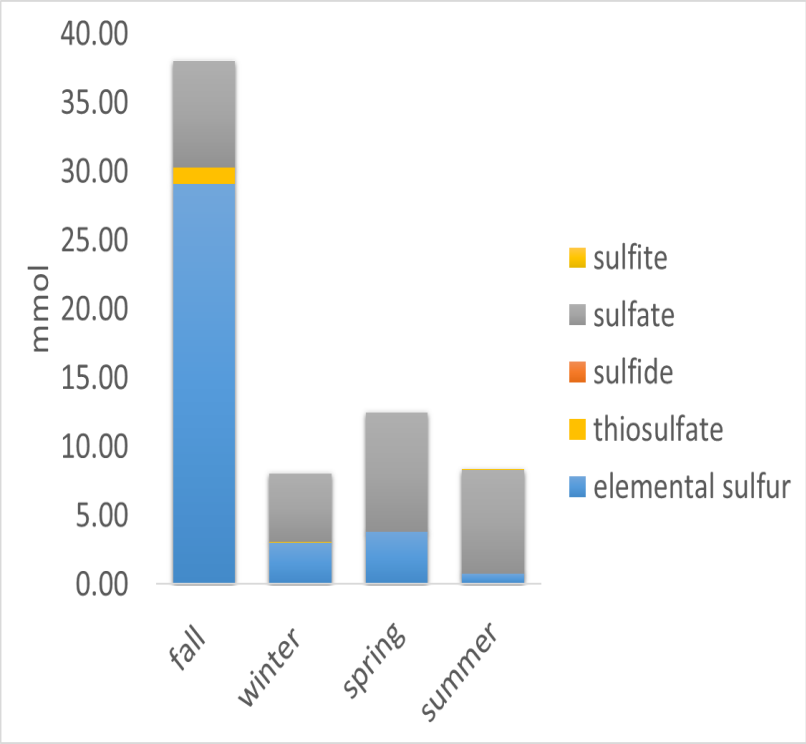
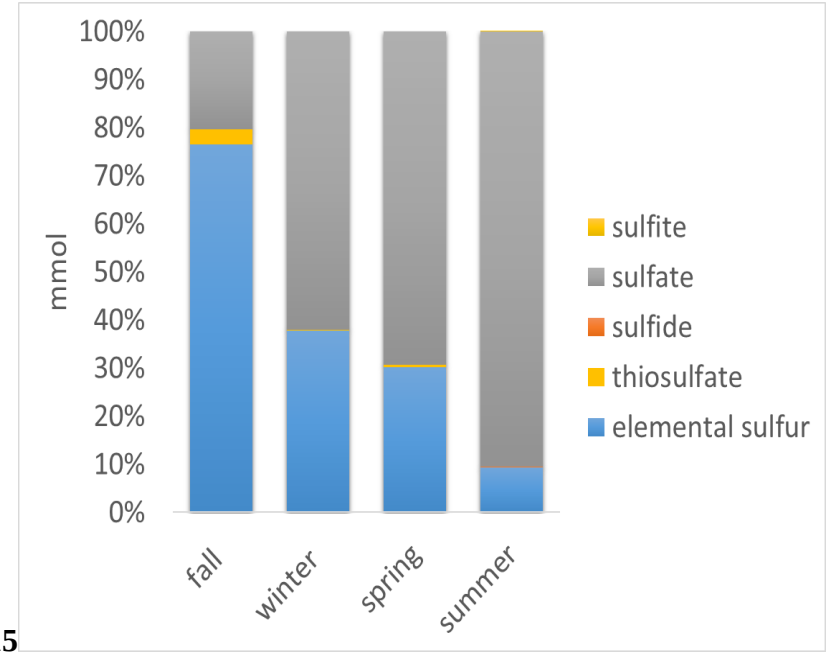


Figure 40: Oxidation Reservoir Hypolimnion Fall, Winter 2014, Spring, Summer



2015

Figure 41: Oxidation Reservoir Hypolimnion Fall, Winter 2014, Spring, Summer 2015 (%)

Figures 28 through 41 are pie charts which depict all of the analyzed sulfur species including elemental sulfur. These charts illustrate the proportion of elemental sulfur in these waters as compared to other species. The graphs are grouped by sample site and it is apparent that in the winter sample times throughout the sites elemental sulfur is the sulfur species in the highest proportions. Elemental sulfur in the winter ranges from 94% to 99% of the species analyzed with the exception of Mine 2 (34%). In the mine 2 samples however sulfur was still in the highest ratio as compared to the other seasons. Referring back to the total sulfur (CSIRO Australia) vs. analyzed sulfur species one can see that the pie charts with the largest gap in unknown sulfur is in the winter months. This may indicate that there are also polysulfide and polythionate reactions which are occurring at this time and affirms our high elemental sulfur results, as elemental sulfur is a reactant in sulfide chain formation. Sulfur percentages decline in spring, with the lowest sample season being summer with percentages between 6 % and 34 %. In the fall elemental sulfur begins to increase once again. One other feature of note is that the diversity of sulfur species peaks in the summer sample time. Sulfate is the analyzed sulfur species in the greatest percentages--between 68% and 91%. However there are also higher percentages of thiosulfate, sulfide and sulfite. This indicates that microbial action is increased in warmer waters and that the sulfur cycling is more active at these times.

4 Experimental assessment of seasonal SOM enrichment sulfur cycling

Microbial acidophilic sulfur oxidizing enrichments and neutrophilic sulfur oxidizing enrichments were conducted in an attempt to grow sulfur oxidizing microbes (SOM/SOB) and sample the solution they were developed in in order to determine their effect on sulfur cycling in the oxidation reservoir and it's inputs. Enrichments were conducted on the same seasonal and spatial scales as SOI samples listed in chapter 3.

The collection for these water samples was done as described in figure 9, 12 and 42. Two hundred and fifty to five hundred mL of water samples were collected in Nalgene bottles for each enrichment. The bottles were sterilized in an autoclave and then EtOH and bleach rinsed and then sealed until the sample was collected. After the water sample was collected these water samples were stored in a refrigerator at ~5 °C until used. They were enriched within a week of returning to Hamilton from Sudbury each sample time. The enrichments were made in a BSC and prior to use, the chamber and all instruments used were cleaned with EtOH and U.V light. Enrichments were stored in 250-1000 mL glass containers, which were autoclaved, rinsed with EtOH and subject to U.V light prior to use. 25 mL of each aqueous mine water sample was added to 250 mL enrichment media 1X (see media recipe appendix #6). The vessel was then sealed and placed in the dark (i.e. enrichments excluded any potential photosynthetic SOM) for ~ 2 weeks at which point it was re enriched following the same procedures and amounts. After a total of ~1 month the enrichments were filtered through 2 μm filter. After each enrichment had finished the two cycle enrichment phase, the eluent was analyzed for pH and sulfur species (sulfide, SOI's, total sulfur and sulfate).

A pre prepared derivatization mixture was made as described in chapter 2, and 50 µL of sample was added to that as soon as filtration was complete. After 30 minutes the reaction was stopped and the vials were frozen at -20 °C until analysis. All of the >60 enrichments for all seasons and sites sampled were analyzed on the HPLC. From that analysis it was determined which enrichments consumed thiosulfate and which ones did not or only partially consumed it. For those enrichments which consumed thiosulfate, the eluent was then subject to further SOI analysis. Those which did not show thiosulfate consumption were not analyzed further. For all the enrichments, sulfide was analyzed for immediately so as to ensure as much sulfide was captured as possible. The remainder of the eluent was stored at 5 °C until further analysis which occurred within 3 days if analysis was called for.

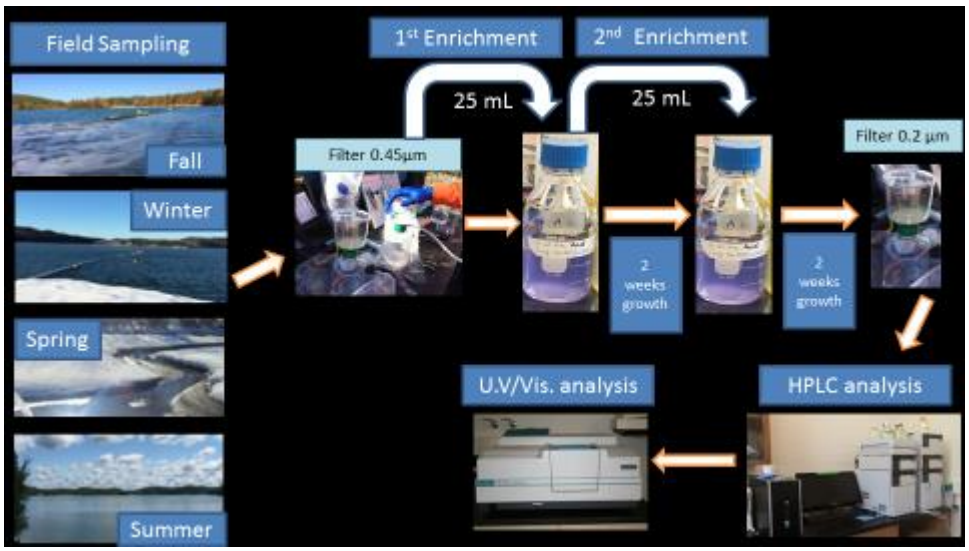


Figure 42: Field sampling methods for derivitizing aqueous samples

All enrichments displayed some visual change which corresponds with a physical change—colour (drop in pH) and/or precipitate (sulfur production)-- within 2 weeks (see figure 42). The amount of floc the colour of the precipitate and the degree to which the pH changed varied by site, and season. Although the products of microbial growth were

visually confirmed in all enrichments, there was a seasonal variation in which enrichments consumed the thiosulfate present in the enrichment media (see table 6). The fact that all sites showed physical signs of microbial growth, with some partially or completely consuming the thiosulfate within one month, indicates that there are likely SOM present in this oxidation reservoir and within its inputs—the conditions for them to start cycling simply need to be provided.

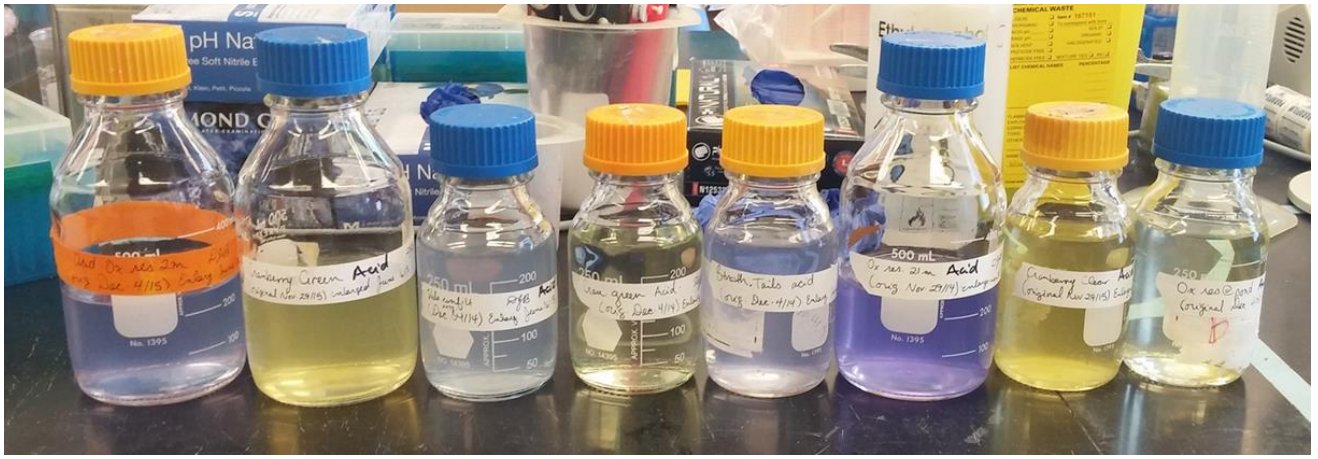


Figure 43: Some example SOx enrichments. The yellow colour represents pH=~3 or less. The blue/purple colour represents pH between 3.5 and 7. Variable amounts of cloudiness can be seen—most likely elemental sulfur, a product of microbial respiration.

Table 9: Acidophilic enrichments by site and season. Orange indicates final pH values between 1 and 3, yellow between 3 and 4, red 4 and 5, blue 5 and 6, green 6 and 8. Start pH was ~7.2. Red numbering indicates complete consumption of thiosulfate, black numbering partial or no consumption of thiosulfate.

site	waste rock 2	waste rock 1	mine 2	mine 3	ox. Res. Epi.	ox res hypo	ox res meta	strath tails	mine 1
sept A	1.94								
nov A	2.75	2.93			3.38	3.48		3.42	3.38
march A		6.13	7.33		3.62	5.76	4.51		
may A			2.17	3.54	6.06	3.74	8.04		5.7

4.1 Seasonal determinants of microbial sulfur cycling: Enrichment media analysis:

Briefly, the enrichments which consumed thiosulfate produced between 0-10 mmols of acid when comparing start pH of the site to final pH of the enrichment (Figure 47). From the 0.032 mols of thiosulfate provided, 31-72% was elemental sulfur, 10-25% was converted into sulfate and < than 1% was sulfide. For the enrichment budget this leaves between 12-60% of unanalyzed SOI's in the enrichment media. These results varied from the bulk water samples which showed an excess of sulfate in the warmer months. In terms of the winter enrichment samples, thiosulfate was not/only partially consumed, however there were pH drops in those enrichments.

Analysis of the enrichment eluent of those samples which consumed thiosulfate showed that, as with the bulk water analysis, there was a disparity in the amount of sulfur

species in solution as compared to the sulfur balance. For the enrichments the sulfur balance was set as the amount of thiosulfate added to solution. This concentration was verified through total sulfur numbers of the initial media and of the final eluent. Total sulfur analysis were performed by CSIRO. As with the bulk water samples, the enrichments show a variation in sulfur species—sulfate and sulfur prevail of the analyzed sulfur species, however as much as a half of the sulfur balance is unaccounted for. This suggests that SOM present in these enrichments, and the bulk water samples are participating in disproportionation reactions in which the end result would be sulfur and sulfate. Referring to figure 47 it is apparent that most of the enrichments did not vary drastically from their original pH. This would imply that the cycling of sulfur in these enrichments would be ~equal with respect to production and consumption of protons.

Referring back to table 2 which shows biotic and abiotic reactions, a likely reaction couple for these enrichments would be the biotic disproportionation of thiosulfate to form elemental sulfur, sulfite and hydroxide (1) $S_2O_3^{2-} + 2\ H_2O \rightarrow S^0 + H_2SO_3 + 2OH^-$. The unstable sulfite could then react with oxygen to form sulfate (2) $H_2SO_3 + 0.5\ O_2 \rightarrow SO_4^{2-} + 2\ H^+$. Microbial oxidation is also possible with iron or oxygen to form sulfate and protons (3) $4Fe^{3+} + S^0 + 3\ H_2O \rightarrow H_2SO_3 + 4\ Fe^{2+} + 4\ H^+$. Iron in the enrichments would be present from the bulk water samples. Microbial oxidation of thiosulfate to form tetrathionate is also likely (4) $2S_2O_3^{2-} + 0.5\ O_2 + 2H^+ \rightarrow S_4O_6^{2-} + H_2O$. The warmer water samples which consumed thiosulfate generated less protons and are therefore more likely to be performing reactions 1 & 2 which create a net neutral balance in regard to protons and results in elemental sulfur, which dominates the sulfur balance for the enrichments (Figure 44). The cooler spring sample waters had a higher tendency to produce protons as compared to the start pH and also generally have more sulfur and unanalyzed sulfur species present (ie: $S_4O_6^{2-}$). The reactions more likely in these waters would be 3 & 4.

Microbial DNA analysis' are necessary to confirm the likely reactions that are occurring in these waters and how the cycling is effected by these microbes seasonally and spatially.

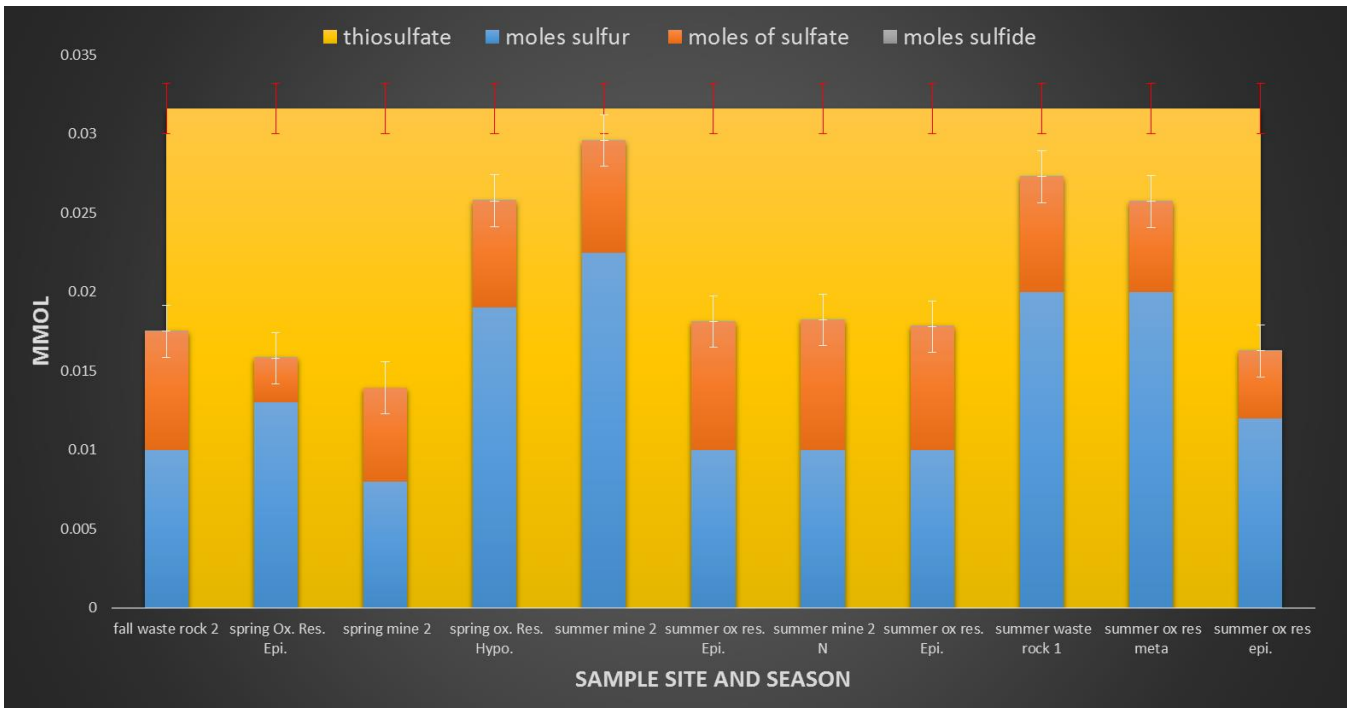


Figure 44: Enrichment sulfur balance—thiosulfate is the known added to the media. All other sulfur species are produced via microbial oxidation/disproportionation reactions. The difference between products and reactants are the SOI's not currently analyzed.

4.2 Environmental controls of enrichment growth and production

Of all of the physicochemical characteristics which were sampled for, none showed any correlation with microbial growth and thiosulfate consumption, with the exception of temperature.

The temperature of the sample sites varied considerably over season and site as mentioned previously (0°C-25°C). Figure 45 shows temperatures at the sites sampled which consumed thiosulfate and produced protons. Figure 46 shows temperatures at the sites sampled which did not/partially consumed thiosulfate with or without proton production. The highest consumption of thiosulfate with production of protons occurred in waters with a temperature of 10°C and higher. Sites with temperatures lower than 10°C did not consume thiosulfate. The temperature requirements for thiosulfate consumption by SOM may be even higher as they may be originating from the inputs (mine1, mine 2, and mine 3) all of which had higher temperatures over all seasons than the waste rock and oxidation reservoirs.

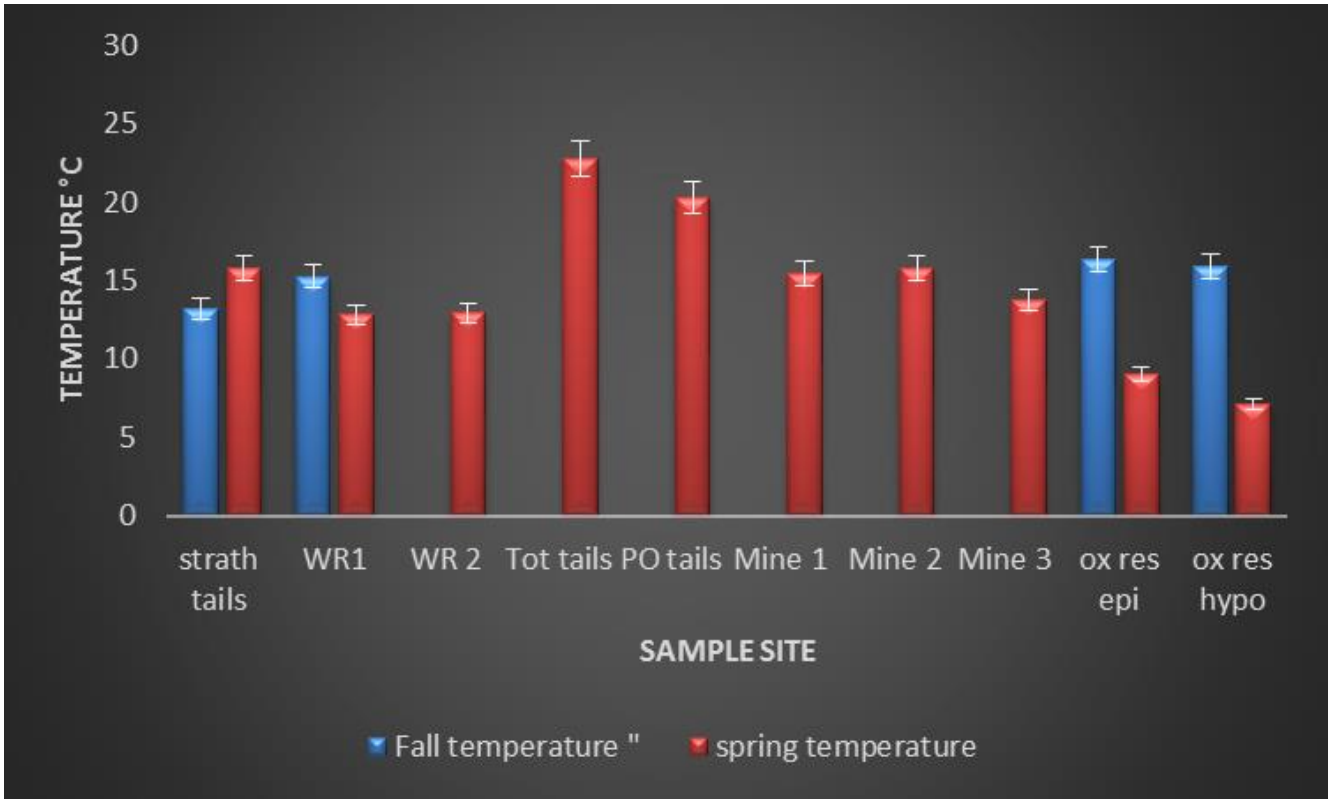


Figure 45: Temperature of the oxidation reservoir and its inputs. All shown sites consumed thiosulfate and generated protons.

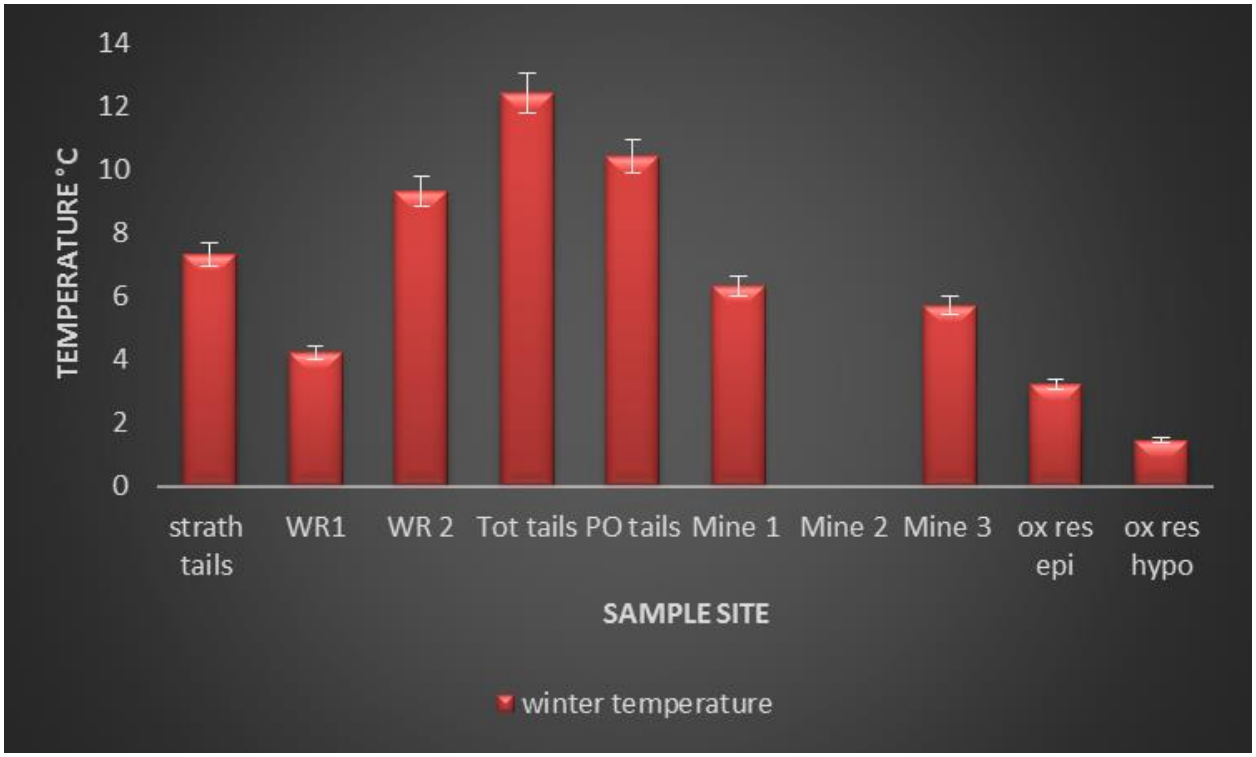


Figure 46: Temperature at the oxidation reservoir and its inputs when thiosulfate was not consumed.

One hypothesis may be that the lower the pH of a site would be, the greater the likelihood that SOM would currently be present. However, throughout the study, the initial pH of the sites did not appear to have a significant effect on thiosulfate consumption and proton production. Sites which consumed thiosulfate had a pH range from ~3.5→~9 which was the approximate range of the site and its inputs. Sites which did not consume thiosulfate (figure 46 shows a sample of these) had initial pH ranges between ~3.5→9.

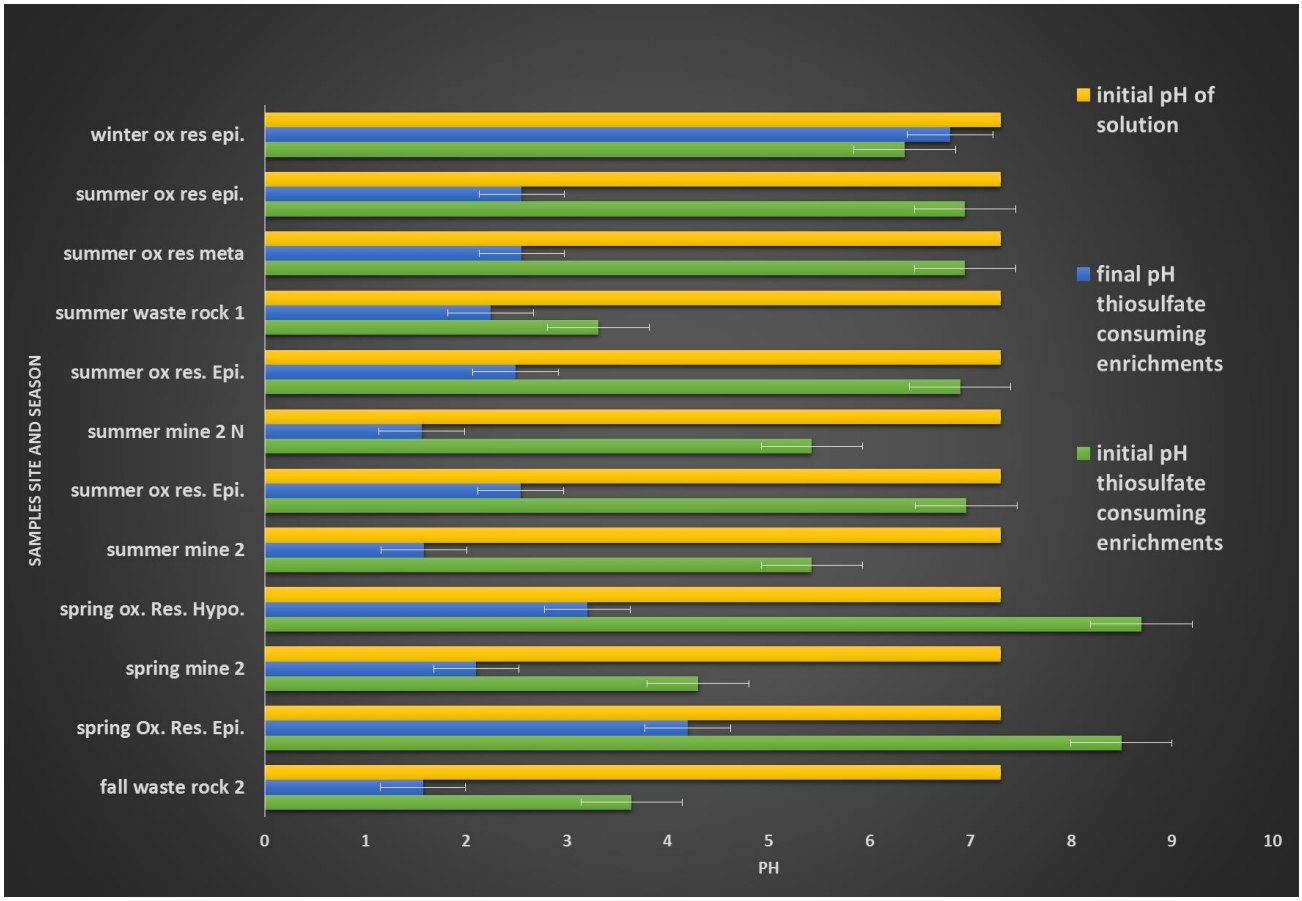


Figure 47: sample site and season vs pH of the starting enrichment solution, pH of the sample site at that season, and pH of the enrichment prior to filtration

Another thought might be that with SOM growth and thiosulfate consumption, significant amounts of protons would be generated in these enrichments. However, that also was not a consistent result for these enrichments. Figure 48 shows the sites which consumed thiosulfate and compares their start pH to their final pH. In many cases the initial pH and the final pH are comparable. In a few the start pH is lower than the final pH. This suggests more than one reaction is occurring in these enrichments and that likely multiple microbes are participating in sulfur cycling in these waters.

Figure 48: Graph of enrichments which consumed thiosulfate. Blue bars indicate the change in proton concentration from initial sample site pH to final enrichment pH

Figure 49 shows the change in pH of two representative sites in four seasons that did not consume thiosulfate. As with the thiosulfate consuming enrichments there is a variation in the initial and final pH. With some showing comparable pH values, some showing proton generation and some showing proton consumption. This result once again demonstrates that there are microbes present in these waters which are capable of creating variable acid generation or consumption conditions if provided with the right nutrients for growth. How these microbes could function and interact with thiosulfate consuming SOMs could provide insight into the management of mine wastewaters.

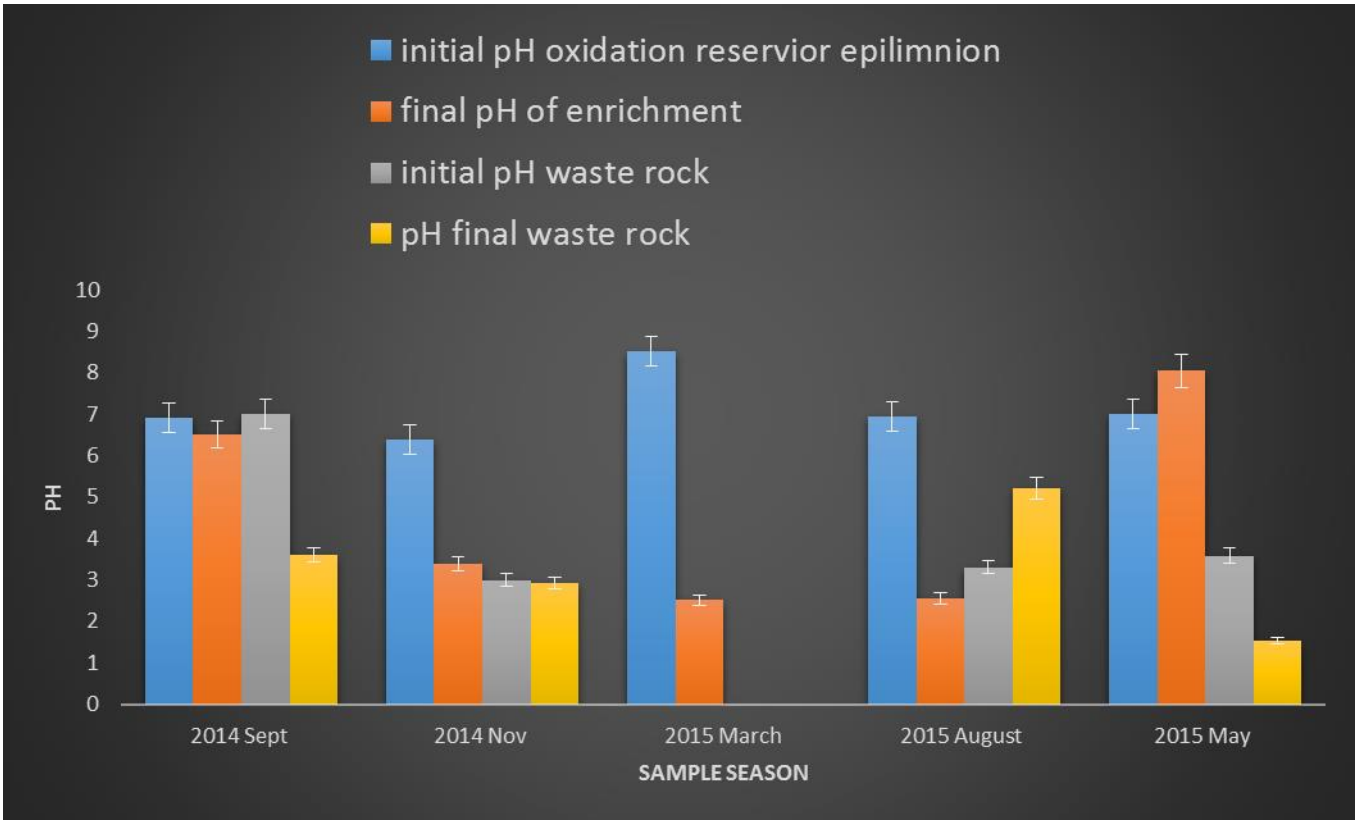


Figure 49: initial pH and final pH of waste rock and oxidation reservoir samples which did not consume thiosulfate

5 Conclusions:

Microbial biogeochemical cycling of sulfur compounds in mining wastewater remains a significant industry challenge and environmental threat. Key impediments to the industry's ability to improve wastewater sulfur management have been securing a better understanding of the specific sulfur oxidation intermediate (SOI) species that occur in wastewaters, as well as microbial transformations of these sulfur species. The objectives of this thesis addressed both of these knowledge gaps in laboratory, methodological, analytical and experimental research.

The development of methods using HPLC to characterize sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$), sulfide (ΣH_2S) as well as elemental sulfur (S^0) enabled assessment of these sulfur compounds in >60 seasonally and spatially varying wastewater samples collected from Sept 2014 to May 2016. Results identified SOIs were present in all wastewater samples and there were seasonal variations in both concentrations and occurrence of specific SOIs. The mass balance analysis of bulk water samples show that the total sulfur concentration varies seasonally in the system. Higher total sulfur occurred during spring and summer (8.4-13.1 mM) with lower (5.3-10.8 mM) total sulfur observed during the fall and winter sampling campaigns. Further, the proportion of the total sulfur pool associated with sulfate, indicative of complete oxidation of sulfur, were highest during spring and summer (75-100%) with a decreasing trend through fall (60-75%) and lowest in the winter under ice (10-20%); suggesting temperature may be an important ecological control on sulfur redox biogeochemistry. Corresponding to the observed decreasing seasonal sulfate trend, an increasing trend in the proportion of unanalyzed sulfur species (e.g. $S_4O_6^{2-}$, S^{2-}_{n+1}, $S_nO_6^{2-}$) was also observed, increasing from 0-25% (spring, summer) to 80-90% under ice. Further, elemental sulfur (S^0), which emerged as an important part of the sulfur cycle in these waters ranging in proportional abundance from 25-99% of the analyzed sulfur species, also increased during the fall and winter (75-99%), compared to 25-65% during the spring and summer.

Enrichment of sulfur oxidizing microbes (SOM) was successful from all >60 water samples collected indicating the presence of these bacteria throughout the system over seasonal scales. However these SOM catalyzed different sulfur transformations consistent with the seasonal SOI characterization results above and indicating that SOM are likely important players in sulfur cycling within mine wastewaters. Consumption of thiosulfate was limited to SOM enrichments from waters which were 10 °C or warmer (i.e. spring/summer) and generated sulfate and unanalyzed SOIs in lower and higher proportions respectively than those observed in summer field samples. Consistent with winter field results evidencing lower concentrations of sulfur and sulfate occurrence, winter SOM enrichments only partially consumed thiosulfate and cycled sulfur through different reactions compared to those catalyzed by warmer SoM enrichments. Further investigation would be necessary into these enrichments to elucidate which microbes are functioning in these enrichments and what nutrients they are cycling.

Analysis of SOI and endemic microbial communities provide a key assessment link in mine environmental management. The new methods that were developed enable more accurate determination of SOI in mining wastewaters. Assessment of SOI within mining waste waters demonstrate that simple H_2S/ SO_4^{2-} measurements will not comprehensively represent sulfur reactions and therefore accurately predict water quality outcomes that occur. Similarly, microbial sulfur metabolism is shown to be possible throughout space and time, but with differing seasonal implications for S cycling in these waters. The inclusion of SOI and SOM understanding into mine wastewater biogeochemical sulfur models will provide prophylactic rather than reactive management strategies.

Studies which investigate endemic microbe and SOI interactions in net neutral mine waste are necessary at this point in mining as remediation and prevention strategies have reached a standstill. Although mine waste is understood and managed to a much higher degree, there are still issues that occur in mine waste—such as the mount polley mine disaster—which suggest that

more still needs to be done. The way to prevent more of these disasters from occurring is not to only treat waters to ensure their neutrality, or secure dangerous waste in a spot which may fail- although these methods are part of mine waste treatment. The way to prevent problems is to understand how, where, why and when problems may initiate prior to any physical or chemical changes in the water system.

The strategy to understand these waters and what microbes are present and how they are cycling sulfur species requires a very comprehensive study of these systems over time. Geochemical data, microbial sequencing data—in both enrichments and bulk waters--and robust SOI analysis methods are a necessity to gather a body of research which may help to solve the puzzle of these complex systems and the complex beings which inhabit them.

The results of this study have made significant progress in the initial explorations of the mine waste puzzle. Not only is this the first study to investigate seasonal and temporal variations in SOI species over time, it is also the first study to authenticate those results and elucidate unanalyzed species via comparisons to total sulfur numbers. This comparison alone helps shed light on the sulfur speciation within mine waters. The sulfur speciation picture allows mine waste management teams to more effectively devise methods of analyzing SOI's as they can understand that thiosalts make up a small fraction of the SOI species that are being cycled in these waters and begin to adjust their analysis so that more comprehensive models can begin to be developed.

In addition--SOI analysis methods were developed and tested. It has been identified that thiosulfate, sulfite and sulfide are able to be analyzed on a reversed phase HPLC system which is equipped with a fluorescence detector. This analysis occurs following a relatively easy derivatization reaction which produces a highly detectable fluorescent species. Elemental sulfur is also able to be analyzed on the same instrument, with a UV detector following a chloroform extraction. The ability to analyze all of these species on the same instrument and following a

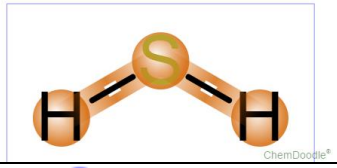
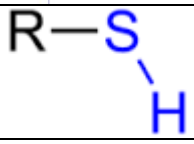
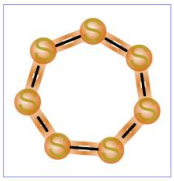
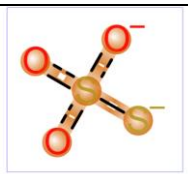
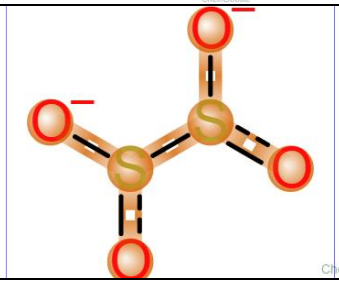
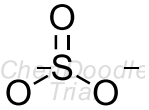
simple reaction and extraction is a significant milestone, greatly cutting down on sample preparation and ensuring that these samples can be analyzed rapidly after collection.

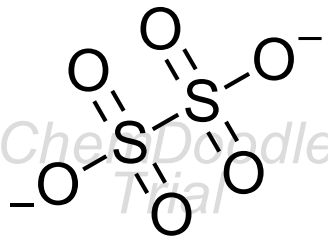
SOI analysis of the mine wastewater does show that SOI's are present in these waters in variable concentrations which correspond to different sample site and time. The data indicates that in the winter months sulfur cycling is much "quieter" with elemental sulfur and unanalyzed sulfur species being the dominant sulfur species in solution. In the warmer waters of the summer months, sulfur speciation is much more diverse which suggests that SOM are more active during these times. Given that the mine inputs have warmer temperatures throughout the season, the possibility of SOM cycling in these waters would be something to further investigate.

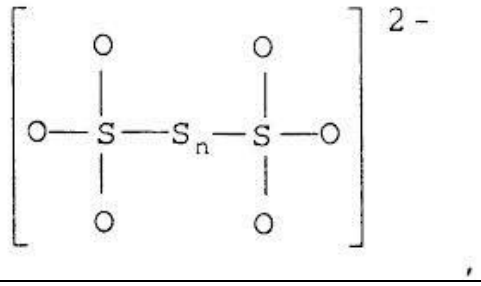
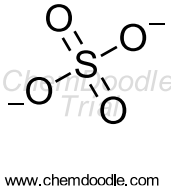
Further studies on these waters are necessary to fully understand this system, especially the role that SOM play in these waters while not currently acid generating. This study provides an excellent foundation to build upon to further elucidate the role of microbes in these systems, how they cycle sulfur and the end result of these cycles over time.

6 Appendix:

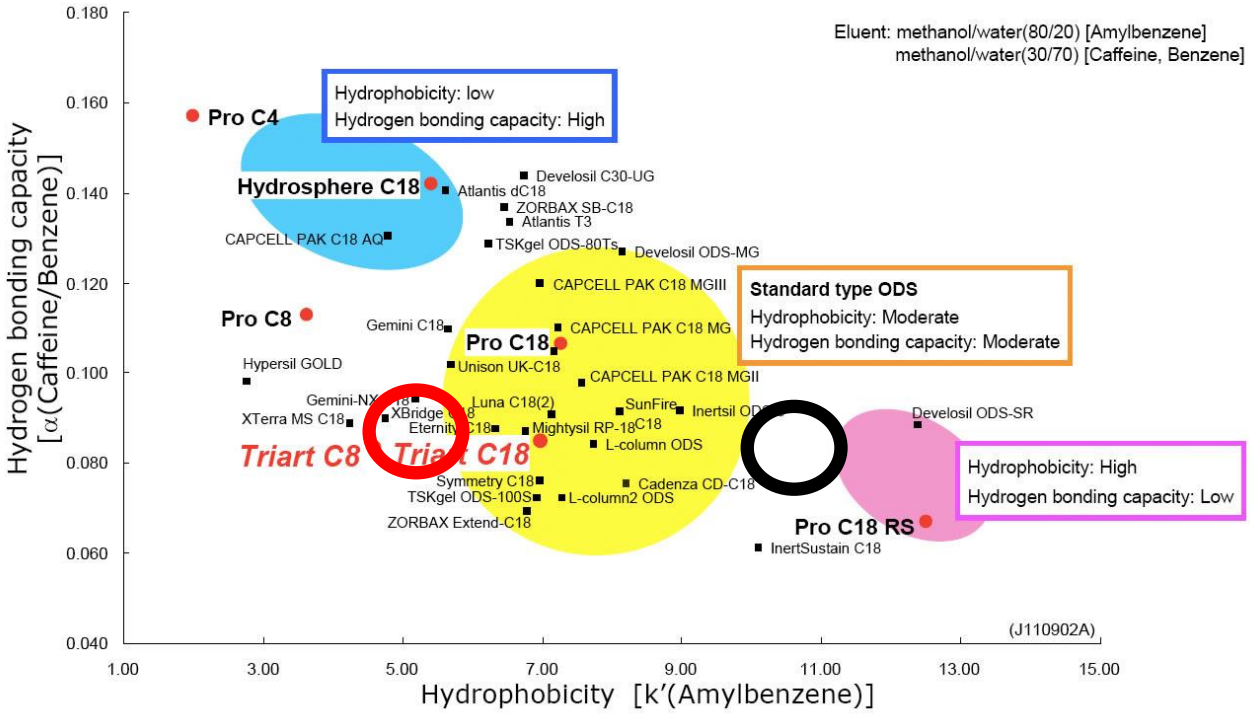
Appendix 1: sulfur species in mine wastewaters

Oxidation	Name	Formula	Molecular structure
-2/-1	Hydrogen sulfide/metal sulfides	H_2S Ie; Fe_2S	
-2	Thiols an	R-SH	
0	Sulfur	S^0 S_8	
2	Thiosulfate	$S_2O_3^{2-}$	
3	Dithionite	$S_2O_4^{2-}$	
4	Sulfite	SO_3^{2-}	

5	Dithionate	S_2O_6	 www.chemdoodle.com

Oxidatio	Name	Formula	Molecular structure
5 and 1	polythionate	$S_{n+2}O_6^{2-}$	
6	sulfate	SO_4^{2-}	

Appendix 2: Column comparisons



(adapted from Ymcamerica.com, 2016)

Reithmeier column in red, our column in black

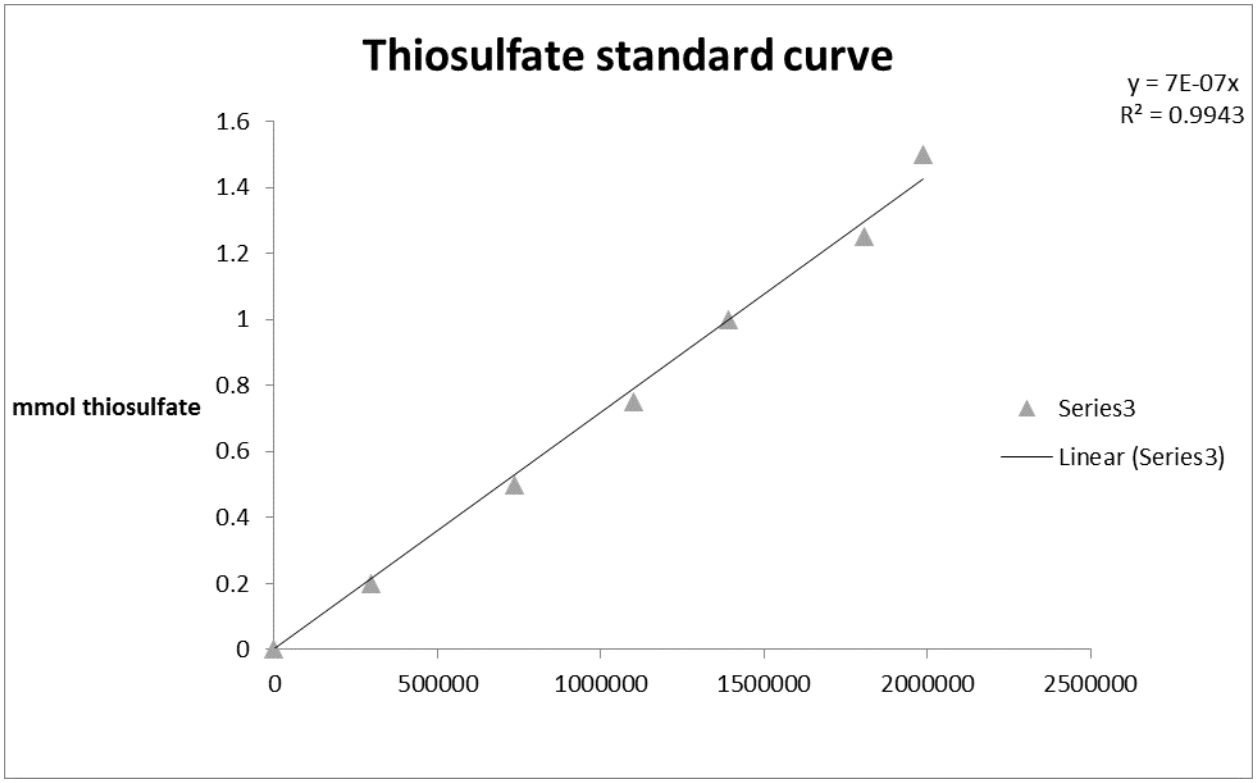
Appendix 3: sulfur species and reaction products

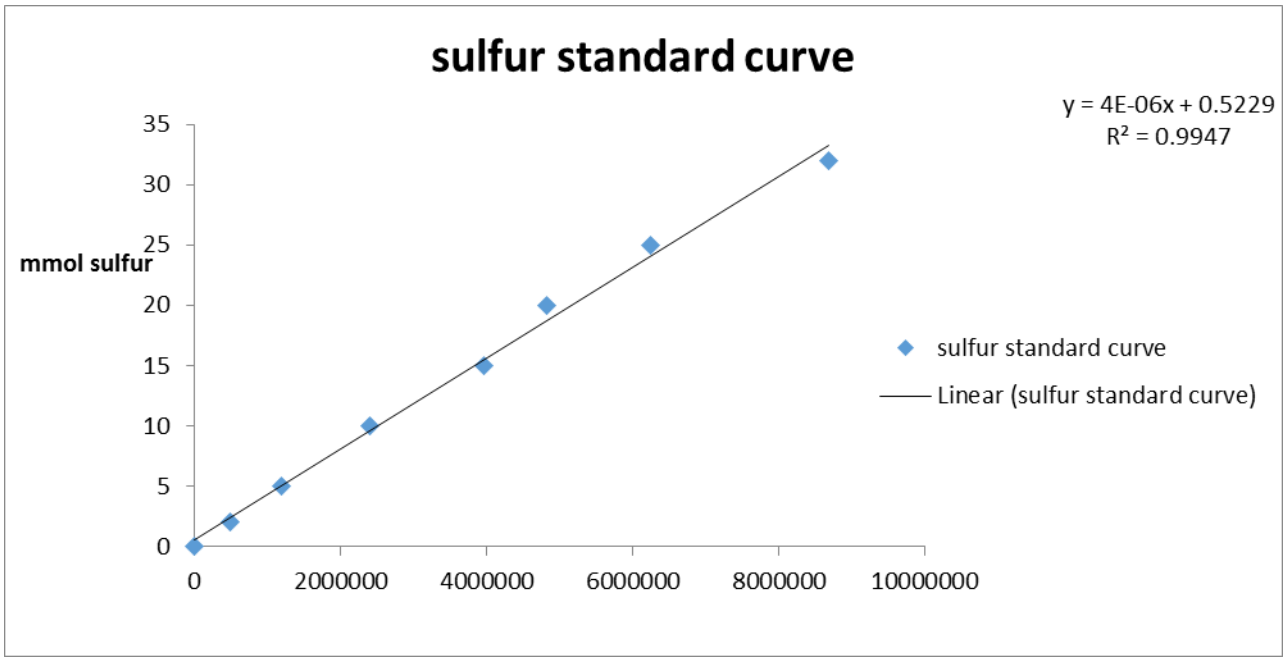
Table 1 Sulfur species occurring in aqueous media. The most common species are printed in bold. [1].

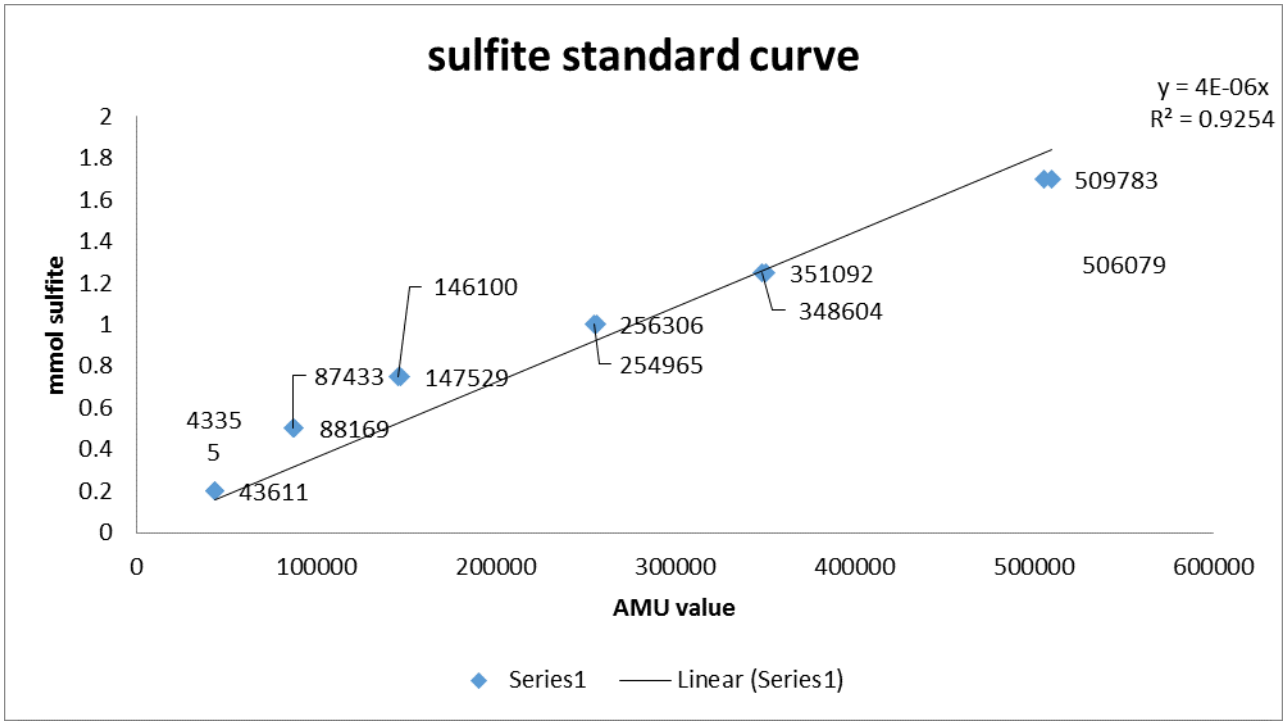
Sulfur species (Y)	Name	Oxidation state	pK	Oxidation products
$S_xO_6^{2-}$ x >= 3	polythionates	0, V		
$S_2O_8^{2-}$	peroxodisulfate	VII	0, 0.9	SO_4^{2-}
$S_2O_7^{2-}$	disulfate	VI		SO_4^{2-}
SO_4^{2-}	**sulfate**	**VI**	**1.98, -3**	**very stable**
$S_2O_6^{2-}$	dithionate	V		
$S_2O_5^{2-}$	disulfite	IV		SO_3^{2-} SO_4^{2-}
SO_3^{2-}	sulfite	IV	1.89, 7.21	SO_4^{2-}
$S_2O_4^{2-}$	dithionite	III	0.35, 2.45	$S_2O_3^{2-}$,SO_3^{2-},SO_4^{2-}
$S_4O_6^{2-}$	tetrathionate	II1/2		SO_4^{2-}
$M_m^{x+}(S_2O_3)_y^{(mx-2y)}$	metal thiosulfate complexes	II		
$S_2O_3^{2-}$	**thiosulfate**	**II**	**0.6, 1.72**	**SO_4^{2-}**
S° & S_8	**elemental sulfur**	**0**		
$CH_3S_xCH_3$	dimethylpoly-sulfide (DMPS)	0,I		
RSH	sulfhydryl thiols	0		
SCN -	thiocyanate	0	-1.8	
S_x^{2-} x >= 2	polysulfides	0, I-		S°, $S_2O_3^{2-}$
HS^-	**sulfide, hydrogen sulfide**	**II-**	**6.99, 12.9**	**$S_2O_3^{2-}$, SO_3^{2-},SO_4^{2-}**

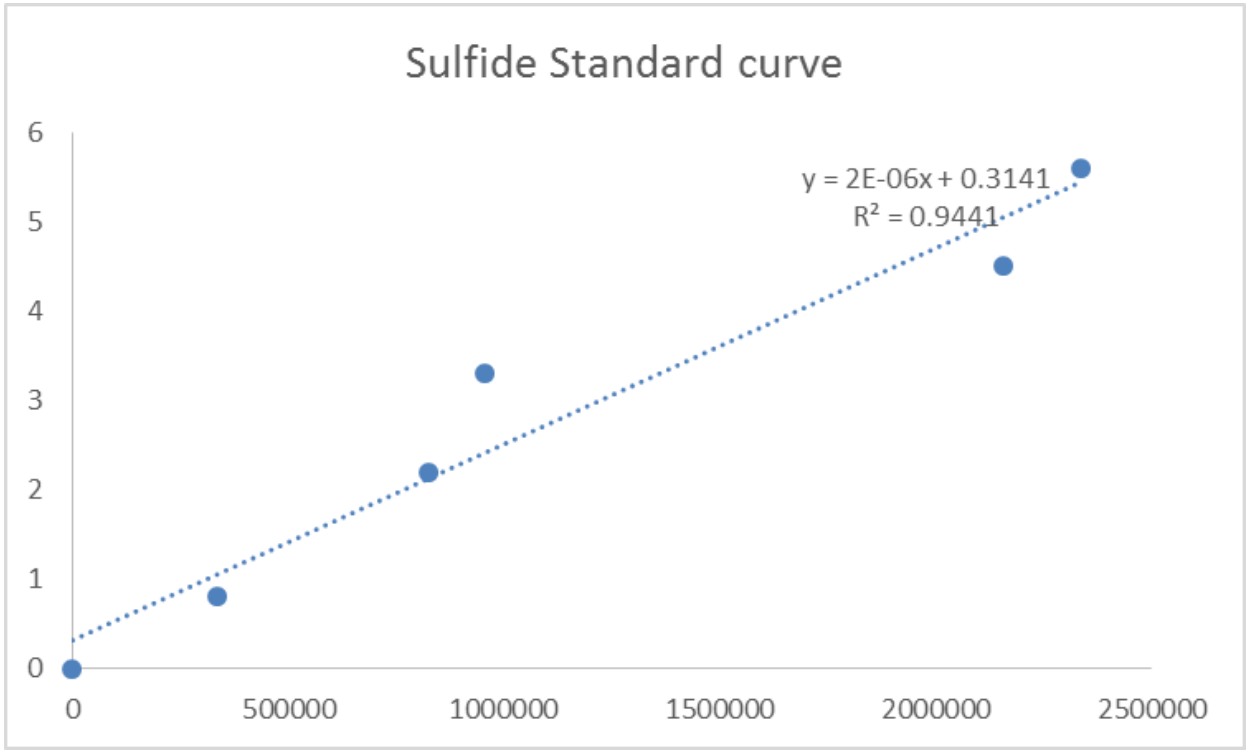
(Keller-Lehmann et al., 2016)

Appendix 4: HPLC standard curves






sulfite standard curve
y = 4E-06x
R² = 0.9254
mmol sulfite
AMU value
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
100000
200000
300000
400000
500000
600000
509783
506079
351092
348604
146100
256306
254965
87433
147529
43355
88169
43611
Series1
Linear (Series1)


Sulfide Standard curve
y = 2E-06x + 0.3141
R² = 0.9441
6
5
4
3
2
1
0
0
500000
1000000
1500000
2000000
2500000

Appendix 5: Media recipe

Neutrophilic sulfur oxidizing bacteria (SOx)

For 1 L of solution:

Part 1:

90 mL of 1.1% (w/v) K_2HPO_4

400 mL tap water

Part 2:

5 g of $Na_2S_2O_3$

90 mL of 0.44% (w/v) NH_4Cl

90 mL of 0.11% (w/v) $MgSO_4$

2.2 mL of solution T

320 mL of tap water

Sterlize the two parts separately and aseptically combine

Add 2-3 drops of phenol red as a colour change agent

Acidophilic sulfur oxidizing bacteria (SOx)

For 1 L of solution

Into 1 L of tap water dissolve:

0.2 g $(NH_4)_2SO_4$

0.25 g $MgSO_4*7H_2O$

0.36 g $CaCl_2*2H_2O$

0.5 g KH_2PO_4

5 g of thiosulfate ($Na_2S_2O_3$)

0.010 g $FeSO_4$

Stir to mix and filter sterilize.

7 References

Akcil, A. and Koldas, S. (2006). Acid Mine Drainage (AMD): causes, treatment and case studies.*Journal of Cleaner Production*, 14(12-13), pp.1139-1145.

Alberta environment and parks, (2015). *Development and validation of analytical methods for elemental sulphur in Alberta soils*.

Amend, J., Edwards, K. and Lyons, T. (2004). *Sulfur biogeochemistry*. Boulder, Colo.: Geological Society of America.

Anon, (2015). *Sulfur*. [online] Available at: http://weppi.gtk.fi/publ/foregsatlas/text/S.pdf [Accessed 17 Apr. 2015].

Antoine.frostburg.edu. (2016). *General Chemistry Online: FAQ: Simple compounds: What is thiosulfate ion, and what is it used for?*. [online] Available at: http://antoine.frostburg.edu/chem/senese/101/compounds/faq/thiosulfate.shtml [Accessed 22 Jul. 2016].

Baker, B. and Banfield, J. (2003). Microbial communities in acid mine drainage. *FEMS Microbiology Ecology*, 44(2), pp.139-152.

Barton, L. (1995). *Sulfate reducing bacteria*. New York [u.a.]: Plenum Press.

Barton, L., Mandl, M. and Loy, A. (2010). *Geomicrobiology*. Dordrecht: Springer.

BELL, F. and BULLOCK, S. (1996). The Problem of Acid Mine Drainage, with an Illustrative Case History. *Environmental & Engineering Geoscience*, II(3), pp.369-392.

Bell, F. and Donnelly, L. (2006). *Mining and its impact on the environment*. London: Taylor & Francis.

Bernier, L. (2008). *SULPHUR BIOGEOCHEMISTRY IN A MINE TAILINGS LAKE*. PhD. McMaster University.

Böttcher, M. and Thamdrup, B. (2001). Anaerobic sulfide oxidation and stable isotope fractionation associated with bacterial sulfur disproportionation in the presence of MnO2. *Geochimica et Cosmochimica Acta*, 65(10), pp.1573-1581.

Brune, D. (1989). Sulfur oxidation by phototrophic bacteria. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 975(2), pp.189-221.

Brunton, T. and Macfadyen, A. (1889). The Ferment-Action of Bacteria. *royal society of london*, [online] 46. Available at: http://rspl.royalsocietypublishing.org/content/46/280-285/542.full.pdf.

Buchanan, D., Coombs, K., Murphy, P. and Chaven, C. (1993). A convenient method for the quantitative determination of elemental sulfur in coal by HPLC analysis of perchloroethylene extracts. *Energy & Fuels*, 7(2), pp.219-221.

Cavemanchemistry.com. (2015). [online] Available at: http://www.cavemanchemistry.com/cavebook/chmetal2.html [Accessed 16 Apr. 2015].

Chen, Y., Joly, H. and Belzile, N. (1997). Determination of elemental sulfur in environmental samples by gas chromatography-mass spectrometry. *Chemical Geology*, 137(3-4), pp.195-200.

Cheng, J., Jandik, P. and Avdalovic, N. (2005). Pulsed amperometric detection of sulfide, cyanide, iodide, thiosulfate, bromide and thiocyanate with microfabricated disposable silver working electrodes in ion chromatography. *Analytica Chimica Acta*, 536(1-2), pp.267-274.

CLINE, J. (1969). SPECTROPHOTOMETRIC DETERMINATION OF HYDROGEN SULFIDE IN NATURAL WATERS1. *Limnol. Oceanogr.*, 14(3), pp.454-458.

Colmer, A. and Hinkle, M. (1947). The Role of Microorganisms in Acid Mine Drainage: A Preliminary Report. *Science*, 106(2751), pp.253-256.

Cutter, G. and Oatts, T. (1987). Determination of dissolved sulfide and sedimentary sulfur speciation using gas chromatography-photoionization detection. *Analytical Chemistry*, 59(5), pp.717-721.

Druschel, G., Hamers, R. and Banfield, J. (2003). Kinetics and mechanism of polythionate oxidation to sulfate at low pH by O2 and Fe3+. *Geochimica et Cosmochimica Acta*, 67(23), pp.4457-4469.

Eliseev, A. (2015). Influence of sulfur compounds on the terrestrial carbon cycle. *Izvestiya, Atmospheric and Oceanic Physics*, 51(6), pp.599-608.

Encyclopedia Britannica. (2015). *mineral | chemical compound :: Semimetals*. [online] Available at: http://www.britannica.com/EBchecked/topic/383675/mineral/80388/Semimetals [Accessed 17 Apr. 2015].

Evangelou, V. (1995). *Pyrite oxidation and its control*. Boca Raton, FL: CRC Press.

Falkowski, P. (2015). *Life's Engines - How Microbes made Earth Habitable*.

Farquhar, J. (2000). Atmospheric Influence of Earth's Earliest Sulfur Cycle. *Science*, 289(5480), pp.756-758.

Finster, K. (2008). Microbiological disproportionation of inorganic sulfur compounds. *Journal of Sulfur Chemistry*, 29(3-4), pp.281-292.

Fischer, E. (1883). Bildung von Methylenblau als Reaktion auf Schwefelwasserstoff. *Berichte der deutschen chemischen Gesellschaft*, 16(2), pp.2234-2236.

FLERE, J. and ZHANG, T. (1998). Sulfur-based autotrophic denitrification pond systems for in-situ remediation of nitrate-contaminated surface water. *Water Science and Technology*, 38(1), pp.15-22.

Fowler, T., Holmes, P. and Crundwell, F. (2001). On the kinetics and mechanism of the dissolution of pyrite in the presence of Thiobacillus ferrooxidans. *Hydrometallurgy*, 59(2-3), pp.257-270.

Freihammer, T. (2016). *Water and Wastewater: Challenges & Opportunities in the Mining Indust…*. [online] Slideshare.net. Available at: http://www.slideshare.net/AECOM/water-and-wastewater-challenges-opportunities-in-the-mining-industry [Accessed 19 May 2016].

Friedrich, C., Rother, D., Bardischewsky, F., Quentmeier, A. and Fischer, J. (2001). Oxidation of Reduced Inorganic Sulfur Compounds by Bacteria: Emergence of a Common Mechanism?.*Applied and Environmental Microbiology*, 67(7), pp.2873-2882.

Friedrich, M. and Finster, K. (2014). How sulfur beats iron. *Science*, 344(6187), pp.974-975.

Gazea, B., Adam, K. and Kontopoulos, A. (1996). A review of passive systems for the treatment of acid mine drainage. *Minerals Engineering*, 9(1), pp.23-42.

Ghosh, W. and Dam, B. (2009). Biochemistry and molecular biology of lithotrophic sulfur oxidation by taxonomically and ecologically diverse bacteria and archaea. *FEMS Microbiology Reviews*, 33(6), pp.999-1043.

Globalreporting.org. (2016). *GRI's History*. [online] Available at: https://www.globalreporting.org/information/about-gri/gri-history/Pages/GRI's%20history.aspx [Accessed 19 May 2016].

Gottschalk, G. (1986). *Bacterial metabolism*. New York: Springer-Verlag.

Gray, N. (1997). Environmental impact and remediation of acid mine drainage: a management problem.*Environmental Geology*, 30(1-2), pp.62-71.

Greenwood, N. and Earnshaw, A. (1984). *Chemistry of the elements*. Oxford [Oxfordshire]: Pergamon Press.

Hardisty, D., Olyphant, G., Bell, J., Johnson, A. and Pratt, L. (2013). Acidophilic sulfur disproportionation. *Geochimica et Cosmochimica Acta*, 113, pp.136-151.

HiQ. (2016). *Flame photometric detector (FPD) | HiQ*. [online] Available at: http://hiq.linde-gas.com/en/analytical_methods/gas_chromatography/flame_photometric_detector.html [Accessed 22 Jul. 2016].

Hubbard, C., Cheng, Y., Engelbrekston, A., Druhan, J., Li, L., Ajo-Franklin, J., Coates, J. and Conrad, M. (2014). Isotopic insights into microbial sulfur cycling in oil reservoirs. *Front. Microbiol.*, 5.

Hurse, T. and Abeydeera, W. (2002). Quantification of sulfur and sulfur-containing compounds in wastewaters by means of a combination of liquid chromatographic methods. *Journal of Chromatography A*, 942(1-2), pp.201-210.

Inap.com.au. (2016). *INAP - The International Network for Acid Prevention*. [online] Available at: http://www.inap.com.au/ [Accessed 19 May 2016].

Johnson, D. (2014). Recent Developments in Microbiological Approaches for Securing Mine Wastes and for Recovering Metals from Mine Waters. *Minerals*, 4(2), pp.279-292.

Johnson, D. and Hallberg, K. (2005). Acid mine drainage remediation options: a review. *Science of The Total Environment*, 338(1-2), pp.3-14.

Jørgensen, B. (1990). The sulfur cycle of freshwater sediments: Role of thiosulfate. *Limnol. Oceanogr.*, 35(6), pp.1329-1342.

Judge, A. (2009). *Periodic Pattern of Human Life*. [online] Laetusinpraesens.org. Available at: http://www.laetusinpraesens.org/musings/period.php [Accessed 16 Apr. 2015].

justice, N., Norman, A., Brown, C., Singh, A., Thomas, B. and Banfield, J. (2014). Comparison of environmental and isolate Sulfobacillus genomes reveals diverse carbon, sulfur, nitrogen, and hydrogen metabolisms. *biomed central genomics*, 15.

Kamyshny, A., Ekeltchik, I., Gun, J. and Lev, O. (2006). Method for the Determination of Inorganic Polysulfide Distribution in Aquatic Systems. *Analytical Chemistry*, 78(8), pp.2631-2639.

Kamyshny, A., Goifman, A., Gun, J., Rizkov, D. and Lev, O. (2004). Equilibrium Distribution of Polysulfide Ions in Aqueous Solutions at 25 °C: A New Approach for the Study of Polysulfides' Equilibria. *Environmental Science & Technology*, 38(24), pp.6633-6644.

Kamyshny, A., Zerkle, A., Mansaray, Z., Ciglenečki, I., Bura-Nakić, E., Farquhar, J. and Ferdelman, T. (2011). Biogeochemical sulfur cycling in the water column of a shallow stratified sea-water lake: Speciation and quadruple sulfur isotope composition. *Marine Chemistry*, 127(1-4), pp.144-154.

Kamyshny, A., Zilberbrand, M., Ekeltchik, I., Voitsekovski, T., Gun, J. and Lev, O. (2008). Speciation of Polysulfides and Zerovalent Sulfur in Sulfide-rich Water Wells in Southern and Central Israel.*Aquatic Geochemistry*, 14(2), pp.171-192.

Keller-Lehmann, B., Corrie, S., Ravn, R., Yuan, Z. and Keller, J. (2016). Preservation and simultaneous analysis of relevant soluble sulfur species in sewage samples. In: *Proceedings of the Second International IWA Conference on Sewer Operation and Maintenance.*.

Konhauser, K. (2007). *Introduction to geomicrobiology*. Malden, MA: Blackwell Pub.

Koofers.com. (2015). *test 1*. [online] Available at: https://www.koofers.com/flashcards/mcb-test-1/review [Accessed 18 Apr. 2015].

Kramer, M. and Cypionka, H. (1989). Sulfate formation via ATP sulfurylase in thiosulfate- and sulfite-disproportionating bacteria. *Arch. Microbiol.*, 151(3), pp.232-237.

KUENEN, J., ROBERTSON, L. and VAN GEMERDEN, H. (1985). Microbial Interactions among Aerobic and Anaerobic Sulfur-Oxidizing Bacteria. *advances in microbial ecology*, 8.

Lackey, J. (1938). The Flora and Fauna of Surface Waters Polluted by Acid Mine Drainage. *Public Health Reports (1896-1970)*, 53(34), p.1499.

Lackey, J. (1939). Aquatic Life in Waters Polluted by Acid Mine Waste. *Public Health Reports (1896-1970)*, 54(18), p.740.

Ledin, M. (1996). The environmental impact of mine wastes -- Roles of microorganisms and their significance in treatment of mine wastes. *Earth-Science Reviews*, 41(1-2), pp.67-108.

Lengeler, J., Drews, G. and Schlegel, H. (1999). *Biology of the prokaryotes*. Stuttgart: Thieme.

Lengke, M., Davis, A. and Bucknam, C. (2010). Improving Management of Potentially Acid Generating Waste Rock. *Mine Water and the Environment*, 29(1), pp.29-44.

Licht, S. and Davis, J. (1997). Disproportionation of Aqueous Sulfur and Sulfide: Kinetics of Polysulfide Decomposition. *J. Phys. Chem. B*, 101(14), pp.2540-2545.

Lichtschlag, A., Kamyshny, A., Ferdelman, T. and deBeer, D. (2013). Intermediate sulfur oxidation state compounds in the euxinic surface sediments of the Dvurechenskii mud volcano (Black Sea).*Geochimica et Cosmochimica Acta*, 105, pp.130-145.

Liebensteiner, M., Tsesmetzis, N., Stams, A. and Lomans, B. (2014). Microbial redox processes in deep subsurface environments and the potential application of (per)chlorate in oil reservoirs. *Front. Microbiol.*, 5.

Lin Ling, B., Dewaele, C. and Baeyens, W. (1990). Application of micro-scale liquid chromatography with fluorescence detection to the determination of thiols. *Journal of Chromatography A*, 514, pp.189-198.

LIZAMA, H. and SUZUKI, I. (1989). Rate Equations and Kinetic Parameters of the Reactions Involved in Pyrite Oxidation by Thiobacillus ferrooxidans. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY,*, [online] 55(11). Available at: http://aem.asm.org/content/55/11/2918.full.pdf.

Loew, O. (1894). THE SYNTHETICAL POWERS, OF MICRO-ORGANISMS.--II. *Science*, ns-23(580), pp.144-145.

Lottermoser, B. (2007). *Mine wastes*. Berlin: Springer.

Meyer, C. (1977). *Sulfur, energy, and environment*. Amsterdam: Elsevier Scientific Pub. Co.

Miura, Y. and Kawaoi, A. (2000). Determination of thiosulfate, thiocyanate and polythionates in a mixture by ion-pair chromatography with ultraviolet absorbance detection. *Journal of Chromatography A*, 884(1-2), pp.81-87.

Miura, Y. and Watanabe, M. (2001). Ion-pair chromatography of polythionates and thiosulfate with detection based on their catalytic effects on the postcolumn azide–iodine reaction. *Journal of Chromatography A*, 920(1-2), pp.163-171.

Montoya, L., Shen, X., McDermott, J., Kevil, C. and Pluth, M. (2015). Mechanistic investigations reveal that dibromobimane extrudes sulfur from biological sulfhydryl sources other than hydrogen sulfide. *Chem. Sci.*, 6(1), pp.294-300.

Moses, C., Kirk Nordstrom, D., Herman, J. and Mills, A. (1987). Aqueous pyrite oxidation by dissolved oxygen and by ferric iron. *Geochimica et Cosmochimica Acta*, 51(6), pp.1561-1571.

Müller, I., Brunner, B. and Coleman, M. (2013). Isotopic evidence of the pivotal role of sulfite oxidation in shaping the oxygen isotope signature of sulfate. *Chemical Geology*, 354, pp.186-202.

Murray, J., Kirschbaum, A., Dold, B., Guimaraes, E. and Miner, E. (2014). Jarosite versus Soluble Iron-Sulfate Formation and Their Role in Acid Mine Drainage Formation at the Pan de Azúcar Mine Tailings (Zn-Pb-Ag), NW Argentina. *Minerals*, [online] 4(2), pp.477-502. Available at: http://www.mdpi.com/2075-163X/4/2/477/htm [Accessed 24 Aug. 2016].

Nageswari, A. (2011). A Sensitive and Simple HPLC-UV Method for Trace Level Quantification of Ethyl p-Toluenesulfonate and Methyl p-Toluenesulfonate, Two

Potential Genotoxins in Active Pharmaceutical Ingredients. *Scientia Pharmaceutica*, 79(4), pp.865-876.

Nancucheo, I. and Johnson, D. (2011). Significance of Microbial Communities and Interactions in Safeguarding Reactive Mine Tailings by Ecological Engineering. *Applied and Environmental Microbiology*, 77(23), pp.8201-8208.

Nancucheo, I. and Johnson, D. (2011). Significance of Microbial Communities and Interactions in Safeguarding Reactive Mine Tailings by Ecological Engineering. *Applied and Environmental Microbiology*, 77(23), pp.8201-8208.

Neculita, C., Zagury, G. and Bussière, B. (2007). Passive Treatment of Acid Mine Drainage in Bioreactors using Sulfate-Reducing Bacteria. *Journal of Environment Quality*, 36(1), p.1.

Nemati, M. and Webb, C. (1997). A kinetic model for biological oxidation of ferrous iron by Thiobacillus ferrooxidans. *Biotechnol. Bioeng.*, 53(5), pp.478-486.

Newton, G., Dorian, R. and Fahey, R. (1981). Analysis of biological thiols: Derivatization with monobromobimane and separation by reverse-phase high-performance liquid chromatography.*Analytical Biochemistry*, 114(2), pp.383-387.

Nordstrom, D. (2000). Advances in the Hydrogeochemistry and Microbiology of Acid Mine Waters.*International Geology Review*, 42(6), pp.499-515.

O'Reilly, J., Dicinoski, G., Shaw, M. and Haddad, P. (2001). Chromatographic and electrophoretic separation of inorganic sulfur and sulfur–oxygen species. *Analytica Chimica Acta*, 432(2), pp.165-192.

Podgorsek, L. and Imhoff, J. (1999). Tetrathionate production by sulfur oxidizing bacteria and the role of tetrathionate in the sulfur cycle of Baltic Sea sediments. *Aquatic Microbial Ecology*, 17, pp.255-265.

Poole, R. (2012). *Advances in microbial physiology*. Amsterdam: Elsevier/Academic Press.

Rabus, R., hansen, T. and Widdel, F. (2006). Dissimilatory Sulfate- and Sulfur-Reducing Prokaryotes. In: M. Dworkin and S. Falkow, ed., *The prokaryotes*, 3rd ed. New York: Springer, pp.659-768.

Radford-Knoery, J. and Cutter, G. (1993). Determination of carbonyl sulfide and hydrogen sulfide species in natural waters using specialized collection procedures and gas chromatography with flame photometric detection. *Analytical Chemistry*, 65(8), pp.976-982.

Radford-Knoery, J. and Cutter, G. (1993). Determination of carbonyl sulfide and hydrogen sulfide species in natural waters using specialized collection procedures and gas chromatography with flame photometric detection. *Analytical Chemistry*, 65(8), pp.976-982.

Reardon, E. and Beckie, R. (1987). Modelling chemical equilibria of acid mine-drainage: The FeSO4-H2SO4-H2O system. *Geochimica et Cosmochimica Acta*, 51(9), pp.2355-2368.

Rethmeier, J., Rabenstein, A., Langer, M. and Fischer, U. (1997). Detection of traces of oxidized and reduced sulfur compounds in small samples by combination of different high-performance liquid chromatography methods. *Journal of Chromatography A*, 760(2), pp.295-302.

Richard, J., Vick, R. and Junk, G. (1977). Determination of elemental sulfur by gas chromatography.*Environmental Science & Technology*, 11(12), pp.1084-1086.

Rickard, D. (2012). *Sulfidic sediments and sedimentary rocks*. Oxford: Elsevier Science Ltd.

Rickard, D. (2015). *Pyrite*.

Rickard, D. and Luther, G. (2007). Chemistry of Iron Sulfides. *ChemInform*, 38(19).

RICKARD, D., GRIFFITH, A., OLDROYD, A., BUTLER, I., LOPEZCAPEL, E., MANNING, D. and APPERLEY, D. (2006). The composition of nanoparticulate mackinawite, tetragonal iron(II) monosulfide. *Chemical Geology*, 235(3-4), pp.286-298.

Rimstidt, J. and Vaughan, D. (2003). Pyrite oxidation: a state-of-the-art assessment of the reaction mechanism. *Geochimica et Cosmochimica Acta*, 67(5), pp.873-880.

Rio Tinto. (2016). *About Rio Tinto*. [online] Available at: http://serc.carleton.edu/microbelife/topics/riotinto/index.html [Accessed 13 May 2016].

S8int.com. (2016). *Those Sophisticated Cave Men--The Lion Cavern the Oldest Mine in the World --Page 20*. [online] Available at: http://www.s8int.com/sophis20.html [Accessed 14 May 2016].

Sardi, F., Manta, B., Portillo-Ledesma, S., Knoops, B., Comini, M. and Ferrer-Sueta, G. (2013). Determination of acidity and nucleophilicity in thiols by reaction with monobromobimane and fluorescence detection. *Analytical Biochemistry*, 435(1), pp.74-82.

Schippers, A. and sAND, W. (1999). Bacterial Leaching of Metal Sulfides Proceeds by Two Indirect Mechanisms via Thiosulfate or via Polysulfides and Sulfur. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, 65(1).

Schulz, H. and Zabel, M. (2006). *Marine geochemistry*. Berlin: Springer.

Shearer, R., Poole, E. and Nowalk, J. (1993). Application of Gas Chromatography and Flameless Sulfur Chemiluminescence Detection to the Analysis of Petroleum Products. *Journal of Chromatographic Science*, 31(3), pp.82-87.

Singer, P. and Stumm, W. (1970). Acidic Mine Drainage: The Rate-Determining Step. *Science*, 167(3921), pp.1121-1123.

Siriraks, A., Kingston, H. and Riviello, J. (1990). Chelation ion chromatography as a method for trace elemental analysis in complex environmental and biological samples. *Anal. Chem.*, 62(11), pp.1185-1193.

Sulfide--USEPA1 Methylene Blue Method2. (2016). 1st ed. [ebook] Available at: http://www.hach.com/sulfide-reagent-set-methylene-blue/product?id=7640198750 [Accessed 21 Jul. 2016].

Tang, K., Baskaran, V. and Nemati, M. (2009). Bacteria of the sulphur cycle: An overview of microbiology, biokinetics and their role in petroleum and mining industries. *Biochemical Engineering Journal*, 44(1), pp.73-94.

THAMDRUP, B., FINSTER, K., WURGLER HANSEN, J. and BAK, F. (1993). Bacterial Disproportionation of Elemental Sulfur Coupled to Chemical Reduction of Iron or Manganese.*APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, [online] 59(1). Available at: http://aem.asm.org/content/59/1/101.full.pdf [Accessed 21 Jul. 2016].

The International Council on Mining and Metals. (2016). *Mining and sustainable development*. [online] Available at: http://www.icmm.com/ [Accessed 19 May 2016].

tuttle, j. and Jannasch, H. (2015). Dissimilatory Reduction of Inorganic Sulfur by Facultatively Anaerobic Marine Bacteria. *journal of bacteriology*, [online] 115(3). Available at: http://jb.asm.org/content/115/3/732.short.

Ubuka, T., Abe, T., Kajikawa, R. and Morino, K. (2001). Determination of hydrogen sulfide and acid-labile sulfur in animal tissues by gas chromatography and ion chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications*, 757(1), pp.31-37.

Vaughan, D. and Craig, J. (1978). *Mineral chemistry of metal sulfides*. Cambridge [England]: Cambridge University Press.

Weir, S., Butler, E. and Haddad, P. (1994). Ion chromatography with UV detection for the determination of thiosulfate and polythionates in saline waters. *Journal of Chromatography A*, 671(1-2), pp.197-203.

Williamson, M. and Rimstidt, J. (1992). Correlation between structure and thermodynamic properties of aqueous sulfur species. *Geochimica et Cosmochimica Acta*, 56(11), pp.3867-3880.

Williamson, M. and Rimstidt, J. (1994). The kinetics and electrochemical rate-determining step of aqueous pyrite oxidation. *Geochimica et Cosmochimica Acta*, 58(24), pp.5443-5454.

Winogradsky, S. (1890). On the nitrifying organisms. *Comptes rendus de l'academie des Sciences,*, [online] 110. Available at: http://202.114.65.51/fzjx/wsw/newindex/wswfzjs/pdf/1890p231.pdf.

Wright, K., Williamson, C., Grasby, S., Spear, J. and Templeton, A. (2013). Metagenomic evidence for sulfur lithotrophy by Epsilonproteobacteria as the major energy source for primary productivity in a sub-aerial arctic glacial deposit, Borup Fiord Pass. *Front. Microbiol.*, 4.

Www2.chemistry.msu.edu. (2015). *Chemical Reactivity*. [online] Available at: https://www2.chemistry.msu.edu/faculty/reusch/virttxtjml/special2.htm [Accessed 17 Apr. 2015].

Wwwbrr.cr.usgs.gov. (2016). *Program Limitations*. [online] Available at: http://wwwbrr.cr.usgs.gov/projects/GWC_coupled/phreeqc/html/final-4.html [Accessed 9 Aug. 2016].

Xia, K., Weesner, F., Bleam, W., Helmke, P., Bloom, P. and Skyllberg, U. (1998). XANES Studies of Oxidation States of Sulfur in Aquatic and Soil Humic Substances. *Soil Science Society of America Journal*, 62(5), p.1240.

Yin, C., Li, H., Liu, H., Zhao, L., Bai, Z., Wang, Y., Zhang, S. and Liu, C. (2014). Study on the formation, determination, and removal of elemental sulfur in ultra-low sulfur gas oil. *Fuel Processing Technology*, 120, pp.16-21.

Ymcamerica.com. (2016). [online] Available at: http://www.ymcamerica.com/columns/images/ymc-triart_c8_selectivity_table_large.jpg [Accessed 7 Jun. 2016].

Zopfi, J., Ferdelman, T. and Fossing, H. (2004). Distribution and fate of sulfur intermediates--sulfite, tetrathionate, thiosulfate, and elemental sulfur--in marine sediments. *Geological Society of America Special Papers*.

.