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A Comparative Study of $\text{SO}_4^{2-}$, $\text{SO}_3^{2-}$ and $\text{S}_2\text{O}_3^{2-}$ as Electron Acceptors in Anaerobic Microbial Systems Containing Sulfate Reducing Bacteria

By

Muhammad Omer Saleem

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

Graduate Department of Chemical Engineering and Applied Chemistry

University of Toronto

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A Comparative Study of $\text{SO}_4^{2-}$, $\text{SO}_3^{2-}$ and $\text{S}_2\text{O}_3^{2-}$ as Electron Acceptors in Anaerobic Microbial Systems Containing Sulfate Reducing Bacteria

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Abstract

Sulfate reducing bacteria (SRB) belong to genera of anaerobic microbes that reduce sulfate and other sulfur oxyanions (SO$_4^{2-}$) to sulfide, through biologically mediated transfer of electrons, from organic (electron) donor to sulfate or other SO$_4^{2-}$. Different sulfur oxyanions, specifically sulfate (SO$_4^{2-}$), sulfite (SO$_3^{2-}$) and thiosulfate (S$_2$O$_3^{2-}$), were used as terminal electron acceptors for mixed cultures containing SRB in lab scale batch reactors, at 35 °C in an anaerobic environment. The mixed cultures were inoculated from sludges of two municipal waste treatment operations. These mixed cultures were observed to reduce all three types of SO$_4^{2-}$ using lactate or glucose as carbon/energy source. The rates of SO$_4^{2-}$ reduction were found to be in the order of $\text{S}_2\text{O}_3^{2-} > \text{SO}_3^{2-} >> \text{SO}_4^{2-}$, which is logical considering the oxidation states of S in these SO$_4^{2-}$. COD/SO$_4^{2-}$ ratio (ratio of electron donor/electron acceptor) was found to have an impact on SRB activity. Theoretically required values of this ratio were calculated based on the stoichiometry for all three SO$_4^{2-}$. Experimental values of optimal COD/SO$_4^{2-}$ ranges for maximum SO$_4^{2-}$ reduction were also determined. It was observed that the theoretically required values of COD/SO$_4^{2-}$ ratios lie within the experimentally observed optimum ranges. The observed optimum ranges of COD/SO$_4^{2-}$ ratios for maximum total SO$_4^{2-}$ removal efficiency were (in g of COD/g of SO$_4^{2-}$ units) 0.5–1.3, 0.6–1.5 and 0.4–1.1 for SO$_4^{2-}$, SO$_3^{2-}$ and S$_2$O$_3^{2-}$ respectively. Similar ranges were observed based on the maximum removal of COD, suggesting that under the optimal conditions, SRB contributed predominantly to the consumption of carbon/energy source.
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### Abbreviations & Conventions Used

- **→** One way reaction
- **=** Reversible reaction
- **SO}_3^{2-}** Generally written formula for different oxyanions of sulfur:
  - Specifically **SO}_4^{2-}, SO}_3^{2-} & S}_2O}_3^{2-}**
- **APB** Acid producing bacteria
- **SRB** Sulfate reducing bacteria
- **MPB** Methane producing bacteria
- **COD** Chemical oxygen demand
- **IC** Ion chromatograph
- **GC** Gas chromatograph
- **FID** Flame ionization detector
- **PFPD** Pulsed flame photo detector
- **psi** Pounds per square inch
Chapter 1: Introduction

Motivation for this research work was a potential application of sulfate reducing bacteria (SRB) in a combined biochemical process for the recovery of valuable metals mainly Co, Ni & Cu. from non-ferrous metal industry smelter slags.

Gbor et al. [1] and Ahmed et al. [2] showed that aqueous SO₂, could be effective in solubilizing metal values from the waste slag. Leaching solution in addition to the dissolved metals contains SO₄²⁻, SO₃²⁻ and S₂O₃²⁻ ions, collectively called sulfur oxyanions (SOₓ³⁻). SRB can potentially be used to reduce these dissolved sulfur oxyanions to sulfide. Produced sulfide can then combined with metal ions for the precipitation of metal values. Precipitated metal sulfides can be recycled back to the foundry for metal recovery. Schematic of proposed process is provided in Figure 1.1.

Figure 1.1: Schematic of overall project application

Metal precipitator in Figure 1.1. serves the purpose of removing the metals as metal sulfides and also limits the concentration of metals, in the solution to be treated in the SRB bioreactor. A high concentration of these metals can have an inhibiting effect on SRB.
Development of a SRB based approach as shown in Figure 1.1 would require knowledge of the behavior of SRB for using different types of sulfur-oxyanions (SO₃⁻) as electron acceptors. Therefore, overall objective of this thesis is to provide this understanding by accomplishing specific objectives:

1. To determine the ability of mixed cultures of SRB to use SO₄²⁻, SO₃²⁻ & S₂O₃²⁻ (collectively written SO₃⁻) as electron acceptors.

2. To compare the reduction of these SO₃⁻ by SRB.

3. To determine the effect of COD/SO₃⁻ ratios for each SO₃⁻ on the SO₃⁻ reduction activity of SRB.
Chapter 2: Literature Survey

2.1 Sulfate reducing bacteria (SRB)

Sulfate reducing bacteria are group of bacteria that use sulfate ion (SO₄²⁻) as a terminal electron acceptor. The process is called sulfate respiration, thereby reducing sulfate to sulfide. However, it has been reported \(^{13,14}\) that some sulfate reducing bacteria can also use sulfite (SO₃⁻⁻) and thio-sulfate (S₂O₃²⁻) as terminal electron acceptors. Therefore, SRB are also termed as sulfide producing bacteria or sulfidogenic\(^{15}\) bacteria. This however, may cause confusion. Another type of bacteria called sulfur reducing bacteria also produce sulfide and will be included in this category of sulfidogenic bacteria. Thus the term sulfate reducing bacteria will be used in this thesis, to refer to the group of anaerobic microbes that are capable of reducing sulfate, sulfite and thiosulfate ions by using them as electron acceptors.

SRB perform dissimilatory sulfate reduction, term used to indicate that during reduction major portion of sulfur is not made a part of the cell structure. This is in contrast to assimilatory reduction in which sulfur is incorporated in the cell structure. From our application point of view (as discussed in Chapter 1), dissimilatory sulfate reduction is of interest. The sulfide produced by dissimilatory sulfate reduction can then be removed as H₂S gas and utilized to precipitate valuable metals.

Most genera of SRB are gram negative, however, some species are also reported to be gram positive\(^{16,17}\). The term gram negative/positive is indicative of the thickness of special protective layer of peptidoglycan, which helps the microbes to survive in dry environments.

The function of dissimilatory sulfate reduction is performed by different species of SRB. Bergey\(^{18}\) provided a convenient way of subclassifying these organisms as below:

Subgroup 1: Spore forming SRB. example: Desulfotomaculum
**Subgroup 2:** Non-spore forming SRB: perform incomplete electron donor oxidation to acetate. example: *Desulfobulbus, Desulfomonas, Desulfovibrio & Thermodesulfovibrio*

**Subgroup 3:** Non-spore forming SRB: perform complete electron donor oxidation to CO$_2$. example: *Desulfo bacter, Desulfobacterium, Desulfo monile, Desulfonema & Desulfosarcina.*

Furthermore, each genus may contain different species of bacteria. For example genus *Desulfo bulbus* of subgroup 2 includes *Desulfo bulbus elongates* and *Desulfo bulbus propionicus*. Each different species of bacteria may have its own characteristic shape, size, capability of utilizing different substrates and other optimum performance parameters like temperature, pressure and pH etc. However, they are all grouped as sulfate reducing bacteria because of their common function of reducing sulfate.

As discussed above, each subgroup of bacteria may contain various species. Therefore, research work using these microorganisms can be carried out in two ways. (i) using pure cultures of SRB containing only selected species of bacteria (ii) using mixed cultures consisting of a community or consortium of bacteria. Both approaches have advantages and disadvantages.

Pure cultures are employed with the objectives related to microbiology or the studies of degradation pathways of certain compounds along with specific enzymes involved. This has the advantage that the behavior of a specific type of microorganism can be studied in isolation.

Use of mixed cultures is favored for industrial applications. In an industrial application, it is usually not feasible to use pure carbon sources like lactate and glucose, etc. for microbial growth. Instead waste streams like sewage and food waste are potential carbon/energy sources. These waste streams already contain various types of
microorganisms and it is difficult to maintain pure culture environment. Mixed cultures, therefore, are more suited in such environments.

In mixed cultures different communities of bacteria work together in harmony, in which that products of one are consumed by the other. This coexistence of different microbial species may sometimes causes problems though. For example, under anaerobic conditions methane producing bacteria (MPB), can exist and compete with SRB for nutrients.

2.2 Environmental applications of SRB

Involvement of SRB in creating problems like pollution of water, soil/sand, oil & gas and injection water for oil wells, corrosion of metal & stonework, food spoilage, blackening of metal based paints, blackening of pulp containing metal traces and many more has been long established. However, recent research shows that SRB constitute the potential group of microbes that can be beneficially used for biological and/or biochemical treatment of polluted wastes. Examples are given below:

2.2.1 Treatment of inorganic waste materials

Burning of fossil fuels produces SO₂. Increasingly strict environmental regulations, prohibit emission of SO₂ in atmosphere. As of 1991 80% of flue gas de-sulfurization (FGD) capacity in United States consisted of wet limestone (calcium carbonate) scrubbing[8]. In this process SO₂ is treated with limestone to produce calcium sulfate (gypsum) and CO₂. This raw gypsum is called flue gas de-sulfurization sludge or FGD sludge. Kaufman et al.[8] showed that SRB can use SO₄²⁻ in FGD sludge as the electron acceptor and sewage waste as the electron donor. A combined chemical and biological method of using the FGD gypsum to produce calcium carbonate and elemental sulfur, with the help of SRB was proposed.

Selvaraj et al.[9] reported a biological process for direct reduction of SO₂ to hydrogen sulfide using SRB eliminating FGD sludge production at the first hand.
Bjørn et al.\textsuperscript{[10]} investigated the overflowing water from open pits of pyrite and coal mine areas. These waste streams contain high sulfate and metals and SRB can be used to treat these polluted streams.

Polyacrylate cylinders with sand bed and a layer of crushed stones at the bottom were filled with acid mine water, supplied with the nutrients to support bacterial growth and incubated for 203 days. After an initial lag phase, pH started to increase and dissolved concentrations of copper, zinc, iron and aluminum were reduced in cylinders. Simultaneously, a black sludge was formed indicating the formation of metallic sulfides.

Wet and dry depositions in presence of sulfuric acid produced from atmospheric SO\textsubscript{2} can attack carbonate stones with subsequent gypsum formation. This decay of calcareous stones is due to the conversion of calcium carbonate into calcium sulfate. In order to remove sulfates from artistic stonework, Ranalli et al.\textsuperscript{[11]} investigated a procedure based upon SRB. Selected species of biomass were applied under anaerobic conditions to sample surfaces. Results showed that sulfate removal was more effective on real stone samples than on artificial gypsum enriched samples.

2.2.2 Treatment of organic materials with SRB

Tetrachloroethene, also known as perchloroethylene (PCE), is a major groundwater contaminant under safe drinking water act in United States. Maximum allowable level of PCE is 5 µg liter\textsuperscript{-1} \textsuperscript{[12]}.

Bagley et al.\textsuperscript{[13]} conducted study on the reductive dechlorination of PCE to trichloroethene and cis-1,2-dichloroethene by employing SRB. For this work, mixed cultures of bacteria from New York wastewater treatment facility were used. These microbes were grown in a basal medium at 35 – 37 °C. To avoid the excessive sulfide buildup, a mixture of N\textsubscript{2} and CO\textsubscript{2} gases was purged through the solution. An overall transformation varied from 92% tetrachloroethene removal in 14 days to 22% removal in 65 days of inoculation.

The effluent generated by a typical fishmeal industry during hydraulic unloading of fish from ships is high in organic content. After initial treatment for removal of fats and
proteins. the effluent contains 4-6 kg COD m⁻² with high salt contents of 1.85 kg SO₄²⁻ m⁻² and 16.2 kg Cl m⁻². According to Aspe et al.\textsuperscript{12} marine sediments and fresh pig manure were assayed as anaerobic inoculum to purify this saline effluent. The marine inoculum incubated at 37° C. showed final methanogenic/sulfate-reducing bacteria ratio of 0.0025. Methanogenic activity was inhibited at COD/SO₄²⁻ ratios lower than 0.5.

The soil and water in many military manufacturing complexes are often contaminated with explosives like 1.3.5-trinitrobenzene (TNB), hexahydro-1.3.5-trinitro-1.3.5-triazine (RDX) and octahydro-1.3.5.7-tetranitro-1.3.5.7-tetraazocine (HMX). Boopathy et al.\textsuperscript{14} investigated the metabolism of these explosive compounds by SRB containing Desulfovibrio spp. It was reported that Desulfovibrio spp. used these explosive compounds as their sole source of nitrogen for growth. The concentrations of TNB, RDX and HMX in the culture media dropped to below the detection limit (<0.5 ppm) within 18 days of incubation. Ammonia was produced from the nitro groups of the explosive compounds in the culture, which was consumed by the microbes as nitrogen source for their bacterial growth and the final concentration of ammonia was < 0.5 mg L⁻¹.

Wilkes et al.\textsuperscript{15} used a mesophilic enrichment culture of SRB isolated from the water phase of North Sea oil tank. Oil from the same tank was used as a sole source of carbon and energy. It was found that SRB specifically depletes certain alkyl benzene in crude oil during growth. The portion of alkyl benzenes remaining in crude becomes selectively enriched in $^{13}$C during the bacterial growth. It was shown that enrichment culture grows on oils of different origin resulting in similar patterns of alkyl benzene depletion.

These applications of SRB are representative of the potential that exists in the applications of these microbes. Although most of the works presented are on experimental or laboratory scale but are indicative of SRB's promising role in industrial applications.
2.3 Effect of various engineering parameters on SRB

The dissimilatory SO$_4^{2-}$ reduction by SRB is effected by many parameters such as temperature, pH, inhibiting substances, types of electron donors/acceptors and COD/SO$_4^{2-}$ ratio etc. It is important to understand the effect of these parameters in relation to SRB activity. Given below is a brief review of literature on this subject:

2.3.1 Inhibition effect of various substances

Air/oxygen is perhaps most common inhibitor of SRB activity as these microbes require anaerobic environment for their growth. The selenite ion is a competitive antagonist of sulfate reduction and the molybdate ion acts as depleting the cellular ATP pool$^{[7]}$. High concentrations of various metals can also inhibit SRB. Actual toxicity of a particular metal depends on conditions, such as type/source of inoculum, cell concentration, pH, temperature, type of carbon sources, types of metal in question and type of system etc.$^{[118]}$. Under conditions similar to the ones used in the current study, it was shown that copper at 80 ppm$^{[17]}$ and cobalt at 70 ppm$^{[118]}$ inhibit the growth of SRB.

Sulfide is also known to be toxic to SRB. sulfide toxicity is closely related to the pH of the system. Different species of sulfide (H$_2$S, HS$^-$ & S$^{2-}$) coexist in equilibrium and the distribution of each species is a function of pH$^{[116]}$. Chin$^{[19]}$ reported that total sulfide concentration of above 150 mg/L effected the growth of SRB as well as sulfate reduction. complete inhibition of SRB activity was observed at 300 mg/L.

2.3.2 Effect of temperature

Similar to other living creatures SRB also operate under optimum temperature for their competitive performance. Based upon favorable temperature ranges, microbes are usually classified into three groups as: psychrophilic ($T < 20 \, ^{\circ}C$), mesophilic ($20 < T < 50 \, ^{\circ}C$) and thermophilic ($T > 50 \, ^{\circ}C$).

Knoblauch et al.$^{[20]}$ studied the effect of temperature on five pure species of psychrophilic SRB. It was concluded that the psychrophilic SRB could be adapted to
permanently low temperatures (below 4 °C) with high relative growth rates and high growth yields.

Kuo\cite{21} designed batch experiments to study the effect temperature on mixed cultures of SRB. Mixed cultures were enriched in SRB from sewage sludge Ashbridges Bay Treatment Plant. Glucose was used as the sole source of carbon/energy. N₂ was purged to maintain an anaerobic environment and to remove the produced sulfide as H₂S. Experiments were conducted at temperatures of 25 °C, 35 °C and 45 °C. Results for the first 140 hours of inoculation showed total reduction of 3.8%, 4.4% and 13.9% for each temperature, respectively. It was concluded for mixed cultures of SRB that at higher temperatures sulfate reduction activity was favored.

2.3.3 Effect of pH

pH of a system effects the distribution of various sulfide species in the system, which can have different toxicity/inhibition affects on SRB\cite{16}.

O'Flaherty et al.\cite{22} showed that pH may also affect the population dynamics of SRB and methane producing bacteria (MPB). Pure culture of both SRB and MPB were inoculated in batch experiments. It was concluded that at pH 7.0 – 7.5, the growth rates of MPB and SRB are similar. Outside this pH range, MPB and SRB have more favorable growth conditions.

2.3.4 Effect of using different carbon sources/electron donors

SRB have ability to use a wide variety of organic compounds as energy and carbon sources. This includes organic acids\cite{16} (like lactate. pyruvate, formate and malate), alcohols\cite{16} (like ethanol. propanol, methanol and butanol), acetic acid\cite{16} and mixture of CO₂ & H₂\cite{6}. Some species of SRB are capable of completely oxidizing the organic substrate while others can only oxidize it to form acetate\cite{6}.

Earlier research on SRB has recommended use of lactate as carbon/energy source\cite{13}. More recently researchers have successfully used synthesis gas\cite{23}. sewage waste\cite{8,9,24}.
food-waste\textsuperscript{13}, sucrose\textsuperscript{25} and glucose\textsuperscript{26,27} as the source of carbon and energy for SRB growth.

\subsection*{2.3.5 Use of different oxyanions as electron acceptors}

It is known that SRB can utilize oxyanions other than $\text{SO}_4^{2-}$, such as $\text{SO}_3^{2-}$ or $\text{S}_2\text{O}_3^{2-}$\textsuperscript{16} and even $\text{SO}_2^{\text{(aq)}}$\textsuperscript{24,28} as electron acceptors. Widdel et al.\textsuperscript{29} and Cypionka et al.\textsuperscript{30} suggested that SRB may first disproportionate the $\text{S}_2\text{O}_3^{2-}$ or $\text{SO}_3^{2-}$ to $\text{SO}_4^{2-}$ (sec. 2.4.1 for further details).

Most work on this subject either reported the occurrence of reduction activity\textsuperscript{13} of different $\text{SO}_x^{3-}$, or focused to investigate the mechanism of reduction. Some work have also focused on the relative effect of using different oxyanions. In a study on the production of $\text{H}_2\text{S}$ in sewer biofilms. Nielsen\textsuperscript{41} reported that sulfide production rates from both $\text{SO}_3^{2-}$ and $\text{S}_2\text{O}_3^{2-}$ fed biofilms were considerably higher than the rate from sulfate fed biofilms.

\subsection*{2.3.6 Effect of COD/So$_x^{3-}$ ratios (competition between MPB & SRB)}

It is suggested that the COD/So$_x^{3-}$ ratio is an important parameter for controlling the growth and cultivation of SRB\textsuperscript{16,31,32,33,34,35}. This ratio is the ratio of electron donor (measured in terms of chemical oxygen demand or COD) to electron acceptor (e.g. $\text{SO}_4^{2-}$, $\text{SO}_3^{2-}$ and $\text{S}_2\text{O}_3^{2-}$). SRB and MPB work under similar anaerobic conditions and usually compete for nutrients and electron donor.

For Sreece\textsuperscript{31} calculated a theoretical COD requirement of 64 g (or 8 electrons) to reduce one mole of $\text{SO}_4^{2-}$ (96 g) based upon the following reaction:

$$8\text{H}^+ + 8\text{e}^- + \text{SO}_4^{2-} \rightarrow \text{S}^{2-} + 4\text{H}_2\text{O} \quad \text{(Eq. 2.1)}$$

Thus, the Eq. 2.1 gives a COD/So$_x^{2-}$ ratio of $64/96 = 0.67$ (g of COD/g of $\text{SO}_x^{2-}$). Actual values of the ratio are often higher than theoretical ones varying from 0.8-1.6. This
difference is because a part of electron donor is also used as carbon source for the biosynthesis of new cells\footnote{164}.

COD/\text{SO}_4^{2-}\text{ ratio can affect the population dynamics of SRB & MPB. It was reported}\footnote{32} that sulfate reducers & methane producers were very competitive in COD/\text{SO}_4^{2-} range of 1.7 to 2.7. However, as the ratio was increased MPB predominated while SRB predominated when COD/\text{SO}_4^{2-} ratio was lowered. For our SRB application (Figure 1.1) we want to shift the dynamic balance of microbial population in favor of SRB because of two reasons.

- Activity of MPB will result in extra COD consumption. This COD will not be used for the production of \text{H}_2\text{S} and is wasted.

- Biomass contribution due to MPB. This biomass is non productive biomass because it is produced without producing the required \text{H}_2\text{S}. Dumping of this biomass would be an unnecessary cost to the process.

Kosinska \textit{et al.}\footnote{34} used pure cultures of \textit{Desulfovibrio desulfuricans} at 38 °C in lab scale batch experiments. This study reported removal of 99%, 85% and 88% \text{SO}_4^{2-} and 25%, 59% and 36% COD at COD/\text{SO}_4^{2-} ratios of 4.7, 2.3 and 1.6 respectively. Yeh\footnote{35} used a waste sludge as electron donor for a SRB system and reported that the minimum COD/\text{SO}_4^{2-} value for the optimum \text{SO}_4^{2-} reduction is greater than 2.0. Other work\footnote{33} using synthetic substrates as electron donor suggested the optimum COD/\text{SO}_4^{2-} value as below 1.0.

This brief overview of different studies on impact of COD/\text{SO}_4^{2-} ratio on SRB activity indicates that these researchers reported mixed results for optimum COD/\text{SO}_4^{2-} ratios for maximum sulfate reduction. Actual value of an optimum COD/\text{SO}_4^{2-} ratio may vary depending upon the type of the system, bacterial source, nutrients etc.
2.4 Chemistry of aqueous sulfur compounds

2.4.1 Disproportionation of $\text{SO}_3^{2-}$ by sulfate reducing bacteria

Disproportionation is a term used for the splitting of one sulfur oxyanion to two new compounds, one is more oxidative than the original sulfur species and the other is more reductive. SRB such as *Desulfovibrio sulfodismutans* and *Desulfovibrio desulfuricans* can perform this unique form of energy metabolism using reduced sulfur compounds. Widdel *et al.* reported disproportionation equations:

$$
\begin{align*}
\text{S}_2\text{O}_3^{2-} + \text{H}_2\text{O} &\rightarrow \text{SO}_4^{2-} + \text{H}_2\text{S} & \Delta G^{\circ} &= -21.9 \text{ kJ} \quad \text{(Eq. 2.2)} \\
\text{SO}_3^{2-} - \frac{1}{2} \text{H}^- &\rightarrow \frac{3}{4} \text{SO}_4^{2-} + \frac{1}{4} \text{H}_2\text{S} & \Delta G^{\circ} &= -58.9 \text{ kJ} \quad \text{(Eq. 2.3)}
\end{align*}
$$

In these reactions, electrons from one sulfur species $\text{S}_2\text{O}_3^{2-}$ or $\text{SO}_3^{2-}$ enter the electron transport chain and eventually reduce molecules of other $\text{SO}_4^{2-}$ to sulfide. The exact enzymology involved in sulfur disproportionation, however, is not yet clearly understood.

Cypionka *et al.* studied the behavior of *Desulfovibrio desulfuricans* and suggested a combined pathway for the disproportionation of $\text{SO}_3^{2-}$ and $\text{S}_2\text{O}_3^{2-}$. Cypionka's model (Figure 2.1) proposed that $\text{S}_2\text{O}_3^{2-}$ is cleaved into elemental sulfur and $\text{SO}_3^{2-}$ that undergoes a further disproportionation to sulfate and sulfide in a second step.

As shown in Figure 2.1, one mole of $\text{SO}_3^{2-}$ is converted to $\frac{1}{4}$ moles of $\text{H}_2\text{S}$ and $\frac{1}{4}$ moles of $\text{SO}_4^{2-}$. On the other hand, one mole of $\text{S}_2\text{O}_3^{2-}$ gives one mole of $\text{SO}_3^{2-}$ and one mole of $\text{S}^0$ which then is converted to $\frac{1}{4}$ moles $\text{SO}_4^{2-}$ and $\frac{1}{4}$ mole $\text{H}_2\text{S}$. Model of Cypionka *et al.* can be summarized as follows:

$$
\begin{align*}
\text{S}_2\text{O}_3^{2-} &\rightarrow \text{S}^0 + \text{SO}_3^{2-} \quad \text{(Eq. 2.4)} \\
\text{SO}_3^{2-} + \frac{1}{2}\text{H}^+ &\rightarrow \frac{1}{4}\text{H}_2\text{S} + \frac{3}{4}\text{SO}_4^{2-} \quad \text{(Eq. 2.5) same as Eq. 2.3} \\
\text{S}^0 + \text{H}_2\text{O} &\rightarrow \frac{3}{4}\text{H}_2\text{S} + \frac{3}{4}\text{SO}_4^{2-} + \frac{1}{2}\text{H}^+ \quad \text{(Eq. 2.6)}
\end{align*}
$$

12
Figure 2.1: A combined pathway for disproportionation of SO$_3^{2-}$ and S$_2$O$_3^{2-}$

Adopted from Cypionka et al. [20]

2.4.2 Aqueous chemistry of sulfide-sulfite-thiosulfate system

A biological system using SRB with different sulfur oxyanions as electron acceptors will produce sulfide species. This sulfide can react inorganically with SO$_3^{2-}$ or S$_2$O$_3^{2-}$. It is therefore important to understand the reactions involved and know the behavior of aqueous sulfide-sulfite-thiosulfate system. Given below is a summary of the work done by Siu[37]:

Reaction between sulfide and thiosulfate

Production of elemental sulfur has been reported by reaction between sulfide and thiosulfate (Kundo et al. [38] and Pai et al.[39]) according to equation:

$$4\text{H}_2\text{S(aq)} + 2\text{S}_2\text{O}_3^{2-} + 4\text{H}^- \rightarrow \text{S}_8(\text{s}) + 6\text{H}_2\text{O} \quad (\text{Eq. 2.7})$$

Experiments conducted by Siu[37] on the study for reaction between sulfide and thiosulfate (Eq. 2.7), showed that this reaction is feasible only at pH range 5 – 7. It
was also proposed using theoretical calculations that reaction between sulfide and thiosulfate becomes thermodynamically unfavorable at pH higher than 8.

**Reaction between sulfide and sulfite**

In neutral to weak alkaline solutions the reaction between sulfite and sulfide leads to thiosulfate. Foerster & Mommsen \[40\] and Urban \[41\] reported that the reaction produced thiosulfate almost predominantly. This reaction was also studied by Neiman *et al.*\[42\] and Barbieri & Majorca\[43\], proposing the same mechanism in which elemental sulfur is first formed which further reacts with sulfite to produce thiosulfate:

\[
2\text{H}_2\text{S}^\text{2+} + \text{SO}_3^\text{2-} + 2\text{H}^- \rightarrow 3\text{H}_2\text{O} + 2^{35}\text{S} + \text{S}
\]  
(Eq. 2.8)

\[
3\text{SO}_3^\text{2-} + 2^{35}\text{S} + \text{S} \rightarrow 2^{35}\text{SSO}_3^\text{2-} + \text{SSO}_3^\text{2-}
\]  
(Eq. 2.9)

Heunisch\[44\] suggested that the overall reaction should be written as:

\[
2\text{HS}^- + 4\text{HSO}_3^- \rightarrow 3\text{S}_2\text{O}_3^\text{2-} + 3\text{H}_2\text{O}
\]  
(Eq. 2.10)

Siu\[37\] reported the occurrence of the reaction between sulfide and sulfite at pH 8 – 9 and suggested that this reaction is very complicated. The products include elemental sulfur, thiosulfate and possibly polythionates & polysulfides depending upon the pH and ratio of two reactants. At moderate to high pH (≥7) when sulfide is the limiting reagent, thiosulfate is a predominant product.

### 2.4.3 pH dependence of aqueous sulfite and sulfide species

Predominant sulfite species in aqueous solution are sensitive to pH changes as shown in Figure 2.2. At pH 7.0, both HSO\text{3-} and SO\text{3-} are major species.

Depending upon the pH of the system, sulfide can also exist as various species like HS\text{-}, H\text{2}S and S\text{-} as reported by Hoa *et al.* (Figure 2.3). This equilibrium distribution of sulfide species is important to understand the dynamics of a SRB reactor system because
different species may have a different toxicity/inhibition effect on the performance of SRB.

**Figure 2.2 : Distribution of sulfur(IV) oxide species as a function of pH**

(Adopted from Brandi et al. [45])

**Figure 2.3 : Distribution of sulfide species with pH**

(Adopted from Hoa et al. [16])
2.5 Biodegradation of glucose

Sulfate reducing bacteria are capable of using a diverse range of carbon sources (as discussed in sec 2.3.4). In this work lactate and glucose were used as carbon and energy sources for SRB growth. It is important to understand the various degradation pathways involved. A hypothetical pathway (Figure 2.4) is reported by Matsui et al.[2b].

![Glucose decomposition pathways with sulfate reduction](Image)

**Figure 2.4**: Glucose decomposition pathways with sulfate reduction

*Adopted from Matsui et al.*[2b]

A detailed list of reactions involving associated bacterial species involved has been provided by Matsui et al.[2b] (Table 2.1).
Table 2.1: Pathways for glucose biodegradation

<table>
<thead>
<tr>
<th>Sr. #</th>
<th>Type of Bacteria</th>
<th>Biochemical Equations</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td>Heterofermentation (Leuconostoc Sp.)</td>
<td>$C_6H_{12}O_6 + H_2O \rightarrow CH_3CHOHCOO^- + CH_3CH_2OH + HCO_3^- + 2H^-$</td>
</tr>
<tr>
<td>b.</td>
<td>Propionic Acid Bacteria (Propionicibacterium Sp.)</td>
<td>$3CH_3CHOHCOO^- \rightarrow 2CH_3CH_2COO^- + CH_3COO^- + HCO_3^- + H^-$</td>
</tr>
<tr>
<td>c.</td>
<td>Acetic Acid Bacteria</td>
<td>$4CH_3CH_2OH + 2H_2O \rightarrow 2CH_3CH_2COO^- + CH_3COO^- + 5H_2 + 3H^-$</td>
</tr>
<tr>
<td>d.</td>
<td>Methanogenic Bacteria (Methanobacterium Sp.)</td>
<td>$4H_2 + HCO_3^- + H^- \rightarrow CH_4 + 3H_2O$</td>
</tr>
<tr>
<td>e.</td>
<td>Sulfate Reducing Bacteria (Desulfobulbus Sp.)</td>
<td>$4CH_3CH_2COO^- + 3SO_4^{2-} \rightarrow 4CH_3COO^- + 4HCO_3^- + 4H^-$ + 3HS^-</td>
</tr>
<tr>
<td>f.</td>
<td>Acetogenic Bacteria</td>
<td>$CH_3CH_2OO^- - 3H_2O \rightarrow CH_3COO^- + HCO_3^- + H^-$ + 3H_2</td>
</tr>
<tr>
<td>g.</td>
<td>Sulfate Reducing Bacteria (Desulfobacter Sp.)</td>
<td>$3SO_4^{2-} + H^- + 4H_2 \rightarrow 3HS^- + 4H_2O$</td>
</tr>
<tr>
<td>h.</td>
<td>Sulfate Reducing Bacteria (Desulfobacter Sp.)</td>
<td>$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$</td>
</tr>
<tr>
<td>i.</td>
<td>Methanogenic Bacteria (Methanaseta Sp.)</td>
<td>$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$</td>
</tr>
</tbody>
</table>

Adopted from Matsui et al.\textsuperscript{[28]}

Above list of degradation pathways from Matsui et al.\textsuperscript{[28]} is not exhaustive. For example above it does not include the classical pathway where SRB (specifically Desulfovibrio) utilize lactate as carbon/energy source. Another account is given by Bagley et al.\textsuperscript{[46]} by developing a mathematical model for different microbial populations in an anaerobic community. A complete list of all possible pathways of glucose with mixed cultures of anaerobic bacteria is out of scope of this work. The purpose of Table 2.1 is to give a general view of glucose degradation with generation of various acids.
Chapter 3: Experimental Work

Experimental work was conducted in two phases. Major differences of the two phases are summarized in Table 3.1 below:

Table 3.1: Major differences for phase 1 & 2 experiments

<table>
<thead>
<tr>
<th>Phase 1</th>
<th>Phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate was used as sole energy &amp; carbon source.</td>
<td>Glucose was used as sole carbon &amp; energy source.</td>
</tr>
<tr>
<td>Inoculum was obtained from Eastern Power Ltd.</td>
<td>Inoculum was obtained from Toronto's Ashbridges Bay Treatment Plant.</td>
</tr>
<tr>
<td>pH of SRB reactors was not controlled.</td>
<td>pH of SRB reactors was controlled around 7.5.</td>
</tr>
</tbody>
</table>

3.1 Materials

3.1.1 Electron donor & electron acceptor

Lactate was chosen as sole energy and carbon source for phase 1 experiments: because most of the researchers\(^{[16], [17]}\) have recommended use of lactate for experimental work. However, for phase 2 work glucose was chosen because ability of SRB to use glucose as sole carbon and energy source has also been reported\(^{[26],[27]}\) and it is much cheaper than lactate.

As discussed in chapter 1, one motive for this work is its probable application as a downstream unit in a process for recovery of valuable metals from industrial slag. Such an environment may contain different \(\text{SO}_4^{2-}\) specifically: \(\text{SO}_4^{2-}\), \(\text{SO}_3^{2-}\) and \(\text{S}_2\text{O}_3^{2-}\). Keeping this in mind, selection of these \(\text{SO}_x^{2-}\) as electron acceptors for this work was made.
3.1.2 Growth media for SRB

Modified Postgate medium C\(^{[7]}\) for SRB growth was used as the growth medium, for its simplicity of preparation and also for its low iron contents. Postgate medium C originally contains sulfate (SO\(_4^{2-}\)) as electron acceptor, however, in our case equimolar quantities of other ions i.e. sulfite (SO\(_3^{2-}\)) and thio-sulfate (S\(_2\)O\(_3^{2-}\)) were used. For phase 1 studies lactate was used as sole carbon and energy source whereas for phase 2, glucose was used instead. Quantity of glucose to be added in a reactor was chosen such as to give same COD value as given by amount of lactate as suggested in original Postgate medium C. To understand the effect of varying COD/SO\(_4^{2-}\) ratio on SRB activity, the amount of glucose was kept constant while the amounts of SO\(_4^{2-}\) were varied. A complete list of nutrient recipe’s for each experiment is given as Appendix 1.

3.1.3 Sources of bacteria

The inoculum sludge for phase 1 work was obtained from Eastern Power. The major process there is the production of methane gas employing MPB. The sludge obtained was then enriched in SRB for one month in a glass flask. This inoculum was continuously fed with fresh solution containing sulfate as electron acceptor, lactate as electron donor and other Postgate medium C nutrients. Continuous purging of N\(_2\) was used to remove the produced sulfide from the system. After enrichment for one month, this reactor was then kept in refrigerator (at 5 °C) as a starting source for phase 1 experiments on sulfate reducing bacteria.

In phase 2 inoculum was obtained from secondary stage anaerobic digester of Toronto's Ashbridges Bay Treatment Plant. Main process there is terminal digestion of organic contents with production of methane gas employing mainly MPB. Sludge obtained was enriched in SRB in glass flask. Inoculum was intermittently fed with fresh nutrient solution containing sulfate as electron acceptor, glucose as electron donor and other Postgate medium C contents. Often pH of the system was checked and adjusted to 7.5. Flask was purged with mixture of N\(_2\) & 5% CO\(_2\) gas to provide anaerobic environment.
Then instead of preserving the inoculum in a refrigerator, it was kept on running and continuously fed with fresh nutrients. This was done in order to avoid the probable effect of dormancy on microbes, caused by the storage of reactor contents in refrigerator. The inoculum for experiments was then taken whenever required, with the help of a plastic syringe directly from inoculum source flask and injected to test reactors.

3.1.4 Chemicals & equipment used
List of various chemical and gases used along with brief information on their purity, purpose and supplier information is provided in Appendix 2.1. while the list of miscellaneous equipment used is given in Appendix 2.2.

3.2 Experimental setup
3.2.1 Overall arrangement
As shown in Figure 3.1. three identical glass reactors were run in parallel as test reactors, for three different species of sulfur oxyanions ($\text{SO}_x^-$). These reactors were enclosed inside an electrically controlled incubator to maintain the temperature. A separately maintained inoculum source (sec. 3.1.3 & 3.3.4), was used as a source of SRB. The sparged gases from these reactors, before disposal to atmosphere were bubbled through a scrubber. Scrubber contained 2N aqueous solution of NaOH to remove any $\text{H}_2\text{S}$. 
3.2.2 Details of test reactor (typical)

Three custom made (spherical shape, glass made) reactors were used for all experimental work on SRB. In each reactor total volume of reactants was kept 3 L.

As shown in Figure 3.2 each reactor was provided with:

- A sparger designed to bubble a) continuous & controlled flow of N\textsubscript{2} gas: to provide anaerobic environment inside the reactor. b) intermittent flow (5% of total flow by volume) of CO\textsubscript{2} gas to lower the pH inside the reactor.

- A dome shaped sparger head, for continuous collection & removal of purging gases from the reactor. Provision was made for the intermittent collection of gas samples, through gas sampling valves. Samples were collected in ½ L volume using gas bladders and were taken away for analysis.
A liquid sampling port was provided for the collection of liquid samples. Port consisted of a ¼ inch Internal Dia hole made into the body of glass reactor and closed using inert rubber stopper. As Elsgaard showed[47] that use of ordinary black color rubber stoppers containing styrene-butadiene may cause a loss of hydrogen sulfide from the system. An inert plastic tube with syringe adaptor connection was inserted through the rubber stopper to facilitate the sample collection using a plastic syringe & sealed glass vials. This method helped to preserve the samples from air oxidation and also to prevent the unwanted oxygen entry in the system.

Magnetic stirrer was used to provide continuous mixing of the reactor contents.
3.3 Selected parameters & procedures

3.3.1 Temperature & pH
Mixed cultures of bacteria obtained as source of inoculum were mainly expected to be of mesophilic type. Literature on SRB recommends an optimum temperature of 35 °C for these types of SRB. Thus for both phase 1 & 2 experiments the temperature was controlled around 35 °C. Reactors containing SRB were placed inside an incubator with digital temperature control with a set-point of 35°C. A separate digital temperature meter was employed to periodically measure the actual temperature inside the incubator. Variation in incubator temperature during the course of experiment was found to be in the range of ± 3°C.

For both phases 1 & 2 the starting pH of the solutions was adjusted to 7.5, as recommended in literature using 1N solutions of NaOH or HCl. For phase 1 experiments, pH was not controlled during the experiment. It was observed that the pH of the solution increased with time because of SRB activity. Subsequently in phase 2 experiments, the pH of system was adjusted to 7.5 after every 24 hours. pH was adjusted to using 1 N NaOH and 5% (by volume) CO₂ gas on as required basis.

For pH measurement Barnant company’s pH meter (Barnant 30), was employed along-with a Cole Parmer’s pH electrode. The pH meter was calibrated at the start of each experiment, using standard pH solutions.

3.3.2 Duration of experiments
Different researchers using SRB systems have used different experimental durations ranging from 1 - 30 days. Our initial experiments were conducted for 12 to 14 days. After examining the SO₄²⁻ reduction patterns of phase 1 experiments, a duration of 7 days was selected for the subsequent work.
3.3.3 Purging gases

For phase 1 experiments, only \( \text{N}_2 \) gas was continuously purged at a continuous flow of 40 mL/min. To provide an anaerobic environment and at the same time to carry away the produced \( \text{H}_2\text{S} \) from the reactor.

Continuous \( \text{N}_2 \) gas purging was also used for phase 2 experiments. In addition an intermittent flow of 5% \( \text{CO}_2 \) was introduced in the later stages of experiments, to lower the pH of solution. Total flow of \( \text{N}_2 \) & \( \text{CO}_2 \) in all cases was kept around 40 mL/min.

3.3.4 Preparation & maintenance of the inoculum source

The inoculum source was once prepared enriched for SRB and continuously maintained during the course of experimental work. The procedure is described as follows:

- Add 1200 mL of de-ionized water in the reactor.
- Sparge, while stirring with a magnetic stirrer, for 5 – 10 min with \( \text{N}_2 \) gas to remove dissolved oxygen.
- Dissolve all the nutrients including electron donor & acceptor.
- Place the reactor in the incubator, for 1 hour to attain the incubator temperature that is 35°C.
- Add 300 mL of biomass from source inoculum as starting SRB source.
- Check the pH of the solution and adjust it to 7.5, by adding 1N solutions of NaOH or HCl on required basis.
- Once prepared the source inoculum is fed, every 5 days by replacing 300 mL of reactor contents with fresh nutrients, consisting of electron donor (lactate for phase 1 & glucose for phase 2), electron acceptor (\( \text{SO}_4^{2-} \)) and other Postgate medium C contents (Appendix 1).
- pH was regularly checked and maintained around 7.5 using 1N NaOH & 1N HCl as required. An average protein concentration of 20 (±5) μg/L was maintained in the source inoculum. Average concentration of sulfate ions in the reactor was maintained around 2500 (±200) mg/L.
3.3.5 Preparation of SRB Test Reactors

Every time a test reactor was prepared for SRB experiments, following procedure was followed:

- Add 2950 mL of de-ionized water in the reactor
- Sparge while stirring, with a magnetic stirrer the reactor, for 5 - 10 min with N₂ gas to remove dissolved oxygen.
- Dissolve all the nutrients including electron donor & acceptor. To avoid air oxidation of SO₃²⁻, after adding all the ingredients in a reactor except Na₂SO₃, purge the reactor contents with N₂ for 10 minutes and then quickly add Na₂SO₃.
- Place the reactor in the incubator, for 1 hour to attain the incubator temperature of 35°C.
- Add 50 mL of inoculum sludge from the separately maintained inoculum source.
- Check the pH of the solution and adjust it to 7.5

3.3.6 Reactor sampling procedure

Gas and liquid samples were collected from all the test reactors every 24 hours (±4 hours). The first set of samples was taken within half hour after adding the source inoculum. Gas samples were collected in three separately marked gas bladders (500 mL volume), from three reactors, by directly connecting gas bladders to gas sampling valves as shown in Figures 3.1 & 3.2. Samples were then taken into lab and analyzed for H₂S & CH₄ gases. Liquid samples were collected (15 mL volume) after thoroughly shaking the reactor, through liquid sampling ports as shown in Figures 3.1 & 3.2, using inert plastic syringes. Samples were then analyzed for pH, chemical oxygen demand (COD), biomass and oxyanions of sulfur (SO₄²⁻). Samples for SO₄²⁻ analysis were immediately preserved by diluting into formaldehyde to prevent air oxidation of SO₃²⁻ (sec. 3.4.1).

3.4 Analytical methods

3.4.1 Dissolved sulfur oxyanions (SO₄²⁻)

To quantify the SO₄²⁻ reduction activity of SRB, liquid samples taken from the reactors were analyzed for the residual SO₄²⁻. Concentrations of different sulfur oxyanions (SO₄²⁻
were determined using a Dionex ion chromatographic (IC) system (DX500) equipped with an analytical column (IonPac AS4A-SC. 4 x 250 mm), a guard column (Ion Pac AG4A-SC. 4 x 50 mm), and an anion self-regenerating suppressor (Model II). An eluent of 1.8 mM Na₂CO₃ + 1.7 mM NaHCO₃ at 2 mL/min provided a good peak separation. The residence times for SO₃²⁻, SO₄²⁻ and S₂O₃²⁻ were 2.4, 3.1 and 6.8 min respectively. The separated anions were measured with a conductivity detector (ED40). The Star Chromatography Workstation software package provided by Varian was used to collect and analyze the data.

Calibration curves were prepared for each of sulfur oxyanion (SO₄²⁻, SO₃²⁻ and S₂O₃²⁻) and are attached as Appendix 3.1.

SO₃²⁻ is a rapidly oxidizable sulfur oxyanion. In order to avoid air oxidation of SO₃²⁻, all samples after taken from the reactors were immediately diluted in 0.4% formaldehyde solution prior to IC analysis. Formaldehyde combines with HSO₃⁻ and forms an organic bisulfite (stable complex) ion according to equation:

\[ \text{H}_2\text{C}=\text{O} + \text{HSO}_3^- \rightarrow \text{H}_2\text{C(OH)}\text{SO}_3^- \quad \text{(Eq. 3.1)} \]

For above reaction to occur, SO₃²⁻ should be in the form of HSO₃⁻. 0.01N HCl was used to lower the pH to desired range.

It was observed that the retention time of PO₄³⁻ was very close to that of SO₄²⁻. FeCl₃ solution was added to precipitate out the PO₄³⁻ ions as FePO₄ which is insoluble. Figure 3.3 shows the effectiveness of the precipitation technique. Chromatogram A is for solution without adding FeCl₃; while chromatogram B is for the solution that is treated with FeCl₃ and filtered before injection to the IC.
Given below is the final version of the procedure developed and used for SO$_4^{2-}$ analysis:

1. Take 25 mL of dilution solution (0.4% formaldehyde + 0.01 N HCl) in 3 flasks, one each for different SO$_4^{2-}$
2. Add 'x' mL of reactor samples into respective dilution flasks
3. Add 0.2 mL of 0.1835 molar FeCl$_3$ solution into each dilution flasks
4. Shake the solutions & allow to stand for 2 – 3 min
5. Filter the solutions using (0.22 μm meter pore size) membrane filters
6. Analyze the filtrate with IC for the presence of different SO$_4^{2-}$

Since the concentration of SO$_4^{2-}$ was changed to vary the electron donor/acceptor ratio, the volume of sample, 'x', was changed from 2 mL to 5 mL to get a dilution that gives a sufficient peak separation of SO$_3^{2-}$ & SO$_4^{2-}$.

### 3.4.2 Biomass

To determine the activity of sulfate reducing bacteria in different experiments it is also important to have a knowledge of their population dynamics that is growth/death/stability. Since protein is an essential component of bacterial cells, its
concentration, can be taken as an indirect representation of the amount of biomass present at a given time. During SRB experiments the growth of SRB was determined as the change in protein using Bradford’s[50] Bio-Rad protein assay method. In this method a differential color change of dye (Bio-Rad) occurs in response to varying concentrations of protein. The spectrophotometer analysis of such a color gives the amount of protein present in the solution in a dye-binding assay. Sigma’s bovine serum albumin standard protein was used to prepare the calibration curve (Appendix 3.2) for protein analysis. Assuming a cell density of $10^{13}$ cells/g of protein. the amount of protein in solutions is converted to number of cell according to the following relation:

$$\text{Number of cells/L} = [2 \times (\text{amount of protein \(\mu g/mL\))] \times 10^{10}$$

Vendor (Bio-Rad) data recommended that protein samples after adding the Bio-Rad dye & before analyzing in spectrophotometer, should be incubated at room temperature for 5 min as the light absorbance will stabilize with time. To ensure data quality experiments were designed to determine the time dependence of color formed by Bio-Rad dye & protein complex. It was observed that the light absorbance decreased significantly even after 5 min (Appendix 4.1). Consequently. all samples were incubated for 15 min before finally analyzed in the spectrophotometer.

To determine the precision. 5 samples were taken from the same reactor at the same time and processed for the protein analysis under same conditions. The relative standard deviation for sulfate. sulfite and thiosulfate reactors were found as 11%, 14% and 22% respectively. This deviation is due to the uncertainty in both sampling and analyzing. Plots for precision of sampling for protein analysis are provided in Appendix 4.2

Given below is the final procedure (adopted from Chin[19]) was used for protein analysis:

1. Using plastic syringes. take 3 samples (1 mL each) from the reactor directly into pre-tagged. 1.5 mL sized plastic vials
2. Centrifuge these vials at 13,000 G for 20 min
3. Discard 0.8 mL of supernatant re-suspend in 1.4 mL of 0.66 N NaOH
4. Digest for 48 hours at 35 °C. to release the protein into the solution
5. Centrifuge again at 5000 G for 5 min
6. Transfer 300 μL of supernatant to a clean test tube
7. Add 200 μL of 2 N HCl
8. Add 4300 μL of de-ionized water
9. Add 200 μL of Bio-Rad Dye
10. Wait for 12-15 min for a stable dye color
11. Measure absorbance in spectrophotometer at 595 nm
12. Find the protein concentration in μg, using the calibration curves from Appendix 3.2.
13. Assuming a cell density of 1013 cells/g of protein, convert the protein concentration to cells/L concentration using relation: Number of cells/L = [2 x (amount of protein μg/mL)] x 10^{10}

3.4.3 Chemical oxygen demand (COD)
COD is a measure of oxidizable organic matter present. A mercury free, commercial COD measurement kit from Chemetrics Inc. was used for this experimental work. The COD kit consisted of pre-filled COD sealed glass tubes. Samples were introduced into the COD tubes (containing mainly an acidic solution of potassium dichromate in the presence of a catalyst silver) and digested for 2 hours at a temperature of 150 °C in a heating block. In the tube oxidizable organic compounds reduced the dichromate ion Cr_{2}O_{7}^{2-} to chromic ion Cr^{3+}. The reagents in the COD tubes changed color corresponding to the amount of chemically oxidizable substances present in the sample. This color is then quantified with spectrophotometer at 620 nm wavelength. The calibration curve for COD measurements as provided by vendor is given as Appendix 3.3.

3.4.4 Detection of H_{2}S & CH_{4} in gaseous phase
Bioreactors for the SRB experiments were expected to contain mainly two different types of bacterial populations of interest. One is methane producing bacteria and other is SRB
which produces H$_2$S. Therefore gas samples collected from SRB reactors were analyzed using a gas chromatograph (GC) for the presence of these two gases.

For the methane detection, a Flame Ionization Detector (FID) was used with following operating parameters:

| Detector Type/Temperature | : FID/290 °C |
| Column Type/Temperature   | : J&W. 0.32 ID. 30 m long, model unknown/100 °C |
| Injector Type/Temperature | : Manual/220 °C |
| Carrier gas (N$_2$) flow  | : 5 mL/min @ 80 PSI pressure |
| Combustion gas (H$_2$) flow | : 30.5 mL/min @ 45 PSI pressure |
| Combustion Air flow       | : 48.5 mL/min @ 60 PSI pressure |
| Column Hold Time          | : 8 min |
| Approx. retention of CH$_4$ | : 5.9 min |
| Lower Detection Limit     | : 90 ppm of CH$_4$ (total sample vol. 500 µL) |

For the H$_2$S detection a Pulsed Flame Photo Detector (PFPD) was used with following operating parameters:

| Detector Type/Temperature | : PFPD/200 °C |
| Column Type/Temperature   | : HP. 0.53 ID. 5 m long, model unknown/120 °C |
| Injector Type/Temperature | : Manual/220 °C |
| Carrier gas (He) flow     | : 8 mL/min @ 60 PSI pressure |
| Combustion gas (H$_2$) flow | : 13 mL/min @ 40 PSI pressure |
| Combustion Air flow       | : 18 mL/min @ 60 PSI pressure |
| Column Hold Time          | : 4 min |
| Approx. retention of H$_2$S | : 3.10 min |
| Lower Detection Limit     | : 90 ppm of H$_2$S (total sample vol. 500 µL) |

For calibration curves of both H$_2$S and CH$_4$ please see Appendix 3.4.
3.5 Repeatability experiment

Before the actual experiments were conducted, a test was carried out to determine the overall repeatability of experiments. Three reactors were run in parallel under the same conditions. In all three reactors glucose was used as electron donor and $\text{SO}_4^{2-}$ as electron acceptor. These results are shown in Figures 3.4 to 3.7. These results show fairly similar trends for $\text{SO}_4^{2-}$ reduction, biomass growth, pH and COD removal in all three reactors. Table 3.2 summarizes the changes in pH and color during the test.
Figure 3.4: Precision experiment pH change with time

Figure 3.5: Precision experiment variation in SO₄²⁻ conc. with time
Figure 3.6: Precision experiment change in soluble COD with time

Figure 3.7: Precision experiment biomass growth in SRB reactor with time
Table 3.2: Summary of pH & color change with time during test runs

<table>
<thead>
<tr>
<th>Time (Hrs)</th>
<th>Sulfate Reactor #1 pH before adjust. = 5.92</th>
<th>Sulfate Reactor #2 pH before adjust. = 5.669</th>
<th>Sulfate Reactor #3 pH before adjust. = 5.87</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH (Obs.)</td>
<td>pH (Adj.)</td>
<td>Color of Reactor Contents</td>
</tr>
<tr>
<td>0</td>
<td>7.59</td>
<td>Transparent</td>
<td>Foam: Black.</td>
</tr>
<tr>
<td>6</td>
<td>5.02</td>
<td>7.60</td>
<td>Blackish Dull</td>
</tr>
<tr>
<td>25</td>
<td>7.59</td>
<td>Black</td>
<td>Black</td>
</tr>
<tr>
<td>45</td>
<td>7.66</td>
<td>Black</td>
<td>Black</td>
</tr>
<tr>
<td>71</td>
<td>7.54</td>
<td>Black</td>
<td>Black</td>
</tr>
<tr>
<td>96</td>
<td>7.68</td>
<td>Black</td>
<td>Black</td>
</tr>
<tr>
<td>125</td>
<td>7.58</td>
<td>Black</td>
<td>Black</td>
</tr>
</tbody>
</table>

Abbreviations:
Obs. = Observed value
Adj. = Adjusted value
ppt. = precipitate(s)
Chapter 4: Results & Discussion

Experimental work was conducted in two phases which differ mainly in carbon and bacterial sources. Other differences between phase 1 and 2 experiments can be found in Table 3.1. Phase 1 experiments were preliminary experiments for understanding the system and design the subsequent phase 2 experiments. Thus the results of phase 1 are presented and discussed only briefly. Discussion of phase 2 results is, however, done in a detailed manner.

4.1 Experiments with lactate as carbon & energy source
Different SO₃²⁻, specifically SO₄²⁻, SO₃²⁻ and S₂O₃²⁻ were used as electron acceptors. Lactate was used as the sole energy and carbon source and other ingredients were the same as recommended in Postgate's medium C (Appendix 1). Experiments were performed in three reactors running in parallel one for each SO₃²⁻ containing mixed bacterial cultures. These cultures were obtained from Eastern Power Ltd. and were pre-enriched in SRB.

Color of reactor contents in all three reactors was initially transparent yellow which turned to opaque black, approximately at the end of 19, 24 and 35 hours for S₂O₃²⁻, SO₃²⁻ and SO₄²⁻ respectively. As iron was included in the nutrient recipe (Postgate medium C), the sulfide produced by SRB combines with iron to form black iron sulfide. Thus, the color change in the reactors is an indication of SRB activity.

4.1.1 Time dependence of biomass & pH
The results of biomass growth and pH data are given in Figures 4.1 a & b. These results show a sharp increase in biomass concentration in all three reactors followed by a subsequent decrease and a final stabilization. Apparently, there is a gradual increase in pH followed by a stabilization period in all three reactors. SRB while reducing SO₄²⁻ produce S²⁻ which combines with available H⁺ to form HS⁻ & H₂S. This H⁺ scavenging effect raises alkalinity of a system and may be responsible for the observed increase in pH in our systems.
Figure 4.1: Phase I - Time dependence of (a) biomass and (b) pH
It is important to understand the impact of system's increasing pH on dynamics of an anaerobic system with mixed culture of anaerobic microbes. In such a system, different communities of bacteria can strive for same nutrient groups. In our case, there are at least two major bacterial groups of interest. SRB and MPB.

Hao et al.\textsuperscript{[16]} reported that at a pH of 7.0-7.5 growth rates of MPB and SRB were similar: below pH 7.0. MPB grew better than SRB while SRB showed a better growth at a pH > 7.5. In our case, the initial pH was adjusted to 7.5 to ensure the advantage of SRB over MPB.

The increase in biomass in first two to three days is attributed mainly due to the growth of SRB. This is consistent with the observed increase in pH which is characteristic of SRB activity. Interestingly, the biomass growth in the SO\textsubscript{4}\textsuperscript{2-} reactor was the lowest while the increase in pH was the least. Although it is a product of SRB activity, sulfide is toxic to bacterial communities including SRB. Its toxicity is however affected by solutions pH. As shown in Figure 2.3, at low pH, sulfide is mostly in un-dissociated form. H\textsubscript{2}S\textsubscript{(aq)}. H\textsubscript{2}S\textsubscript{(aq)} is electrically neutral and is more capable of passing through the cell membrane and hence more toxic\textsuperscript{[31]}. A higher pH, on the other hand, increases the total amount of dissolved sulfides in the solution, due to the increase in the fractions of other species (HS\textsuperscript{-} and S\textsuperscript{2-}) which also have an inhibitory effect on the growth of SRB. It was therefore suggested by O'Flaherty et al.\textsuperscript{[22]} to define two different threshold limits for inhibition studies of sulfides on SRB growth. One for pH values lower than 7.2 based upon undissociated sulfide (H\textsubscript{2}S) and another for higher pH values based on total sulfide concentration.

Another factor which may further complicate the effect of aqueous sulfide on bacteria is the evolution of H\textsubscript{2}S\textsubscript{(gaseous)}. At a lower pH the removal of sulfide via desorption becomes easier due to the greater proportion of sulfide in H\textsubscript{2}S\textsubscript{(aq)} form.

Therefore, it is desirable to control the pH. The literature reported optimum range is 7.5-8.0 \textsuperscript{[17],[22]} at which some sulfide is still in the form of H\textsubscript{2}S. Although H\textsubscript{2}S is soluble in water (2650 mg/L at 35 °C\textsuperscript{[31]}), it is the only sulfide which can be removed by purging
with an inert medium, such as N\textsubscript{2} in our case. If pH is too high, the amount of H\textsubscript{2}S will be too low to be removed via purging and will lead to an accumulation of dissolved sulfides, HS\textsuperscript{-} and S\textsuperscript{2-}. It is believed that the accumulated sulfides in the solution at high pH had contributed the observed subsequent decrease in biomass (Figure 4.1.a). Another limiting factor may be the supply of nutrients in a batch system.

### 4.1.2 Time dependence of individual SO\textsubscript{4}\textsuperscript{2-}

The results of individual oxyanions removal in each reactor are given in Figures 4.2.a, 4.3.a & 4.4.a. In the sulfate reactor only SO\textsubscript{4}\textsuperscript{2-} species was detected. In sulfite and thiosulfate reactors all three species SO\textsubscript{4}\textsuperscript{2-}, SO\textsubscript{3}\textsuperscript{2-} & S\textsubscript{2}O\textsubscript{3}\textsuperscript{2-} were detected. The amount of SO\textsubscript{4}\textsuperscript{2-} detected in thiosulfate reactor was much smaller than sulfite reactor. Most likely the high SO\textsubscript{4}\textsuperscript{2-} content in the sulfite reactor was caused by the air oxidation of the reagent Na\textsubscript{2}SO\textsubscript{3} during reactor preparation. In phase 2 experimental procedures were modified to avoid air oxidation of SO\textsubscript{3}\textsuperscript{2-}. This rectified the problem and phase 2 experiments did not show any unusually high concentration of SO\textsubscript{4}\textsuperscript{2-} in the sulfite reactors.

It is widely accepted\cite{11,29,30,51,52} that SRB are capable of disproportionating SO\textsubscript{3}\textsuperscript{2-} and S\textsubscript{2}O\textsubscript{3}\textsuperscript{2-}. Disproportionation reactions also produces SO\textsubscript{4}\textsuperscript{2-} which is likely responsible for the SO\textsubscript{4}\textsuperscript{2-} detected in the thiosulfate reactor. In aqueous system, SO\textsubscript{3}\textsuperscript{2-} can chemically react with S\textsuperscript{2-}/HS\textsuperscript{-} and generate S\textsubscript{2}O\textsubscript{3}\textsuperscript{2-}. In sulfite reactor S\textsuperscript{2-}/HS\textsuperscript{-} can be produced by SRB which can react with SO\textsubscript{3}\textsuperscript{2-} to generate S\textsubscript{2}O\textsubscript{3}\textsuperscript{2-} (sec. 2.4.2). This explains the observed S\textsubscript{2}O\textsubscript{3}\textsuperscript{2-} in the sulfite reactor. The SO\textsubscript{4}\textsuperscript{2-} detected in the thiosulfate reactor was minor and may be attributed to the following decomposition reaction.

$$S\textsubscript{2}O\textsubscript{3}\textsuperscript{2-} \rightarrow S\textsuperscript{0} + SO\textsubscript{3}\textsuperscript{2-}_{\text{aq}} \quad \text{(Eq. 4.1)}$$

A detailed discussion of possible pathways for the production of different oxyanions in the sulfite and thiosulfate reactors is given in sec. 4.2.4.
Figure 4.2: Phase 1 - (a) individual (b) total SO\textsubscript{3} species, in SO\textsubscript{4} reactor
Figure 4.3: Phase 1 - (a) individual (b) total $\text{SO}_x^{\text{v}}$ species, in $\text{SO}_3^{2-}$ reactor
Figure 4.4: Phase 1 - (a) individual (b) total $SO_x^{1-}$ species, in $S_2O_3^{2-}$ reactor
4.1.3 Time dependence of total $SO_4^{2-}$
Time dependence of total oxyanions in each reactor is given in Figures 4.2.b, 4.3.b & 4.4.b. The total amount of sulfur oxyanions in any SRB reactor is calculated as:

$$\text{moles of total } SO_4^{2-} = \text{moles of } SO_4^{2-} + \text{moles of } SO_3^{2-} + \text{moles of } S_2O_3^{2-}$$

These plots show that the reduction of $SO_4^{2-}$ was high in first five days particularly for sulfite and thiosulfate reactors, consistent with trends in biomass and pH (Figure 4.1.b). Therefore, the rate of reduction was determined for the first five days for all three reactors.

The total $SO_4^{2-}$ removal rate was high for $S_2O_3^{2-}$ (1.8x10$^{-4}$ mol/L hr) and $SO_3^{2-}$ (1.5x10$^{-4}$ mol/L hr) compared to $SO_4^{2-}$ (5x10$^{-5}$ mol/L hr). This shows that the rate of total $SO_4^{2-}$ reduction was much higher in thiosulfate and sulfite reactors as compared to sulfate reactor. Observed order in total $SO_4^{2-}$ rates is $S_2O_3^{2-} > SO_3^{2-} >> SO_4^{2-}$. A detailed discussion of this observation is provided in discussion on phase 2 results.

4.2 Experiments with glucose as carbon & energy source
4.2.1 Design of experiments
4.2.1.1 Importance of COD/$SO_4^{2-}$ ratio
To provide favorable conditions for SRB the ratio of electron donor and electron acceptor needs to be controlled $^{[16],[31]-[34]}$. Chemical oxygen demand (COD) is used as a measure of the amount of energy that can be obtained from a given electron donor such as lactate or glucose. Thus, an important parameter for controlling the growth of SRB is COD/$SO_4^{2-}$ ratio $^{[16],[31]-[34]}$. For SRB, Speece$^{[31]}$ calculated a theoretical COD requirement of 64 g (8 electrons) to reduce one mole of $SO_4^{2-}$ (96 g) based upon reaction given in Eq. 2.1 thus, giving a COD/$SO_4^{2-}$ ratio of 64g/96g = 0.67. This theoretical ratio is the minimum stoichiometric ratio required to reduce all sulfur atoms to $S^{2-}$ level. Actual values are usually higher than theoretical ones varying from 0.8-1.6 since a part of electron donor is also used as carbon source for biosynthesis of new cells. In Speece's reaction $S^{2-}$ was the only sulfide considered suggesting a high pH close to 10. If the
reduction of sulfate is carried out at pH 7 equal amounts of H₂S and HS⁻ will form according to Table 4.5. However, the theoretical COD/SO₄²⁻ ratio will remain the same since the number of electrons involved is the same.

Applying the same approach to the half reduction reactions in Table 4.5 for all three oxyanions of sulfur, theoretical COD/SO₃⁻ ratios were calculated and are given in Table 4.1.

Table 4.1: Theoretical COD/SO₄ₓ⁻ ratios for different SO₄ₓ⁻ at pH 7.0

<table>
<thead>
<tr>
<th>Oxyanions of Sulfur</th>
<th>COD (g)</th>
<th>SO₄²⁻ (g)</th>
<th>COD/SO₄²⁻ (g of COD/g of SO₄²⁻)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO₄²⁻</td>
<td>64</td>
<td>96</td>
<td>0.67</td>
</tr>
<tr>
<td>SO₃²⁻</td>
<td>48</td>
<td>80</td>
<td>0.60</td>
</tr>
<tr>
<td>S₂O₃²⁻</td>
<td>64</td>
<td>112</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Note: above values are calculated for one mole of each SO₄ₓ⁻ for reduction to sulfide

4.2.1.2 Other considerations

Lactate is much more expensive than glucose, which was also used by other researchers [26], [27]. Thus, glucose was chosen as sole energy/carbon source in phase 2 experiments.

The microbes for phase 1 experiments were obtained from Eastern Power where the major process is production of methane gas from waste streams. It was therefore, likely that this bacterial source was initially rich in MPB and contained less SRB. Thus, the sludge produced in Toronto’s Ashbridges Bay Treatment Plant was used as bacteria source in phase 2 experiments (sec. 3.1.3, 3.3.4).

To gain insight into the population dynamics of different species of bacteria specifically SRB & MPB, attempt was made to determine H₂S and CH₄ in the exit gas from the test reactors. In view of the importance of pH discussed in sec. 4.1.1 it was decided to control
the pH of the system instead of just monitoring it. pH was controlled using 1 N NaOH solution and 5% CO₂ purging on as required basis.

4.2.1.3 Conditions of experiments

Six sets of experiments were designed with changing COD/SO₄²⁻ ratios. This ratio was changed by varying the amount of SO₄²⁻ while keeping the amount of COD constant. In each set of experiments, three reactors were run in parallel one for each sulfur oxyanion SO₄²⁻. Given below in Table 4.2 is a description of experimental conditions. Experiments were numbered from G1 to G6 and are arranged in Table 4.2 as G2, G3, G4, G5, G1 and G6 according to the increasing COD/SO₄²⁻ ratio. Detailed contents of recipe for each set of experiment are provided in Appendix 1.

<table>
<thead>
<tr>
<th>Experimental Code</th>
<th>Ratio of Electron Donor/Acceptor</th>
<th>Corresponding COD/SO₄²⁻ Ratios Used</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Units: mol mol</td>
<td>Units: g of COD / g of SO₄²⁻</td>
</tr>
<tr>
<td></td>
<td>For all three Reactors</td>
<td>Sulfate Reactor</td>
</tr>
<tr>
<td>G2</td>
<td>0.16</td>
<td>0.38</td>
</tr>
<tr>
<td>G3</td>
<td>0.21</td>
<td>0.50</td>
</tr>
<tr>
<td>G4</td>
<td>0.32</td>
<td>0.76</td>
</tr>
<tr>
<td>G5</td>
<td>0.53</td>
<td>1.26</td>
</tr>
<tr>
<td>G1</td>
<td>0.64</td>
<td>1.51</td>
</tr>
<tr>
<td>G6</td>
<td>1.28</td>
<td>3.03</td>
</tr>
</tbody>
</table>

Please note that in Table 4.2, the values of COD/SO₄²⁻ ratio in g/g units are calculated as:

(grams of glucose x observed COD/gram of glucose)/(gram of SO₄²⁻)

While values in electron donor/acceptor ratio (mol/mol) units are obtained by dividing moles of glucose used by moles of SO₄²⁻ used. Fixing the value of electron
donor/acceptor ratio will fix the corresponding COD/SO$_4^{2-}$ too. In this thesis, final results are reported in COD/SO$_4^{2-}$ (g/g units) only. While electron donor/acceptor ratio (mol/mol units) is used to experimentally maintain the ratio of electron donor (glucose) and electron acceptor (SO$_4^{2-}$) and also to compare the different SO$_4^{2-}$ on same scale.

### 4.2.2 Change in acidity

The plot of pH in all three reactors against time for experiment G1 is given below in Figure 4.5. This plot was observed to be typical similar plots of all six experiments are provided in Appendix 6.1. very different from phase 1 experiments the pH of all three reactors dropped rapidly from the beginning.

The pH of all reactors was adjusted to around 7.5 every 24 hours and values of pH reported here are the ones before the adjustment. A lower pH means a greater amount of acid was produced during the past 24 hours.

![Figure 4.5: Exp G1 - plot of pH vs. time](image)

The initial drop in pH observed in all reactors could be explained considering the degradation products of glucose (sec. 2.5). The subsequent increase of pH could be attributed to the activity of SRB which scavenges H$^+$. Also at that time the rate of
production of acids by glucose degradation becomes lower than the rate of its consumption. Another reason for the subsequent increase in pH may be the consumption of various acids produced initially (Figure 2.4).

Initial pH drop was much less in the sulfite reactor than in thiosulfate and sulfate. In fact the pH of sulfite reactor was always greater than 7.

This behavior of $\text{SO}_3^{2-}$ may be explained based on aqueous chemistry of sulfide-sulfite-thiosulfate system discussed in sec. 2.4.2. The reaction between $\text{HS}^-$ and $\text{SO}_3^{2-}$ produces $\text{S}_2\text{O}_3^{2-}$ and consumes $\text{H}^+$ at the same time (Eq. 2.10). An overall effect of this reaction is therefore, the removal of $\text{H}^+$ from the system. This argument is supported by the observed accumulation of $\text{S}_2\text{O}_3^{2-}$ in the sulfite reactors, which will be discussed later.

As shown in Figure 2.4. Matsui et al. gave the hypothesized pathways involved in the degradation of glucose with sulfate reducing bacteria in anaerobic environment.

These pathways suggest the formation of acids like lactic acid, propionic acid and acetic acid as the products of glucose degradation. Considering the biochemical equations given in Table 2.1, it is clear that more acid or $\text{H}^+$ could be produced in the biodegradation of glucose as compared to the biodegradation of lactate. This high production of $\text{H}^+$ is responsible for the initial pH drops observed when using glucose as electron donor.

4.2.3 Growth in biomass

The results of the protein/biomass analysis are provided in Figures 4.7 to 4.12. It is important to note that the relative standard deviation in the protein analysis was about 15-20%.

Although the inoculum was first enriched for SRB, other microbiological groups, such as MPB and acid producing bacteria (APB) can coexist under similar conditions. Therefore, an increase in the biomass will be an indicator of the growth of all types of microbes
collectively. Consequently, the quantitative analysis of SRB’s contribution towards overall growth is difficult.

These results suggest that the sulfite reactor grew faster than sulfate and thiosulfate reactors. The sulfite reactor rapidly grew to a maximum within about 24 hours before finally leveling off. The sulfate reactor showed the slowest growth.

![Graph showing biomass growth in SRB reactor vs. time](image)

**Figure 4.6: Exp G1 - biomass growth in SRB reactor vs. time**


Figure 4.7: Exp. G2 - biomass growth in SRB reactor vs. time

Figure 4.8: Exp. G3 - biomass growth in SRB reactor vs. time
Figure 4.9: Exp. G4 - biomass growth in SRB reactor vs. time

Figure 4.10: Exp. G5 - biomass growth in SRB reactor vs. time
In addition, all three reactors in all sets of experiments (G1 – G6) showed a gradual increase in pH and blackening of the reactor contents (because of FeS), confirming the activity of SRB. In other words, the observed protein/biomass growth included a contribution from SRB.

Another interesting comparison can be made here, between the protein/biomass analysis results for phase 1 (Figure 4.1.a) and phase 2 experiments (Figures 4.7 to 4.12). In phase 1, the biomass first increased and then decreased rapidly after about 50 hours. This was attributed to the high pH (> 8.0). In phase 2 experiments, pH was controlled and high pH was avoided. This difference in growth dynamics between phase 1 and phase 2 experiments confirms the importance of pH.

The systems used in this study were very lean in biomass concentration. With higher biomass concentrations microbial communities may have closer interactions in an environment where products of one microbial community can be used by another community. Thus, a thicker biological sludge system is more rigid to pH changes than lean systems such as ours.
The yield of biomass per mole of total $\text{SO}_4^{2-}$ reduced can be determined by dividing the increase in biomass in first 70 hours with the amount of total $\text{SO}_4^{2-}$ removed from the system. These results are provided in Figure 4.12. A high yield represents a case where the activity of SRB is low or vice versa.

As shown in Figure 4.12, the sulfate reactor always gave a higher cell yield than reactors containing sulfite and/or thiosulfate. In other words, in the sulfate reactor the contribution of SRB to the growth of biomass was lower than that in sulfite and thiosulfate reactors. In the sulfate reactor, microorganisms other than SRB were more active as compared to sulfite and thiosulfate reactors. Additional evidence in favor of this argument is the results of gas analysis (sec. 4.2.7) which show lowest $\text{H}_2\text{S}$ production from sulfate reactors. This is consistent with the previous observation that $\text{SO}_4^{2-}$ is the hardest to reduce among three $\text{SO}_x^{2-}$. Moreover, there is a dependency of the yield on COD/$\text{SO}_4^{2-}$ ratio which will be discussed later.

![Graph](image.png)

**Figure 4.12**: Observed cell yield (g cells/mol of total $\text{SO}_4^{2-}$ removed) for 1st 70 hours

Postgate[^1] reported a range of the yield of SRB from 13 – 36 g/mol. This yield is dependent on various growth parameters, for example, type of inoculums used, electron donor/acceptor ratio (COD/$\text{SO}_4^{2-}$ ratio) and amount of nutrients used etc.
4.2.4 Behavior of individual $\text{SO}_4^{2-}$ species

Experimental results for individual $\text{SO}_4^{2-}$ analysis obtained for experiment G1 are typical. For sulfate, sulfite and thiosulfate reactors these results are provided in Figures 4.14a, 4.15a & 4.16a respectively. A complete set of data for all phase 2 experiments (G1 – G6) is provided in Appendix 6.2.

As shown in Figure 4.13a, in sulfate reactors, $\text{SO}_4^{2-}$ was the only $\text{SO}_4^{2-}$ species detected. Figure 4.14a shows that in sulfite reactors all three species of $\text{SO}_4^{2-}$ (i.e. $\text{SO}_4^{2-}$, $\text{SO}_3^{2-}$ & $\text{S}_2\text{O}_3^{2-}$) were detected. There was an accumulation $\text{S}_2\text{O}_3^{2-}$ which reached a maximum after about two days and then started to decrease. In thiosulfate reactors $\text{SO}_4^{2-}$ and $\text{S}_2\text{O}_3^{2-}$ but no $\text{SO}_3^{2-}$ was detected (Figure 4.15a).

It would be advantageous at this point to discuss the possible pathways involved in the formation of various sulfur oxyanions observed in different $\text{SO}_4^{2-}$ reactors.
Figure 4.13: Exp. G1, (a) individual (b) total SO$_4^{2-}$ species in the SO$_4^{2-}$ reactor
Figure 4.14: Exp G1 - (a) individual (b) total SO$_{x}$ species in SO$_{3}^{−}$ reactor
Figure 4.15: Exp G1 - (a) individual (b) total \( \text{SO}_x^2^- \) species in \( \text{S}_2\text{O}_3^2^- \) reactor

Note: Results are for Exp. G1, conducted with Electron donor/acceptor = 0.64 mol/mol
4.2.4.1 $S_2O_3^{2-}$ formation in $SO_3^{2-}$ reactors

The sulfite reactors originally contained no $S_2O_3^{2-}$ ion. But it was detected in all of the experiments (G1 to G6) in $SO_3^{2-}$ reactors. There could be at least two pathways for the formation of $S_2O_3^{2-}$ in sulfite reactors:

- **$S_2O_3^{2-}$ as an intermediate of $SO_3^{2-}$ reduction to $S^{2-}$**: Considering the electron requirement of different sulfur oxyanions on an oxidation state bar as follows:

<table>
<thead>
<tr>
<th>Oxidation States</th>
<th>+6</th>
<th>+4</th>
<th>+2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>← High -------- SO$_4^{2-}$ -------- SO$_3^{2-}$ -------- S$_2$O$_3^{2-}$ -------- Low → (Eq. 4.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electron Req.</td>
<td>8 e⁻</td>
<td>6 e⁻</td>
<td>4 e⁻</td>
</tr>
<tr>
<td>to form $S^{2-}$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

  There could be a possibility of formation of $S_2O_3^{2-}$ as a result of stepwise reduction of $SO_3^{2-}$ in which a given $SO_3^{2-}$ ion instead of getting 4 electrons at a time and reduced to sulfide first gets only 2 electrons and forms $S_2O_3^{2-}$ and then this $S_2O_3^{2-}$ gets 4 more electrons to complete the reduction to sulfide.

  In other words, this mechanism suggests the formation of thiosulfate as an intermediate in the reduction of $SO_3^{2-}$ to sulfide. However, such a stepwise reduction mechanism involving the formation of $S_2O_3^{2-}$ as an intermediate has not been reported in available literature.

- **$S_2O_3^{2-}$ as a product of reaction between HS⁻ & HSO$_3^-$**: Formation of $S_2O_3^{2-}$ (sec. 2.4.2) from inorganic reaction between HS⁻ and HSO$_3^-$ has been reported[37].

  This pathway could also be responsible for the production of $S_2O_3^{2-}$ in sulfite reactor.
It is pertinent to note that the formation of \( S_2O_3^{2-} \) in sulfite reactor was also observed in phase 1 experiment (Figure 4.3). However, the amount of \( S_2O_3^{2-} \) formed in that case was much lower as compared to all of phase 2 experiments. The reason of such behavior could be the pH of system as pH in phase 1 experiments was not controlled and it was observed to increase gradually while in phase 2 experiments (G1 to G6) pH of the system was controlled at 7.5 – 8.0.

Formation of \( S_2O_3^{2-} \) via the inorganic pathway requires the presence of \( HSO_3^- \) species which itself has a dynamic balance with other sulfur(IV) oxide species as a function of pH (Figure 2.2). Considering this factor it could be said that higher the pH (>7), lower is the amount of \( HSO_3^- \) available to react with \( HS^- \) and vice versa.

Given the difference in the amount of \( S_2O_3^{2-} \) formed in phase 1 and phase 2 experiments and the pH based justifications, the second pathway via inorganic reaction is more likely. The reaction between sulfide and \( SO_3^{2-} \) can also produce elemental sulfur\(^{37} \). Sometimes, small clots of yellow color substance were found on the wetted area (of inner walls) of SRB reactors. It occurred more often in sulfite reactors and less often in thiosulfate reactors but never in sulfate reactors. This yellow color material was difficult to clean unless washed with an aqueous solution of \( Na_2S \) and was most likely elemental sulfur. This confirms the importance of inorganic reactions among aqueous sulfur species in the SRB system with \( SO_3^{2-} \) as electron acceptor.

4.2.4.2 Formation of \( SO_4^{2-} \) in sulfite & thiosulfate Reactors

In all experiments of phase 1 and phase 2 (G1 to G6), \( SO_4^{2-} \) was detected during the ion chromatographic analysis of sulfite and thiosulfate reactors. There could be three possible sources for the \( SO_4^{2-} \) found in sulfite and thiosulfate reactors.

- **\( SO_4^{2-} \) formation through disproportionation pathway**: Various literature references have reported the formation of sulfate during the biological reduction of \( SO_3^{2-} \) and \( S_2O_3^{2-} \) (sec. 2.4.1).
**SO₄²⁻ addition as micronutrient**: In all reactors SO₄²⁻, SO₃²⁻ and S₂O₃²⁻, a small amount of SO₄²⁻ was added as micronutrients in the forms of MgSO₄ & FeSO₄ as part of Postgate medium C. This contributed 2.6 x 10⁻⁴ mol/L of SO₄²⁻ to all reactors.

**SO₄²⁻ addition with inoculum**: When introducing the bacterial inoculum from the SRB source reactor to the test reactors. 50 mL of the inoculum was transferred. This would contribute 4.3 x 10⁻⁴ mol/L of SO₄²⁻ to all the reactors.

Table 4.3 is a summary of mass balance of SO₄²⁻. In Table 4.3, the observed SO₄²⁻ concentration is the actual concentration of sulfate ions detected (with ion chromatographic analysis) in any specific SRB reactor (sulfite or thio-sulfate reactors) at the start of experiment. The added sulfate concentrations are calculated values of sulfate ion concentrations being added to the system as micronutrients and with the inoculums.

**Table 4.3: Mass balance on SO₄²⁻ observed vs. added SO₄²⁻ (conc. mol/L)**

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Observed SO₄²⁻ Conc. (mol L)</th>
<th>Added SO₄²⁻ Conc. (mol L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sulfite Reactor</td>
<td>Thiosulfate Reactor</td>
</tr>
<tr>
<td>Phase 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>0.0130</td>
<td>0.0016</td>
</tr>
<tr>
<td>Phase 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>0.0030</td>
<td>0.0028</td>
</tr>
<tr>
<td>G2</td>
<td>0.0021</td>
<td>0.0019</td>
</tr>
<tr>
<td>G3</td>
<td>0.0029</td>
<td>0.0016</td>
</tr>
<tr>
<td>G4</td>
<td>0.0015</td>
<td>0.0053</td>
</tr>
<tr>
<td>G5</td>
<td>0.0018</td>
<td>0.0019</td>
</tr>
<tr>
<td>G6</td>
<td>0.0021</td>
<td>0.0016</td>
</tr>
</tbody>
</table>

If these are only three sources of SO₄²⁻, the mass balance can be described as:
This mass balance equation in conjunction with data in Table 4.3 suggests that disproportionation of SO$_3^{2-}$ and S$_2$O$_3^{2-}$ is likely a source of SO$_4^{2-}$ found in sulfite and thiosulfate reactors.

Published work on disproportionation of S$_2$O$_3^{2-}$ [30], [53] shows an increase in SO$_4^{2-}$ concentration along with a gradual decrease in S$_2$O$_3^{2-}$ concentration. This increase of SO$_4^{2-}$ was observed in some experiments (G2 & G3) in this study. But the increase in sulfate was not significant, suggesting disproportionation was not the predominant mechanism of SO$_3^{2-}$ and S$_2$O$_3^{2-}$ reduction in our systems. Moreover, these previous research works were conducted using selected pure species of SRB namely: *Desulfovibrio desulfuricans* [30] (grown on lactate and thio-sulfate system) and *Thermoanaerobacter finnii* [53] (isolated from oil fields & grown on glucose-thiosulfate system), while our work was conducted using mixed cultures from anaerobic sludge. Only certain species of SRB are reported to perform disproportionation [51].

### 4.2.5 Reduction of total SO$_4^{2-}$

Experimental results for total SO$_4^{2-}$ analysis obtained for experiment G1 are typical. For sulfate, sulfite and thiosulfate reactors, these results are provided in Figures 4.14.b, 4.15.b & 4.16.b respectively. A complete set of plots for all phase 2 experiments (G1 – G6) is provided in Appendix 6.2.

A gradual decrease in total SO$_4^{2-}$ concentrations with time in all three reactors (SO$_4^{2-}$, SO$_3^{2-}$ & S$_2$O$_3^{2-}$) in all experiments (G1 to G6) was observed. As shown in Figures 4.14.b, 4.15.b & 4.16.b, total SO$_4^{2-}$ reduction curves of reactors using SO$_4^{2-}$ and SO$_3^{2-}$ as electron acceptors were observed to be linear as compared to thiosulfate reactor. Total SO$_3^{2-}$ reduction curve of thiosulfate reactor tended to slow down after an initial period of
around 70 hours. It is evident that $\text{SO}_4^{2-}$, $\text{SO}_3^{2-}$ & $\text{S}_2\text{O}_3^{2-}$ were used as electron acceptors by mixed cultures of SRB.

In order to compare the ability of SRB to use different $\text{SO}_4^{2-}$ as electron acceptors, $\text{SO}_4^{2-}$ reduction rate is used. It is basically the slope of line obtained by plotting the molar concentration of total $\text{SO}_4^{2-}$ against time. The rate was determined for first 70 hours only.

A review of actual $\text{SO}_4^{2-}$ plots with time (Figures 4.14.b, 4.15.b & 4.16.b) reveals that the total $\text{SO}_4^{2-}$ reduction curves of sulfate ($R^2 \geq 0.95$) and sulfite ($R^2 \geq 0.96$) reactors were quite linear in the whole duration of experiment. But, the linearity was much worse for the thiosulfate reactor ($R^2 < 0.7$) because most of the reduction was completed in first 70 hours.

Table 4.4 gives the rate of reduction of total $\text{SO}_4^{2-}$ in the first 70 hours for all the experiments. Figure 4.16 is a comparison of rates of reduction for different $\text{SO}_4^{2-}$.

<table>
<thead>
<tr>
<th>Exp. Code</th>
<th>Electron Donor/Acceptor (mol/mol)</th>
<th>Total $\text{SO}_4^{2-}$ Reduction Rates (mol/hour - L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>0.64</td>
<td>$2.15 \times 10^{-5}$</td>
</tr>
<tr>
<td>G2</td>
<td>0.16</td>
<td>$6.79 \times 10^{-5}$</td>
</tr>
<tr>
<td>G3</td>
<td>0.21</td>
<td>$3.80 \times 10^{-5}$</td>
</tr>
<tr>
<td>G4</td>
<td>0.32</td>
<td>$8.19 \times 10^{-5}$</td>
</tr>
<tr>
<td>G5</td>
<td>0.53</td>
<td>$2.69 \times 10^{-5}$</td>
</tr>
<tr>
<td>G6</td>
<td>1.28</td>
<td>$4.93 \times 10^{-6}$</td>
</tr>
</tbody>
</table>
Figure 4.16: Total $SO_\text{x}^{\text{-}}$ reduction rates, for first 70 hours

Figure 4.17: Total $SO_\text{x}^{\text{-}}$ removal efficiency for first 70 hours
Figure 4.16 shows a sequence of reduction rates as: \( \text{S}_2\text{O}_3^{2-} > \text{SO}_3^{2-} \gg \text{SO}_4^{2-} \). The same order can be found if it is based on the removal efficiency (Figure 4.17) defined as:

\[
\text{SO}_4^{2-} \text{ Removal Efficiency (\%)} = \left( \frac{(C_0 - C)}{C_0} \right) \times 100
\]

Where:
\( C_0 \) = Initial mol/L of \( \text{SO}_4^{2-} \) present before the SRB activity started
\( C \) = Final mol/L of \( \text{SO}_4^{2-} \) left after the SRB activity

This order in total \( \text{SO}_4^{2-} \) reduction is in agreement with the results of phase 1 as well as the earlier work \(^{14,16}\). It is logical considering Table 4.5 which gives the theoretical electron requirements of three oxyanions. Apparently, the reduction of one mole of S in \( \text{S}_2\text{O}_3^{2-} \), \( \text{SO}_3^{2-} \) and \( \text{SO}_4^{2-} \) will need 4, 6 and 8 electrons, respectively. It is logical that transfer of fewer electrons as in case of thiosulfate will require less time. Thus, the observed order of reduction rate of different \( \text{SO}_4^{2-} \) is explained based on the oxidation state of sulfur in these \( \text{SO}_4^{2-} \). Nielsen\(^{14}\) also reported the similar results from a study on hydrogen sulfide production in biofilms from sewer systems.

Table 4.5: Electron requirement for half redox reactions (at \( \text{pH} = 7 \))

<table>
<thead>
<tr>
<th>( \text{SO}_4^{2-} )</th>
<th>Reaction</th>
<th>( \Delta G^{0'} ) k J/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfate ( \text{SO}_4^{2-} )</td>
<td>( + 19/2 \text{H}^- + 8 \text{e}^- \rightarrow \frac{1}{2} \text{H}_2\text{S} + \frac{1}{2} \text{HS}^- + 4 \text{H}_2\text{O} )</td>
<td>166.4</td>
</tr>
<tr>
<td>Sulfite ( \text{SO}_3^{2-} )</td>
<td>( + 15/2 \text{H}^- + 6\text{e}^- \rightarrow \frac{1}{2} \text{H}_2\text{S} + \frac{1}{2} \text{HS}^- + 3 \text{H}_2\text{O} )</td>
<td>65.9</td>
</tr>
<tr>
<td>Thiosulfate ( \frac{1}{2} \text{S}_2\text{O}_3^{2-} + 9/2\text{H}^- + 4\text{e}^- \rightarrow \frac{1}{2} \text{H}_2\text{S} + \frac{1}{2}\text{HS}^- + 3/2 \text{H}_2\text{O} )</td>
<td>72.25</td>
<td></td>
</tr>
</tbody>
</table>

Another explanation of the slow reduction of \( \text{SO}_4^{2-} \) as compared to \( \text{SO}_3^{2-} \) and \( \text{S}_2\text{O}_3^{2-} \) can be based upon the mechanism of reduction reaction. According to Hamilton\(^{14}\), sulfate before acting as a terminal electron acceptor for SRB. has first to be activated by a metabolic conversion to adenosine phosphosulfate (APS). This generation of APS involves reaction with ATP. Whereas \( \text{SO}_3^{2-} \) and \( \text{S}_2\text{O}_3^{2-} \) do not require the more energy
demanding activation step of reaction with ATP. A detailed discussion on various enzymes involved in the reduction of sulfur oxyanions is given by Odom[55].

Although some SRB species (sec. 2.4.1) are capable of disproportionating $SO_3^{2-}$ & $S_2O_3^{2-}$, disproportionation does not seem to be a major process for the removal of $SO_3^{2-}$ and $S_2O_3^{2-}$ from the system. If disproportionation is a major process then disproportionation of $SO_3^{2-}$ and $S_2O_3^{2-}$ will form appreciable quantities of $SO_4^{2-}$ according to equations 2.2 & 2.3. As reduction of $SO_4^{2-}$ is a slow process as compared to $SO_3^{2-}$ and $S_2O_3^{2-}$, there would be a net accumulation of $SO_4^{2-}$ in the sulfite and thiosulfate reactors which is not observed. Actually, the observed rate of production of $SO_4^{2-}$ in sulfite and thiosulfate reactors is much lower than the rate of consumption of these oxyanions as shown in Figures 4.2.a, 4.3.a & 4.4.a.

### 4.2.6 Decrease in chemical oxygen demand (COD)

COD tests can provide information about the change in electron donor, but it is important to understand the limitations.

COD values give the amount of oxygen required to chemically oxidize the oxidizable components present. If only the electron donor was oxidizable, this test would be more effective. Our systems may also contain other substances that are chemically oxidizable, such as nutrients and $SO_3^{2-}$. However, this contribution is expected to be very small.

Different species of SRB may differ in their ability of using various electron donors. Chemically oxidizable compounds may not be bio oxidizable. For example, most of SRB species can only partially oxidize the organic compound, such as lactate up to the extent of acetate. Only a few species of SRB have the ability to continue using acetate as electron donor for further oxidation. The ability of SRB to use glucose, pyruvate, lactate, acetate etc. as electron donor varies based upon the type of SRB species in discussion.
Acid producing bacteria (APB) and MPB may also exist in the system and compete with SRB for an energy/electron source that is COD. Thus, a decrease in COD cannot be taken as an indicator that this COD is being used by SRB.

Experimental results for experiment G1 are typical and are presented as Figure 4.18. A complete set of results for experiment G1- G6 are provided in Appendix 6.3.

![Figure 4.18: Exp G1 - plot of soluble cod in SRB reactor vs. time](image)

_Electron donor-acceptor ratio = 0.64 mol/mol_

Irrespective of the electron donor/acceptor ratio and type of SO₃²⁻, all SRB test reactors showed a gradual decrease in COD with time.

In order to compare the removal of COD from the system COD removal efficiency is determined and presented in Figure 4.19 for different COD/SO₃²⁻ ratios. The COD removal efficiency is calculated as:

% COD removal efficiency = \[\frac{(\text{COD}_1 - \text{COD}_2)}{\text{COD}_1}\] * 100

Where:
COD₁ = Initial COD
COD = Final COD after 70 hours

Figure 4.19: COD removal efficiency (%) vs. electron donor/acceptor ratio

As shown in Figure 4.19, the COD removal efficiency was highest in the sulfite reactor followed by the thiosulfate reactor. It was lowest in sulfate reactor which is logical given that reduction rates of both sulfite and thiosulfate were higher than sulfate.

Interestingly, the COD removal efficiency in sulfite reactors was higher than those in the thiosulfate reactors. As discussed earlier, the reduction efficiency of $S_2O_3^{2-}$ was higher than that of $SO_3^{2-}$, in general. The observed higher efficiency in COD removal with sulfite reactor may imply that the activity of bacteria other than SRB was higher in the sulfite reactor than that in the thiosulfate reactors. The following section provides some evidence of activity of MPB.

4.2.7 Formation of H$_2$S and CH$_4$

The mixed cultures used for this work were expected to contain many different types of microbes. Methane producing bacteria (MPB) are one of the microbial community that can coexist within the same system with SRB and compete for similar types of electron donor and nutrients. A better understanding of the competition among SRB and MPB is
needed to enhance the activity of SRB. MPB produces CH₄ while SRB generate H₂S. Thus, analysis of the gas samples obtained from the SRB reactors could provide some information about population dynamics of these two microbial communities. Ideally, the concentrations of CH₄ and H₂S in the exit gas and their change with time will be obtained and used for a quantitative analysis of C and S mass balances.

However, it was found that the detection limits of the GC analysis used were too high for the purpose of quantities analysis. For CH₄, lower detection limit was 90 ppm and for H₂S, it was 80 ppm. Consequently, the concentrations of gases of interest were quantifiable only occasionally. The results of gas analysis are summarized in Tables 4.6 and 4.7. In many cases, the gases were detectable but not quantifiable with confidence. For those cases "CH₄" or "H₂S" were used in Tables 4.6 & 4.7.

Methane gas production (Table 4.6) was observed in all the reactors. Increasing electron donor/acceptor ratios seemed to result in a greater amount of CH₄ produced and more cases where CH₄ was quantifiable. This is expected since a higher electron donor/acceptor ratio means a lesser amount of SO₄²⁻ available for SRB.

An earlier work on SRB using only sulfate as electron acceptor suggested [32] that CH₄ production decreased by lowering COD/SO₄²⁻ ratio and stopped at a COD/SO₄²⁻ ratio (g of COD/g of SO₄²⁻) of 1.0 or below. Possible reasons for the cease of CH₄ production were reported as the lowered availability of carbon source and the high sulfide concentration 160-200 mg/L.

Overall the results of current study agreed with this work. But, production of CH₄ was observed in this study at COD/SO₄²⁻ ratios below 1.0. In addition to the difference in carbon source, the current system might have lower sulfides content. Assuming 100 ppm of H₂S in gas phase the amounts of H₂S(aq) and HS⁻ in solution in equilibrium with the gas phase would be 3 and 20 mg/L respectively, at a pH of 7.5 (Appendix 5). These values, although may not be very accurate, are much lower than those reported [32] in the above study.
Table 4.6: Results of gas analysis for methane conc. ppm

<table>
<thead>
<tr>
<th>Exp. #</th>
<th>Electron donor/acceptor ratio (mole)</th>
<th>SO(_2) (Time Hours)</th>
<th>SO(_3) (Time Hours)</th>
<th>S(_2)O(_3) (Time Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>G2</td>
<td>0.16 CH(_4)</td>
<td>CH(_4)</td>
<td>97</td>
<td>CH(_4)</td>
</tr>
<tr>
<td>G3</td>
<td>0.21 CH(_4)</td>
<td>CH(_4)</td>
<td>97</td>
<td>CH(_4)</td>
</tr>
<tr>
<td>G4</td>
<td>0.32 CH(_4)</td>
<td>CH(_4)</td>
<td>97</td>
<td>CH(_4)</td>
</tr>
<tr>
<td>G5</td>
<td>0.53 CH(_4)</td>
<td>CH(_4)</td>
<td>97</td>
<td>CH(_4)</td>
</tr>
<tr>
<td>G6</td>
<td>1.28 CH(_4)</td>
<td>CH(_4)</td>
<td>97</td>
<td>CH(_4)</td>
</tr>
</tbody>
</table>

Table 4.7: Results of gas analysis for hydrogen sulfide conc. ppm

<table>
<thead>
<tr>
<th>Exp. #</th>
<th>Electron donor/acceptor ratio (mole)</th>
<th>SO(_2) (Time Hours)</th>
<th>SO(_3) (Time Hours)</th>
<th>S(_2)O(_3) (Time Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>G2</td>
<td>0.16 H(_2)S</td>
<td>H(_2)S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>0.21 H(_2)S</td>
<td>H(_2)S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>0.32 H(_2)S</td>
<td>H(_2)S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G5</td>
<td>0.53 H(_2)S</td>
<td>H(_2)S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>0.64 H(_2)S</td>
<td>H(_2)S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G6</td>
<td>1.28 H(_2)S</td>
<td>H(_2)S</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Moreover, MPB were seemingly more active in sulfate reactors than that in the sulfite and thiosulfate reactors, which again is consistent with the trend found earlier on the basis of SRB activity. As shown in Table 4.7, no H\(_2\)S gas was detected in sulfate reactors whereas H\(_2\)S gas was detected in sulfite and thiosulfate reactors. This agrees with the observation made on SO\(_4\)^{2-}\ reduction basis.

Between the sulfite and thiosulfate reactors, the former seemingly produced more H\(_2\)S which is inconsistent with the observation made on the basis of rate of SO\(_4\)^{2-}\ reduction rates. One possible explanation would be that a larger portion of the S\(_2\)O\(_3\)^{2-}\ reduced was converted to elemental sulfur.
There seemed to be a dependence of H₂S production on the electron donor/acceptor ratio which will be discussed in the following section. It should be pointed out that the SO₄⁻ reduction is a necessary but not a sufficient condition for H₂S gas production. To produce H₂S gas, H⁻ are needed to form H₂S(aq). S²⁻ produced by SRB can also react with metal ions to form insoluble metal sulfide such as FeS which is responsible for the black color of SRB reactor. Although no H₂S gas was detected in the exit gas from the sulfate reactor, it does not rule out the activity of SRB. A previous work on H₂S production from biofilms in sewer systems concluded that more H₂S was produced from sulfite and thiosulfate than from sulfate reactors.

4.2.8 Effect of COD/SO₄⁻ ratio

Results of total SO₄⁻ removal and COD removal for experiments (G1-G6) are provided in Appendix 6.4. The effect of COD/SO₄⁻ ratio on the reduction of SO₄⁻ is presented in Figure 4.20 which gives the removal efficiency of total SO₄⁻ for first 70 hours of experiments. as SO₄⁻ reduction was not linear for total duration of experiment.

As shown in Figure 4.20 there seems an optimal range of the COD/SO₄⁻ in which the percentage removal of SO₄⁻ is high. This trend is obvious in the case of thiosulfate. The range is from 0.2 to 0.6 on a mol/mol basis of electron donor/acceptor ratio. Since the SO₄⁻ reduction requires electron donor (COD) as well as electron acceptor (SO₄⁻), an optimal COD/SO₄⁻ ratio is indeed expected. Theoretical requirement of this ratio could be the determined based on the stoichiometry of the reduction reaction assuming if only the reduction reaction consumes carbon/energy sources. In reality, however, this optimal ratio is greater than the one based on stoichiometry, because a part of carbon/energy source is also used building new cells of bacteria.
Figure 4.20: Total $SO_4^{2-}$ removal efficiencies for 1st 70 hours

Figure 4.21: COD removal efficiency for 1st 70 hours
Table 4.8: Theoretical COD/SO₄²⁻ requirements & observed optimal ranges

<table>
<thead>
<tr>
<th>Oxyanions of Sulfur</th>
<th>Optimum COD/SO₄²⁻ Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed Range</td>
</tr>
<tr>
<td></td>
<td>g of COD/g of SO₄²⁻ Units</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>0.5 – 1.3</td>
</tr>
<tr>
<td>SO₃²⁻</td>
<td>0.6 – 1.5</td>
</tr>
<tr>
<td>S₂O₃²⁻</td>
<td>0.4 – 1.1</td>
</tr>
</tbody>
</table>

Table 4.8 gives the theoretically required values of COD/SO₄²⁻ ratios calculated from stoichiometry for SO₄²⁻, SO₃²⁻ and S₂O₃²⁻. Also presented are the corresponding observed optimal ranges for maximum SO₄³⁻ reduction. These values of optimal ranges have been converted from corresponding mol of electron donor/mole of electron acceptor to g of COD/g of SO₄³⁻ basis for all three SO₄³⁻ species. From Table 4.8 it is clear that the observed values of optimal ranges include theoretically expected values determined based on the stoichiometry of reduction reactions. Moreover, the theoretically required values are also on the lower side of the observed optimal ranges as they should be.

A previous work[^32] on sulfate reduction by SRB suggested a COD/SO₄²⁻ value of < 1.7 as favorable for SRB.

Figure 4.21 gives the changes in COD removal with electron donor/acceptor ratio. Again, there is an optimal range in which the COD removal is most effective. More important the range based on COD removal is very similar to that based on SO₄³⁻ reduction that is 0.2 – 0.5 mol/mol basis. This similarity suggests that although there were other types of bacteria, such as MPB and APB, SRB were better contributor to the removal of COD during suggested optimum range of COD/SO₄³⁻ ratio.
Table 4.9: Contribution of SO$_3^{2-}$ reduction towards COD removal

<table>
<thead>
<tr>
<th>Exp. Code</th>
<th>Electron donor / acceptor ratio</th>
<th>% of electrons equivalents used for SO$_3^{2-}$ reduction</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reduction to S$_2^-$</td>
<td>Reduction to S$_0^0$</td>
</tr>
<tr>
<td>G2</td>
<td>0.16</td>
<td>83%</td>
<td>62%</td>
</tr>
<tr>
<td>G3</td>
<td>0.21</td>
<td>22%</td>
<td>16%</td>
</tr>
<tr>
<td>G4</td>
<td>0.32</td>
<td>21%</td>
<td>16%</td>
</tr>
<tr>
<td>G5</td>
<td>0.53</td>
<td>19%</td>
<td>14%</td>
</tr>
<tr>
<td>G1</td>
<td>0.64</td>
<td>21%</td>
<td>16%</td>
</tr>
<tr>
<td>G6</td>
<td>1.28</td>
<td>3%</td>
<td>2%</td>
</tr>
</tbody>
</table>

Table 4.9 gives the list of estimated values of contribution of SO$_3^{2-}$ reduction towards COD removal. Sample calculations for experiment G1 are provided in Appendix 7. The values for SO$_3^{2-}$ reactors are not calculated because of the complexity involved (see Appendix 7). There are two values reported for each SO$_3^{2-}$. One value is for the reduction to S$_2^-$ while the other is for the reduction to S$_0^0$. More details about the theoretical and actual COD usage for SO$_3^{2-}$ reduction to both S$_2^-$ & S$_0^0$ can be found in Appendix 8.

As electron equivalent received by SO$_3^{2-}$ cannot be more than 100%. the calculated 217% contribution of SO$_3^{2-}$ reduction to COD removal suggests that S$_0^0$ was likely another product in addition to S$_2^-$. Indeed it was observed that S$_0^0$ was formed when S$_2$O$_3^{2-}$ and SO$_3^{2-}$ were reduced.

One insight that can be obtained from Table 4.9 is that at lower electron donor/acceptor ratios more percentage of electrons were drawn by SRB. In other words at lower values of electron donor/acceptor ratio SRB’s contribution towards COD removal increased.

Effects of COD/SO$_3^{2-}$ ratio on biomass growth and pH were analyzed but the results were not conclusive. Plots of protein vs. time with electron donor/acceptor ratios are given in Appendices 6.5 & 6.6 respectively.
Chapter 5: Conclusions

Based upon the observations made under a wide range of conditions, following conclusions are drawn.

5.1 From phase 1 experiments

- The SRB containing mixed cultures enriched from the sludge of Eastern Power Ltd., were capable of using \(SO_3^{2-}\) and \(S_2O_3^{2-}\) along with \(SO_4^{2-}\) as terminal electron acceptors. The rate of total \(SO_3^-\) reduction was found to be in the order of \(S_2O_3^{2-} > SO_3^{2-} >> SO_4^{2-}\). This order is the same as the oxidation states of sulfur or electron requirement of these \(SO_x^-\).

- pH was found to be an important parameter affecting the activity of SRB. As expected SRB displayed the ability of \(H^+\) scavenging which raised the pH of the system. When pH was too high, the growth of bacteria was suppressed, which is not likely due to the accumulation of dissolved sulfides at high pH.

5.2 From phase 2 experiments

- With glucose as carbon/energy source, more acid was produced in the system than the lactate fed system. The production of acids sharply lowered the pH of the system and addition of alkali was necessary to control the pH and facilitate the reduction of \(SO_3^-\) by SRB.

- Relatively smaller pH drop was observed in sulfite reactors as compared to sulfate and thiosulfate reactors. This phenomenon is attributed to the chemical reaction between \(SO_3^{2-}\) and biogenic \(S^{2-}\), which is supported by the observed production of \(S_2O_3^{2-}\) in all sulfite reactors. The observed formation of \(S_2O_3^{2-}\) and \(S^0\) in sulfite reactors indicates the importance of chemical interactions between various aqueous sulfur species in such microbial systems.
Disproportionation of SO$_3^{2-}$ & S$_2$O$_3^{2-}$ was possible source of SO$_4^{2-}$ detected in sulfite and thiosulfate reactors. However, disproportionation was unlikely to be a major process for the removal of these sulfur oxyanions.

Mixed cultures of SRB were found to utilize SO$_4^{2-}$, SO$_3^{2-}$ and S$_2$O$_3^{2-}$ as electron acceptors with glucose as carbon/energy source. The rate of total SO$_4^{3-}$ reduction was found in the order of S$_2$O$_3^{2-}$ > SO$_3^{2-}$ >> SO$_4^{2-}$, which was in agreement with the phase 1 results.

Optimal ranges of COD/SO$_4^{3-}$ ratios for total SO$_4^{3-}$ removal were determined for all three SO$_4^{3-}$ as:

- SO$_4^{2-}$ : 0.5 – 1.3 (g of COD/g of SO$_4^{2-}$)
- SO$_3^{2-}$ : 0.6 – 1.5 (g of COD/g of SO$_3^{2-}$)
- S$_2$O$_3^{2-}$ : 0.4 – 1.1 (g of COD/g of S$_2$O$_3^{2-}$)

These observed ranges of COD/SO$_4^{3-}$ ratios cover the theoretically calculated values determined based on stoichiometry.

Similar ranges of COD/SO$_4^{3-}$ ratios were observed for COD removal efficiencies for all three SO$_4^{3-}$. This similarity suggested that SRB were also a contributor to the removal of COD, although other types of bacterial groups such as MPB and APB were present. There was some evidence that this COD removal contribution of SRB increased at lower COD/SO$_4^{3-}$ ratios.
Chapter 6: Considerations for Future Work

Given the potential application of current work in the development of a process to recover valuable metals from SO₄²⁻ containing aqueous media, following are suggested:

6.1 Growing SRB for production of H₂S (g)
It could be more beneficial if instead of bacterial growth the targeted parameter is H₂Sₐ(g) generation itself. This means to determine the optimum pH range, temperature and other conditions for maximum H₂S production. Literature recommended optimum pH range of 7.5 could be beneficial for growth of bacteria itself and not for H₂S production. As pH could indirectly effect the production of H₂S gas by affecting the dynamic balance of different sulfide species (HS⁻, H₂S & S²⁻). Need arises to find the optimum pH for H₂S gas production.

6.2 Use of cheaper & industrially available electron donors
To be economically viable, any industrial application of SRB will likely require use of low cost carbon sources such as biosolids, sewage waste and food waste as electron donor instead of pure sources like glucose or lactate. Use of such waste streams is suggested for future work on sulfate reducing bacteria. Although it could be more complex to understand a system using such a waste organic stream as electron donor, the knowledge acquired for such a system would be readily applicable to industrial uses.

6.3 Optimum pH an important parameter for SRB
As discussed in the results & discussion section pH could be a very important parameter for a microbial system intended to perform dissimilatory SO₄²⁻ reduction. It could affect the dynamics of sulfide species (HS⁻, H₂S & S²⁻) in the system which will change dissolved sulfide concentration and thus can indirectly effect the inhibition of microbial communities in the system. At the same time it may also affect the production of H₂S gas which is the product of interest in our case. It is suggested to study, the effect of pH on the activity of SRB.
6.4 Higher cell/biomass concentration
A low biomass concentration was used for this work. A system with lower biomass concentration is more sensitive to change in environmental parameters, such as pH, temperature etc., which is desirable for lab scale investigations with focus on kinetics and mechanisms. In industrial applications of such a process, a system with low biomass concentration may be more vulnerable to undesirable variations in operating parameters, making the system too sensitive to control and maintain. It is suggested to use a higher concentration of biomass so that system may be more robust to operating parameters.

6.5 Bacterial growth dynamics
In the current study, protein was measured and used as an indicator for the growth of bacterial populations. However, this method does not differentiate among different bacterial populations. Information about growth dynamics of specific group of bacteria, such as SRB or MPB would be very useful to better understand the system.

For example, use of MALDI/TOF (matrix assisted laser desorption/ionization mass spectrometry/time of flight) method can identify even different species of same bacteria, based upon molecular weights of proteins existing in those bacteria. Applying this method for mixed cultures would be very challenging because large number of bacterial species present. This method is currently being used for medical purposes.

6.5.1 Analysis of gas samples
It was realized that the analysis of gas samples, collected from SRB reactors, could be improved, by using more sensitive and optimized gas chromatograph systems. Currently the lower detection limits for methane and hydrogen sulfide were 90 ppm and 80 ppm respectively. Better sensitivity is needed and can be achieved with the GC-PFPD and GC-FID systems.
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Appendix 1  Nutrient Recipe for SRB Growth

1.1 Postgate Mediums C, Original Recipe for SRB Growth

<table>
<thead>
<tr>
<th>Compound</th>
<th>g for 1L</th>
<th>purity</th>
<th>g for 3L</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>0.500</td>
<td>0.990</td>
<td>1.515</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>1.000</td>
<td>0.995</td>
<td>3.015</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>4.500</td>
<td>0.990</td>
<td>13.636</td>
</tr>
<tr>
<td>CaCl₂*2H₂O (see note)</td>
<td>0.040</td>
<td>0.780</td>
<td>0.154</td>
</tr>
<tr>
<td>MgSO₄*7H₂O</td>
<td>0.060</td>
<td>0.980</td>
<td>0.184</td>
</tr>
<tr>
<td>Sodium Lactate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>1.000</td>
<td>3.000</td>
<td></td>
</tr>
<tr>
<td>FeSO₄*7H₂O</td>
<td>0.004</td>
<td>0.990</td>
<td>0.012</td>
</tr>
<tr>
<td>Sodium Citrate*2H₂O</td>
<td>0.300</td>
<td>0.990</td>
<td>0.909</td>
</tr>
</tbody>
</table>

Note: Postgate gives CaCl₂*6H₂O = 0.06 g/L. so calculated equivalent amount of CaCl₂*2H₂O = 0.04 g/L

1.2 Postgate Mediums C, Modified for Different SO₄⁻³

1.2.1 SRB Growth Medium for Phase 1 using Lactate As Electron Donor

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amounts g for 3 L of solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sulfate</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.50</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>3.00</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>13.50</td>
</tr>
<tr>
<td>CaCl₂*6H₂O</td>
<td>0.18</td>
</tr>
<tr>
<td>MgSO₄*7H₂O</td>
<td>0.18</td>
</tr>
<tr>
<td>Sodium Lactate</td>
<td>18.00</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>3.00</td>
</tr>
<tr>
<td>FeSO₄*7H₂O</td>
<td>0.01</td>
</tr>
<tr>
<td>Sodium Citrate*2H₂O</td>
<td>0.90</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>27.00</td>
</tr>
</tbody>
</table>
1.2.2 SRB Growth Medium for Phase 2, using Glucose as Electron Donor

Given below is the SRB growth recipe for Experiment G1. For remaining experiments G2-G6, all the nutrients were kept the same, except the $SO_4^{2-}$ concentration was changed to vary COD/$SO_4^{2-}$ ratio.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sulfate g for 3 L</th>
<th>Sulfite g for 3 L</th>
<th>Thiosulfate g for 3 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.515</td>
<td>1.515</td>
<td>1.515</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>3.015</td>
<td>3.015</td>
<td>3.015</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td>13.636</td>
<td>12.223</td>
<td>15.097</td>
</tr>
<tr>
<td>CaCl$_2$*2H$_2$O</td>
<td>0.154</td>
<td>0.154</td>
<td>0.154</td>
</tr>
<tr>
<td>MgSO$_4$*7H$_2$O</td>
<td>0.184</td>
<td>0.184</td>
<td>0.184</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.919</td>
<td>10.919</td>
<td>10.919</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>3.000</td>
<td>3.000</td>
<td>3.000</td>
</tr>
<tr>
<td>FeSO$_4$*7H$_2$O</td>
<td>0.012</td>
<td>0.012</td>
<td>0.012</td>
</tr>
<tr>
<td>Sodium Citrate*2H$_2$O</td>
<td>0.909</td>
<td>0.909</td>
<td>0.909</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment Code</th>
<th>COD/$SO_4^{2-}$ Ratio Mole / Mole</th>
<th>Weight Added for 3L of solution g</th>
<th>g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G g</td>
<td>Sulfate g</td>
<td>Sulfite g</td>
</tr>
<tr>
<td>G2</td>
<td>0.16</td>
<td>0.38</td>
<td>0.45</td>
</tr>
<tr>
<td>G3</td>
<td>0.21</td>
<td>0.50</td>
<td>0.61</td>
</tr>
<tr>
<td>G4</td>
<td>0.32</td>
<td>0.76</td>
<td>0.91</td>
</tr>
<tr>
<td>G5</td>
<td>0.53</td>
<td>1.26</td>
<td>1.51</td>
</tr>
<tr>
<td>G1</td>
<td>0.64</td>
<td>1.51</td>
<td>1.82</td>
</tr>
<tr>
<td>G6</td>
<td>1.28</td>
<td>3.03</td>
<td>3.63</td>
</tr>
</tbody>
</table>

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## Appendix 2  List of Chemicals & Equipment

### 2.1 List of Chemicals & Gases Used for Experimental Work

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical Formula (Formula Weight)</th>
<th>Purity</th>
<th>Purpose</th>
<th>Supplier</th>
<th>Batch/Lot</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main Nutrients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Potassium Phosphate</td>
<td>KH₂PO₄ (136.09)</td>
<td>99.0%</td>
<td></td>
<td>J1697</td>
</tr>
<tr>
<td>2</td>
<td>Ammonium Chloride</td>
<td>NH₄Cl (53.49)</td>
<td>99.5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Calcium Chloride</td>
<td>CaCl₂·2H₂O (147.02)</td>
<td>78.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Magnesium Sulfate</td>
<td>MgSO₄·7H₂O (246.48)</td>
<td>98.0%</td>
<td>Nutrients for SRB</td>
<td></td>
</tr>
<tr>
<td><strong>Other Chemicals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Yeast Extract</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Ferrous Sulfate</td>
<td>FeSO₄·7H₂O (278.01)</td>
<td>99.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Sodium Citrate</td>
<td>Na₃C₆H₅O₇·2H₂O (294.11)</td>
<td>99.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Glucose</td>
<td>C₆H₁₂O₆ (180.2)</td>
<td>-</td>
<td>Electron Donor for SRB</td>
<td>Sigma</td>
</tr>
<tr>
<td>9</td>
<td>Lactic Acid, Sodium Salt</td>
<td>C₃H₆O₇·Na (112.11)</td>
<td>98.0%</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>10</td>
<td>Sodium Sulfate</td>
<td>Na₂SO₄ (142.04)</td>
<td>99.0%</td>
<td>Electron Acceptor for SRB</td>
<td>ACP Chemicals, Canada</td>
</tr>
<tr>
<td>11</td>
<td>Sodium Sulfite</td>
<td>Na₂SO₃ (126.04)</td>
<td>98.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Sodium Thiosulfate</td>
<td>Na₂S₂O₃ (248.17)</td>
<td>99.5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Formaldehyde Solution</td>
<td>HCHO (30.01)</td>
<td>36.5%</td>
<td>for SO₄²⁻ preservation</td>
<td>BDH Inc. Canada</td>
</tr>
<tr>
<td>14</td>
<td>Sodium Hydro-Oxide</td>
<td>NaOH (40.0)</td>
<td>97.0%</td>
<td>pH balancing agent</td>
<td>Caledone Labs Ltd.</td>
</tr>
<tr>
<td>15</td>
<td>Hydrochloric Acid</td>
<td>HCl (36.5)</td>
<td>6 N</td>
<td></td>
<td>VWR Scientific Canada</td>
</tr>
<tr>
<td>Name</td>
<td>Chemical Formula (Formula Weight)</td>
<td>Purity</td>
<td>Purpose</td>
<td>Supplier</td>
<td>Batch/Lot</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>----------------------------------</td>
<td>--------</td>
<td>-------------------------------</td>
<td>-------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>16  Ferric Chloride</td>
<td>FeCl₃ (162.2)</td>
<td>-</td>
<td>to precipitate PO₄&lt;sup&gt;3-&lt;/sup&gt;</td>
<td>Sigma</td>
<td>127H3447</td>
</tr>
<tr>
<td>17  Bio-Rad Dye for Protein Assay</td>
<td>-</td>
<td>-</td>
<td>for biomass analysis</td>
<td>Bio-Rad Labs</td>
<td>65386A</td>
</tr>
<tr>
<td>18  Protein Standard (Bovine serum albumin)</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Sigma</td>
<td>069H6098</td>
</tr>
<tr>
<td>19  Sodium Carbonate</td>
<td>Na₂CO₃ (105.99)</td>
<td>99.9%</td>
<td>IC eluents</td>
<td>BDH Inc. Canada</td>
<td>120765/80307</td>
</tr>
<tr>
<td>20  Sodium Bicarbonate</td>
<td>NaHCO₃ (84.01)</td>
<td>99.7%</td>
<td></td>
<td>ACP Chemicals, Canada</td>
<td>E1597</td>
</tr>
<tr>
<td>21  COD Reagent kits</td>
<td>-</td>
<td>-</td>
<td>for COD analysis</td>
<td>Chemetrics Inc. USA</td>
<td>54701</td>
</tr>
</tbody>
</table>

**Gases**

<table>
<thead>
<tr>
<th>Name</th>
<th>Formula</th>
<th>Purity</th>
<th>Supplier</th>
<th>Batch/Lot</th>
</tr>
</thead>
<tbody>
<tr>
<td>22  Nitrogen</td>
<td>N₂</td>
<td>99.9%</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>23  Hydrogen</td>
<td>H₂</td>
<td>grade 4.0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>24  Helium</td>
<td>He</td>
<td>grade 5.0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>25  Hydrogen Sulfide</td>
<td>H₂S</td>
<td>3% in N₂</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>27  Compressed Air</td>
<td></td>
<td></td>
<td>Matheson</td>
<td>-</td>
</tr>
<tr>
<td>28  Nitrogen (oxygen free)</td>
<td>N₂</td>
<td>99.9%</td>
<td>BOC gases Canada</td>
<td>-</td>
</tr>
<tr>
<td>29  Carbon Dioxide</td>
<td>CO₂</td>
<td>99.9%</td>
<td>BOC gases Canada</td>
<td>-</td>
</tr>
</tbody>
</table>
### 2.2 List of Miscellaneous Equipment Used for SRB Experiments

<table>
<thead>
<tr>
<th>Equipment Name</th>
<th>Supplier / Model / Spec</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Gas Chromatograph 1</td>
<td>Varian Star / 3800 / PFPD detector</td>
<td>H₂S gas analysis</td>
</tr>
<tr>
<td>2 Gas Chromatograph 2</td>
<td>Varian Star / 3400 / FID detector</td>
<td>CH₄ gas analysis</td>
</tr>
<tr>
<td>3 Ion Chromatograph</td>
<td>Dionex / DX500</td>
<td>SO₄⁻² analysis</td>
</tr>
<tr>
<td>4 Spectrophotometer</td>
<td>Milton Roy / Spectronics 21</td>
<td>Biomass Measurement</td>
</tr>
<tr>
<td>5 COD digester Block</td>
<td>Chemetrics Inc.</td>
<td>COD Measurement</td>
</tr>
<tr>
<td>6 Digital Weighing Machine</td>
<td>ACCULab / V-1mg</td>
<td>weighing</td>
</tr>
<tr>
<td>7 Incubator</td>
<td>PRECISION / dual program, illuminated incubator</td>
<td>Temperature Control</td>
</tr>
<tr>
<td>8 pH electrode</td>
<td>Cole Parmer / #05994-13 AgCl based</td>
<td>pH / temperature measurement</td>
</tr>
<tr>
<td>9 digital pH meter / Temp Gauge</td>
<td>Barnant Company / Barnant 30</td>
<td></td>
</tr>
<tr>
<td>10 Glassware</td>
<td>Misc.</td>
<td>Misc.</td>
</tr>
<tr>
<td>11 Latex Free Plastic Syringes</td>
<td>Becton Dickson &amp; Co / (1.3. 5. 20 &amp; 60 mL sizes)</td>
<td>Anaerobic bio-sludge handling</td>
</tr>
</tbody>
</table>
Appendix 3  Calibration Curves

3.1 Calibration Curves for SO$_{4}^{2-}$ Analysis

3.1.1  Calibration Curve for SO$_{4}^{2-}$ Detection using Ion Chromatograph

<table>
<thead>
<tr>
<th>Conc ppm</th>
<th>Peaks Area</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>average</td>
<td>% deviation</td>
</tr>
<tr>
<td>1</td>
<td>3,520.0</td>
<td>4,123.0</td>
<td>3,821.5</td>
<td>11.2%</td>
</tr>
<tr>
<td>10</td>
<td>46,128.0</td>
<td>46,334.0</td>
<td>46,231.0</td>
<td>0.3%</td>
</tr>
<tr>
<td>100</td>
<td>645,338.0</td>
<td>642,892.0</td>
<td>644,115.0</td>
<td>0.3%</td>
</tr>
<tr>
<td>500</td>
<td>3,672,180.0</td>
<td>3,673,287.0</td>
<td>3,672,733.5</td>
<td>0.0%</td>
</tr>
<tr>
<td>1000</td>
<td>7,441,196.0</td>
<td>7,491,656.0</td>
<td>7,466,426.0</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

\[ y = 7496.2x - 48616 \]
\[ R^2 = 0.9998 \]
### 3.1.2 Calibration Curve for SO$_3$$^2$ Detection using Ion Chromatograph

<table>
<thead>
<tr>
<th>Conc ppm</th>
<th>Area Peaks</th>
<th>% deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>3.040.0</td>
<td>3.153.0</td>
</tr>
<tr>
<td>10</td>
<td>24.090.0</td>
<td>25.103.0</td>
</tr>
<tr>
<td>50</td>
<td>151.550.0</td>
<td>151.671.0</td>
</tr>
<tr>
<td>100</td>
<td>320.304.0</td>
<td>326.713.0</td>
</tr>
<tr>
<td>500</td>
<td>1.955.210.0</td>
<td>1.958.122.0</td>
</tr>
<tr>
<td>1000</td>
<td>3.931.056.0</td>
<td>3.934.656.0</td>
</tr>
</tbody>
</table>

![Graph showing a linear relationship between peak area and Conc. of sulfite ppm](image)

\[ y = 3963.4x - 31818 \]

\[ R^2 = 0.9997 \]
3.1.3 Calibration Curve for $S_2O_3^{2-}$ Detection using Ion Chromatograph

<table>
<thead>
<tr>
<th>Conc ppm</th>
<th>Area Peaks</th>
<th>average</th>
<th>% deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.099.0</td>
<td>3.275.0</td>
<td>3.687.0</td>
</tr>
<tr>
<td>10</td>
<td>34.261.0</td>
<td>34.608.0</td>
<td>34.434.5</td>
</tr>
<tr>
<td>50</td>
<td>174.722.0</td>
<td>176.246.0</td>
<td>175.484.0</td>
</tr>
<tr>
<td>100</td>
<td>377.657.0</td>
<td>376.133.0</td>
<td>376.895.0</td>
</tr>
<tr>
<td>500</td>
<td>2.184.021.0</td>
<td>2.273.480.0</td>
<td>2.228.750.5</td>
</tr>
<tr>
<td>1000</td>
<td>4.774.503.0</td>
<td>4.788.490.0</td>
<td>4.781.496.5</td>
</tr>
</tbody>
</table>

\[
y = 4781.6x - 56903 \\
R^2 = 0.9988
\]
3.2 Calibration Curves for Protein Analysis

<table>
<thead>
<tr>
<th>Protein Conc. (microgram)</th>
<th>Sulfate Reactor</th>
<th>Sulfite Reactor</th>
<th>Thiosulfate Reactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>4.0</td>
<td>0.030</td>
<td>0.020</td>
<td>0.010</td>
</tr>
<tr>
<td>8.0</td>
<td>0.080</td>
<td>0.090</td>
<td>0.060</td>
</tr>
<tr>
<td>12.0</td>
<td>0.150</td>
<td>0.140</td>
<td>0.100</td>
</tr>
<tr>
<td>20.0</td>
<td>0.230</td>
<td>0.210</td>
<td>0.150</td>
</tr>
<tr>
<td>28.0</td>
<td>0.240</td>
<td>0.270</td>
<td>0.220</td>
</tr>
</tbody>
</table>

Absorbance (at 595 nm wavelength)
3.3 Calibration Curve for COD analysis

Equation provided by vendor for calculation of COD is:

$\text{COD (mg/L)} = 22.851 \times (\text{absorbance}) + 1$

Note: the wavelength for this spectrophotometer analysis is 620 nm
3.4 Calibration Curves for GC analysis

3.4.1 Calibration Curve for H₂S Detection using PFPD Detector

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>H₂S ppm</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>660.0</td>
<td>608705</td>
</tr>
<tr>
<td>2</td>
<td>264.0</td>
<td>112693</td>
</tr>
<tr>
<td>3</td>
<td>198.0</td>
<td>84649</td>
</tr>
<tr>
<td>4</td>
<td>132.0</td>
<td>12848</td>
</tr>
<tr>
<td>5</td>
<td>92.4</td>
<td>1115</td>
</tr>
</tbody>
</table>

![Graph for H₂S ppm](image)

\[ y = 1102.5x - 132918 \]
\[ R^2 = 0.9871 \]

3.4.2 Calibration Curve for CH₄ Detection using FID Detector

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>CH₄ ppm</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1639.34</td>
<td>1766.67</td>
</tr>
<tr>
<td>2</td>
<td>1311.48</td>
<td>1361.00</td>
</tr>
<tr>
<td>3</td>
<td>983.61</td>
<td>891.00</td>
</tr>
<tr>
<td>4</td>
<td>655.74</td>
<td>581.00</td>
</tr>
<tr>
<td>5</td>
<td>327.87</td>
<td>398.00</td>
</tr>
<tr>
<td>6</td>
<td>80.00</td>
<td>158.00</td>
</tr>
</tbody>
</table>

![Graph for CH₄ ppm](image)

\[ y = 1.0179x + 11.323 \]
\[ R^2 = 0.9789 \]
Appendix 4 Protein Analysis

4.1 Time Dependence of Bio-Rad Dye Protein Assay for all three reactors
4.2 Precision of Sampling for Protein Analysis

a) Sulfate Reactor

b) Sulfite Reactor

c) Thio-Sulfate Reactor
Appendix 5  Calculations for H$_2$S & HS$^-$ equilibria

Conversion of H$_2$S Concentrations Detected, To HS$^-$ Conc. in solution

1. **Concentration of H$_2$S** :
   100 ppm (assuming average concentration)

2. **Temperature** :
   308.15 °K

3. **Molar Volume** :
   25.27 Litters
   Using $PV = nRT$
   $n = \frac{P \cdot V}{RT}$
   $0.082 L \cdot atm \cdot mol^{-1}$

4. **Henry's Law Constant** $H$ $^{[54]}$
   68590.63 kPa-kg mol
   Using
   $\ln(H) = 3.533 + 0.072437 \cdot T_1 - 11.107656 \cdot 10^{-2} \cdot T_2 - 0.1548 \ln(T) + 0.1442237 \cdot T_1 \cdot \ln(T)$

5. **Mole Fraction of H$_2$S**
   As H$_2$S = 100 0000 ppmv cruise 100 L of H$_2$S L of gas mix
   7.956 x 10$^{-2}$ g of H$_2$S L of mix
   Using
   $\gamma = \frac{m}{M}$
   $\rho = \frac{P}{RT}$
   $H = \frac{P \rho}{n}$
   $m = \frac{P \rho}{n}$
   5.91E-02 mol of H$_2$S mole of N$_2$
   5.91E-02 mol of H$_2$S mole of N$_2$

6. **Solubility of H$_2$S in H$_2$O**
   2.95 mg of H$_2$S L of H$_2$O
   Using
   $\gamma = \frac{m}{M}$
   $\rho = \frac{P}{RT}$
   $H = \frac{P \rho}{n}$
   $m = \frac{P \rho}{n}$
   Converting from mole kg of water to mole L of water

7. **Calculation of Sulfide HS$^-$** $^{[55]}$
   19.6 mg of HS$^-$ L of H$_2$O
   Using
   $\gamma = \frac{m}{M}$
   $\rho = \frac{P}{RT}$
   $H = \frac{P \rho}{n}$
   $m = \frac{P \rho}{n}$

---

<table>
<thead>
<tr>
<th>pH</th>
<th>H$^+$</th>
<th>K$_a$</th>
<th>[H$_2$S]</th>
<th>[HS$^-$/L</th>
<th>[H$^+$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.50</td>
<td>5.16E-08 molar</td>
<td>( \text{assuming average system pH} )</td>
<td>5.16E-08 molar</td>
<td>32.55 - 1519 44/T + (15.672 * (logT 1.011)) - 0.02722 * T)</td>
<td>5.16E-08 molar</td>
</tr>
<tr>
<td>6.04E-07 molar</td>
<td>( \text{assuming } K_a = 32.55 - 1519 44/T + (15.672 * (logT 1.011)) - 0.02722 * T) )</td>
<td>6.04E-07 molar</td>
<td>19.65E-05 molar</td>
<td>19.65E-05 molar</td>
<td></td>
</tr>
</tbody>
</table>

Appendix 6  Experimental Data for Exp. G1 – G6

6.1 Plot of pH Change (Exp-G1 – G6)

Figure 1: Exp G1 - Plot of pH vs Time

Figure 2: Exp. G2 - Plot of pH vs Time
Figure 3: Exp. G3 - Plot of pH vs Time

Figure 4: Exp. G4 - Plot of pH vs Time
Figure 5: Exp. G5 - Plot of pH vs Time

Figure 6: Exp. G6 - Plot of pH vs Time
6.2 Plot of Change In $SO_3^{1-}$ Concentration

![Graph showing change in $SO_3^{1-}$ concentration over time.](image)

Figure 7: Exp. G1- Time dependence of individual $SO_3^{1-}$ species (a) and total $SO_3^{1-}$ (b) in the $SO_4^{2-}$ reactor
Figure 8: Exp. G1 - Time dependence of (a) individual (b) total SO$_x^-$ species in SO$_3^{2-}$ reactor.
Figure 9: Exp G1 - Time dependence of (a) individual (b) total SO\textsubscript{X}\textsuperscript{-} species in S\textsubscript{2}O\textsubscript{3}\textsuperscript{2-} reactor
Figure 10: Exp. G2 - Time dependence of (a) individual (b) total \( \text{SO}_\text{x}^{\text{Y}} \) species in the \( \text{SO}_\text{4}^{\text{2-}} \) reactor
Figure 11: Exp. G2 - Time dependence of (a) individual (b) total $SO_x$ species in $SO_3^{-2}$ reactor
Figure 12: Exp. G2 - Time dependence of (a) individual (b) total SO$_x$ species in S$_2$O$_3$ reactor.
Figure 13: Exp. G3 - Time dependence of (a) individual (b) total $SO_4^{2-}$ species in the $SO_4^{2-}$ reactor
Figure 14: Exp. G3 - Time dependence of (a) individual (b) total $SO_\text{x}^{\text{Y}}$ species in $SO_3^{\text{2}}$ reactor
Figure 15: Exp. G3 - Time dependence of (a) individual (b) total $\text{SO}_x^{\text{V}}$ species in $\text{S}_2\text{O}_3^{\text{V}-2}$ reactor.
Figure 16: Exp. G4 - Time dependence of (a) individual (b) total $\text{SO}_x^\text{Y}$ species in the $\text{SO}_4^{2-}$ reactor
Figure 17: Exp. G4 - Time dependence of (a) individual (b) total $\text{SO}_x$ species in $\text{SO}_3^2-$ reactor
Figure 18: Exp. G4 - Time dependence of (a) individual (b) total SO$_3^-$ species in S$_2$O$_3^{2-}$ reactor
Figure 19: Exp. G5 - Time dependence of (a) individual (b) total $\text{SO}_4^{2-}$ species in the $\text{SO}_4^{2-}$ reactor
Figure 20: Exp. G5 - Time dependence of (a) individual (b) total $SO_3^{2-}$ species in $SO_3^{-2}$ reactor.
Figure 21: Exp. G5 - Time dependence of (a) individual (b) total SO$_x^-$ species in S$_2$O$_3^{2-}$ reactor
Figure 22: Exp. G6 - Time dependence of (a) individual (b) total $SO_3^{\cdot}$ species in the $SO_4^{2-}$ reactor
Figure 23: Exp. G6 - Time dependence of (a) individual (b) total $SO_\text{y}^-$ species in $SO_3^{2-}$ reactor
Figure 24: Exp. G6 - Time dependence of (a) individual (b) total $SO_x^{Y}$ species in $S_2O_3^{2-}$ reactor
6.3 Decrease in Chemical Oxygen Demand (COD) with Time

Figure 25: Exp G1 – Time Dependence of Soluble COD in SRB Reactors

Figure 26: Exp. G2 – Time Dependence of Soluble COD in SRB Reactors
Figure 27: Exp. G3 – Time Dependence of Soluble COD in SRB Reactors

Figure 28: Exp. G4 – Time Dependence of Soluble COD in SRB Reactors
Figure 29: Exp. G5 – Time Dependence of Soluble COD in SRB Reactors

Figure 30: Exp. G6 – Time Dependence of Soluble COD in SRB Reactors
6.4 Effect of COD/SO\textsuperscript{4-} Ratio

6.4.1 Effect of COD/SO\textsuperscript{4-} Ratio on Total SO\textsuperscript{2-} Removal

Figure 31: Total SO\textsubscript{x} Removal with Time (In different SO\textsuperscript{4-} Reactors)

Note: the ratio in these graphs represents the ratio of electron donor/acceptor in mol/mol units

(a) For Sulfate Reactors
(b) For Sulfite Reactors

(c) For Thiosulfate Reactors
6.4.2 Effect of COD/SO$_4^{\text{2-}}$ Ratio on COD Removal

Figure 32: Record of Total COD Removal with Time

Note: the ratio in these graphs represents the ratio of electron donor/acceptor in mol/mol units

![Graph (a) For Sulfate Reactors](image1)

(a) For Sulfate Reactors

![Graph (b) For Sulfite Reactors](image2)

(b) For Sulfite Reactors
For Thiosulfate Reactors
6.5 Effect of COD/SO₄²⁻ Ratio on Protein Growth

Figure 33: Protein Growth with Time

Note: the ratio in these graphs represents the ratio of electron donor/acceptor in mol/mol units

![Graph for Sulfate Reactors](a)

![Graph for Sulfite Reactors](b)
(c) For Thiosulfate Reactors
6.6 Effect of COD/SO$_4$$^{2-}$ Ratio on pH

Figure 34: pH with Time

Note: the ratio in these graphs represents the ratio of electron donor/acceptor in mol/mol units

(a) For Sulfate Reactors

(b) For Sulfite Reactors
For Thiosulfate Reactors
Appendix 7 Sample Calculations, Percentage of Electron Equivalents of COD used for SO$_x^{2-}$ Reduction

### 1. Experimental data for 1st 70 hours basis

<table>
<thead>
<tr>
<th></th>
<th>Reactor</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sulfate</td>
<td>Sulfite</td>
<td>Thiosulfate</td>
<td></td>
</tr>
<tr>
<td>a) Total COD removed g/L</td>
<td>0.442</td>
<td>See note 2</td>
<td>1.364</td>
<td></td>
</tr>
<tr>
<td>b) Actual SO$_x^{3-}$ removed g/L</td>
<td>0.14</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SO$_4^{2-}$</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SO$_3^{2-}$</td>
<td>1.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S$_2$O$_3^{2-}$</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

### 2. Calculations considering reduction to S$^{2-}$

<table>
<thead>
<tr>
<th></th>
<th>Reactor</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sulfate</td>
<td>Sulfite</td>
<td>Thiosulfate</td>
<td></td>
</tr>
<tr>
<td>a) Electron equivalent given by donor /L (= COD/g [61])</td>
<td>0.055</td>
<td>See note 2</td>
<td>0.170</td>
<td></td>
</tr>
<tr>
<td>b) Electron equivalents accepted by SO$_x^{3-}$ for reduction to S$^{2-}$ (estimated using half reactions in Table 4.5)</td>
<td>0.012</td>
<td>0.096</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c) Percentage of electron equivalent used for SO$_x^{3-}$ reduction</td>
<td>21%</td>
<td>56%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 3. Calculations considering reduction to S$^0$

<table>
<thead>
<tr>
<th></th>
<th>Reactor</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sulfate</td>
<td>Sulfite</td>
<td>Thiosulfate</td>
<td></td>
</tr>
<tr>
<td>a) Electron equivalent given by donor /L (= COD/g [61])</td>
<td>0.055</td>
<td>See note 2</td>
<td>0.170</td>
<td></td>
</tr>
<tr>
<td>b) Electron equivalents accepted by SO$_x^{3-}$ for reduction to S$^0$ (estimated using half reactions given in note 4 below)</td>
<td>0.009</td>
<td>0.048</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c) Percentage of electron equivalent used for SO$_x^{3-}$ reduction</td>
<td>16%</td>
<td>28%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Notes

1. Note the sample calculations provided above are only for experiment G1. Calculations for other experiments are similar.

2. Calculations for the sulfite reactor are not provided because of the complexity involved due to the presence of all three species of $SO_3^{2-}$. It was observed that large amount of $SO_3^{2-}$ is converted to $S_2O_3^{2-}$. As discussed in sec 4.2.4.1 this can happen by either of two pathways, namely stepwise reduction of sulfite by SRB and reaction of sulfite and sulfide species. Enough evidence is not available to predict which pathway was predominant. Thus electron equivalent of donor used for $SO_3^{2-}$ reduction in sulfite reactor is not calculated.

3. For thiosulfate reactor it is assumed that only species present is $S_2O_3^{2-}$ although a small quantity of $SO_4^{2-}$ (0.00005 mol/L) was also present.

4. The hypothetical half reactions used for the calculations (in 3.b above) of electron equivalent of COD used for $SO_3^{2-}$ reduction to $S^{0}$ level are:

\[
\begin{align*}
\text{Sulfate:} & \quad 1.6 \text{ SO}_4^{2-} + 4.5 \text{ H}^- + \text{e}^- \rightarrow 1.6 \text{ S}^0 + 2.5 \text{ H}_2\text{O} \\
\text{Sulfite:} & \quad 1/4 \text{ SO}_3^{2-} + 3/2 \text{ H}^- + \text{e}^- \rightarrow 1/4 \text{ S}^0 + 7/4 \text{ H}_2\text{O} \\
\text{Thiosulfate:} & \quad 1/4 \text{ S}_2\text{O}_3^{2-} - 3/2 \text{ H}^- + \text{e}^- \rightarrow 1/2 \text{ S}^0 + 7/4 \text{ H}_2\text{O}
\end{align*}
\]
Appendix 8  Comparison of COD Usage

The graphs presented here provide the comparison of:

1. Actual electron equivalents removed from the system calculated from COD analysis.
2. Theoretical grams of SO$_3^{2-}$ (considering reduction to S$^{2-}$ level) that can be reduced using COD value in 1 above.
3. Theoretical grams of SO$_4^{2-}$ (considering reduction to S$^0$ level) that can be reduced using COD value in 1 above.
4. Actual grams of SO$_3^{2-}$ removed from the system. calculated from the SO$_3^{2-}$ analysis.

(a) For Sulfate Reactors
Electron Donor/Acceptor Ratio (mol/mol)

<table>
<thead>
<tr>
<th>Initial conc.</th>
<th>Th. Rem. for S2-</th>
<th>Th. Rem. for SO</th>
<th>Actually Removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>EI</td>
<td>Th. Rem. for S2-</td>
<td>Th. Rem. for SO</td>
<td>Actually Removed</td>
</tr>
<tr>
<td>0.21</td>
<td>0.32</td>
<td>0.53</td>
<td>0.64</td>
</tr>
</tbody>
</table>

(b) For Sulfite Reactors

Electron Donor/Acceptor Ratio (mol/mol)

<table>
<thead>
<tr>
<th>Initial conc.</th>
<th>Th. Rem. for S2-</th>
<th>Th. Rem. for SO</th>
<th>Actually Removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.18</td>
<td>0.21</td>
<td>0.32</td>
<td>0.53</td>
</tr>
</tbody>
</table>

(c) For Thiosulfate Reactors