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Inhibitory Effect of Copper Precipitates on Anaerobic Biological Sulfate Reduction

by

Shahrokh Shahsavari

A Thesis
Submitted to the Faculty of Graduate Studies
Through Civil and Environmental Engineering
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy at the
University of Windsor

Windsor, Ontario, Canada

2017

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DECLARATION OF ORIGINALITY

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ABSTRACT

Biological anaerobic sulfate reduction to sulfide by sulfate reducing bacteria (SRB) can be performed in a single-stage reactor in which the biological sulfate reduction to sulfide and metal precipitation occur simultaneously, or in two-stage reactors where the two follow sequentially.

The single stage process may be more cost-effective and simpler to operate. However, some factors, such as acidic nature of acid mine drainage (AMD) and the presence of the residual heavy metals in the system may pose an inhibitory and toxic effect on SRB and limit the application of the process. In addition, some studies suggest that beyond a certain level of metal loading, the process of sulfate reduction and the corresponding metal precipitation by the sulfide generated is adversely affected.

In the first part of this study, the effect of different concentrations of copper on anaerobic sulfate reduction in semi continuous stirred tank reactors (SCSTRs) at 35±2ºC was investigated. Four parallel SCSTRs received synthetic wastewater containing copper at various concentrations. They were optimized for pH and were operated at a predetermined COD/$SO4^{2-}$. Reactors receiving lower concentration (< 200 mg/L) of copper showed a very little negative effect in their performance. However, at higher concentrations (> 400 mg/L), performance was inhibited, which could be attributed to the presence of metal precipitates in these reactors. Batch kinetic experiments confirmed this inhibition of the sulfate reduction process in the presence of high concentration of metal precipitates. The cultures withdrawn at various process conditions were analyzed for their respective microbial pattern. It showed that certain concentrations of copper precipitates adversely affected the population of sulfate reducers. Reactors receiving 0 mg/L and 200 mg/L of copper showed more similarity in terms of their respective sulfate reducers' population. The presence of thiosulfate reducers in microbial community may be an evidence for the existence of an alternate pathway in dissimilatory anaerobic sulfate reduction that generates thiosulfate as the intermediate byproducts during the reduction of sulfite to sulfide.

In the second part of the study, two upflow anaerobic hybrid reactors (UAHR) were designed to overcome the inhibition of SRB by the metal precipitates. Two identical UAHRs received simulated wastewater with COD/SO_4^{2-} of 1, where the sulfate concentration was 3040 mg/L. One UAHR was used to represent the single-stage process, and the influent contained metal (copper) in the feed. The other UAHR represented the first stage of the two-stage process. The performance of the two processes was compared over different hydraulic, organic, and sulfate loading rates by varying the HRT between 40 and 2.5 days at a temperature of 33±3°C. The results show that both sulfate reduction and copper precipitation in the single stage process were similar to or better than the two-stage process over the entire duration of the study. The rate of copper removal in the single stage process was found to reach up to two times of that of the two stage process. This suggests that the proposed UAHR configuration was successful in overcoming the inhibition of SRB by the metal precipitates.

In the single stage reactor for which S/Cu was higher than 1, copper was precipitated in the form of CuS. The same happened for the second stage of two stage process once S/Cu was set to be higher than 1. This was confirmed with the equilibrium calculations using MINTEQ speciation model. However, the results of the MINTEQ showed that at S/Cu of less than 1, additional amount of copper is precipitated in the forms of $Cu_3(PO_4)_2$ and CuO.

DEDICATION

This is dedicated to

my proud parents

Mohammad Hossein Shahsavari

and

Akram Azizmohammadi

and

my wife

Maryam Shahabi Far

For their unconditional love, encouragements and supports

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TABLE OF CONTENTS

DECLARATION OF ORIGINALITY……..iii

ABSTRACT……...iv

DEDICATION…….…..vi

ACKNOWLEDGEMENTS……..vii

LIST OF TABLES…….……xv

LIST OF FIGURES……...……..xvii

LIST OF ELECTRONIC APPENDICES…….xxi

LIST OF ABBREVIATIONS……...…….…….xxii

CHAPTER ONE: INTRODUCTION

1.1 Introduction……..1

1.2 Removal of Heavy Metals by Biogenic Sulfate Reduction Process……..……4

1.3 Objectives……5

1.4 Scope of the Work……...6

CHAPTER TWO: LITERATURE REVIEW

2.1 Sources of Heavy Metal Contamination…….…….7

2.1.1 Natural Sources of Heavy Metals……..…….…….7

2.1.2 Anthropogenic Sources of Heavy Metals……..……..8

2.1.2.1 Agricultural Activities……..……...……8

2.1.2.2 Industrial Wastes…….……9

2.1.2.3 Domestic Waste Streams……..……...9

2.1.2.4 Mining Activities…….……...9

2.2 Treatment Options for Wastewater Containing Heavy Metals…..11

2.2.1 Adsorption……...……...12

2.2.2 Membrane Filtration……...……...12

2.2.3 Coagulation-Flocculation……...……14

2.2.4 Ion Exchange……...……..14

2.2.5 Flotation…… ……..15

2.2.6 Electrochemical Treatment……...…….15

2.2.7 Precipitation……...……16

2.2.7.1 Chemical Precipitation……..…….16

2.2.7.2 Biogenic Sulfide Precipitation……..…….20

2.3 Biological Conversion of Sulfate to Sulfide……..21

2.3.1 Effects of pH……...……...23

2.3.2 Sulfide Inhibition……...23

2.3.3 Effects of Temperature……..25

2.3.4 Effects of Hydraulic Retention Time…….25

2.3.5 Chemical Oxygen Demand/Sulfate Ratio…….....26

2.4 Bioreactor Configurations in Anaerobic Sulfate Reduction……..27

2.4.1 Continuously Stirred Tank Reactor……...27

2.4.2 Packed Bed Reactor……...28

2.4.3 Fluidized Bed Reactor……...……28

2.4.4 Gas Lift Reactor…….29

2.4.5 Up-flow Anaerobic Sludge Blanket Reactors……30

2.4.6 Membrane Bioreactor……31

2.4.7 Anaerobic Hybrid Reactor…….32

2.5 Biogenic Sulfide Precipitation of Heavy Metals…….....32

2.5.1 Single Stage Biological Sulfate Reduction Process and Metal Sulfide Precipitation…….…….33

2.5.2 Two Stage Biological Sulfate Reduction Process and Metal Sulfide Precipitation……...35

CHAPTER THREE: EXPERIMENTAL DESIGN AND METODOLOGIES

3.1 Experimental Set-up and Design……...38

3.1.1 Setup 1. Semi-continuous Stirred Tank Reactors (SCSTRs)….....38

3.1.1.1 Phase I. Inoculum Source and Start-up……..38
3.1.1.2 Phase II. Metal Precipitation Experiments…….41
3.1.2 Setup 2. Upflow Anaerobic Hybrid Reactors (UAHRs)……41
3.1.2.1 Phase I. Inoculum Source and Start-up……..41
3.1.2.2 Phase II. Metal Precipitation…….…….…44
3.1.2.3 Determination of Copper and Solids Inventory in UAHRs……...46
3.2 Analytical Methods……47
3.2.1 pH……..47
3.2.2 Oxidation Reduction Potential (ORP)……...……47
3.2.3 Gas production……...47
3.2.4 Sulfate ……48
3.2.5 Total Dissolved Sulfide (TDS)……..48
3.2.6 Alkalinity and Total Volatile Fatty Acids……..48
3.2.7 Total Organic Carbon (TOC)……49
3.2.8 Phosphate Measurement……49
3.2.9 Solids Measurement……...49
3.2.10 Copper Analysis……50
3.3 Genomic Analysis……...……..….50
3.3.1 Sample Collection……..50
3.3.2 DNA Extraction……51
3.3.3 Polymerase Chain Reaction (PCR) and Next Generation Sequencing……51
3.3.4 Statistical Analysis……53
3.4 Precipitate Characterization……...53
3.4.1 Precipitate Concentration……...53
3.4.2 Scanning Electron Microscopy- Energy Dispersive X-ray Spectroscopy……..53
3.4.3 X-ray Diffraction (XRD)……...54
3.4.4 Visual MINTEQ Equilibrium Speciation Model……...54

CHAPTER FOUR: EFFECT OF COPPER PRECIPITATES ON SULFATE REDUCTION IN SEMI-CONTINUOUS STIRRED TANK REACTORS

4.1 Single Stage Copper Precipitation in SCSTRs……...…...……55

4.2 Batch Kinetic Study……..…….62

4.3 Genomic Analysis of Cultures Withdrawn at Different Copper Loading…….……65

CHAPTER FIVE: SULFATE REDUCTION AND COPPER PRECIPITATION IN HYBRID REACTORS: COMPARISON OF SINGLE STAGE AND TWO STAGE PROCESSES

5.1 Reactors Operation……72

5.1.1 Phase I: Start-up……72

5.1.2 Phase II: Process Evaluation…….75

5.1.2.1 40d HRT……76

5.1.2.2 20d HRT……80

5.1.2.3 10d HRT……87

5.1.2.4 5d and 2.5d HRT……...91

5.1.3 Summary of Steady-State Performance of HR1 and HR2……97

5.2 Quantification of Metal Precipitation……..……100

5.2.1 Estimation of Copper Precipitated as Sulfide……..……100

5.2.1.1 Chemical Precipitation……...…..100

5.2.1.2 Copper Precipitation within the Single Stage Process…….104

5.2.2 Comparison of Metal Precipitation in the Single and Two Stage Processes……..106

5.2.2.1 Two Stage Process……...106

5.2.2.2 Single Stage Process……107

5.2.3 Comparison the State of the Research with the Background of Single Stage Metal Removal……113

5.3 Copper Precipitation and MINTEQ Simulation……..117

5.3.1 Group 1 - S/Cu of greater than 1…….……118

5.3.2 Group 2 – CuS formation at S/Cu of less than 1……..…120

5.3.3 Group 3 – Formation of CuS and $Cu_3(PO_4)_2$ at S/Cu of less than 1……....123

5.3.4 Group 4 - Formation of CuS, $Cu_3(PO_4)_2$ and CuO at S/Cu of less than 1……...…….127

5.4 Comparison of Copper and Solids Inventory Inside Single Stage and Two Stage Reactors…….…….132

5.4.1 Distribution of Copper Precipitates Inside the Single Stage Reactors……132

5.4.2 Distribution of VSS Inside the Reactors……..135

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions……..……137

6.1.1 Effect of Copper Precipitates on Sulfate Reduction in Semi-continuous Stirred Tank Reactors……137

6.1.2 Sulfate Reduction and Copper Precipitation in High Rate Reactors…….……...……138

6.2 Future Recommendations……...…….139

APPENDICES

Appendix 1- Calculations……141

Appendix 2- Atomic Absorption Calibration Curves……..143

Appendix 3- Total Carbon (TC) and Inorganic Carbon (IC) Calibration Curves……..……...……145

Appendix 4- SCSTRs Batch Operation Results for Sulfate and VSS…….146

REFERENCES…….……...148

VITA AUCTORIS……..165

LIST OF TABLES

Table 1-1 Maximum Permissible Levels of Important Heavy Metals in Water Bodies….2

Table 2-1 Health Concerns of Various Heavy Metals…….……..11

Table 2-2 Molar Solubility Products of Metallic Compounds…….……..20

Table 3-1 Composition of Growth Medium……...…….……..39

Table 3-2 Experimental Design of Single Stage Copper Precipitation Process…….…...45

Table 3-3 Experimental Design for Two Stage (External) Copper Precipitation Process……...……46

Table 4-1 Distribution of Microorganisms at Different Copper Loading……....…….65

Table 4-2 The Results of Similarity Percentage (Simper) Test on Genomic Data……70

Table 5-1 Summary of Results for Single Stage Process Reactor, HR1……...98

Table 5-2 Summary of Results for Two Stage Process Reactor, HR2……...……...98

Table 5-3 Results of Chemical Precipitation Experiments with S/Cu Molar Ratio>1....101

Table 5-4 Copper Removal Capacity of Two Stage Process……...……107

Table 5-5 Copper Removal Capacity of Single Stage Process……109

Table 5-6 Change in Consumed S^{2-} Molar Concentration to Concentration of Precipitated Copper by Decrease in HRT……111

Table 5-7 Change in the Ratio of Measured S^{2-} to Theoretical Concentration of S^{2-} Calculated from The Concentration of Reduced Sulfate…….……112

Table 5-8 Use of High-Rate Bioreactor in Single Stage Sulfate Reduction and Metal Precipitation Concept……...……114

Table 5-9 Results of The Measurements and MINTEQ Predictions for S/Cu=>1……..118

Table 5-10 Results of Analytical Measurements and MINTEQ Software on Residual Copper and Precipitates Concentration (Initial S/Cu<1 and CuS as the Only Specified Solid Phase)……..……...……120

Table 5-11 Results of Analytical Measurements and MINTEQ for the Experiments with S/Cu<1 and CuS and $Cu_3(PO_4)_2$ as Possible Solid Phases in Equilibrium…….…….124

Table 5-12 Results of Measurements and MINTEQ Predictions for the Experiments with S/Cu<1 and Formation of CuS, $Cu_3(PO_4)_2$ and CuO…….……..……128

Table 5-13 Distribution of Solids in Both Reactors and Copper Distribution in HR1…134

LIST OF FIGURES

Figure 2-1 Solubility of Metal Hydroxide as a Function of pH……..……...…17

Figure 2-2 Solubility of Metal Sulfides and Hydroxides as a Function of pH……..……19

Figure 2-3 Anaerobic Digestion of Organic Matter……...……22

Figure 2-4 Equilibrium Concentration of Sulfide Species as a Function of pH…….…..….24

Figure 2-5 Packed Bed Reactor……..……...……28

Figure 2-6 (A) Up-flow FBR; and (B) Inverse FBR……..…..……..29

Figure 2-7 Gas Lift Reactor…….…….…….……30

Figure 2-8 Up-flow Anaerobic Sludge Blanket Reactor…….…...……31

Figure 2-9 Anaerobic Hybrid Reactor…….…….…….32

Figure 2-10 Single Stage Process Configuration…….....…….……33

Figure 2-11 Two Stage Metal Removal Process……..…….…….…35

Figure 3-1 Semi-Continuous Stirred Tank Reactor (SCSTR) Configuration…….....….40

Figure 3-2 Schematic Diagram of Up-flow Anaerobic Hybrid Reactor……43

Figure 3-3 UAHRs Sections for Solids and Copper Inventory…….…….47

Figure 4-1Variation in pH as a Function of Time of Operation for Semi-Continuous Operation of the Reactors at an HRT of 50 Days……..……56

Figure 4-2 Volatile Fatty Acid Concentration as a Function of Time of Operation for Semi-Continuous Operation of the Reactors at an HRT of 50 Days…….……57

Figure 4-3 Variation in Alkalinity as a Function of Time of Operation for Semi-Continuous Operation of the Reactors at an HRT of 50 Days……...……57

Figure 4-4 Variation in ORP as a Function of Time of Operation for Semi-Continuous Operation of the Reactors at an HRT of 50 Days……...……..…….58

Figure 4-5 Change in Suspended Solids Concentration as a Function of Time of Operation for Semi-Continuous Operation of the Reactors at an HRT of 50 Days…….59

Figure 4-6 Variation in Sulfate Concentration as a Function of Time of Operation for Semi-Continuous Operation of the Reactors at an HRT of 50 Days…….60

Figure 4-7 Variation in the rate of sulfate reduction by the change in the concentration of precipitated copper ……...…….……63

Figure 4-8 Variation in the concentration of VSS (biomass) inside the reactors by the change in the concentration of precipitated copper ……...……...……64

Figure 4-9 Shifts in Microbial Population Observed at (A) Addition of No Copper (B) Addition of 200 Mg/L of Copper (C) Addition of 400 Mg/L of Copper (D) Addition of 600 Mg/L of Copper…….…….67

Figure 4-10 Principal Coordinate Analysis (PCoA) of Genomic Data of the Cultures with Drawn from RC, R1, R2 and R3…….……...…68

Figure 5-1 Variation of PH, ORP, VFA and Bi-Carbonate Alkalinity by Change in Time During the Start Up Phase; A) Single Stage Reactor, HR1 and B) Two Stage Reactor, HR2……73

Figure 5-2 Variation of Sulfate and Total Organic Carbon (TOC) Concentration by Change in Time During the Start Up Phase; A) Single Stage Reactor, HR1 and B) Two Stage Reactor, HR2……...…….75

Figure 5-3 Variation of PH, ORP, VFA and Bi-Carbonate Alkalinity by Change in Time During the Operation at HRT=40 Days; A) Single Stage Reactor, HR1 and B) Two Stage Reactor, HR2……...……..…….……77

Figure 5-4 Variation of Sulfate, Sulfide and TOC Concentrations by Change in Time During the Operation at HRT=40 Days; A) Single Stage Reactor, HR1 and B) Two Stage Reactor, HR2……..……78

Figure 5-5 Variation of pH, ORP, VFA and Bi-carbonate Alkalinity by Change in Time During the Operation at HRT=20 Days; A) Single Stage Reactor, HR1 and B) Two Stage Reactor, HR2……..……82

Figure 5-6 Variation of Sulfate, Sulfide and Toc Concentrations by Change in Time During the Operation at HRT=20 Days; A) Single Stage Reactor, HR1 and B) Two Stage Reactor, HR2…….……84

Figure 5-7 Variation of pH, ORP, VFA and Bi-carbonate Alkalinity by Change in Time During the Operation at HRT==10 Days; A) Single Stage Reactor, HR1 and B) Two Stage Reactor, HR2……...……89

Figure 5-8 Variation of Sulfate, Sulfide and Toc Concentrations by Change in Time During the Operation at HRT=10 Days; A) Single Stage Reactor, HR1 and B) Two Stage Reactor, HR2…….……90

Figure 5-9 Variation of pH, ORP, VFA and Bi-carbonate Alkalinity by Change in Time During the Operation at HRT =5 And 2.5 Days; A) Single Stage Reactor, HR1 and B) Two Stage Reactor, HR2…….……..……93

Figure 5-10 Variation of Sulfate, Sulfide and Toc Concentrations by Change in Time During the Operation at HRT =5 And 2.5 Days; A) Single Stage Reactor, HR1 and B) Two Stage Reactor, HR2…….……..94

Figure 5-11 Variation of the Concentration of Precipitates by the Variation of Precipitated Copper Concentration……..……102

Figure 5-12 XRD Spectra for Externally Formed Precipitate…… 103

Figure 5-13 XRD Spectra for the Sample from Internal Precipitation……..……..105

Figure 5-14 The Relationship Between the Measurement and MINTEQ Results for the Concentration of Formed Precipitates for the Experiments with S/Cu=>1…….……....119

Figure 5-15 The Relationship Between Data Points of (A) Residual Copper (Measured and MINTEQ) and (B)Copper Precipitates Concentrations (Measured and MINTEQ) With The Ideal Data Points Presented by dotted Line……...….……122

Figure 5-16 Correlation Between Measured Residual dissolved copper and MINTEQ Results in Two Different Precipitation Scenarios; Series 1) Formation of CuS and Series 2) Precipitation of CuS and $Cu_3(PO_4)_2$……..125

Figure 5-17 Correlation between Measured Concentration of Precipitates and Prediction by MINTEQ, Series 1) CuS Was Selected in MINTEQ input and Series 2) Both CuS and $Cu_3(PO_4)_2$ Were Selected to Precipitate……126

Figure 5-18 The Relationship of the Correlated Data Points of Analytical Measurement and MINTEQ for Residual dissolved copper Concentrations to the Ideal Situation (Dotted Line), Series 1) CuS Was Selected to Precipitate in MINTEQ Input , Series 2) [CuS+$Cu_3(PO_4)_2$] was Selected to Precipitate and Series 3) [CuS+$Cu_3(PO_4)_2$+CuO] Was Selected…….…….…..129

Figure 5-19 The Relationship of the Correlated Data Points of Analytical Measurement MINTEQ for Copper Precipitates Concentrations to the Ideal Situation (Dotted Line), Series 1) CuS Was Selected to Precipitate in MINTEQ Input , Series 2) [CuS+$Cu_3(PO_4)_2$] Was Selected to Precipitate and Series 3) [CuS+$Cu_3(PO_4)_2$+CuO] Was Selected……...….…130

LIST OF ELECTRONIC APPENDICES

Appendix E1- Culture Maintainance for CSTRs

Appendix E2- Culture Maintainance for UAHRs

Appendix E3- Operation Data for CSTRs

Appendix E4- Operation data for UAHRs

Appendix E5- Copper and Solids Inventory Inside the Reactors

Appendix E6- Sulfate and VSS for CSTRs Batch Kinetic

Appendix E7- Operational Toxonomic Units of Microbial Profile for Different Process Condition

Appendix E8- Precipitation Data, Measurements and MINTEQ Results

Appendix E9- Results of SEM Analysis

Appendix E10- SEM Spectra for S to Cu Greater Than or Equal to 1

Appendix E11- SEM Spectra for S to Cu Less Than 1 and Just CuS to Precipitate

Appendix E12- SEM Spectra for S to Cu Less Than 1 and (CuS+Cu3(PO4)2) to Precipitate

Appendix E13- SEM spectra for S to Cu less than 1 and (CuS+Cu3(PO4)2+ Cuo) to Precipitate

LIST OF ABBREVIATIONS

AAS	atomic absorption spectroscopy
AMD	acid mine drainage
AFR	anaerobic filter reactor
CNT	carbon nanotube
COD	chemical oxygen demand
CSTR	continuous stirred tank reactor
EDAX	energy dispersive analysis X-ray
EDS	energy dispersive (X-ray) spectroscopy
EGSB	expanded granular sludge bed reactor
FBR	fluidized bed reactor
FSS	fixed suspended solids
GLR	gas lift reactor
HR	hybrid reactor
HRT	hydraulic retention time
IC	inorganic carbon
IFB	inverse fluidized bed reactor
MC	master culture reactor
MCL	maximum contamination level
MPB	methane producing bacteria
PBR	packed bed reactor
SCSTR	semi-continuous stirred tank reactor

SEM	scanning electron microscope
SRB	sulfate reducing bacteria
TC	total carbon
ThOD	theoretical oxygen demand
TOC	total organic carbon
TDS	total dissolved sulfide
TS	total sulfide
TSS	total suspended solids
UAHR	upflow anaerobic hybrid reactor
UASB	upflow anaerobic sludge blanket
VFA	volatile fatty acid
VSS	volatile suspended solids
XRD	X-ray diffraction

Chapter 1
Introduction

1.1 Introduction

Metals are essential to our needs and lifestyles, but can create adverse effects on the environment if discharged in high concentrations. At certain levels of concentration, particularly with regard to the heavy metals, they can be toxic to most living organisms including humans. Organ damage, cancer, reduced growth and development, diseases of the nerves as well as brain damage are health problems caused by excessive levels of heavy metals' concentration (Barakat, 2011). Toxic heavy metals are discharged into the environment from various sources. Among the sources of heavy metal pollution, industrial activities such as metal plating, paint production, electronics manufacturing, battery production, etc., pose great hazards to the environment (Das, Gopi Kiran, & Pakshirajan, 2016). One industry that stands out in this regard is the mining industry, because of its large volume of operation and waste production. It is considered as the major source for the discharge of this type of effluent (Johnson & Hallberg, 2005). Acid Mine Drainage (AMD), i.e., highly acidic, sulfate and heavy metal-rich discharge from abandoned mines and the tailings ponds of active mines, is considered to be one of the major sources of heavy metal contamination in the environment (Biswas, 2012; Johnson & Hallberg, 2005; Denys Kristalia Villa Gómez, 2013).

AMD is often caused by pyrite (FeS_2) oxidation but other metal mines also discharge highly acidic wastewater where the ore is a sulfide mineral or is associated with pyrite. In the latter case, pyrite may not be the predominant sulfide and other metallic sulfides of zinc (Zn), copper (Cu), and nickel (Ni) create the acidic waste stream (Environmental Mining Council of British Columbia, 1997). One example is copper mining in which chalcopyrite ($CuFeS_2$), the most common copper (Cu) mineral, it is a copper-iron-sulfide that occurs with a wide range of other sulfide minerals.

The degree of contamination by AMD discharge is difficult to assess precisely, released reports estimate approximately 19000 km of streams and rivers as well as 750 km^2 of lakes in North America, have been seriously contaminated by AMD discharge in 1989

(Johnson & Hallberg, 2005). In the Appalachian Mountains in the eastern United States, more than 12000 km of affected streams have been reported (Fripp, Ziemkiewicz, & Charkavorki, 2000). This issue is more critical in countries such as Canada, where there are huge mineral resources. The Canadian mineral industries produce around 650 million tons of waste per year (Environmental Mining Council of British Columbia, 1997). Almost fifty % of these wastes are waste rock that contains sulfides, heavy metals and other pollutants that cause environmental pollution. The remaining are the mining tailings that still represent a major source of heavy metal contamination (Environmental Mining Council of British Columbia, 1997).

Regulatory agencies such as United States Environment Protection Agency (USEPA) and World Health Organization (WHO) have suggested maximum permissible concentrations for heavy metals in drinking water. Table 1-1 presents these permissible limits for the most important heavy metals (WHO, 2011).

Table 1-1 Maximum permissible levels of important heavy metals in drinking water

Metals	Concentration (mg/L)
Copper	1.0
Zinc	5.0
Nickel	0.2
Lead	0.01
Cadmium	0.005
Chromium	0.05

Unlike organic contaminants, heavy metals are not biodegradable. It is important to decrease the concentration of heavy metals from different waste streams to the allowable levels before their discharge into the environment, specifically to bodies of water. Their recalcitrant nature and consistent presence in the environment have made it more challenging, to find a promising, low cost and efficient option for treatment of heavy metal containing wastewaters like AMD.

The treatment methods applied to wastewaters with heavy metals can be classified in two general categories: physico-chemical processes and biological methods (Akpor & Muchie, 2010; Mallelwar, 2013).

The physico-chemical processes include: chemical precipitation; coagulation-flocculation; flotation; ion exchange; electrochemical precipitation; and membrane filtration. Some of these processes have been widely used to remove heavy metals from industrial wastewaters. Although, physico-chemical processes are efficient, they are usually associated with a high operational cost due to the use of chemicals and high levels of energy consumption (El Bayoumi, 1997; Kurniawan, Chan, Lo, & Babel, 2006).

Chemical precipitation of heavy metals is the most commonly applied process for their removal from metal containing wastewater as it offers the most economical solution in large scale applications (Djedidi et al., 2011). In a precipitation process, soluble heavy metals in wastewater can be precipitated in different forms such as hydroxide, phosphates, carbonates, and metal sulfides. Hydroxide precipitation is a widely applied method of chemical precipitation, however, the method has operational limitations since it generates high volumes of sludge that are characterized by poor thickening and dewatering properties (Peters & Shem, 1993).

Chemical sulfide precipitation has more advantages qualities than that of hydroxide precipitation because of its ability to form highly insoluble precipitates that offer better settling, thickening, and dewatering characteristics. In addition, different solubility products of various metal sulfides at different pH levels could be considered as another advantage since this offers the possibility of selective metal recovery (Tabak, Scharp, Burckle, Kawahara, & Govind, 2003; Denys Kristalia Villa Gómez, 2013). Chemical sulfide precipitation, however, has its own limitations due to transport and handling challenges of sulfide reagents such as Na_2S and CaS as well as their high costs (Mallelwar, 2013). These limitations in the application of chemical sulfide precipitation of heavy metals have led researchers toward using an alternative source of sulfide in sulfide precipitation, i.e., biogenic sulfide produced during the anaerobic sulfate reduction process.

1.2 Removal of Heavy Metals by Biogenic Sulfate Reduction Process

Biological anaerobic sulfate reduction to sulfide by sulfate reducing bacteria (SRBs) is an attractive and cost effective treatment method for neutralization and metal removal from metal laden industrial wastewaters such as AMD. The entire process of biological sulfate reduction and metal removal can be summarized in the following steps (Costa, Martins, Jesus, & Duarte, 2008; Neculita & Zagury, 2008):

$$\text{Organic matter (C, H, O)} + SO_4^{2-} \rightarrow HS^- + HCO_3^- \quad (1\text{-}1)$$

$$M^{2+} \text{ (Metal Cation-}Fe^{2+}, Cu^{2+}, Zn^{2+} \text{ etc.)} + HS^- \rightarrow \text{MS (Metal sulfide)} \downarrow + H^+ \quad (1\text{-}2)$$

Biological sulfate reduction for heavy metal removal from industrial wastewaters has been applied as two major process configurations: the single stage process and the two stage process (Al-Ani, et al., 1995; Villa Gómez, 2013). In a single stage process, both the biogenic sulfate reduction and the metal precipitation happen in a single reactor. In contrast, in the two stage process, biogenic sulfide is generated in the biological reactor and then moved to a second reactor called a chemical reactor that is fed with metal-laden wastewater. Precipitation happens within the chemical reactor.

The single stage process is simpler than the two stage process. Additional advantage of the single stage process is that since the biogenic sulfide produced is removed as insoluble metal sulfide precipitates, the potential for the effect of sulfide toxicity on biogenic sulfate reduction is avoided. However, the metal precipitate formed has been shown to be inhibitory to biogenic sulfate reduction (Utgikar et al., 2002; Kieu et al., 2011). This inhibition has been attributed to the metal precipitate being a physical barrier to SRB for substrate (Utgikar et al., 2002). This inhibition is avoided in the two stage process due to the separation of the biogenic sulfate reduction process stage from the metal precipitation stage. Given the advantages of the single stage process, if the inhibition caused by the metal precipitate can be reduced or eliminated to allow for sulfate reduction rates to be similar to the two stage process, it can be expected to be more cost-effective than the two stage process for the treatment of metal laden industrial wastewaters.

Unlike the effects of dissolved metal concentrations, inhibitory effects due to the formation of metal precipitates have not been well understood and quantified. There have been a limited number of investigations on the effects of metal precipitates on the efficiency of the single stage process. Utgikar, et al. (2002) qualitatively demonstrated that the metal precipitates adversely affect the process in stirred batch reactors. Kieu, et al. (2011) studied the effect of metal loading as an inhibitory process in semi-continuous stirred tank reactors (SCSTR). It should be noted that the differentiation between dissolved and precipitated metals' inhibitory effects is not discussed in their study.

Thus, investigating possible modification of the single stage process to overcome the inhibitory effect of the metal precipitate formed is worthwhile. First, better understanding and quantification of possible inhibitory effects due to the formation of metal precipitates should be helpful in this effort.

Unlike dissolved heavy metals that can adversely affect the microorganisms through the disruption of enzymes, the mechanism of inhibition by precipitates has been reported to be external to the microorganisms (Utgikar et al., 2002). Thus, it can be postulated that the inhibition by heavy metal precipitates is independent of the type of heavy metal. Among different heavy metals, copper, arguably, has the largest application in the industrial sector and copper mining has been reported to generate more than half of the wastes produced during the various metal mining operations (United States Congress, 1988). Thus, in this study copper was selected as the representative heavy metal to evaluate the effect of the metal precipitates on the performance of sulfate reduction process.

1.3 Objectives

The objectives of this study were to:

- Confirm and quantify the inhibitory effect of metal precipitates on a single stage anaerobic sulfate reduction process
- Investigate the alternate process configuration to overcome the inhibitory effect in a single stage process
- Compare the process efficiency with the two stage process

- Evaluate the metal precipitation mechanisms in anaerobic sulfate reduction systems

1.4 Scope of the Work

The scope of the study was to:

- Operate SCSTR reactors at different metal loadings
- Determine the inhibitory effect of metal precipitates on biogenic sulfate reduction
- Quantitatively evaluate the inhibition process
- Establish a new process configuration that considered:
  - Reactor design
  - Establishing an efficient sulfate reduction environment
  - Creating a single stage (internal) metal precipitation
  - The effects of HRT and metal loading rates
  - Evaluating the system's metal precipitation capacity
- Investigate the single stage and two stage metal precipitation processes in parallel
- Compare the single stage and two stage concepts for biogenic sulfate reduction and metal precipitation

Chapter 2

Literature Review

This section discusses the following subjects: the causes of heavy metal contamination of water; the treatment options available to remove heavy metals from wastewater; the biological treatment of wastewater polluted by heavy metals; the unit processes and configurations employed for the biological treatment of wastewater; and the drawbacks that limit the use of biological treatments of metal removal from wastewater and affect their efficiency.

2.1 Sources of Heavy Metal Contamination

Heavy metals are characterized by their high atomic weight and specific gravities, which are more than five times greater than water's (Tchounwou, et al., 2012). The most significant sources of heavy metals in the environment come from natural and anthropogenic sources (Bradl, 2005). The industrial, agricultural, and technological applications of various compounds containing heavy metals have led to environmental problems whereby excess heavy metals have been discharged into the environment. Metals are also discharged into the environment from natural sources. Their excess affects ecological systems and endangers the public health. An understanding of the sources and causes of heavy metal contamination is of great significance to combat these problems.

2.1.1 Natural Sources of Heavy Metals

The geology and geochemistry of any specific region determines the chemistry of its soil and the composition of heavy metals in its environment. Surface and groundwater are natural barriers of heavy metals. Water is chemically influenced by the rocks and soils that comes into contact and react with (Alloway, 1995). Heavy metals are also transported to the atmosphere as gases, aerosols, and particulates; these forms are other sources of heavy metals that are present in the environment (Biegalski, et al., 2013). Natural sources of heavy metals are not the subject of this review as they are not influenced by human activities.

2.1.2 Anthropogenic Sources of Heavy Metals

Heavy metals are released into the environment through human activities (i.e., anthropogenically). The main anthropogenic sources of heavy metals in the environment originate from: agricultural activities; mining activities; mineral processing plants; industrial plants, such as metal refineries; metal finishing; energy production; electronic products; and waste disposal (Alloway, 1999; Bradl, 2005).

2.1.2.1 Agricultural Activities

The world's rapid population growth has led to increased agricultural activities such as the intensive use of fertilizers, pesticides, and other contaminants. Soil amendments such as sewage sludge, animal manure, or dredged sediments are also used for agricultural purposes. Such practices cause soil contamination with heavy metals. The mobility of the heavy metals in these sources can affect soil and subsequently surface and groundwater (Bradl, 2005).

Heavy metals are part of the active compounds of pesticides. An excessive use of pesticides can contribute to the accumulation of heavy metals in farm soil (Gimeno-García, Andreu, & Boluda, 1996). Additionally, fertilizers in any form contain trace amounts of heavy metals. Their repeated application can lead to the accumulation of heavy metals in soil. Among these heavy metals, cadmium (Cd) causes the greatest concern. However, heavy metals such as arsenic (As), lead (Pb), chromium (Cr), nickel (Ni), mercury (Hg), and vanadium (V) may also accumulate in soil through the excessive and repeated use of fertilizers (Mortvedt, 1995). Soil amendments that are meant to enrich the nutritional value of farm soil can also contribute to the contamination of soil with heavy metals. Although there have been efforts to mitigate the release of heavy metals from soil, amendment aids such as mature compost and fresh manure, residual and soluble heavy metal complexes are present in soil and affect bodies of water by leaching into the local and regional groundwater (Walker, Clemente, Roig, & Bernal, 2003).

2.1.2.2 Industrial Wastes

Heavy metals are discharged into the environment through the wastestreams of various industries. Heavy metals such as Cu, Cr, Pb, Zn, Ni, Cd, and V are used by the electroplating, coating, etching, anodizing, and milling industries. The electroplating industry and metal surface treatment processes are considered as significant contributors to the environmental heavy metal pollution problem (Barakat, 2011). Printed circuit board (PCB) manufacturing is also considered a significant source of waste that contains heavy metals. PCBs contain 26 % Cu, Pb, tin (Sn), Cd, and Ni compounds; the waste released from their respective manufacturing plants imposes a risk of heavy metal contamination (Bizzo, Figueiredo, & de Andrade, 2014). Additionally, wood-preserving industries that treat woods with an aqueous solution of Cu, Cr, Zn, and As produce arsenic-bearing wastes (Bhattacharya, Mukherjee, Jacks, & Nordqvist, 2002). Inorganic pigment plants can discharge high concentrations of Cd and Cr while petroleum refineries can release high concentrations of Ni, V, and Cr. These industries generate high volumes of wastestreams that discharge these metals into the environment. The result are adverse effects on ecosystems and great risks to public health such as cancer/tumor development and newborns defects (Barakat, 2011).

2.1.2.3 Domestic Waste Streams

Although not significantly, domestic waste streams are also an important cause of the environmental heavy metal contamination. Household waste streams, institutional wastes, drainage waters, atmospheric depositions, and traffic and building materials can contribute to the discharge of heavy metals into the environment through domestic waste streams. The release of heavy metals such as Cu from tap water and roof runoff also occurs (Sörme & Lagerkvist, 2002). Zn and Hg can be found in the waste streams of operations such as carwashes and dentistry, respectively. Additionally, heavy metals can be discharged through the application of essentials of the human life such as car brake linings, tires, and gasoline (Sörme & Lagerkvist, 2002).

2.1.2.4 Mining Activities

The mining industry is one of the largest producers of heavy metal waste (Johnson & Hallberg, 2005). The total amount of waste generated by a mining activity depends on the

type and the nature of the mine and the size of the operation. In order to extract a valuable mineral, a significant amount of rocks must be excavated and large volume of wastes are produced. In Cu mining, 99 tons of waste rock must be removed to extract one ton of Cu. The amount of waste that is generated in gold mining is even larger (Environmental Mining Council of British Columbia, 1997). Stockpiled waste rocks that contain sulfides, heavy metals, and other pollutants are significant sources of contaminants. Their exposure to water and climatological conditions forms an acidic, sulfate-rich waste stream known as acid mine drainage (AMD) (Environmental Mining Council of British Columbia, 1997). AMD is also discharged from the abandoned mines that are exposed to climatological conditions such as precipitations and temperature changes over the course of the time after their closure (Bless et al., 2008).

Although harmful due to its content, the discharge of wastewater rich in acid and sulfur can have additional adverse effects on the environment and the public health as it often contains high concentrations of heavy metals (Johnson & Hallberg, 2005). Many of the metals in mines are in the form of sulfide and are accompanied by large amounts of iron sulfide (i.e., pyrite [FeS_2], considered a gangue mineral) (Fripp, et al., 2000). AMD causes minerals to oxidize and form acid-soluble minerals (Johnson, 2003). A major cause of the formation of acid is the oxidation of pyrite, as shown in Equation (2-1) (Johnson & Hallberg, 2005).

$$4FeS_2 + 15O_2 + 14H_2O \rightarrow 4Fe(OH)_3 + 8SO_4^{2-} + 16H^+ \quad (2\text{-}1)$$

Metal producing mines with ore that contains sulfide minerals generate metal-containing acidic discharge (AMD). In such cases, the predominant metal ions are commonly Cu, Zn, Ni, Cd, Pb and Cr, but not iron (Fe), that is present in the form of pyrite (United States Congress, 1988). Therefore, depending on the mineralogy of an ore, the nature, characteristics and volume of AMD may vary from site to site.

In general, AMD is characterized by a low pH and high concentrations of various heavy metals and other toxins that adversely affect soil, surface water, and groundwater (Akcil & Koldas, 2006). AMD may form during surface or underground mining operation. The movement of AMD causes adverse effects on surface water. In regards to groundwater, AMD may have less of an adverse effect during active mining as water tables are kept

low by artificial pumping. However, after these mines are closed, their contamination of groundwater can become an unavoidable environmental issue (Naicker, Cukrowska, & McCarthy, 2003).

Copper ore mining and beneficiation plants are major producers of liquid and solid wastes, among other mining activities. A report released by the United States Environmental Protection Agency (USEPA) estimated that copper production processes contributed half of the 40 billion tonnes of waste generated by metallic ore mining and processing industries between 1910 and 1981 (United States Congress, 1988).

2.2 Treatment Options for Wastewater Containing Heavy Metals

Unlike organic contaminants, heavy metals are not biodegradable and are some of the most significant and concerning environmental contaminants. Trace concentrations of heavy metals are required for the function of human organs, but high concentrations can have serious adverse health effects (WHO, 1996). Table 2-1 presents the health problems these metals cause when their concentrations in drinking water exceed the permissible concentrations given in Table 1-1.

Table 2-1 Health concerns of various heavy metals (Barakat, 2011)

Heavy Metal	Health Concern
Copper (Cu)	Liver damage, Wilson's disease, insomnia
Zinc (Zn)	Depression, lethargy, neurological signs like Autism and increased thirst
Lead (Pb)	Fetal brains, diseases of the kidneys, circulatory system, and nervous system
Nickel (Ni)	Dermatitis, nausea, chronic asthma, coughing, human carcinogen
Cadmium (Cd)	Kidney damage, renal disorder, human carcinogen
Chromium (Cr)	Headache, diarrhea, nausea, vomiting, carcinogenic

A variety of treatment technologies have been developed to remove heavy metals from metal-laden wastewater to meet these regulations and standards.

2.2.1 Adsorption

Adsorption is an effective method for the treatment of wastewater contaminated by heavy metals. It is a promising method which allows the possible recovery of sorbents through the application of a suitable desorption process (Fu & Wang, 2011). Applying a granular activated carbon in a fixed bed column can almost completely remove Cd and Pb (Jusoh, Shiung, & Noor, 2007). Researchers have studied the most effective condition for applying acid- and base-modified granular activated carbon and activated carbon fibers at their optimal sorption capacities to remove Cu and Cd from wastewater (Kang, Kim, Choi, & Kwon, 2008). Heavy metal sorption has been carried out using carbon nanotube (CNT) adsorbents. CNTs have the potential to remove heavy metal ions such as Pb, Cd, Cr, Cu, and Ni from industrial wastewater, specifically after the wastewaters have been modified by oxidation agents such as nitric acid (HNO_3) and potassium permanganate ($KMnO_4$) (H. Wang, Zhou, Peng, Yu, & Yang, 2007). The relatively high cost of activated carbon has spurred researchers to find low-cost adsorbents. Agricultural wastes and industrial by-products such as lignin, diatomite, and lignite; and natural substances such as natural zeolite, kaolinite, and montmorillonite have been studied as cheap sorbents (Fu & Wang, 2011). Biosorption has shown promise regarding to removal of heavy metal ions from wastewater. Examples of biosopents include chitinous organisms such as shrimp; crab shells; algal biomasses; and microbial communities such as bacteria, fungi, and yeast (Apiratikul & Pavasant, 2008). Non-living plant materials such as potato peels, seed shells, coffee husks, and citrus peels have been studied as efficient sorbents (Aman, Kazi, Sabri, & Bano, 2008; Amuda, Adelowo, & Ologunde, 2009; Oliveira, Franca, Alves, & Rocha, 2008; Schiewer & Patil, 2008). The sorption of heavy metals can be done with aqueous solutions of microorganisms such as *Bacillus cereus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Fu & Wang, 2011). The use of biosorbents is still in its experimental phase and has not been industrialized due to its limitations such as difficulties in their removal from the system after adsorption.

2.2.2 Membrane Filtration

Membrane filtration is another treatment method used to remove heavy metals from wastewater (Fu & Wang, 2011). Reverse osmosis, ultrafiltration, nanofiltration, and

electrodialysis are membrane processes that efficiently remove heavy metals from wastewater (Fu & Wang, 2011). Ultrafiltration was introduced in 1980 to remove multivalent metal ions from wastewater (Landaburu-Aguirre, García, Pongrácz, & Keiski, 2009). Although it is highly efficient and selective when removing high concentrations of heavy metals from multi-metal contaminated aqueous streams, ultrafiltration has not been yet developed on an industrial scale (Fu & Wang, 2011). Reverse osmosis (RO) is a process that is being used for water desalination (Fu & Wang, 2011). There have been several studies of the process's potential to remove heavy metal ions from wastewater. Ni and Zn can be removed from aqueous solutions with a high degree of efficiency (Ipek, 2005). Mohsen-Nia, et al. (2007) reported that Ni and Cu can be removed from wastewater with more than 99 % efficiency (Mohsen-Nia, Montazeri, & Modarress, 2007). Additionally, RO has been applied with nano-filtration (NF) as an appropriate treatment alternative. The process has been used to remove low concentrations of Cu with high removal efficiencies of 95 % to 99 % (Sudilovskiy, Kagramanov, & Kolesnikov, 2008). This combined process successfully removed high concentrations of 2000 mg/L of Cu (Cséfalvay, Pauer, & Mizsey, 2009). Nanofiltration (NF) is a membrane technology that has been widely investigated in regards to removing heavy metals from aqueous solutions. The process is a low-cost and efficient technology and removes heavy metals ions such as Cu, Ni, and Cr (Fu & Wang, 2011). Another membrane technology is electrodialysis (ED). Its driving force is an electric field across a charged membrane. The membrane can have cation or anion exchange properties. The process is promising in regards to recovering metals from wastewater. Mohammadi et al. (2004) studied the efficiency with which ED removes Pb from wastewater. Process parameters such as feed and product concentration, current density, membrane permselectivity, and flow velocities were investigated to establish an efficient ED process (Mohammadi, Razmi, & Sadrzadeh, 2004). ED has been employed for the removal of trivalent chromium (Lambert, Avila-Rodriguez, Durand, & Rakib, 2006). A study showed the importance of pretreatment to remove suspended solids, fats, and proteins that hinder the effective removal of the Cr. The process is very sensitive to pH adjustments as a low pH will decrease the current efficiency and a high pH may lead to precipitation. ED was successfully used on a pilot scale to remove hexavalent chromium

and produce good quality drinking water (Nataraj, Hosamani, & Aminabhavi, 2007). The process has proven to be promising in regards to recovering Cu and Fe from an aqueous solution during a copper electrowinning process (Fu & Wang, 2011).

2.2.3 Coagulation-flocculation

Coagulation-flocculation is a major wastewater treatment unit operation process that is usually applied prior to sedimentation to remove heavy metals from aqueous solutions (Fu & Wang, 2011). Coagulants such as ferrous sulfate and ferric chloride have been applied to remove impurities from aqueous solutions. El Samrani, et al. (2008) reported high heavy metal removal efficiencies using ferric chloride and a pre-hydrolyzed aluminum coagulant. As coagulants do not effectively remove dissolved substances, it was suggested that a new kind of coagulant be applied to remove soluble heavy metals and insoluble particulates (El Samrani, Lartiges, & Villiéras, 2008). It was suggested that heavy metals such as Pb and Zn be bound to humic acid (HA) to form metal-HA complexes and that coagulation-flocculation be performed with cationic polyelectrolyte polydiallyldimethylammonium chloride as an alternative process (Hankins, Lu, & Hilal, 2006). Coagulating by grafting the sodium xanthogenate group to polyethyleneimine has shown promise in regards to removing the Ni^{2+} ion from wastewater (Chang & Wang, 2007). Flocculation with the conventional flocculants such as polyacrylamide and polyferric sulfate has not been very successful in regards to heavy metal removals. However, the application of a new generation of macromolecules such as those suggested by Chang, et al. (2009), Bratskaya, et al. (2009), and Duan, et al. (2010) has resulted in efficient heavy metal removal (Bratskaya, Pestov, Yatluk, & Avramenko, 2009; Q. Chang, Zhang, & Wang, 2009; Duan et al., 2010). In general, coagulation-flocculation must be followed by other treatment processes to remove heavy metals from wastewater (Fu & Wang, 2011).

2.2.4 Ion Exchange

Ion exchange resins, exchange cations in their structures with heavy metal ions from aqueous solutions. The resins are widely used to remove heavy metals from wastewater due to their high treatment capacity, removal efficiency, and rapid kinetics (Fu & Wang, 2011). Acidic resins with sulfonic acid groups ($-SO_3H$) and carboxylic groups (-COOH)

are the most common ion exchangers used in wastewater treatment processes. During treatment processes, these resins exchange their cations with heavy metal ions present in wastewater, as shown in Equation (2-2) and Equation (2-3):

$$nR - SO_3H + M^{n+} \rightarrow (R - SO_3^-)_n M^{n+} + nH^+ \quad (2\text{-}2)$$

$$nR - COOH + M^{n+} \rightarrow (R - COO^-)_n M^{n+} + nH^+ \quad (2\text{-}3)$$

The efficiency of ion exchange processes depends on parameters such as pH, temperature, contact time, and initial concentration. Synthetic resins, natural zeolites, and silicate minerals such as montmorillonite [$(Na,Ca)_{0.33}(Al,Mg)_2(Si_4O_{10})(OH)_2 \cdot nH_2O$)] show potential in regards to being used for ion exchange processes (Fu & Wang, 2011; Wingenfelder, Hansen, Furrer, & Schulin, 2005).

2.2.5 Flotation

Flotation originated in the mineral processing industry. It is the process of using bubble attachment phenomena to carry particles up to the surface of a solution to be collected and removed (Pryor, 1965). Specific flotation technologies such as dissolved air flotation (DAF), ion flotations, and precipitate flotations have been employed to remove metal ions from aqueous solutions (Fu & Wang, 2011). DAF is a relatively old process that has been applied to remove suspended particles. Ion flotation is a flotation technology that employs specific surfactants to create hydrophobicity at the surface of ionic metal species. Hydrophobic species can then be removed by air bubbles (Fu & Wang, 2011). Polat and Erdogan (2007) optimized the flotation process efficacy in removing Cu, Zn, and trivalent chromium from wastewater. While no more than 75 % of metal removal was achieved at a low pH, almost 90 % of metallic ions were removed at a high pH. This was attributed to the contribution of another flotation technology called precipitate flotation (Polat & Erdogan, 2007). Cd, Pb, and Cu were separated from an aqueous solution using ion flotation and had removal rates of approximately 70 %, 90 %, and 80 %, respectively (Yuan, Meng, Zeng, Fang, & Shi, 2008).

2.2.6 Electrochemical Treatment

The electrochemical treatment of metal-containing wastewater is considered when the recovery of metals is of significance. Also known as electrodeposition, electrochemical

treatment is executed with the deposition of metals on a cathodic electrode's surface. While a large capital investment and a sizeable electricity supply are necessary for the treatment, and have thus narrowed electrodeposition's application in wastewater treatment, the establishment of stringent environmental regulations in the last few decades has caused the treatment to regain consideration as a viable approach (Fu & Wang, 2011). Electrocoagulation (EC) and electroflotation (EF) are alternative technologies that have been widely used in removing heavy metals from wastewater. Electrocoagulation shows promise regarding the removal of heavy metals such as hexavalent chromium (Cr [VI]), Zn, Cu, and Ni from wastewater with initial metal concentrations ranging from 50 mg/L to 5000 mg/L (Heidmann & Calmano, 2008). Additionally, the complete removal of Ni and Zn was achieved by applying a current density of 9 mA/cm^2 during electrocoagulation (Kabdaşlı, Arslan, Ölmez-Hancı, Arslan-Alaton, & Tünay, 2009). Electrofiltration has an effective rate when removing Ni, Cu, Zn, Pb, and Cd from wastestreams. Modified EF processes have been studied to remove Ni and Fe from the metal finishing industry's wastewater (Sun, Miznikov, Wang, & Adin, 2009).

2.2.7 Precipitation

Precipitation is the most widely used treatment technology in regards to removing toxic heavy metals from industrial wastewater before the metals are discharged into bodies of water. The precipitation of heavy metals is divided into two major categories: chemical precipitation and biological precipitation (Mallelwar, 2013). The chemical precipitation of heavy metals is performed by adding a chemical reagent to form an insoluble precipitate of heavy metals. Biological precipitation is a promising precipitation alternative that uses a biogenic sulfide produced during an anaerobic sulfate reduction process to remove heavy metals as an insoluble metal sulfide.

2.2.7.1 Chemical Precipitation

Chemical precipitation is the most traditional and industrially adapted method of removing heavy metals from waste streams. The process is relatively inexpensive and easy to perform (Fu and Wang, 2011). Although a few studies have been carried out on heavy metal precipitation in a phosphate form (Mavropoulos et al., 2002; Pigaga,

Juškėnas, & Selskis, 2002), the precipitation process is mainly performed using the forms of hydroxide, carbonate, and sulfide, as described below.

a. Hydroxide Precipitation

The precipitation of heavy metals in hydroxide form is performed by adding a chemical reagent such as CaO, Ca $(OH)_2$, $Mg(OH)_2$, NaOH, or NH_4OH (Djedidi, et al., 2011). A general equation for the precipitation is below:

$$M^{n+} + nOH^- \leftrightarrow M(OH)_n \tag{2-4}$$

Hydroxide precipitation has some limitations that affect its efficiency. Heavy metals show amphoteric behaviour that results in a high solubility in low and high pH values, as shown in Figure 2.1. This causes a minimum solubility of metal hydroxide at specific pH values (Simon, Meggyes, & McDonald, 2002). The desirable pH range for most of heavy metals is 8.5–9.5; it is required to achieve optimal precipitation.

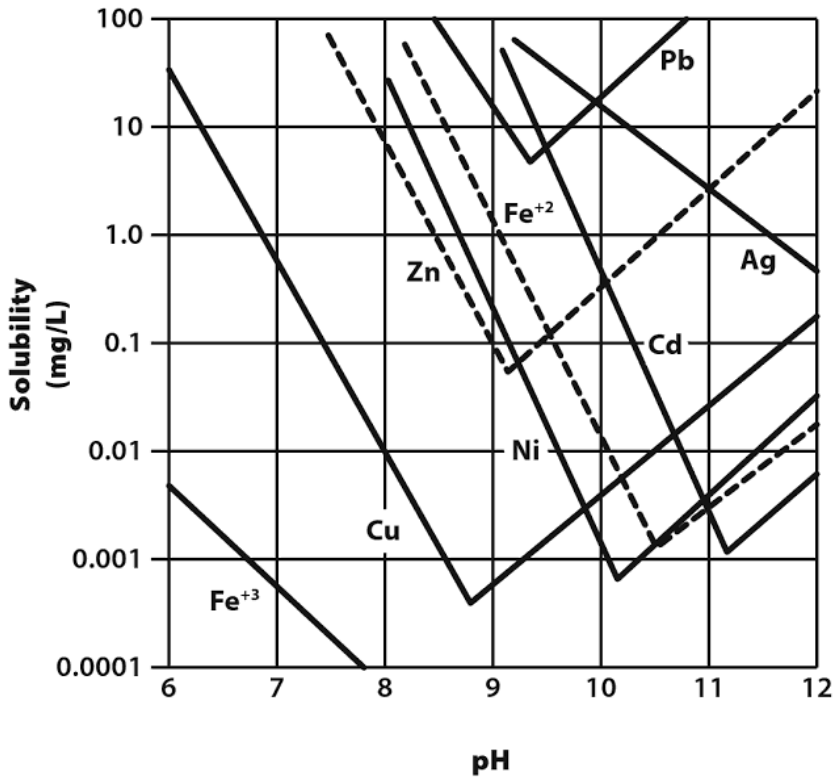


Figure 2-1 Solubility of metal hydroxide as a function of pH (modified after USEPA, 1983)

Lime ($Ca[OH]_2$) is the most common reagent of hydroxide precipitation. Lime is produced from quick lime (CaO). Continuous on-site stirring during hydroxide preparation increases the amount of energy consumed (Armenante, Nagamine, & Susanto, 1998).

Hydroxide precipitation has poor sludge-settling characteristics and produces a high volume of low-density sludge in comparison to other precipitation alternatives (Fu & Wang, 2011). The sludge volume during sulfide precipitation is, for example, 6 to 10 times lower than that of hydroxide precipitation (Huisman, Schouten, & Schultz, 2006). These limitations have resulted in researchers looking for alternative processes that offer more favorable sludge dewatering and thickening characteristics, and which lower the residual soluble metal concentration. The most common alternative processes include carbonate precipitation and sulfide precipitation.

b. Carbonate Precipitation

Carbonate precipitation using soda ash (sodium carbonate) is an alternative to hydroxide precipitation, especially when Pb, Zn, Ni, and Cd are targeted (Patterson, Allen, & Scala, 1977). The hydroxide precipitation of some heavy metals happens when pH values of more than 10 demands a high treatment cost due to the need for a pH adjustment. Carbonate precipitation occurs in lower pH values. Additionally, metal carbonates are denser than metal hydroxides and offer a more favorable solid separation and decent dewatering characteristics (Patterson et al., 1977).

Although carbonate precipitation provides some benefits, it is not as efficient as other methods of precipitation for removing most of the heavy metals in terms of residual heavy metal concentrations (Peters & Shem, 1993). Carbonate precipitation yields a favorable heavy metal removal efficiency as a co-precipitation process for other precipitation techniques such as hydroxide precipitation (Peters, Young, & Bhattacharyya, 1985).

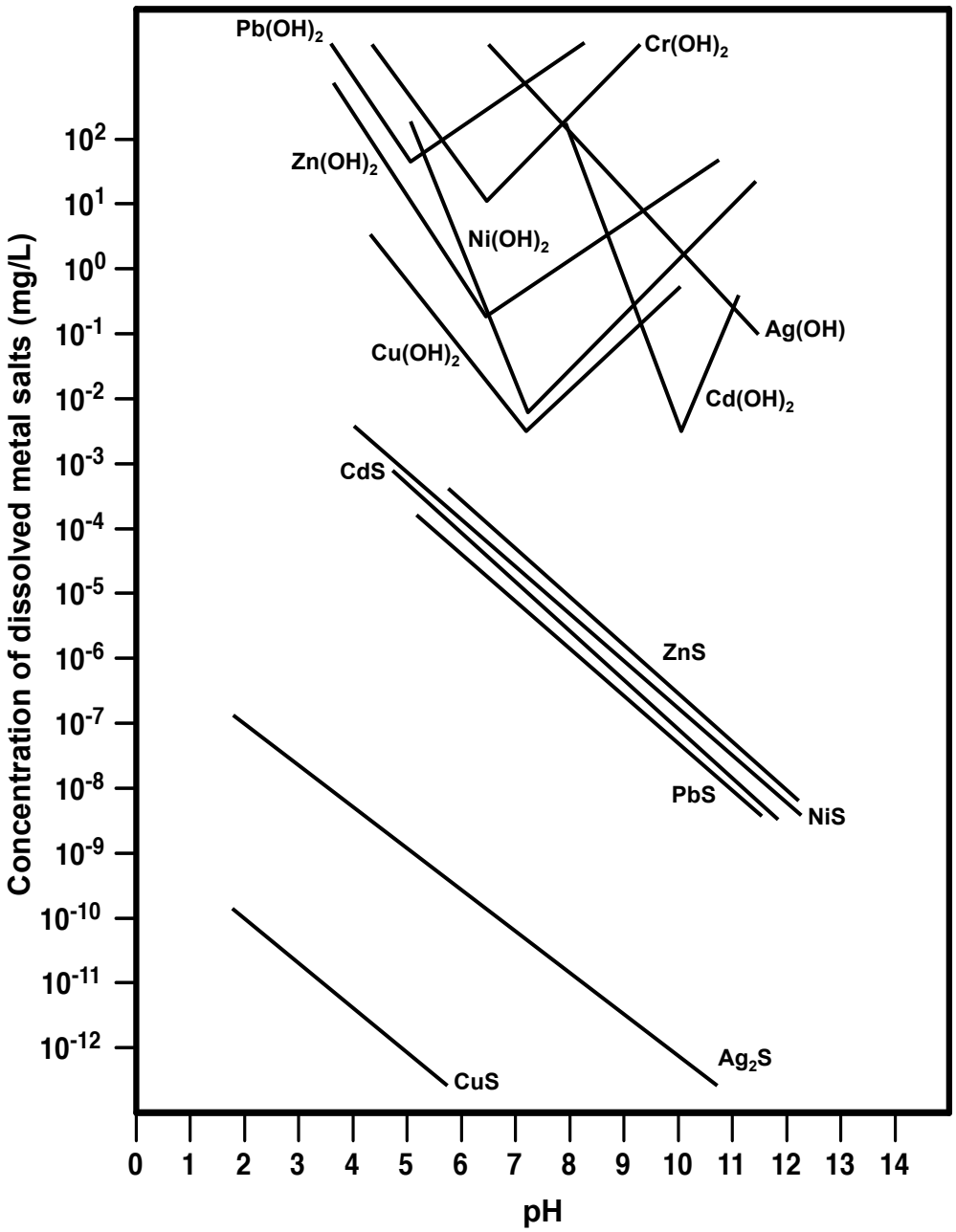


Figure 2-2 Solubility of metal sulfides and hydroxides as a function of pH (modified after USEPA, 1980)

c. Sulfide Precipitation

Sulfide precipitation is an effective alternative to hydroxide precipitation. Sulfide precipitation offers several advantages over other precipitation processes. As previously noted, sulfide precipitation produces less sludge than hydroxide precipitation and results in more favorable dewatering characteristics (Huisman, Schouten, & Schultz, 2006;

Djedidi, et al., 2011). It is an efficient process for removing heavy metals, even at low pH ranges (Peters & Shem, 1993). Figure 2-2 compares the solubility of heavy metal sulfides and hydroxides as a function of pH.

The solubility products of metal sulfides are less than those of hydroxides and carbonates. This increases the chance that the precipitation of heavy metals will be in sulfide form instead of any other form (El Bayoumy, 1997; Villa Gómez, 2013). Table 2-2 shows the solubility products of various heavy metal precipitates (El Bayoumy, 1997). Selectively removing heavy metals to enhance their recovery is a benefit of the sulfide precipitation process (Peters & Shem, 1993).

The low solubility of heavy metal sulfides yields low residual metal concentrations, resulting in a high efficiency treatment process. Metal sulfide precipitation is less affected by complexes and chelating agents than are hydroxide processes (Peters & Shem, 1993).

2.2.7.2 Biogenic Sulfide Precipitation

Chemical sulfide precipitation has some disadvantages that limit its application. There are concerns with regards to its sulfide toxicity (Peters & Shem, 1993) and the generation of hydrogen sulfide (H_2S) gas fumes in acidic conditions (Fu & Wang, 2011).

Table 2-2 Molar solubility products of metallic compounds (El Bayoumy, 1997)

Compounds	Solubility Product
$Cu(OH)_2$	$1.0x10^{-19}$
$Ni(OH)_2$	$6.5x10^{-18}$
$Pb(OH)_2$	$3.0x10^{-16}$
$Zn(OH)_2$	$1.2x10^{-17}$
$CuCO_3$	$1.0x10^{-10}$
$NiCO_3$	$6.6x10^{-9}$
$PbCO_3$	$3.3x10^{-14}$
$ZnCO_3$	$1.4x10^{-11}$
Cu_2S	$3.0x10^{-48}$
CuS	$6.0x10^{-36}$
NiS	$1.0x10^{-28}$
PbS	$1.0x10^{-28}$
ZnS	$2.0x10^{-24}$

The process of sulfide precipitation has to be performed in a precisely controlled environment and, as a result, it is an expensive heavy metal removal method. The application of expensive and difficult to handle chemicals such as sodium sulfide (Na_2S), as noted in the literature (Mallelwar, 2013), is another limitation of the method. For the past few decades, heavy metal precipitation using a biogenic sulfide, produced during a biological sulfate reduction with sulfate reducing bacteria (SRB), has been considered an effective and economically viable alternative to chemical sulfide precipitation. The fundamentals of the biological conversion of sulfate to sulfide, the engineering aspects of the process, the process limitations, and available alternatives to address the process limitations are discussed below.

2.3 Biological Conversion of Sulfate to Sulfide

Sulfate-reducing bacteria (SRB) are a specific group of anaerobes that under certain environmental conditions oxidize a carbon source to reduce the present sulfate ions (SO_4^{2-}) to sulfide by a dissimilatory metabolism (Villa Gómez, 2013). This is shown in Equation (2-5), where CH_2O is an electron donor (Neculita & Zagury, 2008):

$$SO_4^{2-} + 2CH_2O \rightarrow H_2S + 2HCO_3^- \quad (2\text{-}5)$$

SRB are prokaryotes that are found in all environments as they tolerate extreme conditions. They can be heterotroph, with an electron donor that is an organic compound, or autotroph, with an electron donor of hydrogen and a carbon source of carbon dioxide (CO_2) (Villa Gómez, 2013). Other than the presence of an electron donor and an electron acceptor (sulfate), the most favorable conditions for SRB are a low oxidation reduction potential (ORP) of less than -100 mV (Postgate, Kent, Robson, & Chesshyre, 1984), a pH ranging from 6–9 (Widdel & Bak, 1992), and a temperature ranging from 31–43ºC (Okabe, Nielsen, Jones, & Characklis, 1995). Widdel (1988) classifies SRB as two major groups: (1) Those that completely oxidize the carbon source to CO_2; and (2) Those that produce acetate, CO_2, and H_2 by oxidizing the carbon source. SRB are usually involved in the last mineralization stage, but they are also capable of initially fermenting sugars and amino acids (Villa Gomez, 2013).

To better understand the anaerobic sulfate reduction process, knowledge of the concept of anaerobic digestion is necessary. Different micro-organisms are involved in the anaerobic digestion process and, more significantly, competition between methane-producing bacteria (MPBs) and SRB adversely affects the efficiency of the sulfate reduction process (Sharma & Biswas, 2010). A major challenge of maintaining an efficient sulfate reduction process is in inhibiting methanogens to divert reducing equivalents toward the reduction process. Figure 2.3 shows the stages of the anaerobic digestion of organic matter and the rule of SRB during the process.

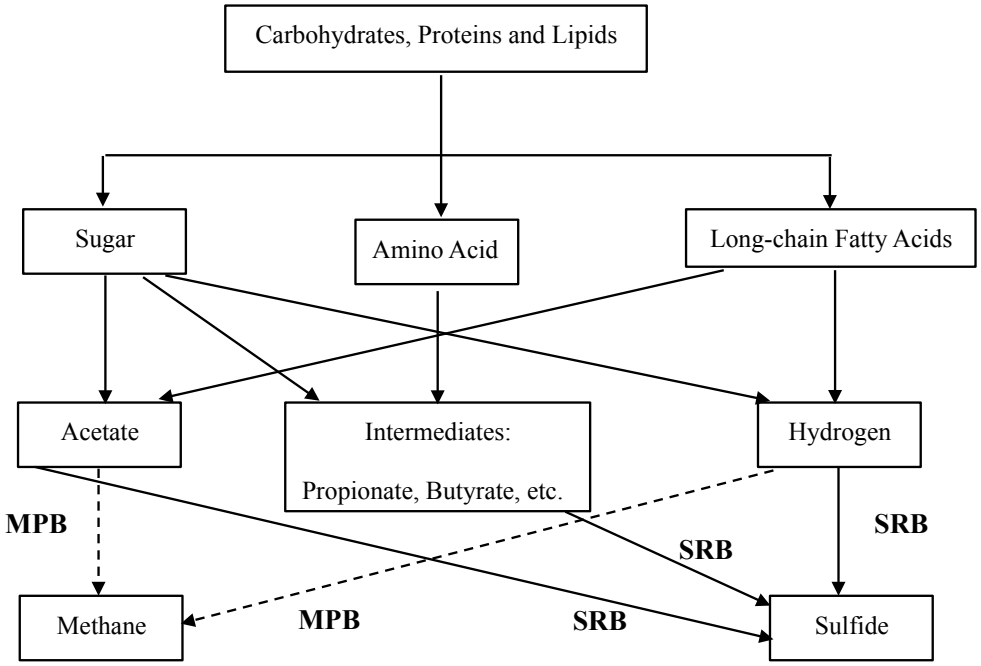


Figure 2-3 Anaerobic digestion of organic matter (modified after Gujer & Zehnder, 1983)

A group of hydrolytic micro-organisms, acidogens, acetogens, methanogens, and sulfate reducers complete the anaerobic digestion process in four steps: acidogenesis; acetogenesis; methanogenesis; and/or sulphidogenesis (Bagley & Brodkorb, 1999; A. Veeken, Kalyuzhnyi, Scharff, & Hamelers, 2000). As a result of complete mineralization, the major end products of the process are methane or sulfide, biomass, water, and carbon dioxide (CO_2). In the anaerobic treatment of sulfate-rich wastewater such as AMD, sulfate acts as an alternative terminal acceptor. In such conditions methane is not formed as a major end product. However, competition between the different substrates (electron donors) through the co-existence of MPBs and SRB cannot be avoided. It affects the

sulfate reduction process. In any sulfate reduction reactor, maintaining a proper equilibrium between acid production and acid consumption is of great significance as doing so can prevent the accumulation of volatile fatty acids (VFAs) inside the reactor; and VFAs cause the system to fail (Biswas, 2012). Competition between SRB and MPBs is governed by parameters such as pH, temperature, hydraulic retention time (HRT), and chemical oxygen demand/sulfate (COD/SO_4^{2-}) ratio. These factors are important in regards to the efficiency of sulfate reduction to sulfide, and they are discussed below.

2.3.1 Effects of pH

The optimal pH ranges 7.3–7.6 and 6.5–7.8 have been reported for most species of SRB and MPBs, respectively (Vogels, Kejtjens, & Van der Drift, 1988; Widdel & Bak, 1992). SRB generally have a higher growth rate at higher pH values than MPB (Omil, Elferink, Lens, Pol, & Lettinga, 1997). The pH value at which SRB can survive is 10 while the pH value at which MPBs can survive is 8.5 (Biswas, 2012). Other than the direct effect that pH has on the growth of SRB and MPBs, indirectly pH has effects that result in an inhibitory concentration of sulfide on these micro-organisms and those effects should be considered.

2.3.2 Sulfide Inhibition

The accumulation of a high concentration of sulfide inside the anaerobic reactor can inhibit both sulfate reduction and methane production processes. The mechanism of sulfide inhibition is not yet understood. It may occur through a denaturation of essential enzymes (PNL Lens, Visser, Janssen, Pol, & Lettinga, 1998); the deficiency of the cells' required metallic nutrients due to metal sulfide precipitation (Reis, Almeida, Lemos, & Carrondo, 1992), and an unavailability of essential reactants to the enzymes due to a physical barrier caused by a metal sulfide formation (Utgikar, et al., 2002).

Generally, the sulfide in water and wastewater is classified as one of three forms (Polo Christy, 2001):

i. Total sulfide (TS) forms, which include dissolved forms of bisulfide (HS-), sulfide (S^{2-}), and hydrogen sulfide (H_2S) and insoluble forms of metal sulfides;
ii. Dissolved forms, which include ionized species such as HS-, S^{2-}, and free H_2S; and

iii. Unionized forms, which include H_2S gas.

Figure 2-4 shows the equilibriums of various sulfide species as a function of pH. According to this figure, H_2S is the dominating form of sulfide with a pH range of 6–7 whereas HS- has a pH range of 8–9.

Although unionized H_2S can act as a neutral molecule to permeate the cell membrane and inhibit the sulfate reduction process, some research has suggested that inhibition occurs due to an accumulation of total sulfide (Biswas, 2012; Choi & Rim, 1991).

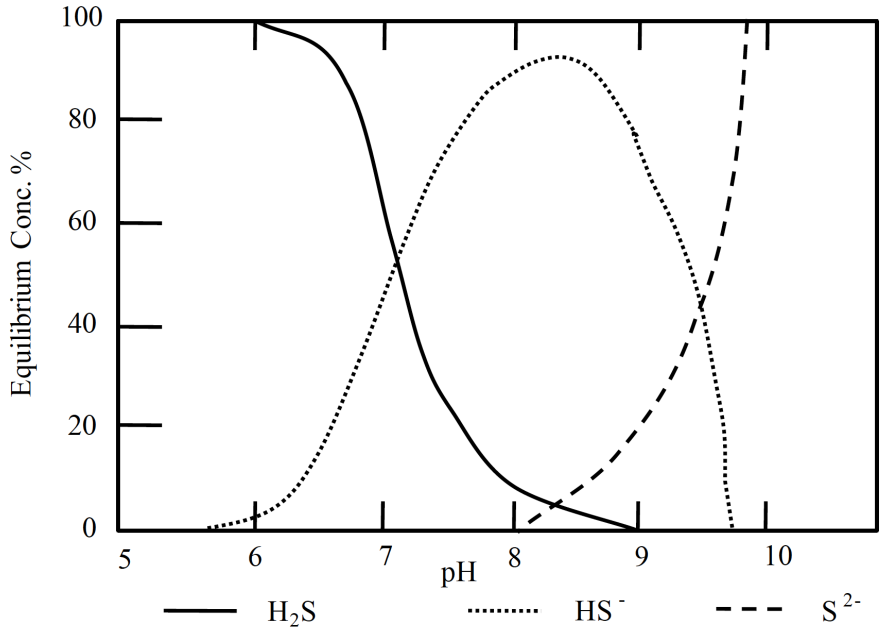


Figure 2-4 Equilibrium concentration of sulfide species as a function of pH (Hao et al., 1996)

A total sulfide concentration of 160 mg/L to 200 mg/L has been reported to inhibit SRB (McCartney & Oleszkiewicz, 1993). Elsewhere, the total sulfide has been suggested as SRB's inhibitor while the MPB are inhibited by the presence of free hydrogen sulfide (Hilton & Oleszkiewicz, 1988). The process's pH, the type of electron donor, the mechanism of sludge retention, and the type of the anaerobic reactor have been suggested as important factors in the form of sulfide that inhibits the activity of SRB (Polo Christy, 2001). A 185 mg/L concentration of unionized sulfide could cause a 50 % inhibition of SRB that utilizes a lactate-sulfate substrate at a pH of 8, while the same consortium could tolerate concentrations below 300 mg/L at a pH of 7. A consortium of acetate-consuming

SRB can tolerate free and total sulfide concentrations of 100 mg/L and 800 mg/L, respectively (Omil, et al., 1997). Visser, et al. (1996) concluded that at a pH of 7 or more total sulfide causes an inhibition of granular sludge, while unionized H_2S is responsible for the inhibition process in the same condition for suspended sludge (Visser, Pol, & Lettinga, 1996). A 50 mg/L to 130 mg/L concentration of unionized H_2S results in a 50 % inhibition in SRB activity (Lens, et al., 1998). This is a pH-dependent factor for granular sludge as a 50 % inhibition occurs with 250 mg/L of H_2S at a pH range of 6.4–7.2. The same inhibition cannot be avoided at a pH range of 7.8–8 with just a 90 mg/L concentration of H_2S (Biswas, 2012).

2.3.3 Effects of Temperature

Competition between SRB and MPBs is governed by changes in temperature. Sulfide reduction is less sensitive to temperature shocks than methanogenesis. However, this effect may be a function of factors such as type of sludge; exposure time to certain temperature shocks; and the temperature change (Polo Christy, 2001). SRB are less sensitive even at a temperature shock of 65°C for 8–9 hrs when in granular sludge containing SRB and MPBs (Visser, Gao, & Lettinga, 1993). Although an optimal temperature of 31–43ºC has been reported for most types of SRB (Okabe, et al., 1995), some special genera were known to tolerate a temperature as high as 70ºC.

2.3.4 Effects of Hydraulic Retention Time

In general, a long hydraulic retention time (HRT) is favorable for SRB. Isa, et al. (1986) demonstrated that an HRT increase from 12 hours to 10 days in a high-rate anaerobic reactor led to a sulfate reduction of approximately 8 % (Isa, Grusenmeyer, & Verstraete, 1986). Polo Christy (2001) reported that a decrease in HRT results in a lower effluent sulfide concentration and a biomass washout. This is in agreement with Singh, et al. (1996) findings where a more noticeable washout of granular sludge occurred during the operation of an up-flow anaerobic sludge blanket (UASB) reactor at an HRT of 3 hours than at HRTs of 4 hours and 6 hours (Singh, Harada, & Viraraghavan, 1996). Additionally, researchers found that despite the inhibitory concentration of sulfide, a sulfate reduction and a decrease in COD occurred when an anaerobic contactor was operated for a long HRT (Choi and Rim, 1991).

2.3.5 Chemical Oxygen Demand/Sulfate Ratio

A chemical oxygen demand/sulfate (COD/SO_4^{2-}) ratio indicates the ratio of electron donors (COD) to electron acceptors (SO_4^{2-}) in an anaerobic environment. It is a key factor in the metabolic pathways of dissimilatory sulfate reduction. Several studies have reported completely different metabolic pathways for sulfate reduction based on different COD/SO_4^{2-} (Uberoi & Bhattacharya, 1995; Colleran, et al., 1994; McCartney & Olesziewicz, 1991). SRB are more flexible in terms of their metabolic possibilities than MPBs (Stefanie, Visser, Pol, & Stams, 1994). Organic substrate oxidization levels are governed by COD/SO_4^{2-} levels in influents (Lens, et al., 1998).

The importance of this ratio increases as the COD/SO_4^{2-} in wastewater decreases. Theoretically, each mole of sulfate (96 g) needs 8 moles of electrons to be reduced; they can be derived from a suitable electron donor. As each mole of electron is equivalent to 8 g of COD, the total theoretical COD of 64 g is required to reduce 96 g of sulfate. Reduction of sulfate with SRB follows the reaction shown in Equation 2-6 (P Lens, Vallerol, Esposito, & Zandvoort, 2002):

$$SO_4^{2-} + 8e + 4H_2O \rightarrow S^{2-} + 8OH^- \qquad (2\text{-}6)$$

Theoretically, in waste streams with a COD/SO_4^{2-} of 0.67, there is sufficient sulfate available for SRB to completely remove organic matter as COD (Rinzema, Lettinga, & Wise, 1988). In situations in which there is an insufficient amount of substrate in the wastewater, the addition of extra substrate is required for a decent sulfate reduction (Omil, Lens, Visser, Hulshoff Pol, & Lettinga, 1998). Part of the organic substrate is taken by MPBs, so a proper balance needs to be found between the organic substrate and the SO_4^{2-}; this balance is of great significance in establishing an efficient anaerobic sulfate reduction process.

Choi and Rim (1991) reported that SRB out-competed MPBs at COD/SO_4^{2-} of less than 1.7 (i.e., in sulfate rich conditions) while they actively compete at COD/SO_4^{2-} ranging between 1.7 and 2.7. With a COD/SO_4^{2-} of more than 2.7 (i.e., with a sulfate-limiting condition), MPBs out-compete SRB (Choi and Rim, 1991). This is consistent with the findings of Colleran, et al. (1994), which stated that sulfidogenesis increased when the COD/SO_4^{2-} decreased from 1.9 to 1.2. Freese and Stuckey (2004) reported a possible shift

towards sulfate reduction when the COD/SO_4^{2-} decreased from 2 to 1. These findings reveal that sulfidogenesis and methanogenesis activities are mainly governed by the influence of the COD/SO_4^{2-}. Despite the inverse relationship between sulfidogenesis activity and the COD/SO_4^{2-}, the sulfate reduction rate improves when the COD/SO_4^{2-} is raised (Erdirencelebi, Ozturk, Ubay Cokgor, & Ubay Tonuk, 2007; S. Lopes, X. Wang, M. Capela, & P. Lens, 2007; A. Wang, Ren, Wang, & Lee, 2008).

2.4 Bioreactor Configurations in Anaerobic Sulfate Reduction

Bioreactors are classified based on the biomass retention mechanism inside their systems. Various bioreactor types have been used to anaerobically reduce sulfate such as: continuously stirred tank reactors (CSTRs); packed bed reactors (PBRs); gas lift reactors (GLRs); up-flow anaerobic sludge blanket reactors (UASBs); fluidized bed reactors (FBRs); and membrane bioreactors are the most widely applied process configurations. The mechanisms, benefits, and drawbacks of each configuration are discussed below.

2.4.1 Continuously Stirred Tank Reactors

Continuous stirred tank reactors (CSTRs) run with a uniform agitation mixture of reactants. They can run in a continuous mode in which reactants and products flow continuously or semi-continuously. In the semi-continuous mode, the reactor is fed in specific time intervals based on a hydraulic retention time. In such cases, it is assumed that the reactor feed is uniformly distributed inside the reactor and that the products have the same composition as in the tank. Stirred tank reactors can also be run in a batch mode in which they are fed with the reactants once and stopped after a particular time. Stirred tank reactors are reliable, consistent, and easy to operate. However, they need a high solid retention time (SRT) to achieve an efficient reduction of sulfate. Another drawback of CSTRs is an active biomass washout (Villa Gómez, 2013). White and Gadd (2000), Kieu, et al. (2011), and Biswas (2012) have studied how effective stirred tank reactors are in regards to the sulfate reduction process (Kieu, Muller, & Horn, 2011; White & Gadd, 2000).

2.4.2 Packed Bed Reactors

A packed bed reactor (PBR) typically has a chamber containing sand media, polymer rings, and pellets that retain biomass. The feed flows through the pellets across the length of the chamber and the feed and biomass interaction alters the composition of the substances. Figure 2.5 presents a schematic of a PBR.

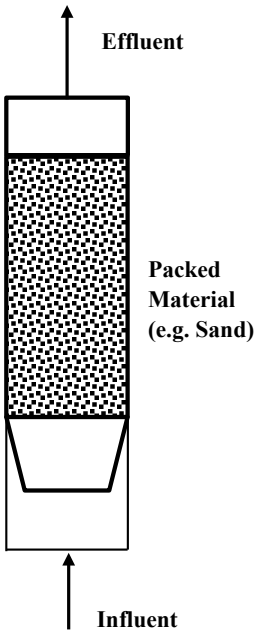


Figure 2-5 Packed bed reactor (Villa Gómez, 2013)

Packed bed reactors require a high solid retention time that results in high reactor volumes. They flexible in that they can be operated in both up-flow and down-flow modes. Despite these benefits, PBRs are usually associated with operational problems such as clogging. They also need a high pressure to pump the feed (Villa Gómez, 2013). Examples of applications of packed bed reactors for anaerobic sulfate reduction can be found in several research works (El Bayoumi, 1997; Jong & Parry, 2003; Kolmert & Johnson, 2001).

2.4.3 Fluidized Bed Reactors

The construction of fluidized bed reactors (FBRs) is based on the biofilm development of carrier materials. The carrier materials are fluidized by a recirculation of an effluent from either the top of the reactor to the bottom (i.e., up-flow FBR) or from either the bottom of

the reactor to the top of the reactor (i.e., inverse FBR). Figure 2-6 shows schematics of these configurations. FBRs result in the retention of a biomass inside the reactor; the process is facilitated with a short hydraulic retention time and concludes with an efficient anaerobic sulfate reduction. Different support materials such as iron chips, synthetic polymers, and silicate minerals have been applied as up-flow fluidized bed reactors (A. Kaksonen, Riekkola-Vanhanen, & Puhakka, 2003; D. K. Villa Gómez, 2013).

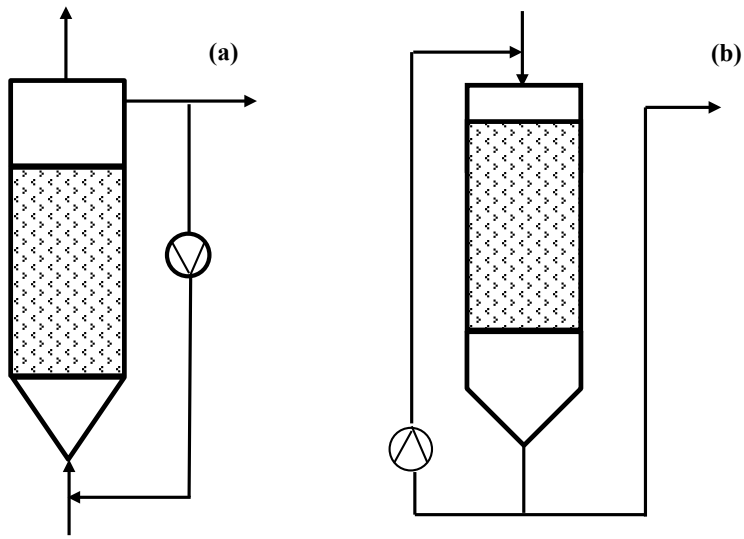


Figure 2-6 (a) Up-flow FBR; and (b) Inverse FBR (Villa Gómez, 2013)

In an inverse FBR, carrier materials must have a lower density than water. Polyethylene spheres and rings are the materials most widely used to carry the biofilm in inverse fluidized bed reactors (Castilla, Meraz, Monroy, & Noyola, 2000; Celis-García, Razo-Flores, & Monroy, 2007; Gallegos-Garcia, Celis, Rangel-Méndez, & Razo-Flores, 2009). Some of the advantages of the fluidized bed reactors are: a favorable retention of biomass; a lack of clogging and channeling problems; and a low risk of shock loads (Villa Gómez, 2013).

2.4.4 Gas Lift Reactors

Gas lift reactors (GLRs) are gas-liquid or gas-liquid-solid pneumatic contactors that work on the basis of fluid circulation. The fluid cyclically moves through channels built for specific purposes (J. C. Merchuk, 2003). A stream of gas is used to agitate the content of the reactors and, more importantly, to facilitate the exchange of material between the gas

phase and the medium. Reaction products are removed during an exchange with the gas phase (J. Merchuk & Gluz, 1999). The most significant advantages of this reactor are a high loading of solids and a favourable transfer of mass with efficient mixing and a controlled liquid flow (Vunjak-Novakovic, Jovanovic, Kundakovic, & Obradovic, 1992). Additionally, a high rate biological kinetics can be obtained when using H_2 as an electron donor. Each GLR has an operational drawback in that the gaseous substrates inside each GLR need a high pressure (Villa Gómez, 2013). A schematic diagram of a GLR is shown in Figure 2-7.

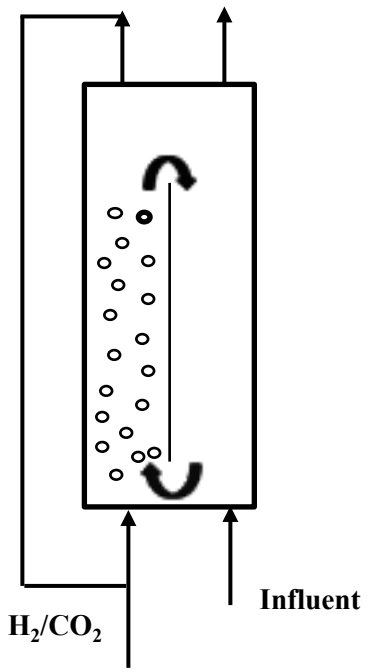


Figure 2-7 Gas lift reactor (Villa Gómez, 2013)

2.4.5 Up-flow Anaerobic Sludge Blanket Reactors

Up-flow anaerobic sludge blanket reactors (UASBRs) are generally cylindrical-shaped reactors in which an influent is suspended solids and bacterial activity and growth form a sludge. A sludge blanket is comprised of agglomerated micro-organisms of about 1–3 mm in diameter that resist to being washed out due to their specific weights. The influent is fed from the bottom of the reactor and flows upward. The micro-organisms break down organic compounds and release gas due to their activity. The gas mixes the sludge and an equilibrium of up-flow velocity and settling speed results in a suspended sludge blanket.

Sloped walls at the top of the reactor deflect the materials that reach the top and as a result a clear influent is withdrawn at the point above the sloped wall (Figure 2-8).

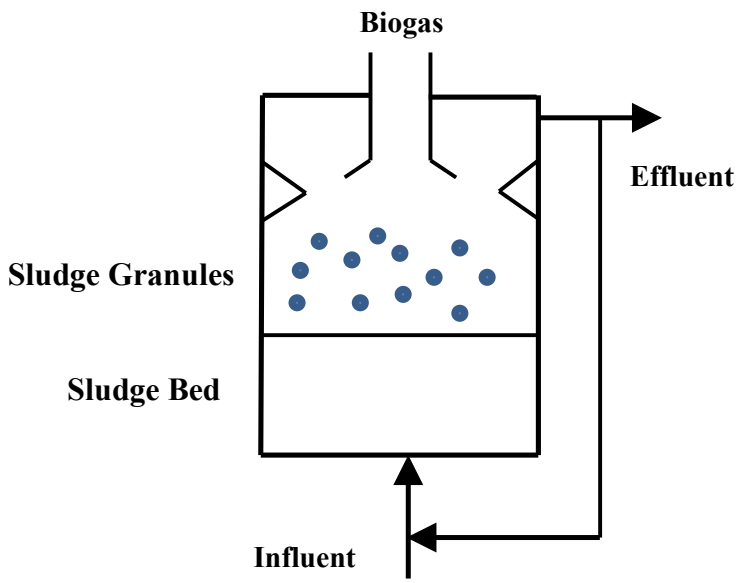


Figure 2-8 Up-flow anaerobic sludge blanket reactor (Sasse, 1998)

UASBRs do not require carrier materials and do not experience clogging. However, a biomass washout may occur and they are susceptible to the influent characteristics; these drawbacks limit their applications (Villa Gómez, 2013).

2.4.6 Membrane Bioreactors

Membrane bioreactors (MBRs) combine a membrane filter with a suspended growth bioreactor (Judd, 2010). Membrane reactors can be immersed, (i.e., submerged) whereby a filter is installed in the main bioreactor vessel, or external (i.e., side stream), where a filtration unit is installed on the reactor externally. A high rate of sulfate reduction can be achieved using membrane bioreactors, even with a high salinity that is a result of a high biomass retention. However, MBRs face some operational drawbacks (Vallero, Lettinga, & Lens, 2005). The most significant drawbacks that limit the application of MBRs are that they need a periodic backwash of the membrane surface (Vallero et al., 2005) and that a high cost is incurred when overcoming trans-membrane pressure (Villa Gómez, 2013).

2.4.7 Anaerobic Hybrid Reactors

Anaerobic hybrid reactors (AHRs) combine the advantages of fixed-bed and sludge blanket reactors (Henry, Donlon, Lens, & Colleran, 1996). Carrier materials such as pall rings are used to retain a biomass film in the reactor upper portion while a granular sludge bed may be developed in a matrix-free section in the reactor lower portion. Figure 2-9 shows a hybrid reactor's structure.

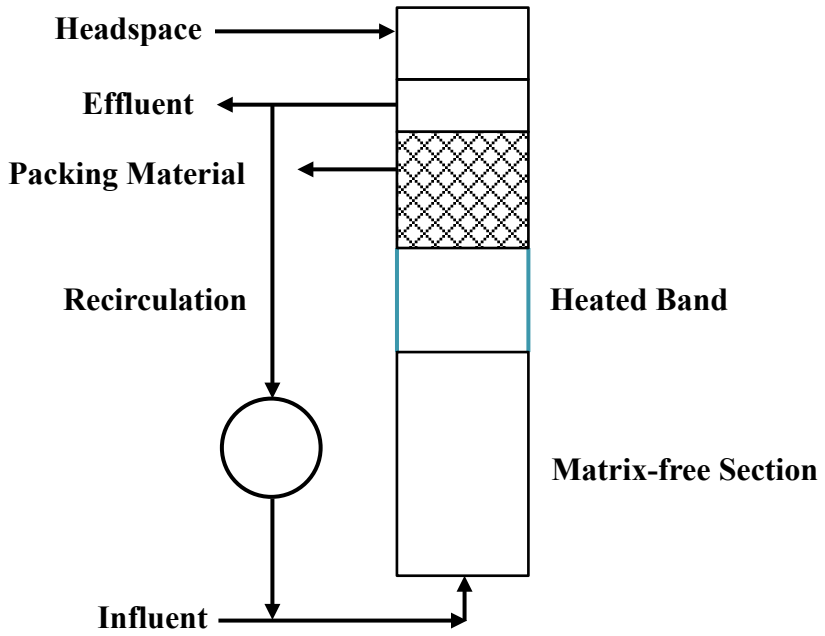


Figure 2-9 Anaerobic hybrid reactor (Mallelwar, 2013)

A biofilm develops on packing materials and polishes the wastewater that flows through. The packing materials retain the suspended sludge that flows upward and prevent the active washout of the biomass (Henry, et al., 1996). The hybrid reactor design has successfully treated industrial wastewater containing sulfate (S. Colleran & Pender, 2002; Mallelwar, 2013).

2.5 Biogenic Sulfide Precipitation of Heavy Metals

The biogenic sulfide precipitation of heavy metals is a viable heavy metal removal process, specifically when wastewater laden with heavy metals contains a considerable amount of sulfate. Examples of such wastewater are waste streams released by metal refineries and AMD (Villa Gómez, 2013). The biogenic sulfate production process in

Equation 2-6 is followed by the formation of highly insoluble metal precipitates, shown in Equation 2-7, where M^{2+} represents Zn^{2+}, Cu^{2+}, Pb^{2+}, and Ni^{2+}.

$$H_2S + M^{2+} \rightarrow MS\ (s) + 2H^+ \qquad (2\text{-}7)$$

The processes of reducing biological sulfate to sulfide and precipitating metals using the resulting biogenic sulfide can occur simultaneously. This can be done in single stage or two stage processes. Single stage and two stage processes are used in this study to assess and compare the efficiency of removing heavy metals from wastewater. The following sections are overviews of the processes as well as the advantages and disadvantages associated with them.

2.5.1 Single stage Biological Sulfate Reduction Process and Metal Sulfide Precipitation

The single stage process is a configuration in which both the biological reduction of sulfate to sulfide and the precipitation of metals simultaneously occur in a single reactor. Figure 2-10 is a schematic of the single stage process.

The application of the single stage process has been studied by several researchers. Hammack and Edenborn (1992) used a PBR to effectively remove Ni from wastewater (R. W. Hammack & Edenborn, 1992). The same bioreactor type was used by others to precipitate metal ions such as Cu, Zn, Ni, Fe, manganese (Mn), magnesium (Mg) and aluminium (Al) (I. S. Chang, Shin, & Kim, 2000; Jong & Parry, 2003; La, Kim, Quan, Cho, & Lee, 2003).

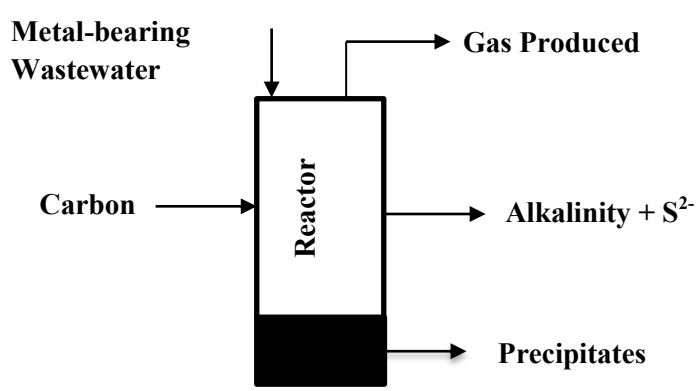


Figure 2-10 Single stage process configuration

Hammack, et al. (1994) efficiently removed more than 90 % of Cu, Zn, Mn and Al by treating a wastewater containing metal mixture with a gas lift reactor (Hammack, Edenborn, & Dvorak, 1994). An anaerobic filter reactor (AFR) was applied to treat wastewater with high concentrations of mixed metals (approximately 1900 mg/L). Almost 99 % of each metallic ion was removed (Steed et al., 2000). Other types of reactors such as fixed bed and up-flow sludge blankets have been applied in a single stage configuration (Gallegos-Garcia et al., 2009; Kaksonen et al., 2003; Sierra-Alvarez, Karri, Freeman, & Field, 2006). Villa Gómez (2013) studied the application of simultaneous sulfate reduction and metal precipitation in inverse fluidized bed (IFB) reactors. Single stage approaches to sulfate reduction and metal precipitation have also been used in pilot plants and full scales. Pilot scale anaerobic filters were installed to treat a metal-laden waste stream drained from the New Jersey Zinc Company's zinc smelter residue dumps in Palmerton, Pennsylvania (U.S.A.), (Dvorak, Hedin, Edenborn, & McIntire, 1992). Additionally, the Budel Company's zinc refinery in Budel-Dorplein (The Netherlands) used up-flow anaerobic sludge blanket reactors to remediate metal-polluted groundwater (Scheeren, Koch, & Buisman, 1993).

Single stage treatment processes are simple, low-cost alternatives to treat metal-containing wastewater. Micro-organisms are not affected by the dissolved sulfide that is present during these processes as the generated sulfide is taken by the dissolved heavy metals of the wastewater to form the precipitates. However, the process may not be viable for the treatment of highly acidic wastewater (Kaksonen & Sahinkaya, 2012). The same situation occurs in the treatment of highly saline wastewater when the operating range of bacteria is not obtained (Huisman et al., 2006). A high concentration of heavy metals that have dissolved in highly acidic wastewater is toxic to micro-organisms as it deactivates the enzymes by disrupting their functional groups and denaturing their proteins (Utgikar et al., 2001). Additional concerns regarding the application of the single stage process are operational problems such as system plugging that occurs as a result of the production of the high volume of sludge and seasonal fluctuations of feed streams that affect bioreactor performance (Govind, Kumar, Puligadda, Antia, & Tabak, 1997). The simultaneous presence of SRB and metal precipitates inside the reactor is an additional drawback of the single stage process as it makes the withdrawal of precipitates from the bioreactor

impossible; this results in a loss of biomass (Kaksonen & Sahinkaya, 2012). It is viewed as a system deficiency in terms of the system's ability to recover metals (Villa Gómez, 2013). The formation of metal precipitates inside the reactor may have an inhibitory effect on the activity of SRB (Utgikar et al., 2002). This may occur due to precipitates coating the SRB surface; the coating blankets the cells and halts the sulfate reduction process.

2.5.2 Two stage Biological Sulfate Reduction Process and Metal Sulfide Precipitation

A two stage process has been suggested to overcome limitations, such as low pH and high metal concentration, that are associated with the single stage process. The two stage process has been designed somehow SRB are not exposed to such inhibitory conditions (Al-Ani, Henry, & Prasad, 1996). The two stage process employs two separate reactors. One is a biological reactor in which sulfide is produced through sulfate biological reduction to sulfide. The second one is a chemical reactor, or contactor, where the sulfide is transferred from the biological reactor to precipitate heavy metal from receiving wastewater. Figure 2-11 is a schematic flowsheet of the two stage process.

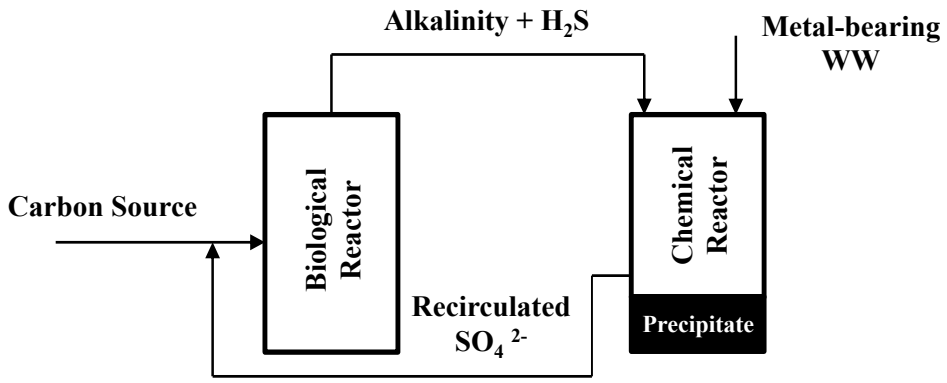


Figure 2-11 Two stage metal removal process (Al-Ani, et al., 1995)

A variety of studies have evaluated the two stage process of removing heavy metals from wastewater. Govind, et al. (1997) suggested a combination of an SRB reactor, precipitators, and clarifiers to selectively remove the metal sulfide. In their suggested system, the hydrogen sulfide (H_2S) and alkalinity produced by the SRB reactor would

transfer to the precipitators; there the selective removal of heavy metals would occur with a pH optimization. A pilot-scale study was conducted by Foucher, et al. (2001) to selectively recover Zn and Cu from a real mine effluent. It may have been one of the first large-scale applications of the two stage process. It used a fixed bed column reactor fed by a H_2/CO_2 mixture to generate the H_2S and alkalinity required to regulate the pH for selective metal recovery (Foucher, Battaglia-Brunet, Ignatiadis, & Morin, 2001). Tabak, et al. (2003) later suggested multiple stages of chemical reactors for the selective recovery of heavy metals at different pH levels by using biogenic sulfide that was withdrawn from a biological reactor. Instead of adjusting the pH, they suggested the precise control of the pS (pS = -log (S^{2-}) in the chemical reactor to better facilitate the selective recovery of heavy metals using the biogenic sulfide (Tabak & Govind, 2003). This process has been described in detail in the literature (Esposito, Veeken, Weijma, & Lens, 2006; A. H. Veeken, Akoto, Pol, & Weijma, 2003). The two stage process was commercialized by the Paques BV Company (EL Balk (The Netherlands)) and a full-scale plant was installed to remove heavy metals from a North American zinc mine's drainage water (Huissman, 2006). Alvarez, et al. (2007) used the H_2S produced by an up-flow anaerobic packed bed reactor to precipitate the metal in a metal-containing effluent that was collected from an active mine in Bolivia. They reported a close to complete removal of Ca and had Cu and Zn removal efficiencies of more than 90 % (Alvarez, Crespo, & Mattiasson, 2007). The two stage process was applied using an anaerobic sulfate reduction bioreactor inoculated with a mixed SRB culture and a metal sulfide precipitator that received the effluent of the bioreactor. It treated wastewater containing Mg, Fe, Cu, and Ni. A close to complete removal of Fe, Cu, and Ni was achieved. The system's efficiency in removing Mg was zero (Cao, Zhang, Mao, Fang, & Yang, 2009). Mallelwar (2013) applied the sulfidogenic effluent of a hybrid reactor to remove Cu from a synthetic wastewater.

In the the two stage process, pH and concentrations of heavy metals in receiving wastewater are easier to control than in the single stage process (Johnson and Hallberg, 2005). The two stage process allows the selective recovery of heavy metals through conducting the necessary controls inside the chemical reactor. However, it requires higher investment of capital and has the higher operational costs than the single stage

process due to its additional unit. Additionally, dissolved sulfide permanent presence in the biological reactor can have a toxic effect on SRB and may decrease the rate of sulfate reduction.

Chapter 3

Experimental Design and Methodologies

This chapter presents details of the experimental configurations as well as the design and methodologies applied in order to accomplish the objectives of the research. The source of the inoculum, composition of the substrate, culture maintenance, the structure of the experimental configuration, analytical measurement protocols along with the experimental designs are represented and discussed in this chapter.

3.1 Experimental Set-up and Design

During this study, experiments were carried out in different phases using two different setups and configurations as noted in the following:

Setup 1. Completely stirred tank reactors (CSTRs)

Phase I. Start-up

Phase II. Metal Precipitation Experiments

Setup 2. Up-flow anaerobic hybrid reactors (UAHRs)

Phase I. Start-up

Phase II. Metal Precipitation

3.1.1 Setup 1. Semi-continuous Stirred Tank Reactors (SCSTRs)

3.1.1.1 Phase I

a. Inoculum Source

The source of inoculum was grayish black sludge with a distinctive odor of H_2S obtained from anaerobically digested sludge from a Municipal Wastewater Treatment Plant, in Chatham, Ontario. This sludge was used to develop an enriched SRB culture required for anaerobic sulfate reduction experiments.

b. Growth Medium

A simple medium composition suggested by Mallelwar (2013) was used for the growth of SRB. Composition of medium are presented in Table 3-1.

Table 3-1 Composition of growth medium (Mallelwar, 2013)

Name	Chemical Formula	Concentration (mg/L)
Sodium sulfate	Na_2SO_4	4500
Dextrose	$C_6H_{12}O_6$	2850
Ammonium chloride	NH_4Cl	1000
Potassium phosphate monobasic	KH_2PO_4	100
Potassium phosphate dibasic	K_2HPO_4	400

Glucose was used as a carbon source (i.e., substrate) and sodium sulfate was applied as sulfate source. The growth medium was prepared using tap water. The theoretical oxygen demand (ThOD) of the substrate was calculated according to the following reaction:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad (3\text{-}1)$$

According to Equation 3-1, 180 mg/L of substrate (glucose) is equivalent to 192 mg/L of ThOD that corresponds to total ThOD of 3040 mg/L for 2850 mg/L of substrate used in growth medium composition. For a simple substrate like glucose that is easily oxidized, the amount of ThOD is expected to be similar to that of chemical oxygen demand (COD). The concentration of 4500 mg/L of sodium sulfate contains 3040 mg/L of sulfate (SO_4^{2-}). Therefore, for the growth medium with the composition presented in Table 3-1, COD/ SO_4^{2-} of 1 is obtained.

c. Culture Maintenance Reactor

Two parallel and completely stirred tank reactors (CSTR), referred to as MC1 and MC2, each with 4 liters of working volume, were used to maintain the required culture. The schematic of the reactors is shown in Figure 3-1.

Each reactor was inoculated with a mixture of 2 liter of inoculum source and 2 liter of the growth medium with the composition given in Table 3-1. These reactors were operated in a semi-continuous mode at an HRT of 40 days at room temperature of 22±2ºC as

optimized by Biswas et al. (2012). To maintain the HRT, a certain volume of the reactor, known as semi-continuous completely stirred tank reactor (SCSTR), was replaced with the fresh growth medium in an appropriate time interval as per calculation given in Appendix 1.

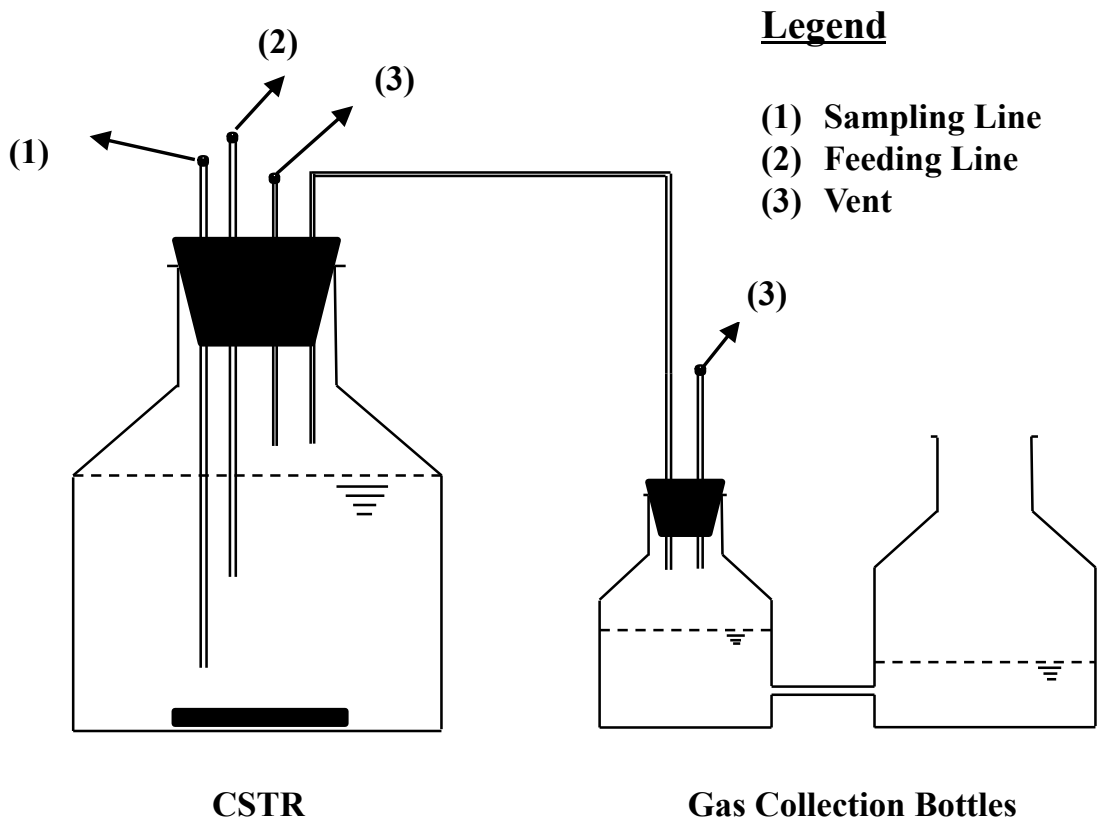


Figure 3-1 Semi-continuous stirred tank reactor (SCSTR) configuration

The SRB activity and reactor performance were closely monitored. The parameters included: pH; oxidation-reduction potential (ORP); volatile fatty acids (VFA); alkalinity; sulfate concentration; and total organic carbon (TOC). The reactor reached a steady-state after 50 days and the inoculum for the current study was obtained after 100 days of operation. The results of the operation of MC1 and MC2 are presented in Appendix E1.

3.1.1.2 Phase II. Metal Precipitation Experiments

Four parallel 600 ml experimental SCSTRs, with the same configurations illustrated in Figure 3-1, were set up using enriched and identical inoculum from the 4 L MC reactor described in Part (c), section 3.1.1.1. The culture, after the transfer, was flushed with nitrogen for 5 minutes to remove any oxygen from possible exposure to air during the transfer process. These reactors were operated at a hydraulic retention time of 50 days to lower the possible biomass washout from the reactors. HRT of 50 days was maintained by replacing 85 ml of their contents with synthetic feed every 7th day. The feed composition was the same as that used for the MC reactor except for varying the Cu concentration and the addition of a chelating agent. Reactor RC served as a control with the feed Cu = 0 mg/L. The synthetic wastewater fed to the reactors (termed R1, R2, and R3) included Cu concentrations of 200 mg/L, 400 mg/L, and 600 mg/L while maintaining the concentration of other ingredients the same as in the RC and MC reactors.

A stock solution of 1200 mg/L of Cu created by dissolving copper chloride in water and a double-concentrated medium were prepared. Also prepared for each reactor was 100 ml of feed that maintained the required Cu concentration and nutrients by mixing appropriate amounts of Cu stock solution and medium. Water was added to compensate for the volume deficit where necessary. Sodium citrate was added as a chelating agent to prevent metal precipitation in the feed (Kieu et al., 2011). Similar concentration (450 mg/L) was added to the feed for all four reactors. Before feeding the reactors, the feed solutions were flushed with nitrogen to remove dissolved oxygen.

3.1.2 Setup 2. Up-Flow Anaerobic Hybrid Reactors (UAHRs)

3.1.2.1 Phase I

a. Inoculum Source

The inoculum source for the column reactors consisted of three separate parts: (1) anaerobic sludge from municipal wastewater treatment plant in Chatham (Ontario) as described in Part (a), section 3.1.1.1; (2) granular anaerobic sludge from an industrial (ethanol) wastewater treatment plant, Chatham; and (3) stored SRB culture collected

during the culture maintenance for the CSTR reactors as described in Part (c), section 3.1.1.1.

b. Culture Maintainance

In a lidded container, 6 L of stored active SRB cultures were mixed with 4 L of Chatham anaerobic sludge that was sieved with a 50 Mesh laboratory sieve to remove the coarse particles. The content of container was then dispensed between two jars labeled as C1 and C2, each with the volume of 10 L. To obtain a highly active SRB culture, reactor A1, with the same configuration as shown in Figure 3.1, was started by mixing of 1 L of granular sludge, 1 L of anaerobic sludge and 2 L of stored culture collected from the operation of MC1 and MC2 (Part (c), Section 3.1.1.1). The content of A1 was passed from the 50 Mesh sieve and undersize was returned to the A1. All A1, C1 and C2 were monitored for their pH, ORP, VFA and bi-carbonate and alternately fed with the different concentrations of nutrients presented in Table 3-1. Details of monitoring the cultures and feeding procedure have been given in Table Appendix E2. After 10 days 2.5 L of stored culture from the operation of MC1 and MC2 was dispensed between C1 and C2 and 400 ml of culture from A1 was added to each jar to enhance their sulfate reduction activity. A1, C1, and C2 were monitored and fed for more 20 days as presented in Appendix E2 and another 400 ml of A1 was added to each of C1 and C2. Active sulfate reductions were observed in C1 and C2 after a total of 5 weeks.

c. Up-flow Anaerobic Hybrid Reactor (UAHR) Configuration

Figure 3-2 shows a schematic of UAHR reactor configuration. UAHR is a combination of a packed bed reactor and a sludge blanket reactor. The reactor used in the current study had a height of 120 cm and internal diameter of 100 mm. The total volume of the reactor was 9.8 L and liquid volume was set at 8.2 L. The column was fabricated using PVC and the middle 1-foot section was made of stainless steel to use the heating band (tape-type by BriskHeat, Columbus, OH, U.S.A.) that maintained the temperature of 33±3ºC inside the reactor. The temperature was regulated using a T130P temperature control system (Advance Greenhouse Controls, Buffalo Junction, VA, USA) and was continuously monitored using the installed digital thermometer. The total of 100 Polypropylene pall rings with a diameter of 25 mm and specific surface area 233 m^2/m^3 were used as a

packing material to support the formation of biofilm. Pall rings occupied 20 % of the column volume. Experimental set up had the following components:

A peristaltic pump with two separate lines was used for the recirculation and feeding to the reactor. The recirculation rate was adjusted at 60 ml/min (Oktem, Ince, Sallis, Donnelly, & Ince, 2008). This maintains an up flow velocity of 0.456 m/h inside the column. Gas production was measured using simple liquid displacement in gas collection bottles.

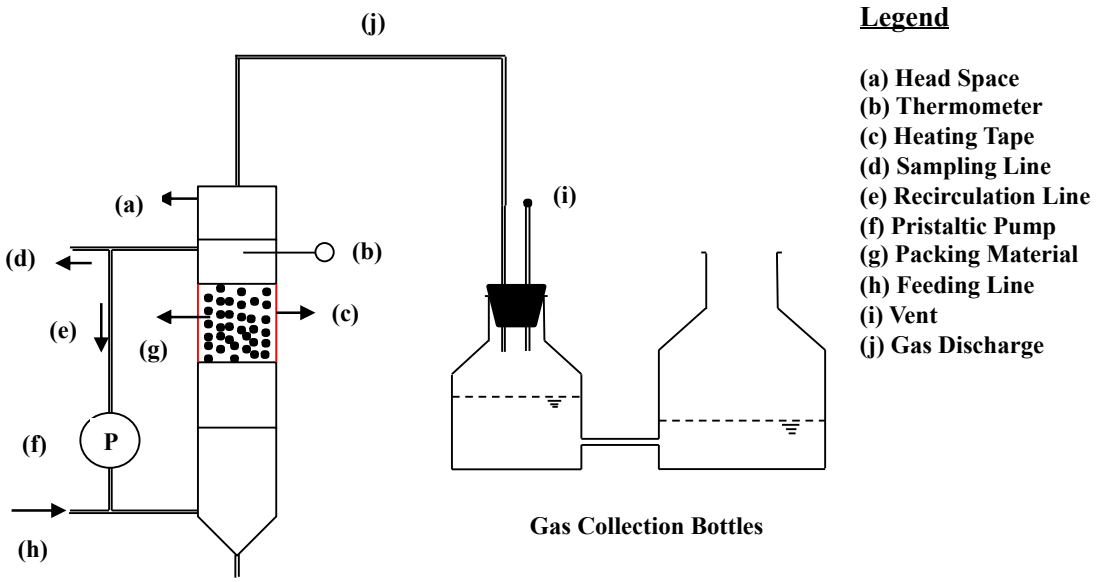


Figure 3-2 Schematic diagram of up-flow anaerobic hybrid reactor

d. UAHRs Start-up

Contents of C1 and C2 (Part (b), Section 3.1.2.1) were completely mixed together in a large lidded flask with an inlet and an outlet. The content of the flask was flushed with the nitrogen gas to remove the air and ensure of maintaining the anaerobic environment. Two UAHRs, labeled as HR1 and HR2, were flushed with nitrogen gas for 15 min to purge the oxygen and were then seeded with the content of the flask. The deficit to reach the operating volume of 8.2 L was compensated by the fresh growth medium with the composition given in Table 3-1. The head space of the reactors was flushed by nitrogen gas to further minimize the exposure to the air and maintain an anaerobic environment. Both UAHRs were monitored for their pH, ORP, sulfate, VFA, and bicarbonate for the

first two days and in the second day 600 ml of the reactors content was replaced with 400 ml of active culture from the A1 (Part (b), Section 3.1.2.1) and 200 ml of medium with the composition of 2g/l NH_4Cl, 0.2 g/l KH_2PO_4, 0.8 g/l K_2HPO_4, 4.8 g/l Na_2SO_4 and 6 g/l glucose. The reactors were monitored until Day 7 by when they were started to be fed semi-continuously at the HRT for 40 days by replacing 1430 ml of their contents with the fresh growth medium (Table 3-1) every 7th day. Both reactors were operated until Day 56 by which time near to complete sulfate reduction was perceived for each feeding cycle in both reactors. The acquired data of the start-up phase has been presented in Appendix E4.

3.1.2.2 Phase II; Metal Precipitation

a. Single Stage (Internal) Process

For the single stage metal precipitation, the feed of HR1 was modified with different concentrations of Cu at the varying HRT. HR2 was used as a control and operated at the same HRT without the addition of Cu. The addition of Cu was started at the concentration of 200 mg/L and increased gradually to reach its maximum of 1500 mg/L so that no residual Cu was measured inside the reactor. The Cu addition was stopped once the residual Cu concentration was detected inside the reactor. To prevent Cu precipitation in the feed of the reactors, sodium citrate was added as a chelating agent at different concentrations. In order to keep the pH of the reactors in the optimum range of 6.7±0.2, the feed to the reactors were modified with the additional bi-carbonate in the form of $NaHCO_3$. The hydraulic retention time (HRT) was changed when steady state reactor performance was perceived. A steady state was assumed to be achieved when the changes in the operational parameters of the reactors were within ±5 % of the average value for three consecutive feeding cycles. Experimental design of single stage (internal) Cu precipitation is given in Table 3-2.

Chemical precipitation was also conducted externally to evaluate the additional copper removal capacity of the reactor effluent due to the presence of different concentration of residual sulfide after each run of internal precipitation experiments. Withdrawn effluent from the reactor was mixed with a copper stock solution in volumetric 1:1 basis, so that 3 different copper concentrations of 300, 600 and 1200 mg/L were maintained while the

concentration of NH_4Cl, KH_2PO4, and K_2HPO4 remained similar to that of the growth medium at 1000 mg/L, 100 mg/L, and 400 mg/L, respectively.

Table 3-2 Experimental design of single stage copper precipitation process

Time Period (Days)	HRT (Days)	Added to Growth Medium to Modify the Feed		
		Copper Concentration (mg/L)	Additional Bi-Carbonate (mg/L)	Sodium Citrate (mg/L)
56-105	40	200	0	480
105-141	20	200	0	480
141-169	20	600	450	875
169-191	10	600	600	875
191-197	5	600	750	875
197-212	5	1000	1100	1675
212-218	5	1500	1100	1675
218-223	2.5	1500	1100	1675

b. Two stage (External) Process

To evaluate the two stage metal precipitation process, the effluent withdrawn from the reactor HR2 at different HRT was mixed with a stock solution containing $CuCl_2$, NH_4Cl, KH_2PO_4, and K_2HPO_4. Sodium Citrate was also added to prevent the precipitation of Cu in stock solution. The effluent was mixed with the Cu solution of 3:1, 1:1, and 1:3. The concentration of stock solution components was chosen so that for each volumetric ratio, the mixture of the Cu concentration of 300 mg/L, 600 mg/L, and 1200 mg/L were maintained while the concentration of NH_4Cl, KH_2PO_4, and K_2HPO_4 remained similar to that of growth medium at 1000 mg/L, 100 mg/L, and 400 mg/L, respectively.

For the reactor HR1, by which the single stage Cu precipitation was carried out, the residual sulfide was detected by a decrease in HRT to 2.5 days. Thus, the external Cu precipitation was also carried out by mixing the effluent of HR1 with the Cu stock solution just in volumetric ratio of 1:1. External Cu precipitation was repeated in some HRTs for the effluent of HR1 due to the change in residual sulfide concentration by change in influent Cu concentration during the internal Cu precipitation process. Table 3-3 presents the experimental design for a two stage (external) Cu precipitation.

Table 3-3 Experimental design for two stage (external) copper precipitation process

Reactor	HRT (Days)	Mixture of Effuluent and Copper Solution Characteristics		
		Eff./ Cu Sol. (v/v)	S^{2-} Concentration (mg/L)	Copper Concentration (mg/L)
HR2	20	3 to 1	405	300, 600 and 1200
		1 to 1	270	300, 600 and 1200
		1 to 3	135	300, 600 and 1200
HR2	10	3 to 1	411	300, 600 and 1200
		1 to 1	274	300, 600 and 1200
		1 to 3	137	300, 600 and 1200
HR2	5	3 to 1	405	300, 600 and 1200
		1 to 1	270	300, 600 and 1200
		1 to 3	135	300, 600 and 1200
HR2	2.5	3 to 1	300	300, 600 and 1200
		1 to 1	200	300, 600 and 1200
		1 to 3	100	300, 600 and 1200
HR1	20	1 to 1	230	300, 600 and 1200
HR1	20	1 to 1	163	300, 600 and 1200
HR1	10	1 to 1	172	300, 600 and 1200
HR1	5	1 to 1	174.3	300, 600 and 1200
HR1	5	1 to 1	120	300, 600 and 1200
HR1	5	1 to 1	70	300, 600 and 1200
HR1	2.5	1 to 1	25	300, 600 and 1200

3.1.2.3 Determination of Copper and Solids Inventory in UAHRs

Solids and Cu inventory inside the reactors, were determined for different reactors sections as shown in Figure 3-3. The content of each section was discharged into containers and representative samples from each container were withdrawn in triplicates to perform solids analysis and Cu measurement for each. The results of analysis have been presented in Appendix E5.

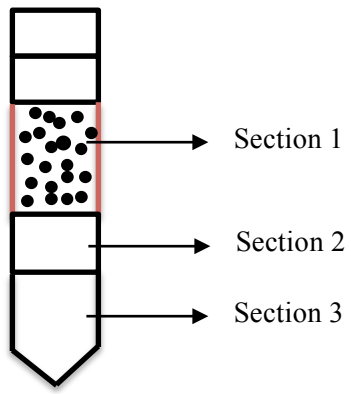


Figure 3-3 UAHRs sections for solids and copper inventory

3.2 Analytical Method

3.2.1 pH

For all the reactors pH was measured immediately after sampling. The pH was measured using a pH electrode and Oaklon pH meter according to standard methods for the examination of water and wastewater (APHA, 2005). The electrode was calibrated for pH buffers 4 and 7 on a daily basis. The electrode was rinsed, blotted dry, and then was placed into a beaker and the sample was directly discharged into the beaker and then the reading was recorded. The pH value was used as an indicator of the reactors performance and internal environmental conditions.

3.2.2 Oxidation-Reduction Potential (ORP)

An Orion 9678BNW Sure-Flow Oxidation/ Reduction Potential (ORP) probe was used to measure the ORP as per the Standard Methods (APHA, 2005). The probe was placed into the beaker containing the sample and reading was taken after 5 minutes of contact time of the probe and sample until the equilibrium was maintained. There was a limited amount of change observed in the measured ORP level.

3.2.3 Gas Production

Gas production was measured by liquid volume displacement in the calibrated aspirator bottles connected to the head space of the reactors (Biswas, 2012). The aspirator bottles were filled with NaCl-saturated water to avoid the dissolution of the gas transferred from

the reactors. The difference between initial and final levels of the liquid inside the bottles indicated the amount of gas produced over a certain period of time.

3.2.4 Sulfate

The gravimetric method given in protocol 4500-SO_4^{2-} D of standard methods (APHA, 2005) was used to measure the sulfate concentrations in the influent and the effluent of the reactors. This method is applicable to analyze the samples with high sulfate concentration (> 10 mg/L). Sulfate ions were precipitated using 100 g/l barium chloride solution and the weight of dried precipitate was used to calculate the concentration of the sulfate. Samples were filtered using glass microfiber filters and sulfate measurements were conducted in duplicates of 10 ml samples.

3.2.5 Total Dissolved Sulfide (TDS)

The total dissolved sulfide was measured according to the 4500-S^{2-} C iodometry method given in standard methods (APHA, 2005). To avoid the loss of volatile sulfide, samples were not filtered. Samples pretreatment were carried out to eliminate the interference caused by sulfite, thiosulfate and other soluble substances that may react to iodine. Sulfide analyses were carried out in triplicates and the coefficient of variation of less than 5 % was observed during the measurements.

3.2.6 Bicarbonate Alkalinity and Volatile Fatty Acids (VFA)

A direct titration method was used to determine the alkalinity and volatile fatty acid (VFA) concentrations (DiLallo & Albertson, 1961). The measurements were carried out in triplicate on non-filtered samples with a volume of 10 ml each. The total alkalinity was measured by the titration of samples to pH 4 using 0.01N sulfuric acid and the volume of acid was noted. The pH of sample was then adjusted to 3.3–3.5 by adding more 0.01N H_2SO_4 and was boiled for 3 minutes. After cooling to room temperature, the pH was adjusted to 4 by 0.05 N NaOH and titration continued until pH of 7 was obtained. The amount of NaOH required to increase the pH from 4 to 7 was used to determine the volatile acid alkalinity (VFA). VFA is measured as mg/L of acetic acid. The difference between total alkalinity concentration and volatile acid alkalinity yields the bicarbonate alkalinity as mg/L of $CaCO_3$. According to the procedure given by DiLallo and Albertson

(1961), for the volatile fatty acid concentrations of more than 180 mg/L, correction factor of 1.5 was used to determine the total VFA concentration as acetic acid.

3.2.7 Total Organic Carbon (TOC)

The TOC was measured with a TOC analyzer (Shimadzu TOC-V_{CSH}) according to the protocol 5310 B given in the standard methods for the examination of water and wastewater (APHA, 2005). The analyzer was calibrated using standard solutions of total carbon (TC) and inorganic carbon (IC) and calibration curves were prepared. Calibration curves for TC and IC, have been shown in Appendix 3. Samples for TOC measurements were filtered using glass microfiber filters (Whatman 934-AH). Prior to the analysis, samples were diluted to the acceptable measurement ranges using Milli-Q water (Millipore Co., Van Nuys, CA, U.S.A.) and the pH of samples reduced to less than 2 by acidification with concentrated H_2SO_4. The samples were then analyzed for TC and IC. TOC values were calculated by subtracting IC values from TC and multiplying by respective dilution factors. A coefficient of variance (CV) of < 2 % was set for duplicate injections. TOC was measured in duplicates for the samples with the volume of 10 ml.

3.2.8 Phosphate Analysis

Calorimetry was used to measure the concentration of the phosphate as described in method 4500-P C. Vanadomolybdophosphoric Acid Calorimetry in standard methods for the examination of water and wastewater (APHA, 2005). A kit with the code 3655-SC (LaMotte, Chestertown, Maryland, U.S.A.) for determination of the high concentration of phosphate (0 – 70 mg/L) was used to prepare the samples. A Smart 3 portable calorimeter (LaMotte, Chestertown, Maryland, U.S.A.) was used for the direct reading of phosphate concentration in prepared samples. The readings multiplied with the respective dilution factor to measure the phosphate concentration in the samples.

3.2.9 Solids Measurement

The solids concentration was measured in accordance with method 2540D given in standard methods (APHA, 2005). Liquid samples were filtered using glass microfiber filters (Whatmann 934-AH). Filter paper was dried at 103–105ºC for one day. The retained mass on the filter paper was measured for the difference between the weights of

filter paper after drying and before sample filtration. This procedure yields the amount of total suspended solids (TSS). The filter paper was then ignited at 550ºC for 2 hours. The mass lost as a result of the ignition indicates the amount of volatile suspended solid (VSS). The difference between TSS and VSS results the amount of Fixed Suspended Solids (FSS).

3.2.10 Copper Analysis

The concentration of Cu in both liquid and solid phases was measured using atomic absorption spectrophotometry (AAS). An AA-6800 atomic absorption analyzer (Shimadzu Co., Kyoto, Japan) was used to determine copper concentration.

Liquid samples were filtered using 0.22µm syringe filter to remove any solid particles. The pH of the samples must be below 3 to free all chelated forms of the Cu (Maillacheruvu, 1993). The concentrated HNO_3 was used when necessary. Standard calibration curve at the range of 0 mg/L to 10 mg/L was developed prior to each run of samples Cu analysis. Examples of calibration curves have been shown in Appendix 2. The samples were diluted using Milli-Q water in order to measure concentration for diluted samples in the range of 0 to 10 mg/L range. The actual Cu concentration was calculated by multiplying the dilution factor with the Cu concentration of diluted sample.

Solid samples were digested using nitric acid according to standard methods 3030E (APHA, 2005). The resulted solution was then diluted to 100 ml using Milli-Q water and the procedure performed for the liquid samples was carried out to measure the Cu concentration. All the Cu analysis was performed in triplicate.

3.3 Genomic Analysis

Genomic analysis was carried out to determine the change in the microbial composition by the variation in the process conditions.

3.3.1 Sample Collection

Samples from different reactors were collected at the end of CSTRs operation and immediately transferred to 15 ml plastic centrifuge tubes in triplicate. The centrifuge

tubes were capped and labeled to be identified for their respective process condition and stored in the freezer at -20ºC until the analysis.

3.3.2 DNA Extraction

DNA extraction was performed according to the protocol suggested by Chaganti et al. (2012). In this process 0.4 ml of thawed, well-mixed contents of the centrifuge tube were transferred to a 2 ml tube containing approximately 250 mg of zirconia/silica beads, 0.4 mL cetyl trimethyl ammonium bromide (CTAB) extraction buffer (20 % (wt/vol) CTAB (Sigma-Aldich, Toronto, Canada) in 1.4 M NaCl with 480 mM potassium phosphate buffer at pH 8.0) and 0.4 ml of phenol-chloroform-isoamyl alcohol (25:24:1 (pH 8.0)). Three cycles of freeze-thaw (-80ºC) were carried out on the mixture. A Thermo Savant Bio 101 Fast Prep FP 120 homogenizer (Savant Instruments, Holbrook, U.S.A.) was used to lyse the microbial cells for 45 s at a 6.5 speed setting. To obtain clear phase separation, the homogenized samples were centrifuged at 4ºC for 10 min at 16000 x g. The clear supernatant was re-extracted using an equal volume of chloroform-isoamyl alcohol (24:1) in 1.5 mL centrifuge tubes and was then centrifuged for 10 min at 10000 x g. Isopropanol solution was added to the extract (2/3 vol) to precipitate the nucleic acid. The mixture was incubated at room temperature for 10 min and was centrifuged at 4ºC for 20 min at 10000x g. The precipitate was removed from the supernatant and was washed using 70 % (Vol/Vol) ice-cold ethanol, and then air-dried and re-suspended in 50 µL of sterile Milli-Q water for polymerase chain reaction (PCR) and next generation sequencing.

3.3.3 Polymerase Chain Reaction (PCR) and Next Generation Sequencing

PCR was carried out in two steps on all DNA samples targeting the V9 region of the 18S-rRNA gene. The first step of PCR was carried out to amplify the targeted DNA. In the second step, a short-cycle PCR was designed to ligate identifying sequences ("barcodes"). The required adaptor sequences for the ION Torrent Next-Generation Sequencing (NGS) library was ligated in the second step of the PCR.

The first step of PCR was performed according to the following thermocycling protocol:

1. Initial denaturing stage at 94ºC for 2 minutes

2. 27 cycles of: denaturing at 94ºC for 15 s; annealing at 55ºC for 15 s; elongation at 72ºC for 30 seconds
3. Elongation step at 72ºC for 7 minutes
4. A hold step at 4ºC.

To create the barcode library the following steps were performed in the second step, short cycle, PCR:

1. Initial denaturing stage at 94ºC for 2 minutes
2. 8 cycles of: denaturing at 94ºC for 15 s; annealing at 60ºC for 15 s; elongation at 72ºC for 30 seconds
3. Final elongation step at 72ºC for 7 minutes
4. A hold step at 4ºC.

The barcoded PCR amplicons were then pooled, visualized on an agarose gel and then excised from the gel and purified using a Qiagen MinElute gel extraction kit. An Agilent 2100 Bioanalyzer equipped with a High Sensitive DNA chip (Agilent Technologies, Mississauga, Canada) were used to determine DNA concentration and fragment size distribution of the samples obtained from the combination of the products of both steps of PCR.

The next generation sequencing was carried out on a "318" microchip using an Ion Torrent Personal Genome Machine ("PGM"; Life Technologies, Carlsbad, CA, U.S.A.). The polyclonal and low-quality sequences were removed from sequence reads within the PGM software. Non-informative sequence data were also trimmed from the sequences that matched the PGM 3′ adaptor. The resulting filtered sequence data were checked for reading quality before rRNA detection, rRNA clustering and assignment of taxonomic identification as described in Meyer et al. (2008). Operational taxonomic units (OTUs) were determined using the SILVA small subunit database (SSU) (Yilmaz et al., 2013) by clustering similar sequences based on a 97% similarity threshold. The OTUs represented only by either a single (singleton) or two (doubleton) sequences in the whole library, i.e., samples control and treated were excluded from the data before metagenomic species detection. Rarefaction curves were established for both the initial and post-culture samples. These curves represent the relationship between the number of sequence reads

and the number of recovered taxes. The match between full taxonomic characterization of the samples and sequence read numbers is determined using these curves.

3.3.4 Statistical Analysis

To evaluate the similarity and variations of microbial profile of the culture under different process conditions, statistical analysis such as similarity %age test (SIMPER) and principal component analysis (PCoA) on complex microbial datasets obtained from PCR and next generation sequencing was carried out using PAST software package (Hammer, Harper, & Ryan, 2001).

3.4 Precipitate Characterization

3.4.1 Precipitate Concentration

Fixed suspended solid (FSS) concentration was used to represent the concentration of the precipitates resulted from precipitation experiments. The concentration of FSS was determined according to the method described in section 3.2.9.

3.4.2 Scanning Electron Microscopy- Energy Dispersive X-ray Spectroscopy

Scanning Electron Microscopy- Energy Dispersive X-ray Spectroscopy (SEM/ EDS) was performed to determine the quantitative elemental analysis of the precipitates. SEM/ EDS analyses were carried out using an FEI Quanta 200 FEG Environmental SEM, with an EDAX Octane Plus SDD detector (Silicon Drift Detector), running the TEAM Software, V. 4.1 (FEI, Hillsboro, Oregon, U.S.A.). Samples of the SEM were prepared by the filtration of the precipitates using glass microfiber filters (Whatmann 934-AH). The filter papers were dried and tested with compressed air to ensure the sample was fully attached and would not be sucked into the device during the vacuum. Low vacuum mode with a chamber pressure of 70 Pa and the voltage of 18 kV was used.

The mapping technique was used for the analysis in which the SEM moves the beam pixel by pixel across the sample, and collects EDS data from each pixel in the field of view, and compiles it into a visual representation of the elements present in the whole area.

3.4.3 X-ray Diffraction (XRD)

An AXRD Benchtop Powder Diffractometer (PROTO Mfg. Ltd., Oldcastle, Ontario, Canada) was applied to determine the specific phases that present in the precipitates sample using X-ray diffraction. Samples of precipitates were dried and powdered to analyze using the analyzer. The samples were placed in the circular sample holder and a glass microscope slide was used to flatten each sample's surface. The sample holder was then magnetically attached to the diffractometer. Standard samples of any specific phase were run using the analyzer and their spectra X-ray spectra were obtained. The spectrum of the unknown sample was compared to the standard spectra to determine the presence of each phase.

3.4.4 Visual MINTEQ Equilibrium Speciation Model

Visual MINTEQ software version 3.1 (https://vminteq.lwr.kth.se/download/) was used to predict the Cu speciation and solubility equilibria for different experimental conditions. The chemistry of precipitation experiments matrices, experiments temperature and pH of the matrices were used as input to the software. The concentration of solid phases was calculated for different precipitation scenarios by choosing the oversaturated solutes as precipitates species. Also the concentration of residual Cu was calculated for each case. The results of MINTEQ software were then compared to those obtained from experimental data.

Chapter 4

Effect of Copper Precipitates on Sulfate Reduction in Semi-continuous Stirred Tank Reactors

Inhibition of sulfate reduction by SRB due to precipitation of metals has been suggested as a limitation of the single stage process. However, its impact on sulfate reduction has not been fully quantified. The effect was qualitatively demonstrated by Utgikar et al. (2002) in batch serum bottle studies. In a more recent study by Kieu et al. (2011), a decrease in sulfate reduction at higher metal loadings in a single stage, semi-continuous stirred tank reactors (SCSTRs) was attributed to a combination of soluble and precipitated metal sulfides (Kieu et al., 2011; Utgikar et al., 2002).

The aim of this chapter is to investigate if metal precipitates alone can significantly impact sulfate reduction process in a single stage process. The study was carried out using semi-continuous stirred tank reactors (SCSTR) operated at 37ºC using synthetic wastewater containing various concentrations of Cu.

4.1 Single stage Copper Precipitation in SCSTRs

The four reactors were started using the same SRB enriched culture obtained from the MC reactor. The reactors were fed semi-continuously (once per week) to maintain an HRT of 50 days. The feed to reactors R1, R2, and R3 was modified to include Cu concentrations of 200 mg/L, 400 mg/L, and 600 mg/L respectively. Reactor RC served as the control with no Cu added to the feed. The maximum copper concentration of 600 mg/L was chosen, with the expectation that the sulfide produced will still be sufficient to precipitate copper to its solubility limit. The residual low concentration of dissolved copper is then not expected to adversely affect the microorganisms. The results for the 98 days of operation of these reactors are presented in Figures 4-1 through 4-6. The results for the variation in pH are presented in Figure 4-1.

The higher pH of around 7.6 for all the reactors effluents at the first feeding (Day 7) as compared to 7.4 for the startup culture from the MC reactor may be attributed to the stripping of CO_2 and H_2S during sparging with nitrogen at the start of the experiments. While the pH settled in at around 6.8 in RC and R1 by Day 28, it continued to decline in

R2 and R3 reaching a value of 6.4 by Day 35. While many SRB have been shown to tolerate a pH range of 6 to 9, the pH range for optimum growth is frequently reported in the range of 6.2–7.5 (S. I. C. Lopes, X. Wang, M. I. Capela, & P. N. L. Lens, 2007; Willow & Cohen, 2003). In order to minimize the impact of pH on the performance of the reactors, additional alkalinity in the form of sodium bicarbonate was added to the feed of all reactors starting from Day 35 onwards to achieve a target pH of 6.8 ± 0.2. Additional alkalinity at a concentration of 1750 mg/L (as $CaCO_3$) was mixed with the feed on Day 35, Day 42, and Day 49 was sufficient to achieve the pH in the target range for reactors RC and R1. No further addition of alkalinity was required to maintain the pH within the range of the two reactors for the remainder of the experiment (until Day 98). For reactors R2 and R3, regular addition of alkalinity at a concentration of 1750 mg/L (as $CaCO_3$) with the feed starting on Day 35 allowed for the pH to increase to and stabilize at around 6.5 by Day 56.

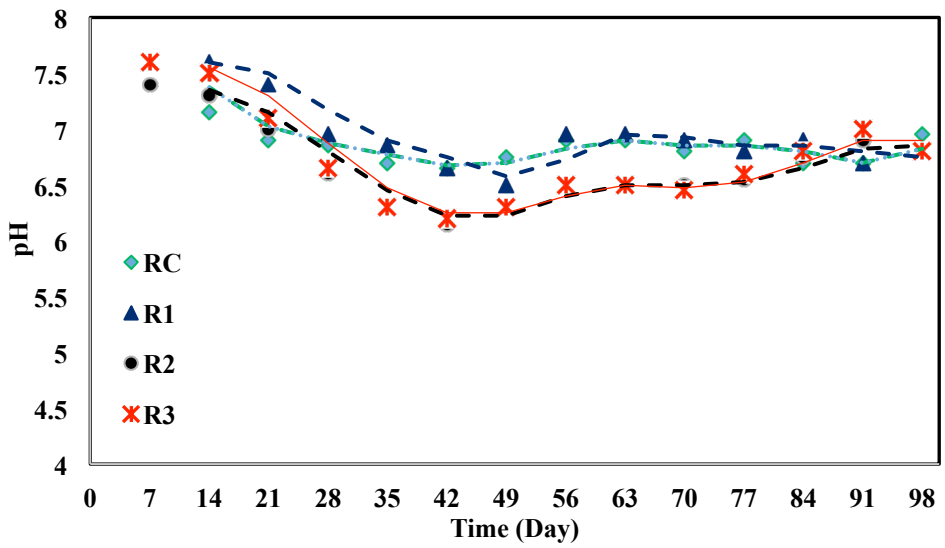


Figure 4-1 Variation in pH as a function of time of operation for semi-continuous operation of the reactors at an HRT of 50 days

The increase in alkalinity added to 2630 mg/L on Day 70 allowed the pH to reach the target range of 6.8 ± 0.2. The same level of the added alkalinity was needed to maintain the pH in the target range for the remainder of the experiment (until Day 98). Figures 4-2

and 4-3 show the concentration of volatile fatty acids (VFA) and alkalinity inside the reactors during the study period.

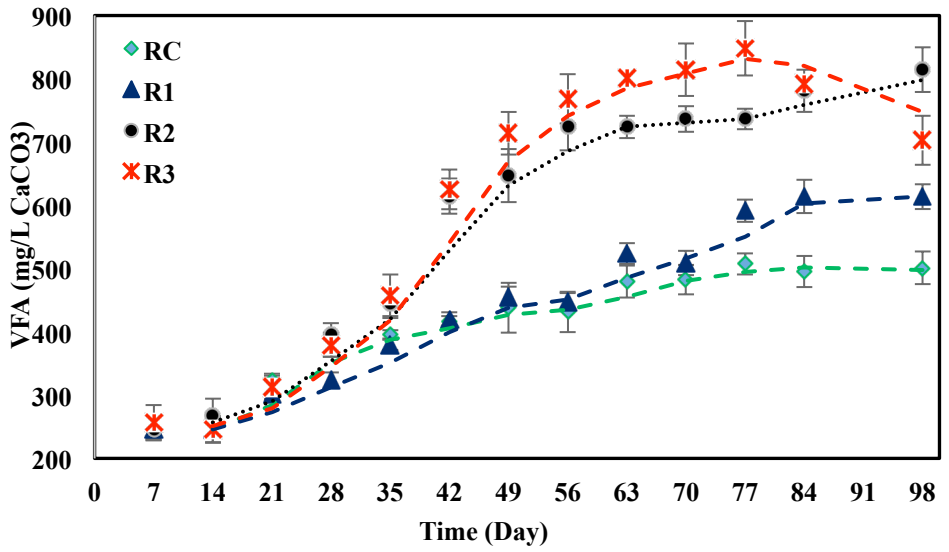


Figure 4-2 Volatile fatty acid concentration as a function of time of operation for semi-continuous operation of the reactors at an HRT of 50 days. Data presented are average of measurements from duplicate samples and error bars represent the range.

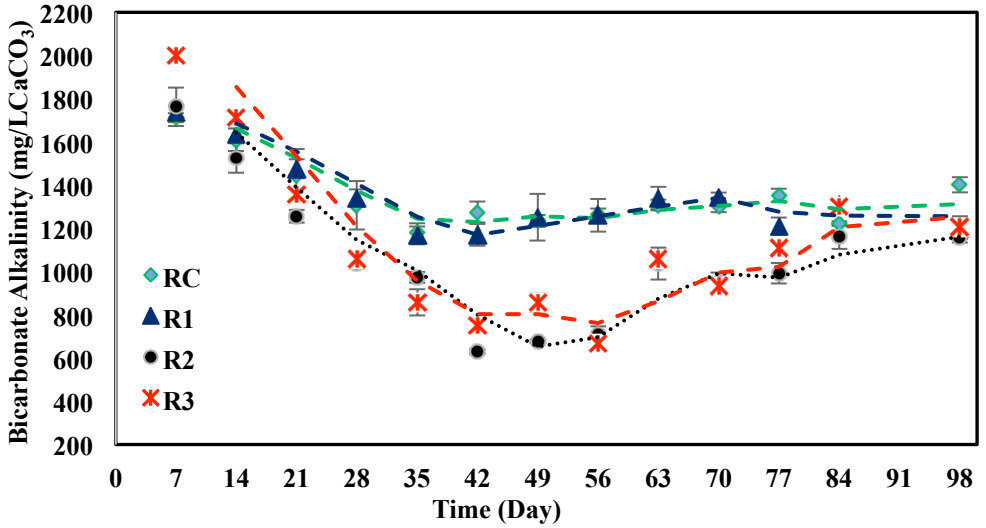


Figure 4-3 Variation in alkalinity as a function of time of operation for semi-continuous operation of the reactors at an HRT of 50 days. Data presented are average of measurements from duplicate samples and error bars represent the range

Variation in effluent ORP values for all reactors is presented in Figure 4-4. A steady ORP value of -320±30 mV was maintained throughout the entire study period for RC, R1 and

R2, and until Day 77 for R3, which is within the range of -100 mV to -500 mV, reported to be suitable for the growth of SRB (L. A. Bernardez, L. R. de Andrade Lima, E. B. de Jesus, C. L. Ramos, & P. F. Almeida, 2013). Subsequently, ORP values were seen to increase steadily in R3, reaching a value of almost +150 mV by Day 98.

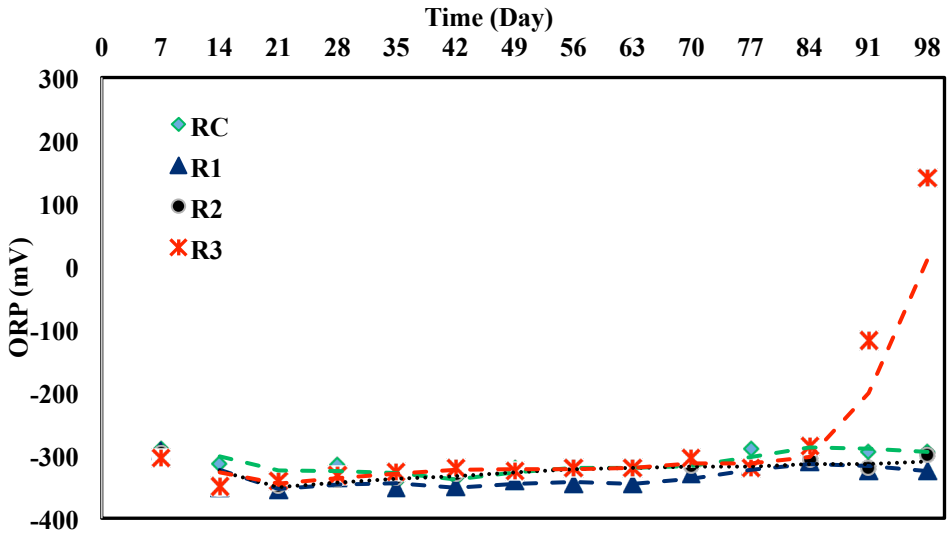


Figure 4-4 Variation in ORP as a function of time of operation for semi-continuous operation of the reactors at an HRT of 50 days

At the start of the study, the suspended solids (SS) concentration in the effluent for all reactors was about 350 mg/L (Figure 4-5). The SS concentration in the control reactor (RC), which is essentially biomass, appears to decline reaching a steady-state concentration of about 200 mg/L by Day 49 and maintained at that level afterwards. In the reactors receiving Cu in the influent (R1, R2, and R3), the SS concentration was observed to increase with time and increasing influent Cu concentrations, which can be attributed to precipitation of Cu inside the reactors. In all of these reactors, the rate of increase was more rapid in the beginning and then gradually declining to approach a quasi steady state of 400 mg/L, 600 mg/L, and 850 mg/L by Day 56 in R1, R2, and R3, respectively. In R1, a similar level (400 mg/L to 500 mg/L), with some fluctuations, was maintained for the remainder of the study. In R2 and R3, a trend of increasing SS concentrations was observed between Day 63 and Day 91 reaching a concentration of about 815 mg/L in R2 and 1132 mg/L in R3 by Day 91. No Cu was detected in the effluent (measured by atomic absorption spectrophotometer; Appendix E3) of either of

reactors R1 and R2 during the study suggesting all the added Cu was precipitated inside the reactors resulting in the higher observed SS concentrations. Although no Cu was observed in the effluent of R3 until Day 70, residual Cu concentration was measured in the effluent for this reactor from Day 77 onwards indicating incomplete precipitation of the influent Cu (Appendix E3).

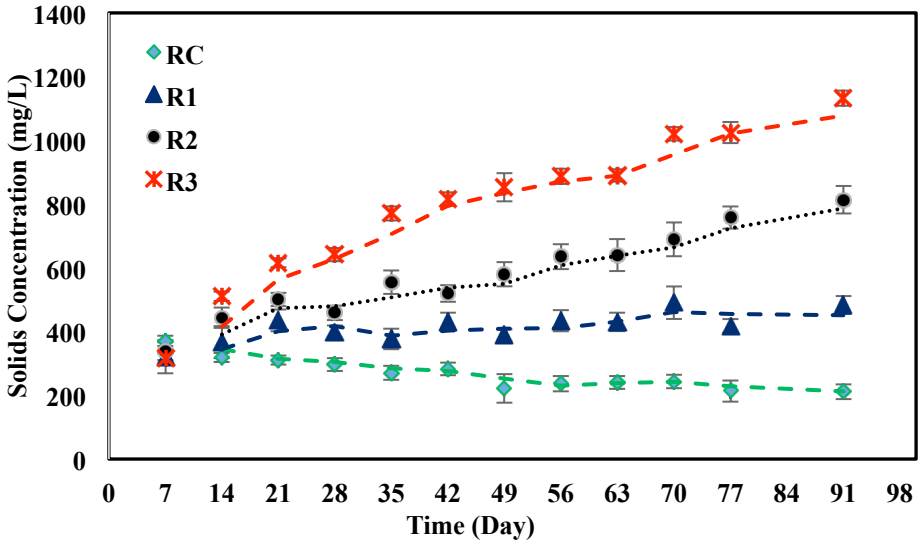


Figure 4-5 Change in suspended solids concentration as a function of time of operation for semi-continuous operation of the reactors at an HRT of 50 days. Data presented are average of measurements from duplicate samples and error bars represent the range

The results show that effluent sulfate concentration increased in all reactors as they adjusted and acclimatized to the operating conditions. As depicted in Figure 4-6, sulfate concentration in RC and R1 showed a very similar trend and reached the steady state concentration of 1300±80 mg/L after 91 days for both corresponding to sulfate reduction of 55 %. For reactor R2, the trend was similar to RC and R1 until Day 21 of operation beyond which rapid increase in effluent sulfate was observed. The steady state sulfate concentration of 1950±50 mg/L was reached after Day 70. This corresponds to a lower sulfate reduction of 38.7% compared to 55% in the control (RC). For R3, the trend for variation in sulfate concentration was similar to others until Day 14. Subsequent to that, effluent sulfate steadily increased to reach a value of 2310 mg/L, corresponding to a sulfate reduction of 24% by Day 91. There was no indication of sulfate concentration reaching a steady state.

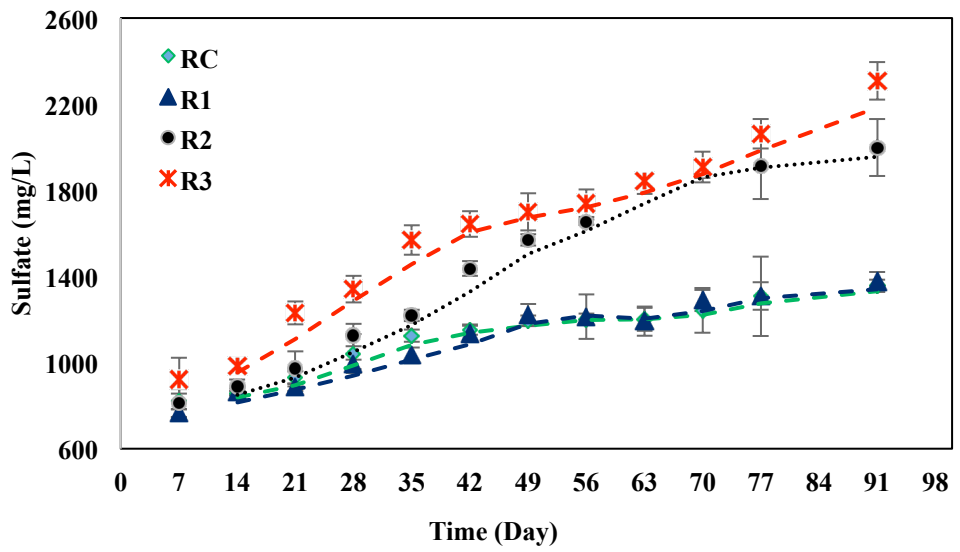


Figure 4-6 Variation in sulfate concentration as a function of time of operation for semi-continuous operation of the reactors at an HRT of 50 days. Data presented are average of measurements from duplicate samples and error bars represent the range

The pH is seen to decline much more rapidly in reactors R2 and R3 during the first 35 days of operation, before addition of external alkalinity was initiated. Higher VFA levels or defection of alkalinity could be possible reasons for the trend observed. Figure 4-2 shows that VFA levels were similar in all the four reactors during the first 35 days of operation. However, there was a faster decline in alkalinity in R2 and R3 as compared to RC and R1. The feed composition for all the reactors was the same except for the amount of Cu added as $CuCl_2$. Since $CuCl_2$ is an acidic salt, the higher consumption of alkalinity and the faster decline in pH in R2 and R3 may be attributed to the greater acidity due to the higher concentration of $CuCl_2$ added to their feed solutions.

Sulfate concentrations were fairly similar (750 mg/L to 1200 mg/L) in reactors RC, R1, and R2 for the first 35 days of operation (Figure 4-6). This suggests that the lower pH of 6.4 in R2 compared to about 6.8 in reactors RC and R1 did not have a significant impact on the sulfate reduction process. However, in R3, significantly higher sulfate concentrations were observed, as compared to the other reactors from Day 21. As shown in Figure 4-1, the pH values in R3 were very similar to those in R2. Although sulfate concentrations in the two reactors were very similar until Day 14, sulfate concentration in R3 on Day 21 and Day 28 were 1230 mg/L and 1340 mg/L as compared to 970 mg/L and 1120 mg/L in R2 (Figure 4-6). The trend seems to be closely following the trend

observed for SS concentration (Figure 4-5). The SS concentrations for R2 and R3 were similar to each other on Day 14. However, the SS concentration in R3 on Day 21 and Day 28 were 620 mg/L and 640 mg/L as compared to 500 mg/L and 480 mg/L in R2 (Figure 4-5).

Data for reactors R1 and R2 from Day 35 through to Day 56 shows a similar correlation (Figure 4-5 and Figure 4-6). The difference in SS concentration of RC (no Cu) and other reactors (R1, R2, and R3) is indicative of the amount of metal precipitated. Both of these data sets show a trend of increasing sulfate concentrations with increasing SS concentrations or alternatively that sulfate reduction is inhibited by the presence of SS or metal precipitates. In the case of reactors R1 and R2 from Day 35 to Day 56, the sulfate reduction could also have been affected by the higher VFA levels in R2 as compared to R1 (Figure 4-2). However, for the trend for R2 and R3 from Day 14 to Day 28, as discussed above, the VFA levels were similar (Figure 4-2). It can be deduced, therefore, that the inhibition in sulfate reduction can only be attributed to the presence of SS or metal precipitates. It is thus believed that the inhibition of sulfate reduction observed from the data for reactors R1 and R2 from Day 35 to Day 56, as discussed above, is also largely due to the presence of precipitated metal and that the increasing VFA levels may be the consequence.

The decrease in the concentration of VFA in R3 after Day 77, can be attributed to decrease in the activity of sulfate reducing bacteria and consequent reduction in the rate of organic matter (glucose) utilization (Bertolino et al., 2012).

Similar steady state sulfate reductions in RC and R1 (Figure 4-6) throughout the semi-continuous operation suggests that accumulation of metal precipitates in the range of 200 mg/L to 300 mg/L (Figure 4-5) does not significantly affect sulfate reduction. A similar interpretation may be made from the SS and sulfate data for reactor R2 until Day 21 and R3 until Day 14 (Figure 4-5 and Figure 4-6) when sulfate reduction is observed to be similar to that in the control (RC) at SS concentrations in the range of 200 mg/L to 300 mg/L. However, higher levels of sulfate in R2 and R3 were observed later as compared to the control (RC) (Figure 4-6). At that time, the SS concentration of metal precipitates ranged between 500 mg/L to 800 mg/L (Figure 4-5). Since no soluble Cu was measured

in the effluents of R2 and R3 (until Day 70), the higher levels of sulfate in their effluents could be attributed to the inhibition of the sulfate reduction process by the higher concentration of SS or metal precipitates. This suggests that the concentration of SS or metal precipitates have to be higher than a threshold value (around 300 mg/L in the current study) to significantly affect the sulfate reduction process. In R3, increasing sulfate concentrations (Figure 4-6) and ORP values (Figure 4-4) were observed after Day 70 suggesting failure of the sulfate reduction process. Measurable concentrations of residual Cu were also observed the reactor effluent during this period (data not shown). With the highest concentration of Cu in the feed (600 mg/L), the concentration of metal precipitates and the impact on sulfate reduction (and the sulfide produced as a result) was the highest amongst all the reactors (Figures 4-5 and 4-6). The results suggest that by Day 70, the reduction in the amount of sulfide produced was not sufficient to precipitate all the influent soluble copper. Toxicity resulting from the soluble Cu concentration may be responsible for complete cessation of sulfate reduction observed (Utgikar et al., 2001; Kieu et al., 2014).

4.2 Batch Kinetic Study

Batch kinetic data were obtained from the analysis of the samples taken from the reactor on a daily basis for 10 days after a one-time feeding. Data of sulfate concentrations and their variation over the course of the time have been given in Appendices E6 and 4. Reactor RC entered its steady state sulfate concentration of 1380±20 mg/L after three days of operation. The same amount of steady state in effluent sulfate concentration of R1 was achieved but at a slower rate than that of the RC. For reactor R2 steady state concentration of 1930±30 mg/L for effluent sulfate happened after Day 4. The reactor receiving 600 mg/L of copper (R3) showed a low amount of sulfate reduction rate as its effluent sulfate decreased to a steady state concentration of 2400 mg/L from its initial value of 2500 mg/L after 6 days. The lowest sulfate reduction rate for R3 may be attributed to the toxic effect of residual dissolved copper that appears in the reactor effluent after Day 77 onward.

For RC, R1 and R2 in which the sulfate reduction rates were considered to be influenced only by the accumulation of precipitates, the initial linear range of the data (first 3 data

points) was used to calculate the rate of sulfate reduction. The change in variation of sulfate reduction rate for added copper concentration have been shown in Figure 4-7. The sulfate reduction rate is observed to be highest for RC at 104.5 mg/L/d, which progressively reduces to 60.9 mg/L/d, 49.6 mg/L/d for R1 and R2.

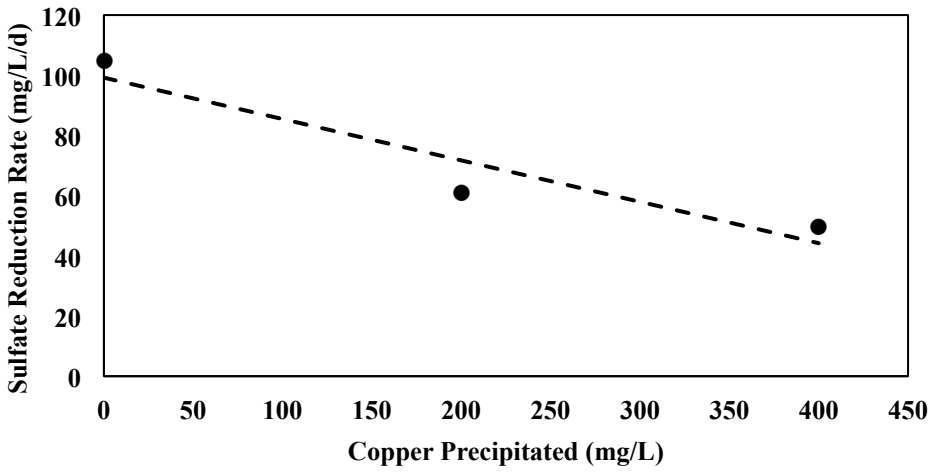


Figure 4-7 Variation in the rate of sulfate reduction by the change in the concentration of precipitated copper

The significant reduction in sulfate reduction (42 %) in R1 compared to the control (RC) shows that the sulfate reduction process is significantly affected even at the lower SS or metal precipitate concentrations of 200 mg/L to 300 mg/L. Thus, contrary to the interpretation based on the semi-continuous operation of the reactors, there may not be a threshold concentration of metal precipitates in order for the sulfate reduction process to be significantly affected. Batch operation data (Appendix 4) show that despite a lower rate of sulfate reduction, sulfate concentrations in R1 were similar to the control (RC) by Day 7. This may explain the similar levels of sulfate reduction in RC and R1 (Figure 4-6), and, therefore, the lack of inhibition observed during the semi-continuous operation of the reactors. The higher HRT of 50 days during the semi-continuous operation allowed for sulfate reduction in R1 to "catch up" with that in RC and thus no difference in their performance was observed.

The concentration of volatile suspended solids (VSS) was also measured as an indicator of biomass concentration inside the reactors. Data for VSS and their variation over the

course of time have been presented in Appendices E6 and Appendix 5, respectively. The variations of VSS in daily basis at different process condition are presented in Figure 4-8.

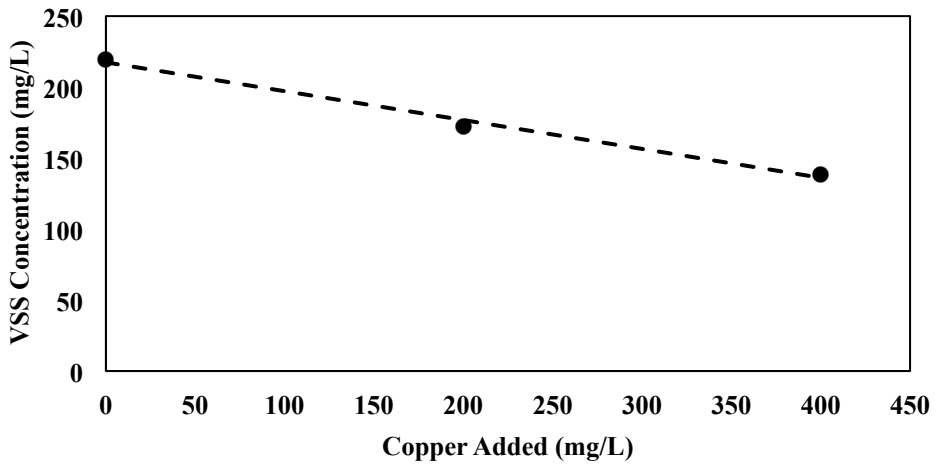


Figure 4-8 Variation in the concentration of VSS (biomass) inside the reactors by the change in the concentration of precipitated copper

Biomass concentrations for RC, R1 and R2 were found to be 219.6±5.9 mg/L, 172.5±3.8 mg/L and 138.3±2.9 mg/L, respectively. Considering the average concentration, amount of biomass inside the reactors was measured at 131.8 mg, 103.5 mg and 83 mg. These data suggest a steady drop in the amount of biomass by the accumulation of solids inside the reactors.

The rate of sulfate reduction was normalized by the appropriate amount of biomass in each reactor to evaluate the sulfate reduction activity at different concentration of Cu fed to the reactors which result in different amounts of precipitate inside each reactor. SO_4^{2-}/ VSS for reactors RC, R1 and R2 were calculated as 0.48 mg SO_4^{2-}/mg VSS.d, 0.35 mg SO_4^{2-}/mg VSS.d and 0.36 mg SO_4^{2-}/mg VSS.d.

4.3 Genomic Analysis of Cultures Withdrawn at Different Copper Loading

In a previous study by Utgikar et al. (2012), the inhibition of biogenic sulfate reduction in the presence of metal precipitates was deemed to be external to the microorganisms and attributed to the metal precipitate being a physical barrier to SRB for substrate. Thus increasing amounts of metal precipitates from RC to R3 is expected to affect the microbial population but not much change is expected in the composition of this population.

Microbial community analysis was carried out using Polymerase Chain Reaction (PCR) and next generation sequencing techniques to determine Operational Taxonomic Unit (OTU) for the investigation of the possible effect of the copper removal process on microbial community profile. The results have been presented in Appendix E7. The distribution of different microorganisms for various experimental conditions (i.e., the change in the concentration of copper in the influent from 0 to 600 mg/L) was calculated from the OTU numbers and results have been given in Table 4-1.

Table 4-1 Distribution of microorganisms at different copper loading

		Distribution(%)			
		RC	**R1**	**R2**	**R3**
Microorganisms	***Uncultured bacteria***	1.39	3.57	6.51	71.74
	***Trichococcus* sp**	20.65	4.44	2.22	2.89
	***Proteiniphilum* sp**	27.86	44.24	26.73	5.38
	***Bacteroidetes* sp**	17.07	10.69	55.30	5.52
	***Petrimonas sulfuriphila*sp**	16.49	16.46	2.19	0.46
	***Dethiosulfovibrio salsuginis* sp**	1.14	16.11	5.69	1.60
	***Uncultured Ruminobacillus* sp**	0.03	0.11	0.03	9.50
	Uncultured Synergistetes bacterium	0.06	0.51	0.04	1.06
	Uncultured Firmicutes bacterium	7.20	3.48	1.09	1.02
	***Geobacter* sp**	6.51	0.24	0.14	0.35
	Others	1.61	0.15	0.06	0.48
Overal		100.00	100.00	100.00	100.00

To visualize the effect of the change in influent copper concentration on the composition of the microbial community, the results of microbial distribution at different copper loading rates has been depicted in Figures 4-9 (a) to (d).

The results show that the although there were some differences, the microbial community composition was fairly similar between the control (RC; no copper), R1(copper = 200 mg/L), and R2 (copper = 400 mg/L) but distinctly different in R3 (copper = 600 mg/L). As explained in Section 3-3-1, the samples for microbial community analysis were collected towards the end of the experimental run (Day 90). At that time, RC, R1 and R2 were maintaining an environment conducive to sulfate reduction and were actively reducing sulfate (Figure 4-6). In R3 however, the higher copper concentration and metal precipitation is seen to have led to a progressive shutdown of sulfate reduction and a complete change in the environmental conditions (Figure 4-4). This can explain the distinctive change in the microbial community composition.

To visualize the similarities and variations between the microbial profiles data, the application of metric multidimensional scaling technique known as principal coordinate analysis (PCoA) has been suggested as the best option (Schütte et al., 2008). Figure 4-10

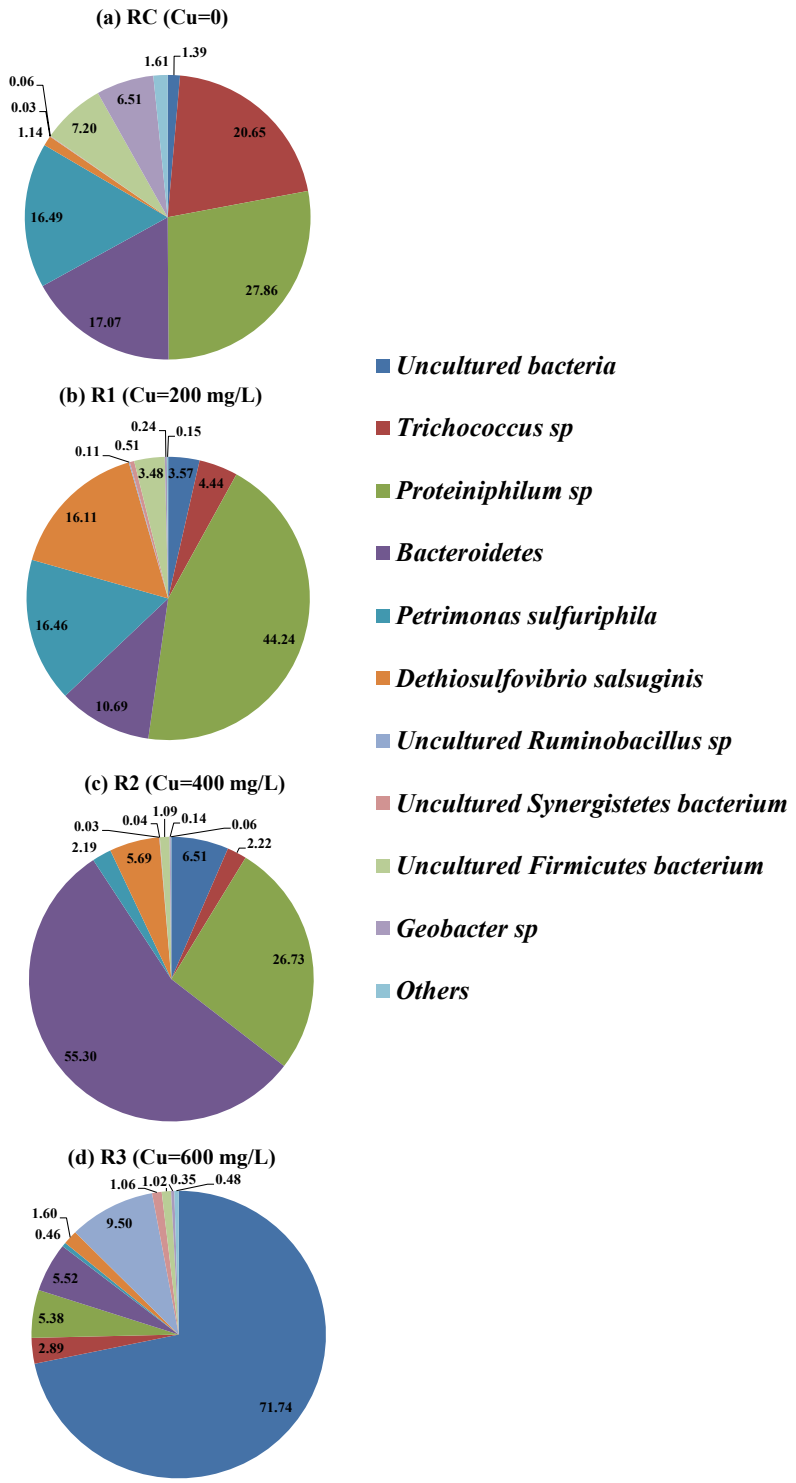


Figure 4-9 Shifts in microbial population observed at (a) addition of no copper (b) addition of 200 mg/L of copper (c) addition of 400 mg/L of copper (d) addition of 600 mg/L of copper

shows the results of conducting PCoA on microbial profiling of different operational conditions using the PAST software package.

PCoA analysis of data revealed that close to 89% of the variance in data set can be explained by the first two coordinates. As shown in Figure 4-10, a progressive change in similarity of the cultures is quite obvious. RC showed the highest similarity to R1 in comparison to other cultures as the two have been clustered to each other (Figure 4-10). These two cultures vary from the cluster of cultures from R2 along the vertical axis, which represents the 2nd principal coordinates that explains only 18.31% of total variance.

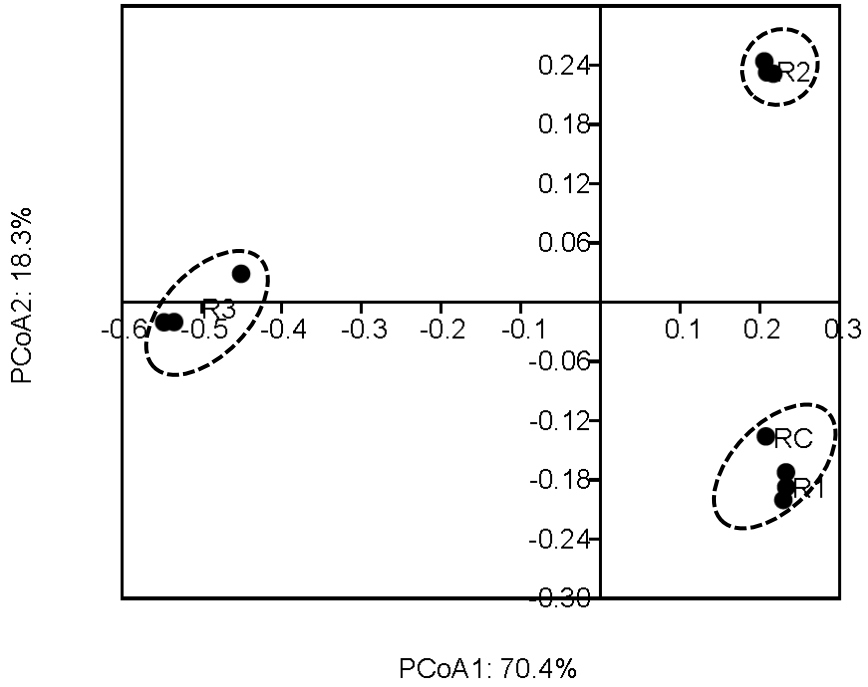


Figure 4-10 Principal coordinate analysis (PCoA) of genomic data of the cultures with drawn from RC, R1, R2 and R3

The cluster of cultures from R3 is well separated from the other three (RC, R1 and R2). This is attributed to a distinct change in the microbial community composition due to cessation of sulfate reduction and resulting shifts in environmental conditions (Figure 4-4).

Since no residual copper was measured in solution in R1 and R2, all the copper present in the influent was precipitated. The progressive reduction in the rate of sulfate reduced

from RC to R1 and R2 was attributed to the mass transfer limitation of substrate availability to the microbial community. Based on that, no effect on the microbial community composition was expected. However, the PCoA analysis presented in Figure 4-10 shows that this was not entirely true, with some progressive change observed from RC to R1 to R2. Similarity %age (SIMPER) test may be a useful statistical tool to evaluate the contribution of different components of a dataset on overall dissimilarity. SIMPER test was conducted using the PAST software package on the same genomic dataset. The results have been presented in Table 4-2.

The community in RC, R1, and R2 are dominated by hydrolytic and acid forming bacteria (*Trichococcus* sp. and *Bacteroidetes*). Only two of the identified species (*Petrimonas* and *Dethiosulfuvibrio salsuginis*) are known to part of the sulfate reduction pathway to sulfide.Among the microorganisms that contribute to the overall dissimilarity, *Petrimonas sulfuriphila* sp is the only species that is capable to reduce the sulfate to hydrogen sulfide under the anaerobic condition (Van Eerten-Jansen et al., 2013). As presented in table 4-2, this species contributes in 12 and 16% of overall dissimilarity of RC to R1 and R2, respectively. This can be a reason of decrease in sulfate reduction rate from RC to R1 and R1 to R2.

Fractional distribution of microorganisms in RC and R1 revealed that the two are quite similar in terms of their respective population of *Petrimonas sulfuriphila* sp. Significant decrease in distribution of this species was observed in R2 (Table 4-1). The adverse effect of certain concentrations of residual heavy metals on *Petrimonas sulfuriphila* sp. has been reported in the literatures (H. T. Kieu, Horn, & Müller, 2014). However, in this study no residual copper was measured during the entire course of the experiments. Thus, there seems to be some adverse effect of copper precipitates that can possibly change the population of the *Petrimonas* microorganisms.

Table 4-2 The results of similarity percentage (SIMPER) test on genomic data

		RC to R1	RC to R2	RC to R3	R1 to R2	R1 to R3	R2 to R3
Overal Dissimilarity (%)		39.5	49.1	79.1	43.7	77.4	76.36
		Contribution of Different Especies to Dissimilarity (%)					
Type of Microorganism	***Trichococcus* sp.**	29.3	20.7	8.5	1.0	1.1	1.3
	Bacteroidetes	17.8	32.7	4.7	66.1	1.8	22.2
	Dethiosulfovibrio salsuginis	12.8	4.0	-	7.0	4.4	1.4
	Petrimonas sulfuriphila	11.6	16.1	8.2	12.1	5.6	-
	***Uncultured Geobacter* sp.**	8.0	5.5	2.6	-	-	-
	***Proteiniphilum* sp.**	5.2	5.8	10.4	5.8	11.2	8.5
	Uncultured Firmicutes bacterium	3.8	4.5	1.8	1.7	-	-
	Uncultured Firmicutes bacterium	3.6	2.5	1.2	-	-	-
	***Geobacter* sp.**	2.3	1.6	-	-	-	-
	Uncultured bacteria	1.0	4.6	53.0	5.7	65.6	56.3
	***Uncultured Ruminobacillus* sp.**	-	-	7.0	-	8.7	7.8
Overal Contribution (%)		95.6	97.9	97.4	99.4	98.2	97.6

Dethiosulfovibirio salsuginis is an strictly anaerob capable of reducing thiosulfate to sulfide and does not reduce sulfate to sulfide directly (Diaz-Cardenas, Lopez, Patel, & Baena, 2010). This species require thiosulfate to be survived. No analytical measurement of thiosulfate was carried out in the current study. However, the loss of sulfide at different ranges of 45% to approximately 80% during the anaerobic sulfate reduction has been reported by the researchers (Jong & Parry, 2003; H. T. Q. Kieu, Muller, & Horn, 2011; Mallelwar, 2013). Part of this sulfide loss may be attributed to the alternate pathway during anaerobic sulfate reduction that contributes in the formation of thiosulfate (Kobayashi, Tachibana, & Ishimoto, 1969; Suh & Akagi, 1969).

As presented in Table 4-1, distribution of *Dethiosulfovibirio salsuginis* sp. increased from RC to R1 and decreased in R2 compared to that of R1. The fluctuation in the population trend was unexpected and exact reason of that is unknown. However, that might be attributed to the level of the contribution of the alternate pathway of thiosulfate production at each process condition.

Chapter 5

Sulfate Reduction and Copper Precipitation in High Rate Reactors: Comparison of Single and Two-Stage Processes

Experiments using two up-flow anaerobic hybrid reactors (UAHR), HR1 and HR2 were conducted in 2 Phases. In Phase I, the two hybrid reactors were started as described in Chapter 3, Section 3.1.2. The purpose of Phase I or start-up phase was to operate HRI and HR2 at a hydraulic retention time (HRT) of 40 days in a semi-continuous mode (feed once every 7 days) under similar operating conditions and identical feed composition (no copper). The reactors were monitored over the course of the time to allow them to reach similar performance or baseline for the monitored parameters (pH, alkalinity, VFA, sulfate, TOC). Phase II was carried out to evaluate and compare the single stage and two stage process configurations for the treatment of AMD, as discussed in Section 3.1.2.2. HR2 was operated to represent the first stage (biological sulfide production) of the two stage process, and the feed composition remained the same as that used during Phase I. For HR1, during Phase II, the feed composition was modified to allow for the evaluation of the single stage process for the treatment of acid mine drainage (AMD), as discussed in Section 3.1.2.2a Copper was added to the feed to represent the presence of heavy metals in AMD, and a summary of the modified feed compositions over the course of Phase II are presented in Table 3-2.

5.1 Reactors Operation

5.1.1 Phase I: Start-up

The reactors were inoculated with the enriched culture maintained according the protocol given in Section 3.1.2.1 (a). After 8 days of the inoculation the reactors by enriched culture, reactors started to be operated at HRT of 40 days with the influent COD/SO_4^{2-} of 1 and sulfate concentration of 3040 mg/L. Reactors were closely monitored for important parameters such as pH, ORP, sulfate, Bicarbonate and VFA concentration. Figure 5-1 shows the variation of pH, ORP, Volatile Fatty Acids (VFA) and bicarbonate alkalinity during the start-up phase.

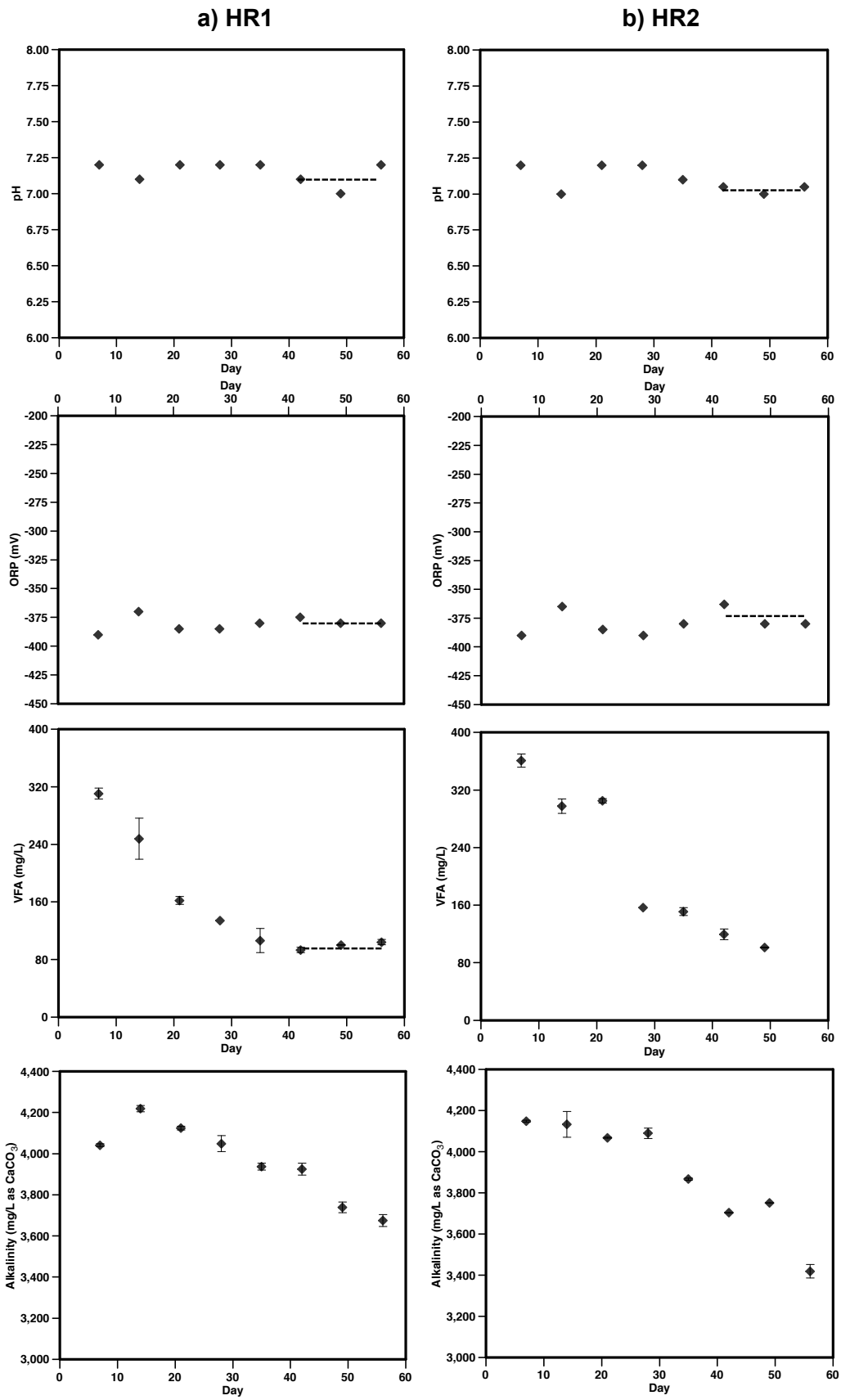

a) HR1
b) HR2
pH
ORP (mV)
VFA (mg/L)
Alkalinity (mg/L as CaCO3)
Day

Figure 5-1 Variation of pH, ORP, VFA and bi-carbonate alkalinity by change in time during the start up phase; a) single stage reactor, HR1 and b) two stage reactor, HR2. For VFA and alkalinity, each data point represents average of the measurements for three samples and error bars represent the associated standard error.

There was little change in the pH and ORP values for both HR1 and HR2 and the values were quite similar for the entire duration of Phase I. Steady-state values of 7.1±0.09 and -380±3.5 for pH and ORP for HR1 were very similar to 7.1±0.04 and -378.6±9.7 for HR2. The values are within the optimum pH range of 6.2-7.5 (S. I. C. Lopes et al., 2007; Willow & Cohen, 2003) and ORP range of -100 to -500 mV (L. Bernardez, L. de Andrade Lima, E. de Jesus, C. Ramos, & P. Almeida, 2013) reported in many species of SRB in literature. As an important operational parameter, in this study, the pH of both reactors was trying to be adjusted at targeted pH value of 6.8±0.2 as suggested by Mallelwar (2013).

For total VFA, an increase in levels from ~ 165 to ~350 mg/L was observed between Days 0 and 7. This may be attributed to the inability of SRBs to consume all of the VFA produced by the faster growing acid forming bacteria at the beginning of reactor operation. More time allowed the SRBs to grow in population resulting in the decline in VFA levels observed between Day 14 and 28. Not much variation in bicarbonate alkalinity values was observed for HR1 and HR2 during the entire Phase I, reaching approximately similar steady-state values of 3867±111 and 3774±84 mg/L respectively.

Variation of the effluent sulfate concentration and Total Organic Carbon (TOC) has been presented at Figure 5-2.

Both reactors showed very similar trends of sulfate conversion at the beginning of start-up phase. Figure 5-2 shows the sulfate concentration in the effluent of both reactors reaches to almost zero from the initial values of 127.6 and 150.2 mg/L for HR1 and HR2 respectively. This means that the sulfate conversion of more than 99% happened at the end of the first feeding cycle and continued for the entire of start-up phase.

TOC removal rates for both HR1 and HR2 also showed a similar trend for an entire start-up phase (Figure 5-2). Initial TOC concentrations for HR1 and HR2 were approximately 220 and 240 mg/L that dropped to around of 95 mg/L at Day 21. These correspond to the decline of VFA after Day 7 of the operation. The concentrations of TOC for both reactors

entered a steady state condition for the remaining of Phase I. TOC levels of 103±16 mg/L and 106±15 mg/L were achieved for HR1 and HR2 respectively.

Similar performance of two hybrid reactors during the start-up phase provided an appropriate baseline for the next phase of the experiments.

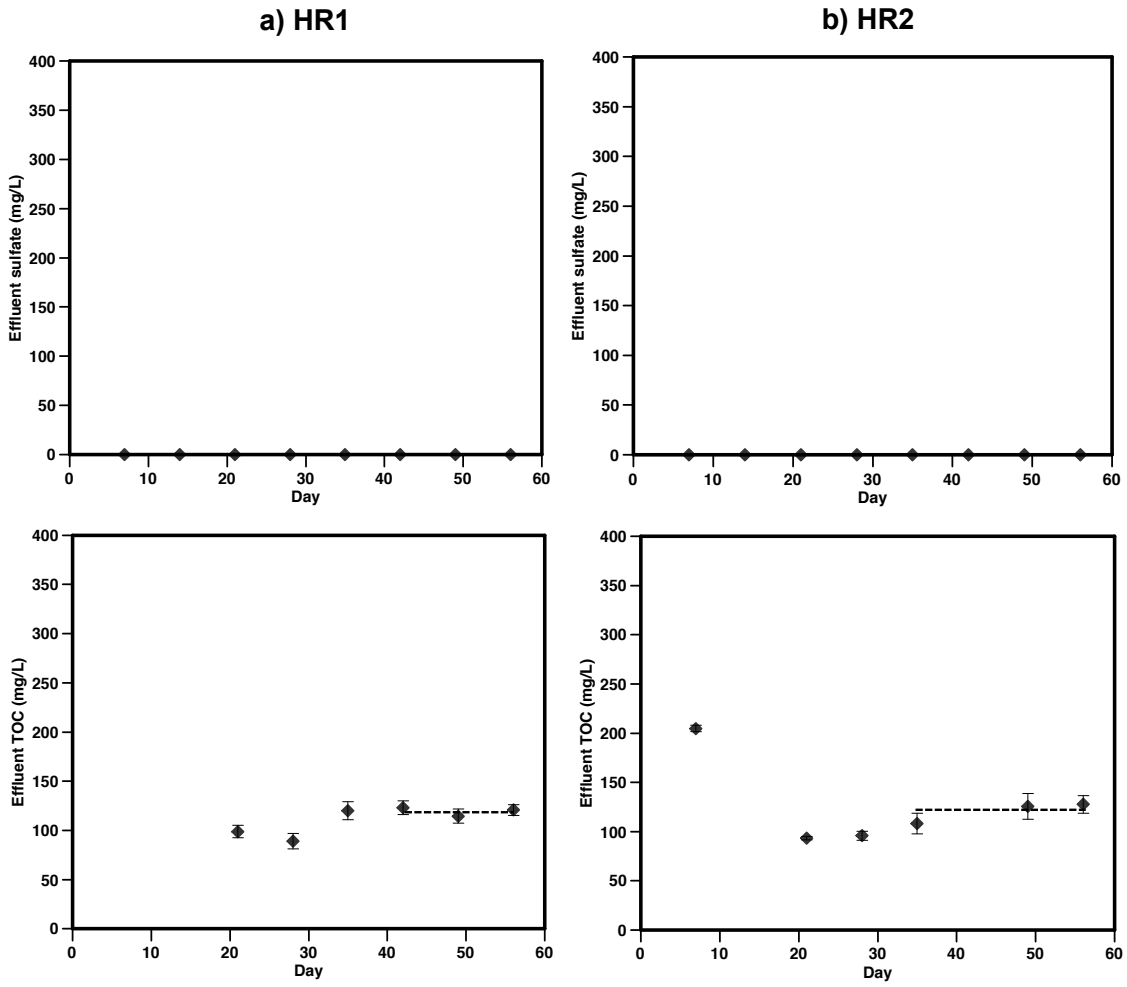


Figure 5-2 Variation of sulfate and total organic carbon (TOC) concentration by change in time during the start up phase; a) single stage reactor, HR1 and b) two stage reactor, HR2. Data presented are average of measurements from duplicate samples and error bars represent the range

5.1.2 Phase II: Process Evaluation

Phase II of the study was conducted to evaluate and compare the performance of single stage and two stage processes for the treatment of AMD, as discussed in Section 3.1.2.2.

5.1.2.1 40 d HRT

From Day 56 onward, the feed of HR1 was modified to include 200 mg/L of copper and the reactor was operated till it was deemed to have reached a steady-state (Day 105). All other operating conditions (including HRT = 40 days) were the same as in the start-up phase (Phase I).

Figure 5.3 shows the variation of pH, ORP, VFA and bicarbonate alkalinity at HRT of 40 days. For HR1, pH of the reactor started to decrease from 7.2 at Day 84 and reached a steady state level of 6.9±0.1 based on data collected at Day 88 till 105. This can be attributed to addition of copper salt with the influent of reactor from Day 56 that increases the acidity of influent. This did not affect the reactor pH initially, but with the continuous addition of influent started to drop the pH since Day 84. The level of ORP in HR1 remained unchanged during this period in comparison to that of Start-up Phase (Figure 5-3a). Steady state ORP of -382±10 mV was reached based on data collected between Days 91 and 105. Decrease in the VFA concentration in HR1 from 100 mg/L to concentration of 87±8 mg/L can be attributed to the acidity added with the influent of the reactor. For bicarbonate alkalinity, a gradual decrease from 3675 mg/L to 3140 mg/L was observed between Days 56 – 91, after which the values stabilized to reach a steady-state value of 3130±10 mg/L between Days 91 and 105. This decline in alkalinity may also be attributed to the acidity introduced into the feed by the copper salt.

For the entire period of time, changes on the level of ORP was not significant in HR2 in comparison to that during the start-up Phase (Figure 5-3b).

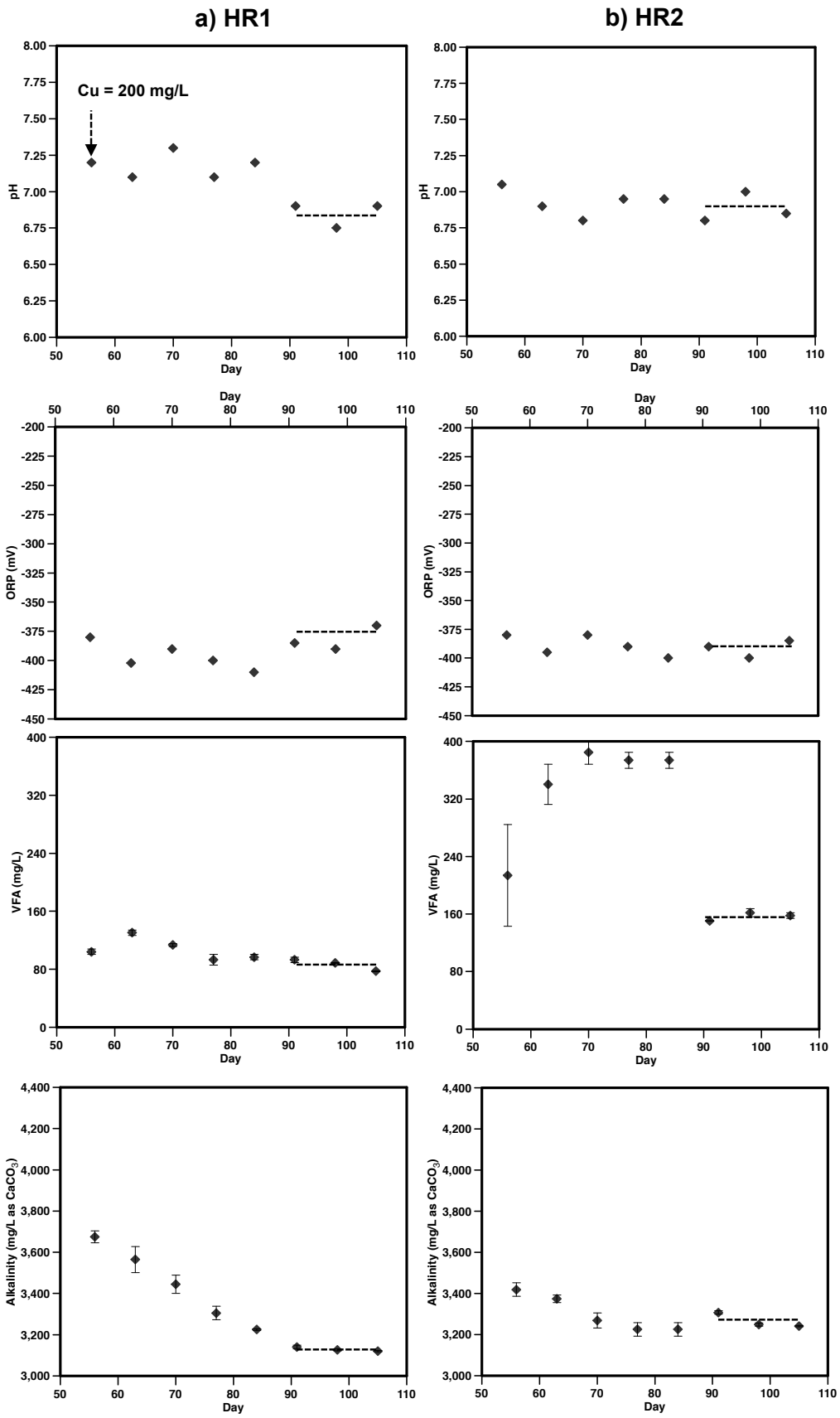

a) HR1
b) HR2
Cu = 200 mg/L
pH
ORP (mV)
VFA (mg/L)
Alkalinity (mg/L as CaCO3)
Day

Figure 5-3 Variation of pH, ORP, VFA and bi-carbonate alkalinity by change in time during the operation at HRT=40 days; a) single stage reactor, HR1 and b) two stage reactor, HR2. For VFA and Alkalinity, each data point represents average of the measurements for three samples and error bars represent the associated standard error.

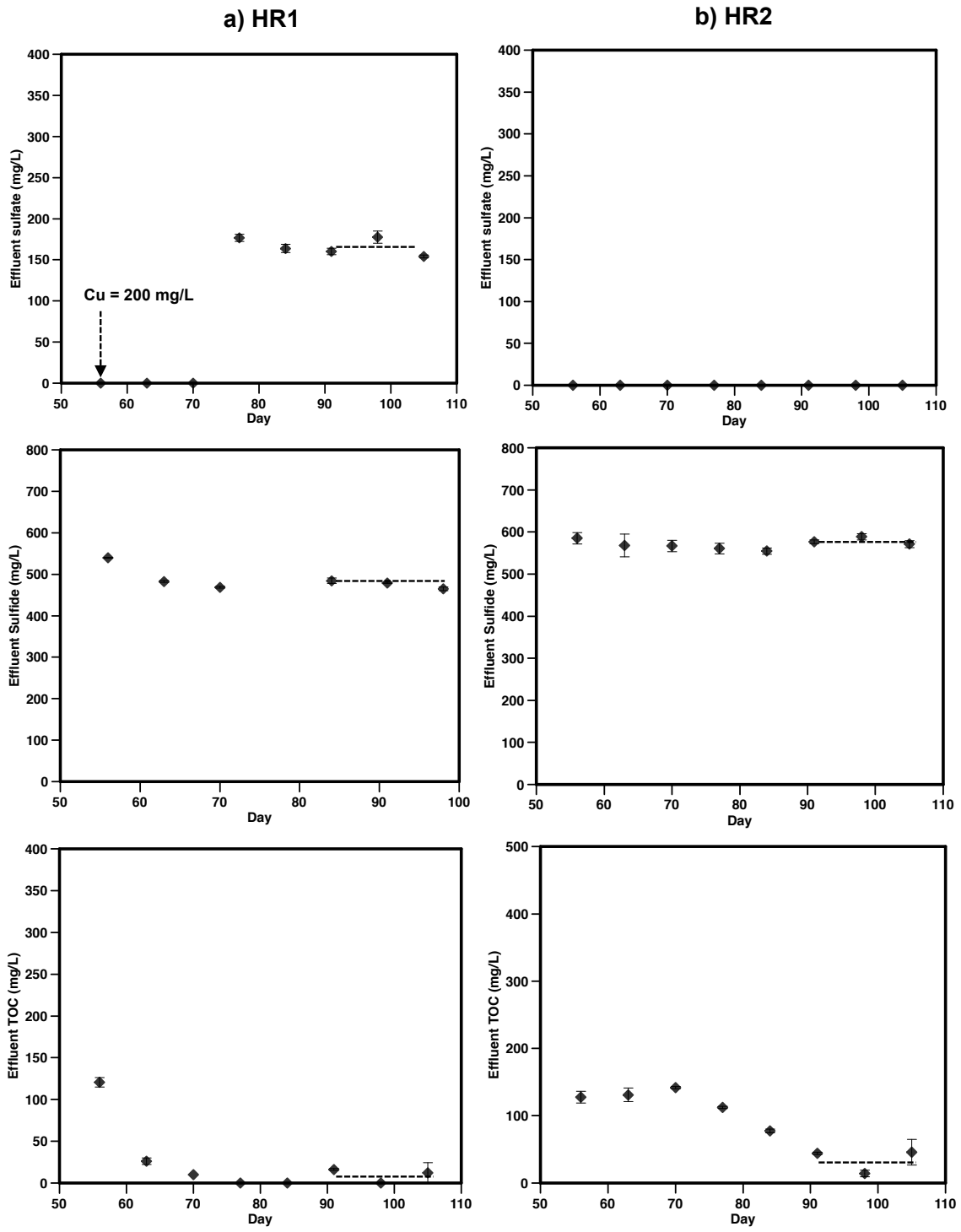


Figure 5-4 Variation of sulfate, sulfide and TOC concentrations by change in time during the operation at HRT=40 Days; a) single stage reactor, HR1 and b) two stage reactor, HR2. For sulfate and TOC, data presented are average of measurements from duplicate samples and error bars represent the range. For sulfide, average of triplicates was used and error bars represent the associated standard error.

During the operation of HR2 at HRT of 40 days, not much changes were observed in the reactor's ORP level and ORP of -393±6 mV was perceived for this time period (Figure 5-3b). The pH of the reactor dropped to 6.8 at Day 70 from its level of 7.1 at Day 56. The reason for this is not known since all the operational parameters remained unchanged compared to start-up phase. A pH fluctuation was also observed from Day 84 till Day 98 during which the pH declined from 6.95 to 6.8 and raised back to 7. The pH of the reactor reached a steady state of 6.9±0.1 at the end of the time period. As shown in Figure 5-3b, concentration of alkalinity decreased from 3420 to 3270 mg/L for the period of Day 56 till Day 70 while for the same time the concentration of VFA increased from 214 mg/L to 385 mg/L. Further, consumption of produced VFA by SRB decreased the level of VFA to 151 mg/L at Day 91 and a steady state VFA concentration of 157±6 mg/L was reached based on data collected from Day 91 till Day 105. A steady-state bicarbonate concentration of 3256±36 mg/L was also perceived based on the alkalinity measurement carried out from Day 91 till Day 105.

Sulfate was non-detectable in HR1 from Day 56 to Day 70. Slight increase in sulfate concentration to 177 mg/L at Day 77 was observed and a sulfate concentration of 164±12 mg/L was reached between Days 91 and 105 (Figure 5-4a). Given the complexity of the biogenic sulfate reduction process and the multitude of parameters that affect it, it is possible that small changes in the operating conditions may be responsible for such variations. Similar shifts were also observed in reactor HR2 (Figure 5-4b) and other studies in our Group from time to time.

Sulfide values declined from 540 mg/L on Day 56 to 468.6 mg/L on Day 70. The values then stabilized to reach a steady-state concentration of 486±10 mg/L during the time period of 91 till 105 Days. The decline in sulfide concentration can be attributed to loss due to precipitation of the added copper in the form of copper sulfide. No measurable

concentrations of copper was observed in the reactor effluent. Close to complete degradation of added TOC was observed during the entire period, reaching a concentration of 10±8 mg/L in the similar time frame of 91 to 105 days (Figure 5-4a).

No sulfate was detectable for the entire time period of HR2 operation at the HRT of 40 Days (Figure 5-4b). Effluent sulfide concentration showed no shift, maintaining a steady state value of 573±17 mg/L by Day 105. The slight increase in concentration of total organic carbon (TOC) from Day 56 to 70 corresponds to increase in VFA level at the same time. TOC concentration decreased from Day 70 and no steady state was perceived by the end of operation at HRT of 40 Days (Figure 5-4b).

5.1.2.2 20 d HRT

From Day 105 till Day 173, both reactors were operated at a HRT of 20 Days. The concentration of copper in HR1 influent was maintained at 200 mg/L (similar to that for HRT of 40 Days) until Day 141 by when the influent copper concentration increased to 600 mg/L. All other conditions remained unchanged. Results of operation under described changes are presented in Figure 5-5 and 5-6.

For HR1, no major change in the reactor pH was observed until Day 141. The pH was observed to decline from 6.9 to 6.7 Between Day 141 and 148. This can be attributed to increase of the acidity of influent by the increase in copper concentration to 600 mg/L which was initiated at Day 141. To enhance the buffering capacity and to maintain the optimum targeted pH value of 6.8±0.2, the addition of 420 mg/L (as $CaCO_3$) excess alkalinity in the form of $NaHCO_3$ with the influent of HR1 was initiated from Day 148. This resulted in an increase in the level of pH from 6.7 at Day 148 to 6.9 at Day 159, when the addition of excess alkalinity stopped. The pH declined to the lowest level of 6.6 at Day 162 and the addition of alkalinity initiated again from Day 166 at the concentration of 600 mg/L (as CaCO3) from Day 166. The pH level of HR1 reached a steady state of 6.7±0.1 for the time period of Day 166 till 173 (Figure 5-5a). Similar to HR1, for HR2, the level of pH did not show any significant change for the period of Day 105 till 131. From Day 131 pH started to decrease reaching its lowest of 6.7 at Day 138. The addition of alkalinity at a concentration of 300 mg/L (as CaCO3) with the influent of HR2 was initiated at Day 138. The concentration of excess alkalinity increased to 420

mg/L (as CaCO3) to prevent the higher decrease of pH from the targeted pH value. No alkalinity added with the reactor influent once the trend of pH variation was deemed to maintain the target pH. For HR2, a steady state pH of 6.70±0.03 was reached for the period of Days 166 till 173 (Figure 5-5b).

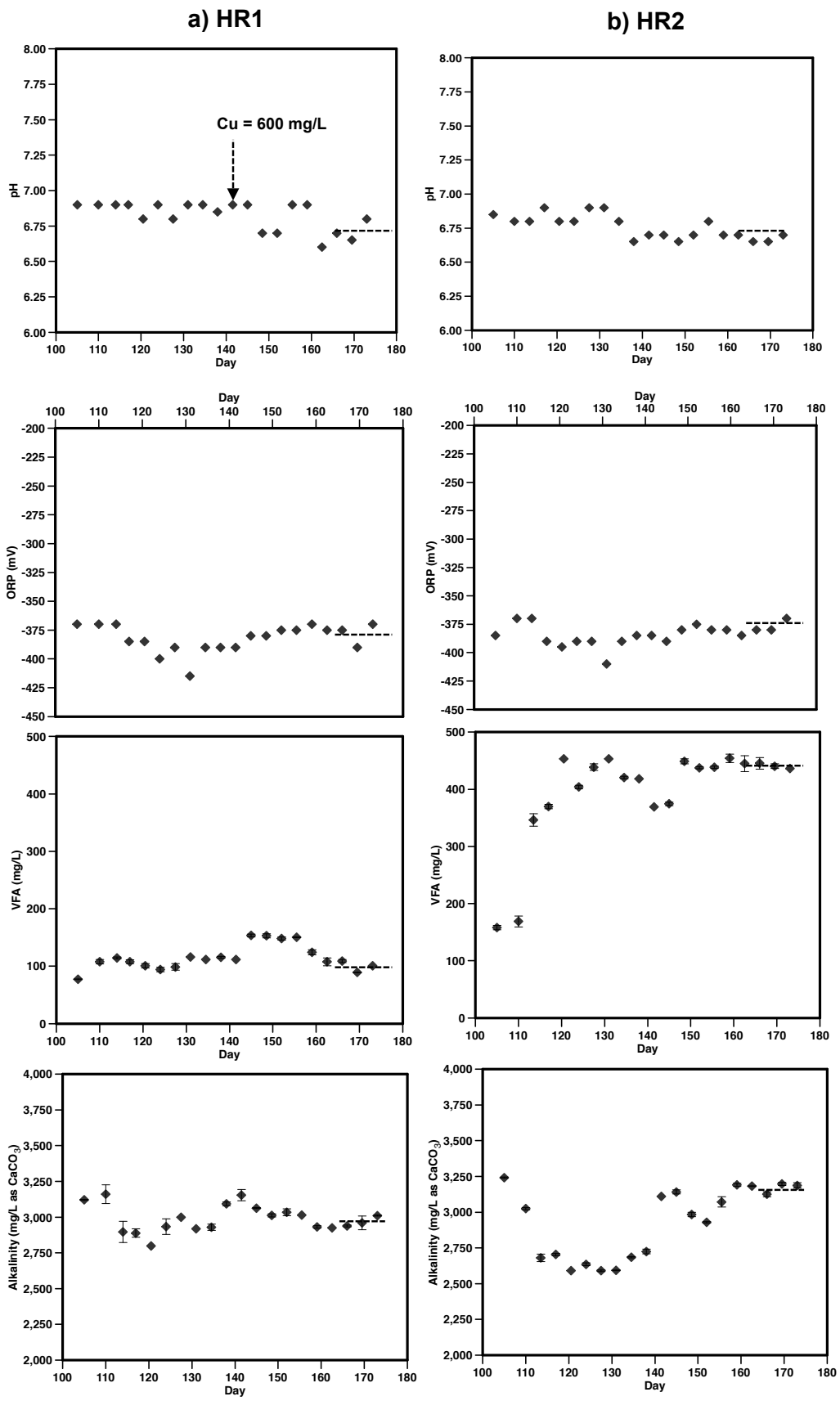

a) HR1
b) HR2
Cu = 600 mg/L
pH
ORP (mV)
VFA (mg/L)
Alkalinity (mg/L as CaCO3)
Day

Figure 5-5 Variation of pH, ORP, VFA and Bi-carbonate alkalinity by change in time during the operation at HRT=20 Days; a) Single Stage Reactor, HR1 and b) two stage reactor, HR2. For VFA and alkalinity, each data point represents average of the measurements for three samples and error bars represent the associated standard error.

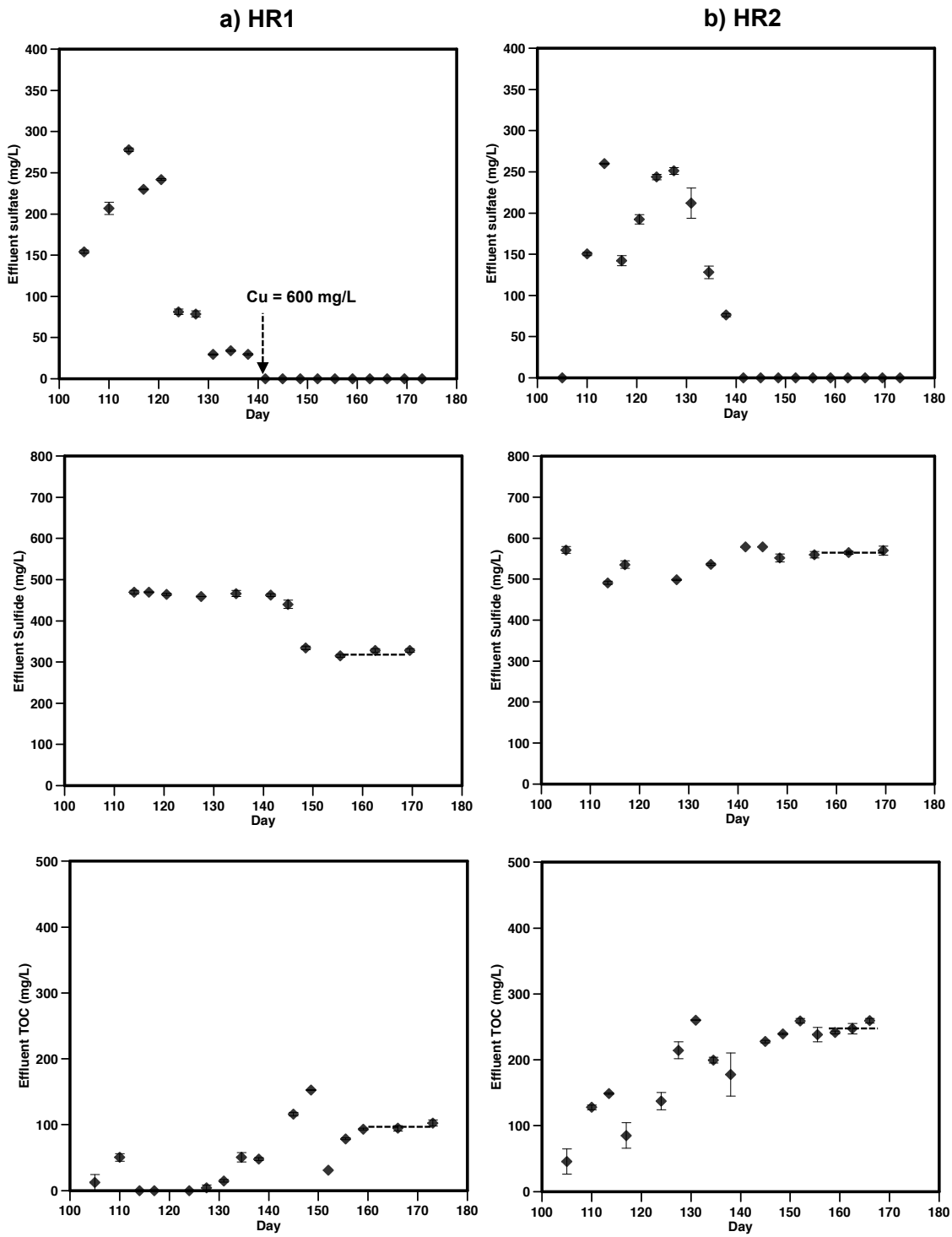


Figure 5-6 Variation of sulfate, sulfide and TOC concentrations by change in time during the operation at HRT=20 Days; a) single stage reactor, HR1 and b) two stage reactor, HR2. For sulfate and TOC, data presented are average of measurements from duplicate samples

and error bars represent the range. For sulfide, average of triplicates was used and error bars represent the associated standard error.

For HR1, no substantial of variation was observed in the alkalinity of the reactor until Day 141 beyond which the combination of generated alkalinity and extra alkalinity added with the influent of the reactor reached a steady state level of 2969±37 mg/L (Figure 5-5a). In general, no significant change in the concentration of VFA was observed in the entire of period of time for HR1 suggesting that microorganisms were able to take up the organic carbon. VFA concentration of HR1 slightly increased from 77 mg/L at Day 105 to 115 mg/L at Day 114. This initial increase might be attributed to the increase of organic loading rate. Another increase in the concentration of VFA occurred between Day 141 and 145 that can be attributed to the increase in the acidity of influent. A concentration of 100±10 mg/L of VFA was measured for HR1.

As shown in Figure 5-5b Decline in alkalinity concentration at the effluent of HR2 from Day 105 till 114 can be attributed to the shorter HRT at this period that lowers the contact time of SRB with the organics for bicarbonate generation.

By the addition of excess alkalinity from Day 138 onward, concentration of alkalinity in the effluent of HR2 started to increase and eventually, steady state level of 3170.4±9.0 mg/L was reached (Figure 5-5b).

Concentration of VFA increased from 158 mg/L at Day 105 to 453 mg/L at Day 120. This can be attributed to the increase of organic loading rate by decrease in HRT at Day 105. The concentration of VFA then reached a steady state of 441±4.5 mg/L based on the measurements carried out at Day 166 till Day 173.

The effluent sulfate concentration of both HR1 and HR2 showed approximately similar trends during the operation (Figure 5-6a and b). For HR1, reactor sulfate concentration increased from 154 at Day 105 to its highest level of 277.9 mg/L at Day 114. This can be attributed to the increase in the reactor sulfate loading rate. Once the microorganisms acclimate to the new condition and start to uptake the sulfate, the effluent sulfate began to decrease until almost 99% of sulfate reduction was achieved during the operation of reactor at HRT of 20 Days. Similarly in HR2, an increase in sulfate concentration was observed from almost zero at Day 105 to approximately 250 mg/L by Day 127. By

acclimation the organisms to new condition, sulfate concentration started to decrease and almost complete sulfate reduction was perceived for HR2. Given the increase in VFA concentration at the same period in HR2, initial increase in sulfate concentration might be also attributed to the dominance of acid producers over sulfate reducers. The fluctuation in sulfate concentration that was observed between Day 110 and Day 120 can be attributed to the self-oscillating coexistence of methane producers (MPB) and SRB. That may lead to a dominance period for MPB that results in lowering the sulfate reduction efficiencies in such circumstances (Parkin, Lynch, Kuo, Van Keuren, & Bhattacharya, 1990; Vavilin, Vasiliev, Rytov, & Ponomarev, 1994).

Sulfide values in the effluent of HR1, started to decline from 463 mg/L at Day 141 to 335 mg/L at Day 148 (Figure 5-6a). This decline in the effluent sulfide concentration is attributed to increase in influent copper concentration from 200 mg/L to 600 mg/L that needs more sulfide to precipitate copper as copper sulfide. No measurable concentration of copper was observed in the reactor effluent.

Sulfide concentration reached a steady-state value of 327±2 mg/L for the period of Day 166 till Day 173. The role of increase in the influent copper concentration can be better understood by the observation of effluent sulfide data of HR2. For HR2, from Day 105 to 141, there was a slight variation in the amount of producing sulfide that can be assigned to the shifts in the rate of sulfate conversion during the same period. The values then stabilized to reach a steady-state sulfide concentration of 564.8±4.95 mg/L for the measurements carried out between Day 166 and Day173 (Figure 5-6b).

For HR1, the initial increase of TOC concentration from Day 105 till day 110 may be attributed to the increase of organic loading rate. Almost complete degradation of TOC was observed from Day 114 to Day 127, when the TOC started to increase until flatting out at 50 mg/L by Day 134. This might be due to the lower activity of methane producer during the mentioned period of time. The higher increase in the concentration of TOC was observed from Day 141 till Day 148. This can be attributed to the increase in the concentration of added sodium citrate from 134 to 355 mg/L (as carbon). By decrease in the carbon content of used sodium citrate to 293 mg/L at Day 148, decline in the amount of TOC was observed. However, the level of TOC increased again from Day 152 to Day

159 in spite of the use of same concentration of citrate. The reason for this change is not known. By adjusting the citrate concentration to 244 mg/L (as carbon) at day 159, TOC concentration reached to a level of 97±5 mg/L (Figure 5-6a).

TOC degradation in HR2 steadily decreased for the entire of time period and did not reach steady state (Figure 5-6b). This could be expected considering that no sodium citrate was added with the influent of HR2.

5.1.2.3 10 d HRT

The results of operation of HR1 at the HRT of 10 Days, are presented in Figures 5-7a and 5-8a. Decrease in the pH of HR1 was observed immediately after the change of the HRT to 10 Days (Figure 5-7a). Since the acidity of the reactor influent remains unchanged, the drop in the level of pH can be attributed to a decrease in the HRT that increases the sulfate and organic loading rate.

From Day 178, bicarbonate alkalinity of 600 mg/L (as $CaCO_3$) in the form of $NaHCO_3$ was added with the influent of the reactor to maintain the target pH of 6.8±0.2 . This could help to prevent the further drop in the pH level and a steady state pH of 6.6±0.01 was maintained. ORP of the reactor remained in the similar level of that during the operation at HRT of 20 Days and was perceived to be at steady state level of -376.7±5.8 mV (Figure 5-7a). For HR2, the addition of extra alkalinity of 435 mg/L (as CaCO3) could stabilize the level of pH and a steady state pH of 6.6±0.03 was reached (Figure 5-7b).

The decrease in HRT to 10 d did not affect the sulfate reduction and by Day 190, close to the complete sulfate reduction, was observed for HR1 (Figure 5.8a). Sulfide concentration had a steady state value of 336.9±20.9 mg/L, which was not significantly different than that which was perceived by the end of operation at HRT of 20 Days (327.1±1.7 mg/L). This was expected due to the unchanged concentration of influent copper for both time periods. The sulfate concentration of HR2 showed a similar trend to that of HR1. It slightly increased from zero at Day 173 to approximately 110 mg/L at Day 175 by when sulfate concentration started to gradually decrease until Day 185. From Day 185 until the end of the time period, no sulfate was detected in reactor effluent (Figure 5.8b). This change in sulfate concentration could be attributed to the time

required by SRB to be acclimated to the new condition due to the increase of the influent sulfate loading rate. The sulfide concentration initially decreased to 529 mg/L due to the mentioned decrease in the sulfate reduction. However, sulfide concentration increased after Day 185 and reached a steady state value of 562.7±22.3 mg/L by Day 191.

For HR1, The TOC and the VFA levels increased with the increase in the organic load as shown in Figures 4.11 and 4.12. Effluent TOC increased 287.4 mg/L at Day 175 from 102 mg/L at Day 173. By acclimation of the organisms to new condition, TOC concentration decreased gradually reaching a steady state value of 217.4±11.9 mg/L. Similar trend happened to VFA level that also increased from Day 173 to 175 then followed by a gradual decrease until reaching a steady state concentration of 456±4 mg/L. TOC concentration slightly increased in HR2 from Day 173 till Day 178 by when it decreased in much higher rate reaching to 249 mg/L from 438 mg/L. Since not much of change was observed in the concentration of VFA, the reason for such decline in TOC concentration is unclear. Reactor HR2 maintained a steady state TOC and VFA concentration of 249.6±10.5 mg/L and 522±3 mg/L, respectively.

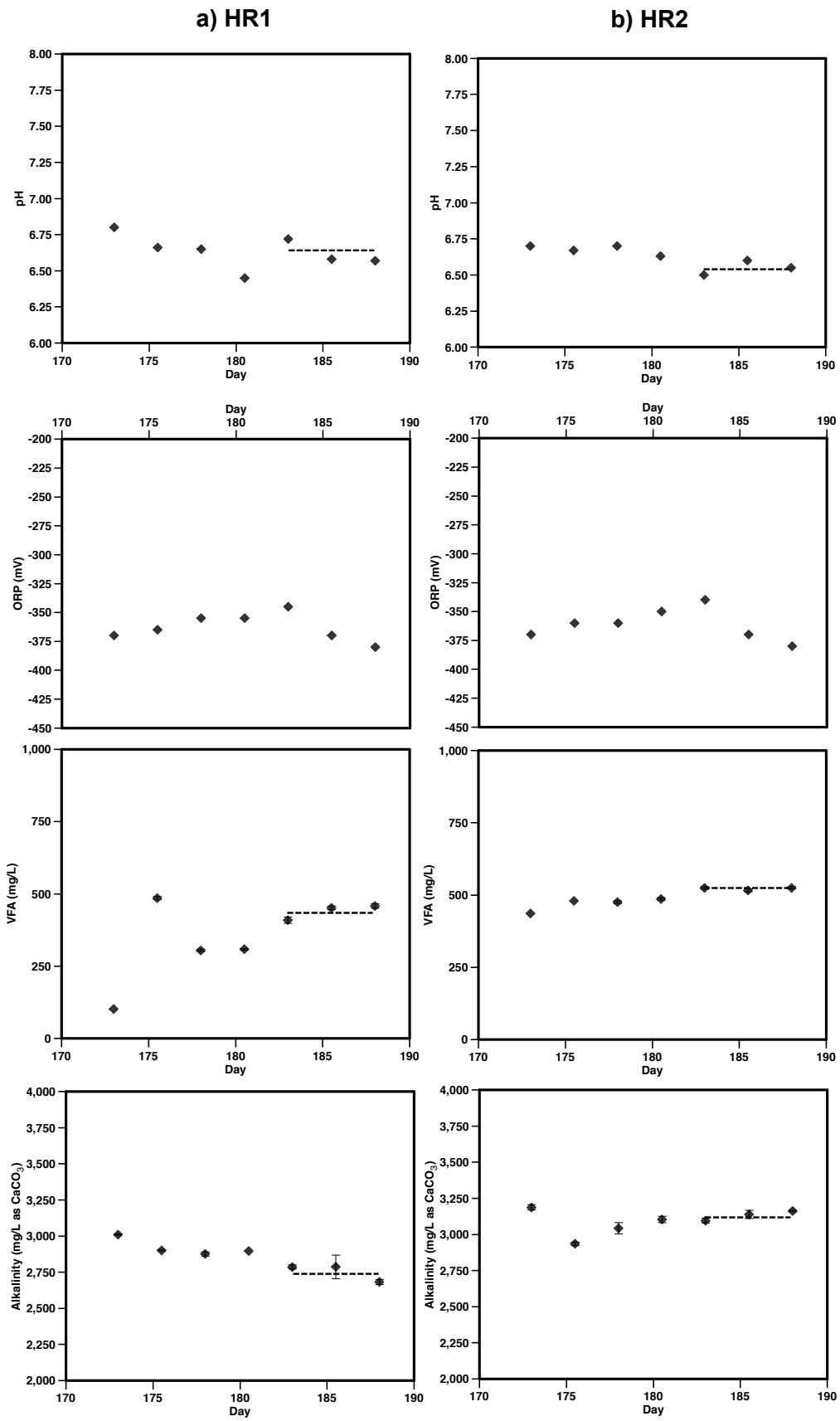

a) HR1
b) HR2
pH
ORP (mV)
VFA (mg/L)
Alkalinity (mg/L as CaCO3)
Day

Figure 5-7 Variation of pH, ORP, VFA and Bi-carbonate alkalinity by change in time during the operation at HRT=10 Days; a) single stage reactor, HR1 and b) two stage reactor, HR2. For VFA and alkalinity, each data point represents average of the measurements for three samples and error bars represent the associated standard error.

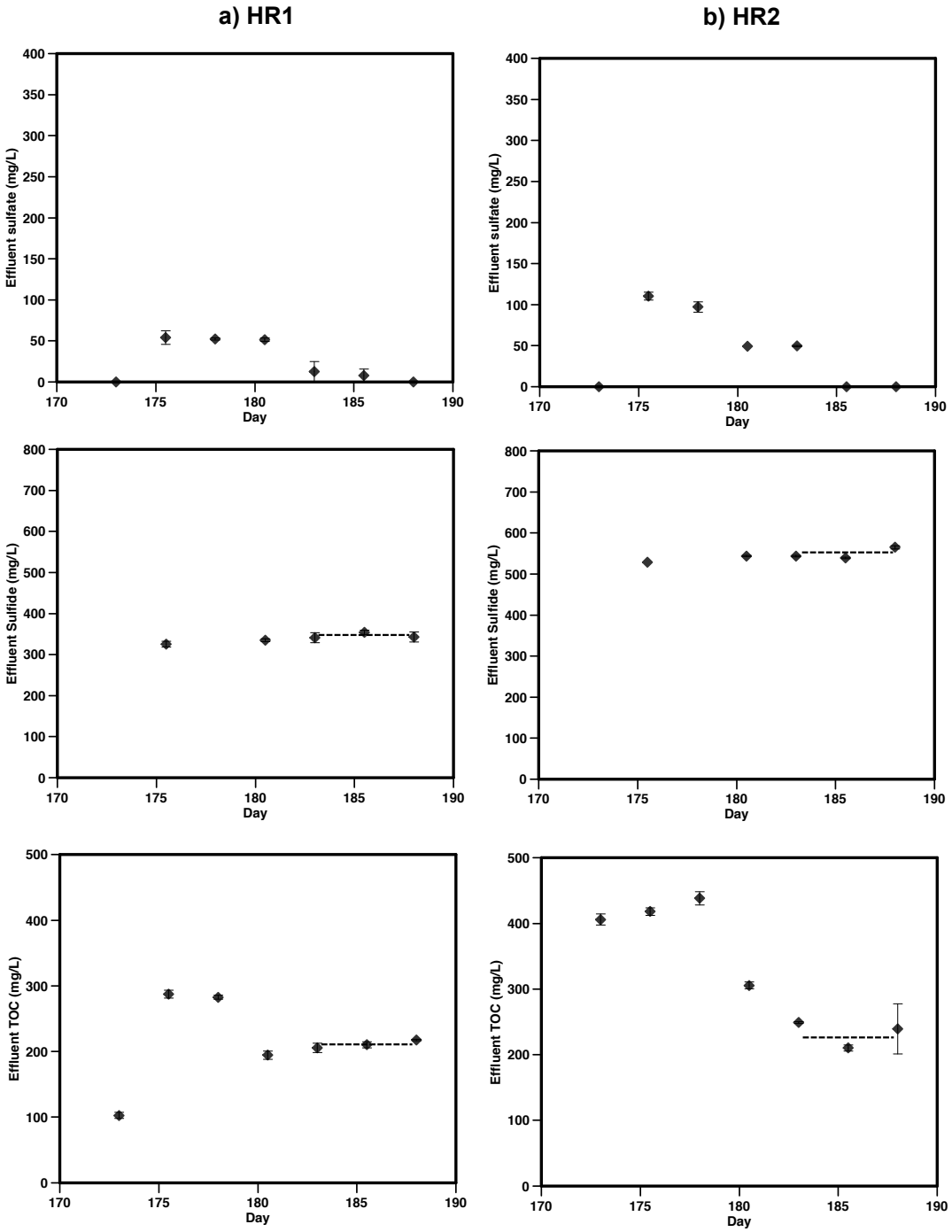


Figure 5-8 Variation of sulfate, sulfide and TOC concentrations by change in time during the operation at HRT=10 Days; a) single stage reactor, HR1 and b) two stage reactor, HR2. For sulfate and TOC, data presented are average of measurements from duplicate samples and error bars represent the range. For sulfide, average of triplicates was used and error bars represent the associated standard error.

5.1.2.4 5 d and 2.5d HRT

Reactors were operated at HRT of 5 Days from Day 191 till 219 and at a HRT of 2.5 Days from Day 219 till day 223. The concentration of copper in reactor influent that was maintained at 600 mg/L (similar to that for HRT of 10 Days) changed to 1000 and 1500 mg/L at Days 198 and 213 respectively. All other conditions remained unchanged.

The amount of alkalinity added to the influent of HR1 increased to 750 mg/L (as CaCO3) at Day 192, however pH of the reactor declined to 6.4 by Day 194. This could be attributed to the increase in the organic loading rate due to the decrease in HRT. Although the pH of reactor remained unchanged from Day 194 to Day 198, further drop in pH level was expected due to the increase of acidity of influent by an increase in the influent copper concentration from 600 mg/L to 1000 mg/L at Days 198. To prevent this pH decline, concentration of the added alkalinity increased to 1100 mg/L (as $CaCO_3$) at Day 198 and was continued at the same dose for the remaining of operation. By increase in the influent copper concentration from 1000 to 1500 mg/L at Day 213, no noticable change was observed in the level of pH and it seemed the total of generated and added alkalinity was able to stabilize the pH level. Under the recent operational changes, a steady state pH of 6.32±0.03 was reached based on data collected from Day 217 till 219. By decreasing the HRT to 2.5 Days, pH gradually decreased from 6.3 on Day 219 until reaching a steady state level of 5.6±0.05 by the end of the operation (Figure 5-9a). This decline can be attributed to the decline in the level of generated alkalinity by SRB as a result of decrease in HRT.

For HR2, gradual decline in the level of pH from 6.6 at Day 191 to 6.4 at Day 195, forced to increase in concentration of additional alkalinity from 750 mg/L (as CaCO3) to 1100 mg/L for the same period of time. This could stabilize the pH and a steady state pH of 6.7±0.05 was reached for HR2 (Figure 5.9b). By Decrease in HRT of reactor from 5

Days to 2.5, the level of pH started to decline from 6.65 on Day 219 and a steady state level of 6.34±0.01 was perceived by the end of the operation.

During the operation of reactor at HRT of 5 days, VFA concentration of HR1 gradually increased from 570 mg/L at Day 191 and reached a steady state level of 1463±9 mg/L. The increasing trend in VFA concentration was continued at a substantially lower rate by decrease in HRT to 2.5 days. The steady state concentration of 1694±12 mg/L was reached by the end of operation due to the increase in organic loading rate (Figure 5-9a). Increase in VFA concentration is mainly attributed to the increase in organic loading rate. Not much change was observed in the concentration of alkalinity from Day 191 till Day 213 and the addition of excess alkalinity was able to compensate for the deficit of generated alkalinity by SRB during the mentioned period of time. However, from day 213 wth increase in the acidity of influent the amount of alkalinity began to decline and no steady state was perceived. This decline continued at HRT of 2.5 Days reaching the minimum of 1125 mg/L at Day 222 and eventually, the steady state alkalinity level of 1104±6 mg/L was reached (Figure 5-9a).

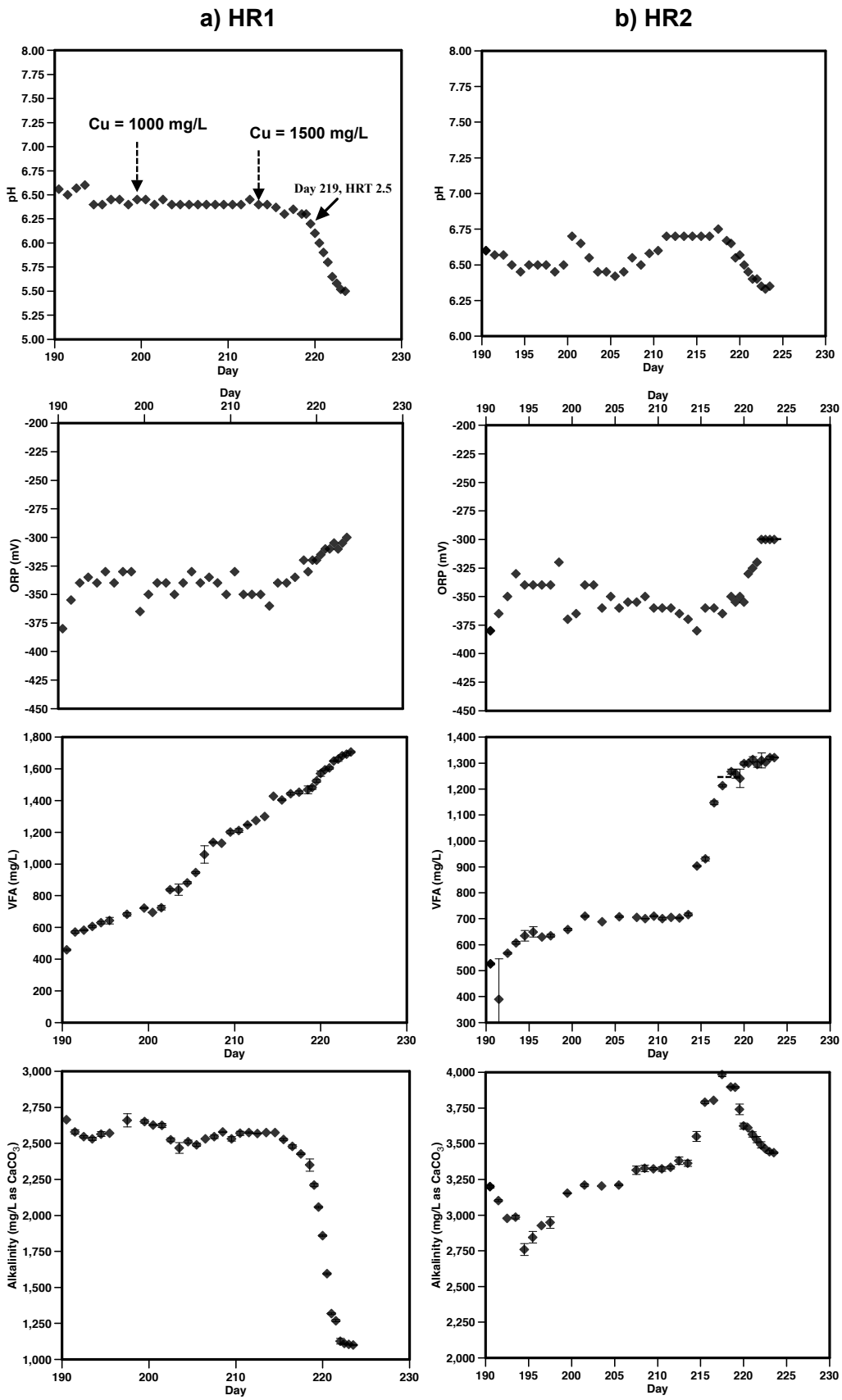

a) HR1
b) HR2
Cu = 1000 mg/L
Cu = 1500 mg/L
Day 219, HRT 2.5
pH
ORP (mV)
VFA (mg/L)
Alkalinity (mg/L as CaCO3)
Day

Figure 5-9 Variation of pH, ORP, VFA and Bi-carbonate alkalinity by change in time during the operation at HRT=5 and 2.5 Days; a) single stage reactor, HR1 and b) two stage reactor, HR2. For VFA and alkalinity, each data point represents average of the measurements for three samples and error bars represent the associated standard error. For some data points error bars are smaller than symbols, therefore not shown.

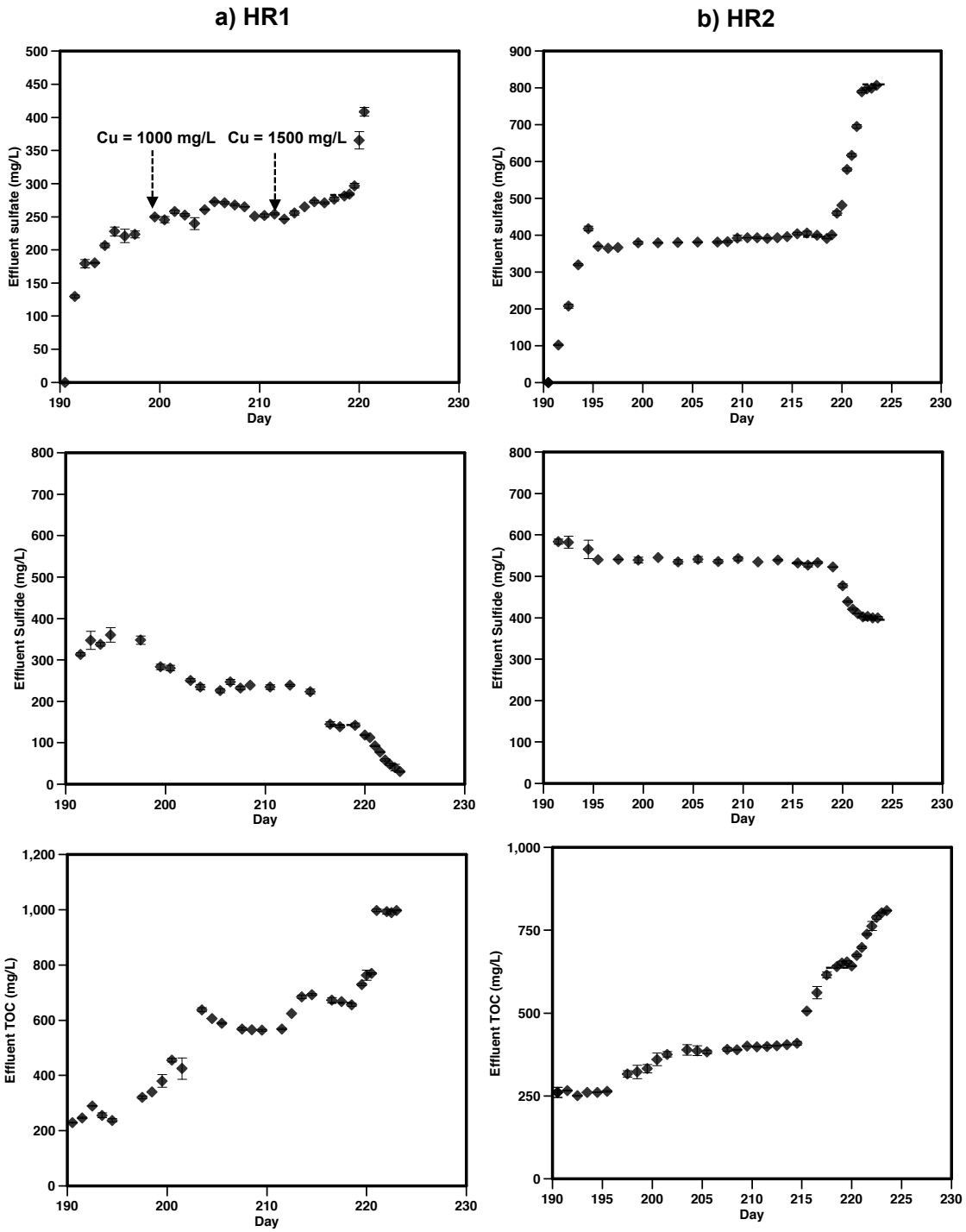


Figure 5-10 Variation of sulfate, sulfide and TOC concentrations by change in time during the operation at HRT=5 and 2.5 Days; a) single stage reactor, HR1 and b) two stage reactor, HR2. For sulfate and TOC, data presented are average of measurements from duplicate samples and error bars represent the range. For sulfide, average of triplicates was used and error bars represent the associated standard error.

For HR2, VFA increased from 390 mg/L at Day 191 to 710 mg/L at Day 201. This can be attributed to the increase of organic loading rate. Another increase in the amount of VFA was observed to begin at Day 213. This may be attributed to the effect of the addition of carbon in the form of citrate to the influent of HR2 at 467 mg/L (as carbon) that initiated from Day 213 to minimize the difference between the influents' matrices of reactors HR1 and HR2. By decrease in HRT to 2.5 Days, no cosiderable variation observed in VFA concentration and steady state VFA concentration of 1315±10 mg/L was maintained for HR2 (Figure 5-9a).

Although, the pH levels for both reactors were lower than the targeted pH value of 6.8±0.2, this low pH did not seem to have much impact on the rate of sulfate reduction. An increase in the concentration of sulfate of HR1 happened during its early operation, at HRT of 5 Days, and reached to 227 mg/L at Day 195 (Figure 5.10a). This may be attributed to the increase of sulfate loading rate. By acclimation the organisms to new condition, sulfate concentration stabilized and reached a steady state of 280.8±3.8 mg/L. Further increase in sulfate concentration occurred when the HRT was decreased to 2.5 Days. Concentration of sulfate increased from 284 mg/L at Day 219 to 822 mg/L at Day 222. A steady state sulfate concentration of 873±44 mg/L was perceived from data collected during the last three feeding cycles. During the operation with HRT of 5 Days, three distinctive steady state levels of sulfide were perceived. By Day 198, it was possible to maintain a steady state sulfide concentration of 348.8±11.3 mg/L. This was approximately equal to that at the end of reactor operation at HRT of 10 Days. From Day 198, the influent copper concentration was increased to 1000 mg/L. The sulfide concentration decreased gradually and steady state sulfide concentration of 237.3±2.4 mg/L was measured at Day 213. By then the influent copper concentration was increased to 1500 mg/L. By the end of reactor operation at HRT of 5 Days (Day 219) steady state sulfide concentration of 142.1±2.9 mg/L was achieved (Figure 5.10a). Decline in the

sulfide concentration is attributed to the sulfide uptake by copper ion to form the copper sulfide precipitates. When HRT was reduced from 5 Days to 2.5 Days, sulfide concentration decreased from 142.4 at Day 219 to 30.1 mg/L at Day 223. No steady state condition was reached (Figure 5-10a). Since the influent copper concentration remained unchanged (the value being the same as in the previousrun), this drop in sulfide concentration can only be attributed to the decrease in sulfate reduction rate which changed from 92% to 78%. No copper was found during the duration of the entire operation.

For HR2, Effluent sulfate concentration increased to 418 mg/L by Day194 from its level of 100 mg/L at Day 191. This may be attributed to the increase in sulfate loading rate. The concentration of sulfate then started to be stabilized from Day 195 and reached a steady state value of 397.3±5.6 mg/L by Day 219 (Figure 5.10b). The HRT was changed to 2.5 Days and the influent sulfate loading rate was increased. This decreased the rate of sulfate reduction. The steady state effluent sulfate concentration of 801.3±4.9 mg/L was reached for HR2. This also shows an increase of 400 mg/L reactor sulfate content when compared to the results obtained at HRT of 5 Days. In contrast, sulfide concentration decreased until Day 195 by when it stabilized to reach a steady state level of 527.6 ±5.5 mg/L. The lower sulfide concentration compared to that obtained during the operation at HRT of 10 Days can be attributed to lower sulfate reduction rate. Increased reduction in sulfate concentration was observed during the operation of the reactor at HRT of 2.5 Days. This dropped the sulfide concentration of HR2 from 522.4 mg/L at the beginning of the run to a steady state level of 401.1±1.96 mg/L, at the end of the operation (Figure 5.10b).

An initial increase of TOC in HR1 from Day 191 till Day197 could be attributed to the increase of organic loading rate by decrease in HRT from 10 Days to 5 Days. Another increase in the level of TOC that was initiated at Day 198 could be attributed to the increase of the amount of the carbon from 244 mg/L to 321 mg/L, which was added to the reactor influent as citrate. No significant TOC variation was observed after an increase in the citrate carbon concentration to 467 mg/L at Day 213, and steady state TOC concentration of 650±21 was reached. By changing HRT to 2.5 Days and increasing

the organic loading rate, TOC concentration increased from 650 mg/L to 998 mg/L at Day 221 while the reactor maintained a steady state TOC concentration of 994±4 mg/L.

The addition of 467 mg/L of carbon in the form of citrate with the influent of HR2 from Day 213 increased the reactor's TOC concentration from 404 mg/L to 615 mg/L. The steady state concentration of 635±18 mg/L was reached as shown by the data collected between the time period of Day 217 and Day 219 (Figure 5.10b).

By decreasing the HRT to 2.5 Days, TOC concentration of HR2 rose gradually reaching a steady state concentration of 800.4±10.6 mg/L. This may be attributed to the increase in the influent organic loading rate.

5.1.3 Summary of Steady-State Performance of HR1 and HR2

The overall steady state results from the operation of HR1 and HR2 reactors have been shown in Tables 5-1 and 5-2, respectively. Near complete sulfate removal was observed in both reactors at HRT of 40, 20 and 10 Days, respectively. When the HRT decreased to 5 Days, sulfate reduction in HR1 and HR2 was 92 and 88%, respectively. A further decrease of HRT to 2.5 Days dropped the sulfate reduction rate of both reactors to approximately 78%. This was expected since the influent loading rate was higher and the decrease in the time required for biomass to uptake the substrate, effectively. Several researchers observed increase in sulfate reduction as the HRT levels were increased. Isa et al (1986) reported an increase in sulfate reduction from 65 to 98 by changing the HRT from 0.5 to 10 Days for an influent sulfate concentration of 1500 mg/L. Chang et al. (2000) reduced 75% of total influent sulfate concentration of 2850 mg/L by a packed bed reactor operated at HRT of 20 Days. Similar observationhas been reported by Nagpal et al.(2000), who observed a decrease in sulfate reduction rate from 35 hours to 5 hours for an influent sulfate concentration of 2500 m/L Nagpal, Chuichulcherm, Peeva, & Livingston, 2000). The concentration of influent in the latter study ranged from 1920 to 2400 mg/L. Mallelwar (2013) examined similar reactor configuration to the current study where the HRT was reduced from 50 to 7 Days; decrease in the sulfate reduction from 99% to 58% was observed . This trend was similar to that observed in the current study. However, the sulfate conversion of 58% at the lowest HRT of 7 Days was lower than 78% reported in the present study for the HRT of 2.5 Days.

Table 5-1 Summary of results for single stage process reactor, HR1

Parameter	HRT (Days)							
	40	20	20	10	5	5	5	2.5
Copper (mg/L)	200	200	600	600	600	1000	1500	1500
Influent Copper Loading ($mgL^{-1}d^{-1}$)	5	10	30	60	120	200	300	600
SO_4^{2-} removal (%)	>95	>99	>99	>99	93.5±1.5	92±1	92±0.5	78±1
Total sulfide (mg/L)	486±10	463±4	327±2	337±21	348.8±11.3	237.3±2.4	142±6	NS
Total VFA (mg/L)	87±8 (NS)	113±2	100±10 (NS)	456±4	NS*	1273±27	1463±9	1694±12
TOC (mg/L)	10±8 (NS)	61±20 (NS)	97±5	217±12	NS	NS	650±21	994±4
TOC removal (%)	~99	95±1.6	91.9±0.4	81.9±1	NS	NS	44.6±0.8	17.8±0.3

*No Steady State

Table 5-2 Summary of results for two stage process reactor, HR2

Parameter	HRT (Days)				
	40	20	10	5	2.5
SO_4^{2-} removal (%)	>99	>99	>99	88.5±0.5	79±0.5
Total sulfide (mg/L)	573±17	565±5	563±22	528±6	401±2
Total VFA (mg/L)	157±6	441±4.5	522±3	1463±9	1315±10
TOC (mg/L)	NS	NS	249.6±10.5	635±18	800.4±10.6
TOC removal (%)	NS	NS	78.7±0.4	47.5±2	33.3±0.9

For HR2, for which no sulfide was taken up by copper precipitation, no changes were observed at the level of sulfide even when the HRT changed from 40 Days to 10 Days. However, the observed drop of sulfide concentration from 552 mg/L at HRT of 10 Days to 538 mg/L and 403 mg/L at HRT of 5 and 2.5 Days, respectively, could be related to the decrease in sulfate conversion from >99% to the levels of 89 and 79% at HRT of 5 Days and 2.5 Days, respectively.

Anaerobic sulfate reduction in high rate bioreactor to produce the biogenic sulfide for metal sulfide precipitation from the waste water has been investigated. Colleran et al. (1994) reported a sulfide production rate of 415 $mgL^{-1}d^{-1}$ using a packed bed reactor (PBR) with an influent sulfate concentration of 3430 mg/L (Emer Colleran, Finnegan, & Lens, 1995; E Colleran, Finnegan, & O'Keeffe, 1994). Almost similar rate of biogenic sulfide production of 386 $mgL^{-1}d^{-1}$ was reported for an upflow anaerobic sludge blanket (UASB) reactor receiving 3000 mg/L of sulfate (Polo Christy ,2001). El Bayoumy et al. (1996) used an upflow anerobic fixed film (UAFF) reactor to convert 2200 mg/L of sulfate. A maximum of 70 $mgL^{-1}d^{-1}$ of biogenic sulfide production rate was acheived in an upflow anaerobic hybrid reactor (UAHR) by Mallelwar et al. (2013). In this study almost two-fold increase was achieved for a similar configuration.

The higher sulfide production rate was obtained in the UAHR used in the current study in comparison to other high rate systems except for PBR used by Colleran et al.(1994) and UASB applied by Polo Christy (2001). However, hybrid reactors require the higher HRT to maintain the high level of sulfate reduction compared to others.

In UAHR used in current study, 79% of sulfate fed to the system was converted at HRT of 2.5 days. This was lower than that of 93% reported by Greben (2000) for PBR system at HRT of 1.4 Days. Also current UAHR was able to yield 161 $mgL^{-1}d^{-1}$ of sulfide at its lowest HRT of 2.5 Days which was lower than that of 386 $mgL^{-1}d^{-1}$ reported for UASB configuration at HRT of 0.5 Days (Polo Christy, 2001).

5.2 Quantification of Metal Precipitation

Biological sulfate reduction produces sulfide that is used to precipitate copper in the single or two stage processes being investigated in the current study. Depending on the matrix, it is possible that additional copper may be precipitated due to the presence of other ions in solution. Therefore, in order to determine the effectiveness of the processes in their ability to precipitate copper or to compare them, it is important to determine the amount of copper precipitated with the sulfide produced. Precipitation of copper in the form of sulfide was evaluated for both single stage and two stage metal removal concept. For the single stage process, reactor HR1 was fed with different concentration of copper and the amount precipitated with sulfide inside the reactor was evaluated. The potential for the residual sulfide, if any, in HR1 effluent to precipitate copper was evaluated using batch chemical precipitaton experiments as described in Section 3.1.2.2b. The ability of the sulfide produced in stage 1 (HR2) of the two stage process to precipitate copper in stage 2 (chemical precipitation) was evaluated by running batch experiments as described in Section 3.1.2.2b. Efficiencies of copper sulfide precipitation for both concepts were estimated and compared together. The precipitates resulted from the precipitation experiments were also characterized using Scanning Electron Microscope and Energy Dispersive Spectroscopy (SEM/EDAX) and X Ray Diffraction (XRD) to identify the other copper precipitate species that were possibly be formed. The visual MINTEQ model was also applied as a powerful speciation model to simulate the precipitation experiments to further evaluate the precipitation process.

5.2.1 Estimation of Copper Precipitated as Sulfide

In the presence of sulfide, different forms of copper sulfide complexes, including CuS, Cu_2S etc., can possibly be formed. Determination of the type of precipitated copper sulfide is of significance to estimate the capacity of the process for the precipitation of copper.

5.2.1.1 Chemical Precipitation

The database of solubility of copper sulfide complexes availalble in literature and MINTEQ and the matrix of the feed suggests that copper will only precipitate with sulfide if the sulfide to copper (S/Cu) molar ratio is greater than 1. In order to determine

the type of copper sulfide precipitate formed during chemical precipitation, data from batch chemical precipitation experiments (Section 3.1.2.2b) where S/Cu molar ratio was greater than 1 were chosen. Table 5-3 presents data from these experiments. The concentration of precipitated copper was calculated as the difference of initial and residual copper concentration and the concentration of produced precipitate was quantified gravimetrically using the fixed suspended solid (FSS) concentration measurements.

Table 5-3 Results of chemical precipitation experiments with S/Cu molar ratio >1

Initial Copper (mg/L)	Copper Remaining (mg/L)	Copper consumed (mg/L)	Precipitate Concentration (mg/L)
300	1.35	298.65	418.5
600	0.68	599.33	918.0
300	0.90	299.10	505.8
300	0.77	299.23	532.8
300	0.54	299.46	422.1
300	1.37	298.63	400.0
600	0.69	599.32	871.0
300	0.91	299.09	438.0
300	0.57	299.43	410.4
300	1.33	298.67	420.0
600	0.66	599.34	982.0
300	0.89	299.11	406
300	0.58	299.42	395.1
300	1.35	298.65	418.5
600	0.68	599.33	972
300	0.90	299.10	459
300	1.00	299.00	453.6
600	0.50	599.50	918
300	0.67	299.33	459

Among different copper complexes precipitated due to the charachteristics of the matrix applied in chemical precipitation, copper sulfides offer the lowest solubility (International Union of Pure and Applied Chemistry 2007), therefore, in the availability of enough sulfide to react with copper, copper sulfide complexes are the only form of copper compound that are expected to precipitate.

Data given in Table 5-3 is plotted in Figure 5-11 and subjected to a linear regression passing through the origin. The regression equation of y=1.52x translates to an S/Cu

molar ratio of 1.04 which is very close to one. This indicates that the copper sulfide complex formed during the second-stage chemical precipitation of metal is copper (II) sulfide (CuS). The high R-squared value of 0.96 suggests that the composition of precipitate did not vary with the change in HRT and hydraulic loading over the duration of 220 days.

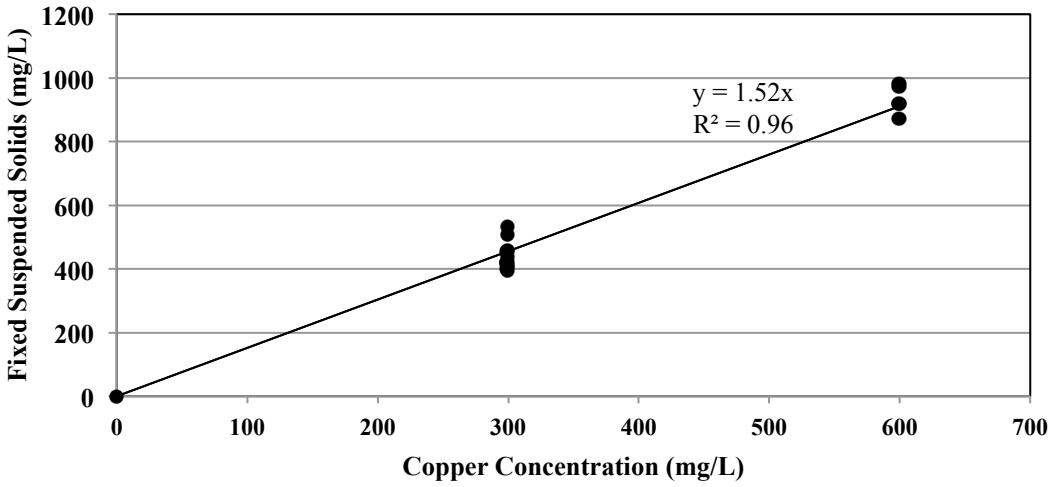


Figure 5-11 Variation of the concentration of precipitate by the variation of precipitated copper concentration

During the 220 days of the reactors operation, samples from the batch second stage chemical precipitation experiments (Section 3.1.2.2 (b)) with copper stock solution concentration of 1000 mg/L mixed with the first stage effluent in a volumetric ratio of 1:1 were collected and stored in a closed container under nitrogen at 4ºC. With first stage effluent sulfide concentration of > 500 mg/L for the entire study (Table 2, Appendix E4), the S/Cu ratio for these samples was always >1 and only copper sulfide complexes were expected to be formed. At the end of the experiments, the samples collected during the course of the study were mixed and a representative sample of the precipitate was characterized using XRD to further investigate the copper sulfide complex formed. The XRD spectrum of the precipitate is shown in Figure 5-12. The resulted peaks for the precipitate sample were compared to standard peaks of pure CuS, $Cu_3(PO_4)_2$ and a 2:1(w/w) mixture of Pure CuS and $Cu_3(PO_4)_2$.

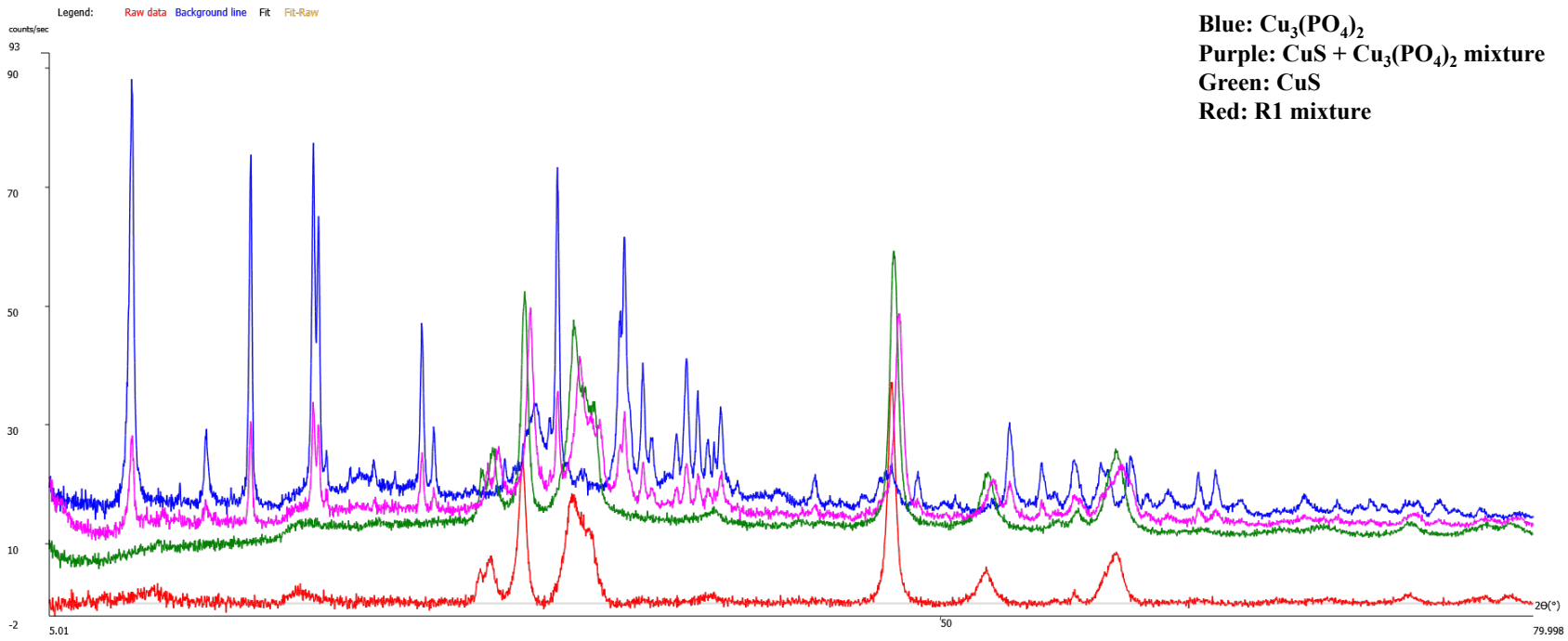


Figure 5-12 XRD spectra for externally formed precipitate

The peaks observed in the precipitate from the experiments is seen to be a perfect match with the pure CuS suggesting that CuS is the copper sulfide complex formed during the second stage chemical precipitation. This is in agreement with the findings from the gravimetric analysis of the precipitates presented in Figure 5-11. The absence of any other peaks suggests that no other form of copper precipitate was formed including copper phosphate. This confirms the assumption that under the conditions of the experiment (S/ Cu >1), only copper sulfide complexes were precipitated.

Precipitation of copper in the form of CuS in a two stage copper precipitation process has also been cited elsewhere. Mallelwar et al. (2013) conducted two stage copper precipitation process with the effluent withdrawn from a hybrid anaerobic bioreactor. They found that at the similar pH to the current study, the molar ratio of sulfide to precipitated copper is approximately 1 which is close to 1.04 of this study.

5.2.1.2 Copper Precipitation within the Single Stage Process

In all the experiments conducted with the single stage process (HR1), varying amounts of residual sulfide were always present in the reactor effluent. The database of solubility of copper sulfide complexes availalble in literature and MINTEQ suggests that copper will only precipitate with sulfide under these conditions. To characterize the type or types of copper sulfide complexes formed within the single stage process, samples of the precipitate collected from the bottom of single stage reactor at the end of reactor operation was analyzed using X-Ray Diffraction (XRD). XRD analysis were also conducted on commercially available pure CuS, $Cu_3(PO4)_2$, and a 1:1 mixture of both. Figure 5-13 shows the results of the analysis.

The results show an almost perfect match between the XRD spectrum of the precipitate from the single stage process with that of pure CuS. This suggests that copper precipitated as CuS in the single stage process in the current study. The results also indicate that no additional copper was precipitated with the phosphate presented in the matrix.

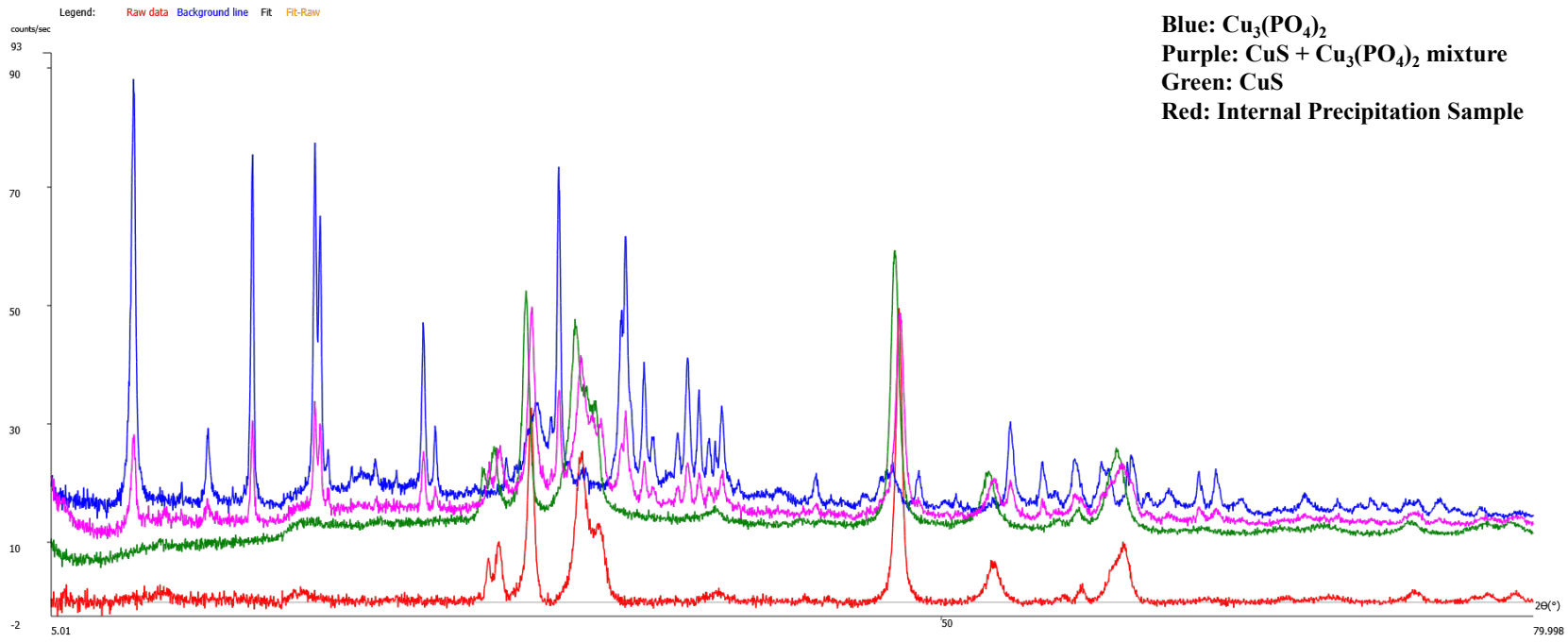


Figure 5-13 XRD spectra for the sample from internal precipitation

Internal formation of copper (II) sulfide (CuS) during a single stage copper precipitation process has been suggested by various researchers. The formation of fine and colloidal CuS was observed during the treatment of the mixed metal-contained wastewater by the biogenic sulfide (Bhagat, Burgess, Antunes, Whiteley, & Duncan, 2004). Jin et al. (2007) analyzed the precipitates resulted from the addition of copper to an active sulfate reducing microcosms using energy- dispersive X-ray diffraction analysis. The results showed that the predominant formation of CuS (Jin, Drever, & Colberg, 2007). Qiu et al. (2009) applied an electro-dispersive X-ray spectroscopy for determination of the elemental composition of copper precipitate produced by biogenic sulfide generated by the isolates of Citrobacter Sp.(Qiu et al., 2009). The S to Cu ratio of 1.07 was obtained which is fairly close to 1.04 resulted from the simulation suggested in this study. Characterization of the precipitates produced from mine waste by isolates of sulfate reducing microorganisms by XRD showed the formation of covellite (CuS) and chalcopyrite ($CuFeS_2$) (Ikkert, Gerasimchuk, Bukhtiyarova, Tuovinen, & Karnachuk, 2013). Characterization of precipitates of copper with biogenic sulfide using a X-ray absorption fine structure spectroscopy also confirmed the formation of CuS (Villa-Gomez et al. 2013).

5.2.2 Comparison of Metal Precipitation in the Single and Two Stage Processes

In this section the ability of two stage and single stage process for the precipitation of copper in the form of CuS is discussed.

5.2.2.1 Two Stage Process

For two stage process, steady state concentration of residual sulfide at each HRT was measured and the copper removal capacity of two stage reactor for each HRT was calculated using S/Cu ratio obtained from the correlation (see Section 5.2.1.1). Table 5-4 presents the quantification of copper precipitation capacity in the form of CuS for two stage copper precipitation concept.

Table 5-4 Copper removal capacity of two stage process

HRT (Days)	Residual S^{2-} (mg/L)	Copper Removal Capacity	
		Concentration (mg/L)	Rate ($mgL^{-1}d^{-1}$)
40	573	1102	27.5
20	565	1087	54.3
10	563	1083	108.3
5	528	1015	203.1
2.5	401	771	308.5

Not much of variations were observed in the precipitated copper concentrations by decrease in HRT from 40 to 5 Days. Concentration of removed copper decreased from 1015 to 771 mg/L by decrease in HRT from 5 to 2.5 Days. This can be attributed to the significant decrease in the concentration of biogenic sulfide at HRT of 2.5 Days. However decrease in HRT, increases the rate of copper removal as given in Table 5-4. The maximum copper removal rate of 308.5 $mgL^{-1}d^{-1}$ was obtained at HRT of 2.5 Days in spite of minimum precipitated copper concentration of 771.2 mg/L at the same HRT.

5.2.2.2 Single Stage Process

In the single stage reactor, copper in the reactor feed is precipitated inside the reactor. To determine the ability of the sulfide generated to precipitate copper, the concentration of copper in the reactor feed was gradually and systematically increased in steps during the course of the experimental run, starting with a concentration of 200 mg/L. The step increases were continued till there was residual sulfide present to accommodate those changes. This ensured During the same period of time, the HRT was gradually decreased from 40 Days to 2.5 Days, while allowing the reactor to reach steady-state at each copper loading and HRT. At any given copper loading and HRT, no residual copper was measured in the effluent showing that all the copper added was precipitated inside ther reactor. The presence of residual sulfide represents additional capacity of the process to precipitate copper that has not been utilized. The potential of this sulfide to precipitate additional copper was estimatedusing the correlation discussed in Section 5.2.1.1. The sum of internally precipitated copper and estimated potential of residual sulfide to precipitate was used to represent the total copper removal capacity of the single stage

process. Table 5-5 presents the estimated total copper removal capacity of a single stage process at various HRTs and copper concentrations.

Table 5-5 Copper removal capacity of single stage process

HRT (Days)	Cu Removed Internally (mg/L)	Effluent Residual S^{2-} (mg/L)	Additional Cu Removal Capacity (mg/L)	Total Cu Removal Capacity	
				Concentration (mg/L)	Rate of Removal ($mgL^{-1}d^{-1}$)
40	200	486	943.7	1143.7	28.8
20	200	463	899	1099	55.0
20	600	327	635	1235	61.8
10	600	337	654.4	1254.4	125.4
5	600	348.8	677.3	1277.3	255.5
5	1000	237.3	460.8	1460.8	292.2
5	1500	142	275.7	1775.7	355.1
2.5	1500	30	58.3	1558.3	623.3

The total copper removal capacityof the single stage process increased from 1144 to 1780 mg/L concomitant with increase in copper concentration of the feed from 200 mg/L to 1500 mg/L (Table 5-5). During the period, the HRT of the feed was reduced from 40 days to 5 days. However, for the same copper concentration of 1500 mg/L in the feed, decrease in HRT from 5 days to 2.5 days resulted in a decrease of total copper removal capacity from 1780 mg/L to 1560 mg/L. This reduction may be attributed to the significant decrease in the efficiency of sulfate reduction and concomitant sulfide production by a decrease in HRT from 5 to 2.5 days, as discussed in Section 5.1.3. However, the rate of copper removal at the lower HRT of 2.5 days was still higher than that at HRT of 5 days.

The results of copper precipitation capacity of two stage and single stage processes, presented in Tables 5-4 and 5-5 are compared. For HRT of 40 days to 10 days close to complete sulfate reduction observed for both single stage and two stage processes, as summarized in Section 5.1.3. At lower HRTs of 5 Days and 2.5 days, although sulfate reduction was lower (about 92% and 78% respectively), it was still similar between the two processes. Based on the above, similar total copper removal capacities were expected for the two processes.comparable and efficiency of the anaerobic sulfate reduction process in both reactors decreased to almost 92% and 78%, respectively (Table 5-1 and Table 5-2). However, the results presented in Tables 5-1 and 5-2 show that the capacity of the single stage process to precipitate copper was higher than the two stage process by a factor of 1.05 at HRT of 40 days which gradually increased to a factor of about 1.75 and 2 at HRTs of 52.5 days respectively. To examine a plausible explanation for the significantly higher metal removal capacity of the single stage process, the data collected during the course of the experimentation was reexamined. As explained in Section 3.1.2.2a, the metal loading to the single stage process was gradually increased from 200 to 1500 mg/L in steps. At each step and HRT, the reactor was operated till a new steady level for sulfide was reached. This data is summarized in Table 5-6. For each step change in copper concentration (ΔCu, mg/L), the change in residual sulfide concentration (ΔS^{2-}, mg/L) and their molar ratio were calculated and included in Table 5-6. XRD analysis of the precipitate from the single stage reactor (HR1) (Section 5.2.1.2) and fixed solids measurements from batch chemical precipitation experiments show the

precipitation of copper as CuS for which the molar ratio of S/ Cu is 1. The same is supported by results from other studies (Bhagat et al. 2004; Jin et al. 2007; Qiu et al. 2009; Ikkert et al. 2013).

Table 5-6 Change in consumed S^{2-} molar concentration to concentration of precipitated copper by decrease in HRT

HRT (Days)	Cu (mg/L)	ΔCu (mg/L)	S^{2-} (mg/L)	ΔS^{2-} (mg/L)	$\Delta S^{2-}/\Delta Cu$ (Molar)
40	0	200	570	84	0.84
	200		486		
20	200	400	463	133	0.67
	600		330		
5	600	400	348.8	111.5	0.56
	1000		237.3		
5	1000	500	237.3	95.3	0.38
	1500		142		

For CuS to be formed, $\Delta S^{2-}/\Delta Cu$ ratio is expected to be 1. However, the $\Delta S^{2-}/\Delta Cu$ molar ratios following all the step changes in copper concentration are < 1 (0.38 - 0.84; Table 5-6). This shows that at each of these step changes, more copper was precipitated than that can be accounted for by the change in sulfide concentration. Identification of CuS as the only identified form of copper precipitates in the single stage reactor (Section 5.2.1.2) indicates the availability of additional sulfide that is not accounted for the difference. The theoretical availability of a sulfur pool to account for this possibility from a mass balance perspetive is explored in Table 5-7. Knowing the mass of copper precipitated, the mass of sulfide (S^{2-}) needed to account for the formation of CuS was calculated. The total estimated sulfide production was then calculated by adding the sulfide removed with CuS to the measured residual dissolved sulfide that remains in the effluent of the reactor after each Cu loading. This total sulfide can be used to estimate the balance of sulfur at each stage of the reactor operation. The results of the calculations have been presented in Table 5-7.

Table 5-7 Change in the ratio of actual S^{2-} to theoretical concentration of S^{2-} calculated from the concentration of reduced sulfate

HRT (Days)	SO_4^{2-} Reduced (mg/L)	Expected S^{2-} (mg/L)	Cu Removed Inside (mg/L)	S^{2-} required (mg/L)	Residual S^{2-} (mg/L)	Total S^{2-} (mg/L)	$S^{2-}/S^{2-}(SO_4^{2-})$
40	3040	1013	-	-	-	570	0.56
40	3040	1013	200	100	486	586	0.58
20	3040	1013	200	100	463	563	0.56
20	3040	1013	600	300	327	627	0.62
10	3040	1013	600	300	337	637	0.63
5	2797	932	600	300	348.8	648.8	0.70
5	2797	932	1000	500	237.3	737.3	0.79
5	2797	932	1500	750	142	892	0.96

The results show that when no copper added with the influent of the reactor, which is similar to the two-stage process, only about 56% of total S estimated from measured sulfate reduction was recovered as sulfide. This reduced recovery of sulfide is not unusual and is widely reported in anaerobic biological sulfate reduction literature related to wastewater treatment (Jong & Parry, 2003; H. T. Kieu, Müller, & Horn, 2011; Mallelwar, 2013). Jong and Perry (2003) examined an anaerobic packed bed reactor to remove the mixture of heavy metals. They reported a significant loss of approximately 80% in the concentration of sulfur and stated that only 3.5% of this loss is related to the formation of metal sulfide. In the study conducted by Kieu et al. (2011) in CSTRs, the loss of sulfur was 25% of total initial sulfur by approximately 6% of total loss contributing in the precipitation of metal sulfide. Mallelwar (2013) reported the concentration of 450 mg/L of sulfide in an optimal operation of a two-stage reactor with the same configuration and influent sulfate level to the current study. This “loss” in sulfide could be explained by the formation of intermediates, particularly trinthionate and thiosulphate, as the growing evidence in microbiological literature is confirming. As far back as 1969, Suh and Akagi (1969) reported the reduction of sulfite to thiosulfate by *Desulfovibrio vulgaris* microorganisms and Kobayashi et al. (1969) suggested the formation of trithionate and thiosulfate as the intermediates of the pathway of sulfite reduction to hydrogen sulfide. The possible formation of trithionate and thiosulfate as

free intermediates in dissimilatory sulfate reduction was hotly debated over several decades hence, as indicated in a review by Hansen (1994). Since then, many studies have confirmed the existence of a trithionate pathway and enzymes in support of that pathway and formation of the intermediates (Santos, Venceslau, Grein, Leavitt, Dahl, Johnston and Pereira, 2015). The results presented in Table 5-7 show that if more of the sulfate reduced was diverted to the formation of sulfide with reducing residual sulfide concentrations, there is enough S pool available to theoretically account for the formation of CuS. The total S estimated to be recovered as sulfide from measured sulfate reduction would then range between 56 – 96% as shown in the last column of Table 5-7.

5.2.3 Comparison the State of the Research with the Background of Single Stage Metal Removal

Application of high-rate bioreactors for the simultaneous sulfate reduction and metal precipitation has been well investigated since the early 1990s. Table 5-8 presents a summary of applied bioreactor configuration on single stage concept and comparison the results with the outcome of the current study.

The hybrid bioreactor in the current study was capable to remove up to 11.8 meq/L of metal yielding the maximum removal rate of 4.72 $meqL^{-1}d^{-1}$. This rate of metal removal was higher than any other studies except than that reported by Kaksonen et al. (2004) who used a fluidized bed reactor (FBR) at the low hydraulic retention time of approximately 0.254 Day to remove the total Fe and Zn at the rate of 7.3 $meqL^{-1}d^{-1}$ (Kaksonen, Franzmann, & Puhakka, 2004). However, the current system was able to treat the wastewater containing almost 12 $meqL^{-1}$ of metal that was much higher than 4.6 $meqL^{-1}$ fed to the mentioned FBR. Among all the configurations reported in Table 4-10, anaerobic filter reactor (AFR) investigated by Steed et al. (2000), was capable to treat higher influent sulfate (5000 mg/L) and metal (13.5 $meqL^{-1}$) concentration. However, the higher HRT required with AFR that decreases the rate of metal removal may limit the application of such bioreactors.

Table 5-8 Use of high-rate bioreactor in single stage sulfate reduction and metal precipitation concept

Reactor Type	HRT (Days)	Inf. SO_4^{2-} (mg/L)	Sulfate Reduction (%)	Metal Fed to the Bioreactor			Removal Rate ($meqL^{-1}d^{-1}$)	Reference
				Metal Ion	Total Concentration (meq/L)	Rate ($meqL^{-1}d^{-1}$)		
PBR[1]	20	2580	~50	Zn	6.1	0.31	0.24	Chang et al., 2000
				Cu				
				Fe				
				Mn				
AFR[2]	8.5	5000	-	Fe	13.5	1.59	1.54	Steed et al., 2000
				Zn				
				Mn				
				Cu				
				Cd				
				As				
				Pb				
FBR[3]	0.5	2000	45	Fe	1.31	2.61	2.6	Glombitza, 2001
				Al				
				Zn				
				Ni				
PBR	0.67	2500	-	Cu	12.87	19.1	0.53	Jong and Parry, 2003
				Zn				
				Ni				
				As				
				Fe				
				Mg				
				Al				

Cont.

Reactor Type	HRT (Days)	Inf. SO_4^{2-} (mg/L)	Sulfate Reduction (%)	Metal Fed to the Bioreactor			Removal Rate ($meqL^{-1}d^{-1}$)	Reference
				Metal Ion	Total Concentration (meq/L)	Rate ($meqL^{-1}d^{-1}$)		
PBR	4	1500	95	Ca	8.96	2.27	2.26	La et al., 2003
				Mg				
				Fe				
				Zn				
				Mn				
				Al				
				Cu				
				Cd				
FBR	1	1000-2200	85	Zn	3.42	3.42	3.42	Kaksonen et al., 2003
				Fe				
FBR	1	2000	85	Zn	7.3	7.3	7.3	Kaksonen et al., 2004
				Fe				
UASB[4]	1	700	-	Cu		1.035	1.029	Sierra-Alvarez et al., 2006
				Zn	1.035			
				Ni				
EGSB[5]	0.35	4980	-	Cu	0.035-0.525	0.1-1.5	0.1-1.5	Sierra-Alvarez et al., 2007

Cont.

Reactor Type	HRT (Days)	Inf. SO_4^{2-} (mg/L)	Sulfate Reduction (%)	Metal Fed to the Bioreactor			Removal Rate ($meqL^{-1}d^{-1}$)	Reference
				Metal Ion	Total Concentration (meq/L)	Rate ($meqL^{-1}d^{-1}$)		
EGSB[5]	0.35	4980	-	Cu	0.035-0.525	0.1-1.5	0.1-1.5	Sierra-Alvarez et al., 2007
IFB[6]	1	3125	50	Fe	4.64	4.64	4.55	Gallegos-Garcia et al., 2009
				Zn				
				Cd				
IFB	1.16	1000	17-76	Zn	0.225	0.216	0.216	Villa Gòmez et al., 2013
				Cu				
				Pb				
				Cd				
UAHR[7]	40-2.5	3040	78-99	Cu	1.58-11.8	0.04-4.72	0.04-4.72	Current Study

[1] Packed Bed Reactor
[2] Anaerobic Filter Reactor
[3] Fluidized Bed Reactor
[4] Up-flow Anaerobic Sludge Blanket
[5] Expanded Granular Sludge Bed
[6] Inverse Fluidized Bed Reactor
[7] Up-flow Anaerobic Hybrid Reactor

5.3 Copper Precipitation and MINTEQ Simulation

Similar to other heavy metals, copper sulfide is expected to preferentially precipitate under conditions of excess sulfide due to its low solubility. Under condtitions of limited supply of sulfide, other copper salts may precipitate depending on matrix conditions including the concentrations of the competing ions and the pH. To evaluated the ability of sulfide produced in the first stage of the two stage process to precipitate copper, batch precipitation experiments were conducted using different ratios of the effluent from the first stage and stock copper solutions of different concentrations as described in Section 3.1.2.2. The experiments resulted in several different ratios of sulfide to copper and pH. The residual dissolved copper concentration and the amount of copper precipitate formed (as fixed solids) were measured for each of the experiments.

Visual MINTEQ software was used to determine the precipitates speciation and equilibrium of solid-dissolved phase. Composition of the matrices of the precipitation experiments, pH and temperature were used as input for MINTEQ software and outputs of the MINTEQ for residual dissolved copper and concentration of precipitates species were compared to data obtained from the actual measurements. All MINTEQ inputs, measurement results and software outputs have been given in Appendix E8.Based on the results of MINTEQ simlations, the precipitation experiments were divided into four groups;

1. Experiments with S/Cu molar ratio > 1, for which only CuS was predicted to precipitate
2. Experiments with S/Cu molar ratio < 1 for which CuS was still the only copper precipitates species expected to be formed.
3. Experiments with S/Cu < 1 where the precipitation of $Cu_3(PO_4)_2$ was predicted in addition to CuS
4. Experiments with S/Cu < 1 where the precipitation of $Cu_3(PO_4)_2$ and CuO was predicted in addition to CuS

For each of the four groups, the measured fixed solids and residual dissolved copper concentrations are compared against MINTEQ predictions in the following sections.

5.3.1 Group 1 - S/Cu > 1

For this group of experiments, MINTEQ model specified CuS as the only possible solid phase in the equilibrium. Therefore by the selection of CuS as the possible precipitates, the concentration of residual dissolved copper and formed precipitates was calculated by MINTEQ. The actual concentration of the precipitates and residual dissolved copper were measured using the analytical protocols described in Chapter 3. All the results of measurements and MINTEQ outputs have been presented in Table 5-9.

Table 5-9 Results of the measurments and MINTEQ results for S/Cu=>1

Cu (mg/L)	S^{2-} (mg/L)	S/Cu	pH	Residual Cu, measured (mg/L)	Residual Cu, MINTEQ (mg/L)	Measured Precipitates (mg/L)	MINTEQ Precipitates (mg/L)
300	405	2.70	6.8	0.00	0.00	465.0	451.2
600	405	1.35	6.4	0.00	0.00	1020.0	906.4
300	270	1.80	6.7	0.00	0.00	562.0	453.2
300	411	2.74	6.6	0.04	0.00	444.4	453.2
600	411	1.37	6.4	0.13	0.00	967.8	906.2
300	274	1.83	6.6	1.89	0.00	486.7	453.2
300	398.7	2.66	6.5	0.17	0.00	466.7	453.2
600	398.7	1.33	6.2	3.52	0.00	1091.1	906.4
300	265.8	1.77	6.4	0.08	0.00	451.1	453.2
300	405	2.70	6.4	4.06	0.00	465.0	453.1
600	405	1.35	6.3	5.94	0.00	1080.0	906.2
300	270	1.80	6.4	2.27	0.00	510.0	453.1
300	300	2.00	6.1	2.15	0.00	504.0	453.1
600	300	1.00	5.9	3.42	5.50	1020.0	898.6
300	200	1.33	6.3	3.52	0.00	425.0	453.1
300	230	1.53	6.7	5.20	0.00	592.0	453.1
300	163	1.09	6.3	2.01	0.00	468.9	453.1
300	172	1.15	6.2	6.28	0.00	456.0	453.1
300	174.3	1.16	6.3	11.57	0.00	439.0	453.1

According to data given in Table 5-9, the residual dissolved copper concentration calculated by MINTEQ was almost zero for all the cases. The measured dissolved copper concentrations were < 5 mg/L for 15 of the 19 experiments and thus in good agreement with MINTEQ results.

Figure 5-14 shows the correlation between the measured values of fixed solids concentration as representative of precipitates with precipitates concentrations resulted from the MINTEQ simulation. The measured precipitate concentrations are also in good agreement with MINTEQ predictions with data points scattered around the ideal correlation represented by the dotted line.

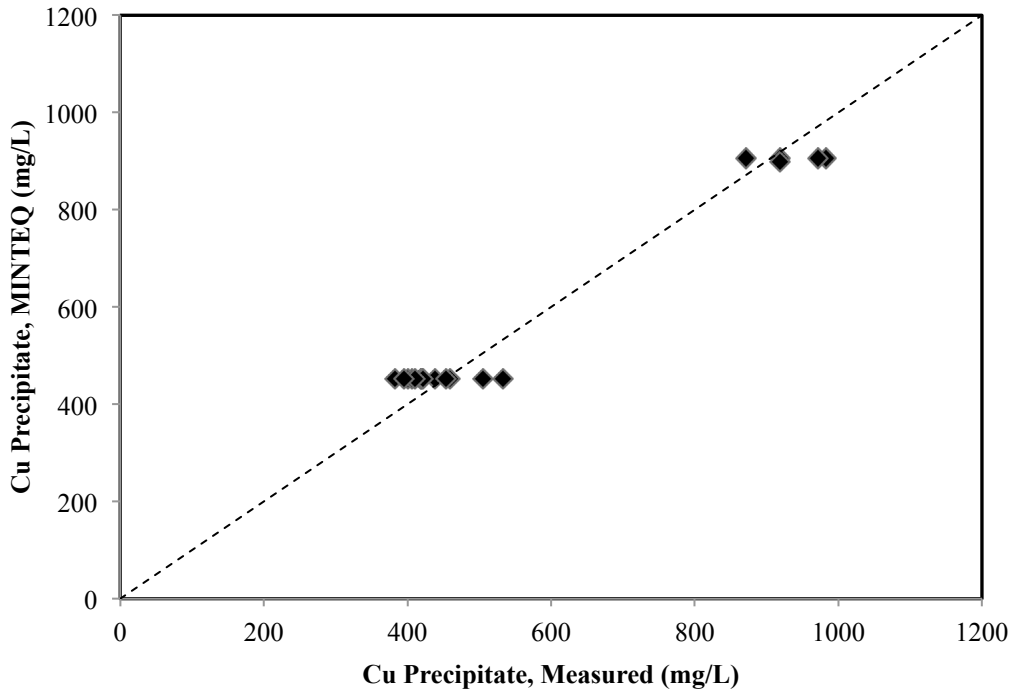


Figure 5-14 The relationship between the measurement and MINTEQ results for the concentration of formed precipitates for the experiments with S/Cu=>1

Scanning Electron Microscope (SEM) was also used to characterize some of precipitates resulted from the experiments of this group. Different areas of the samples of precipitates were mapped for the elemental quantifications by SEM/EDAX. The results of the SEM analysis and corresponding spectra have been given in Appendices E9 and E10. The intense peaks of Cu and S were observed from the spectra provided by the analysis and no significant peaks were identified for the other elements.

The atomic Cu/S ratios for the precipitates on the basis of elemental quantification during the analysis were found to be in the range of 1.1-1.2. The results have been presented in Appendix E9 of the elecronic appendices.

5.3.2 Group 2 – CuS Formation at S/Cu < 1

For these group of experiments, S^{2-} limiting condition leads to presence of dissolved residual dissolved copper after the precipitation of CuS. However, MINTEQ model did not specify any other form of possible solid phase except CuS. Table 5-10 presents the results of the measurement of residual dissolved copper and formed precipitates concentrations against MINTEQ outcomes for both parameters.

Table 5-10 Results of analytical measurements and MINTEQ software on residual dissolved copper and precipitates concentration (initial S/Cu<1 and CuS as the only specifide solid phase)

Cu (mg/L)	S^{2-} (mg/L)	S/Cu	pH	Residual Cu, measured (mg/L)	Residual Cu, MINTEQ (mg/L)	Measured Precipitates Concentration (mg/L)	MINTEQ Precipitates Concentration (mg/L)
600	270	0.90	6.0	37.00	65.30	831.0	808.4
300	135	0.90	6.4	41.90	32.60	450.0	404.2
600	274	0.91	6.1	39.40	57.30	701.1	820.4
300	137	0.91	6.6	16.10	28.80	408.9	410.2
600	265.8	0.89	6.0	80.50	73.60	758.9	795.8
300	132.9	0.89	6.2	9.76	36.90	432.2	397.4
600	270	0.90	5.8	35.62	65.30	740.0	808.3
300	135	0.90	5.9	41.10	32.60	415.0	404.2
600	200	0.67	5.8	147.49	204.80	670.0	599.0
300	100	0.67	6.1	58.40	102.40	440.0	299.5
600	230	0.77	6.1	157.70	145.30	780.0	688.3
300	120	0.80	6.2	69.40	62.70	400.0	359.0
300	70	0.47	6.1	122.80	162.50	310.0	209.3

Figure 5-15(a) and (b) show the data points from analytical measurements and MINTEQ run for the residual dissolved copper concentration and formed precipitates concentration, respectively. The relationship between these data points to dotted line was considered to validate the MINTEQ results.

As shown in Figure 5-15, both data points of residual dissolved copper concentration and precipitates concentration suggests relatively good fit to the dotted line with the better fit of precipitates concentration data. In general, the results of MINTEQ model for residual

dissolved copper concentration were higher than those obtained from the measured residual dissolved copper. For the precipitate concentration MINTEQ resulted in lower values than those obtained from the measurements. No significant variations were observed between the measured values of the precipitate concentrations with the simulation results. This might be attributed to the variation of equilibrium constant during the experiments compared to that used in MINTEQ run.

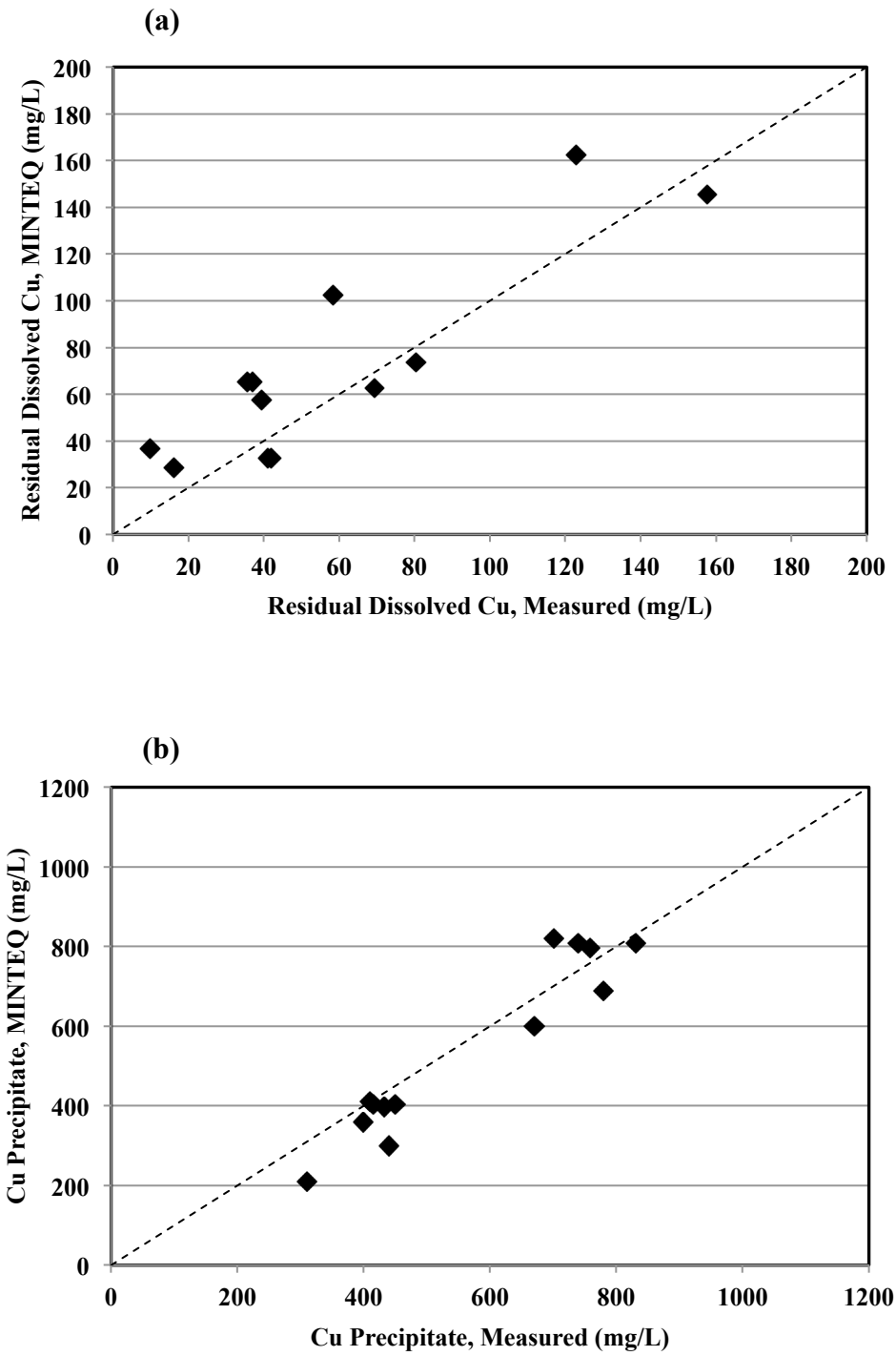


Figure 5-15 The relationship between data points of (a) residual dissolved copper (measured and MINTEQ) and (b) copper precipitates concentrations (measured and MINTEQ) with the ideal data points presented by dotted line

Therefore the results of correlation verifies the formation of CuS as the individual form of copper precipitates. Mapping of the precipitates by SEM/EDAX were conducted to characterize the precipitates of those experiments for which the high differences between the prediction and measurement results were observed. The concentration of phosphates were also measured to evaluate the possible contribution of the initial phosphate in the precipitates. The results of analysis have been presented in Appendix E9.

According to data from SEM/EDS analysis, no phosphorus was observed in elemental quantification of different area of the samples. This can also be verified by the results of residual phosphate analysis where no significant changes in the concentration of residual phosphate in comparison to its initial value of approximately 280 mg/L were observed. These provide enough evidences to reject the possibility of formation of the copper phosphate.

The atomic Cu/S ratio in the range of 1.1-1.25 was obtained for various precipitates from the SEM/EDAX analysis that can be indicative of the formation of CuS during the sulfide precipitation of copper. It was found out that the formation of CuS occurs in the pH levels higher than 5.8.

5.3.3 Group 3 - Formation of CuS and $Cu_3(PO_4)_2$ at S/Cu < 1

For this Group, Copper phosphate, $Cu_3(PO_4)_2$ was predicted to precipitate in addition to CuS in MINTEQ simulations. Results from fixed solids and dissolved copper mesurements and corresponding MINTEQ simulations for this Group are summarized in Table 5-11.

To evaluate the different precipitation scenarios data points were created by correlation of analytical measurments and MINTEQ for both residual dissolved copper concentration and precipitate concentration. The relationship of this data points to their possible ideal situation (i.e., dotted line) were then evaluated.

Table 5-11 Results of analytical measurments and MINTEQ for the experiments with S/Cu<1 and CuS and $Cu_3(PO_4)_2$ as possible solid phases in equilibrium

Cu (mg/L)	S^{2-} (mg/L)	S/Cu	pH	Residual dissolved copper Concentration (mg/L)			Precipitates Concentration (mg/L)		
				measured	MINTEQ if CuS↓	MINTEQ if ($CuS+Cu_3(PO_4)_2$)↓	Measured	MINTEQ if CuS↓	MINTEQ if $CuS+Cu_3(PO_4)_2$↓
300	25	0.17	5.6	210.0	252.2	211.2	170.0	74.9	155.9
600	135	0.45	5.1	306.1	768.0	321.3	510.0	404.2	431.6
600	137	0.46	5.5	128.5	330.9	317.4	508.9	410.2	436.7
600	132.9	0.44	4.6	273.3	339.2	320.0	528.9	397.4	434.3
600	135	0.45	5.6	264.8	334.7	324.5	570.0	404.2	424.6
600	100	0.33	5.2	351.6	404.5	319.4	580.0	299.5	469.3
600	163	0.54	5.7	149.4	279.0	211.8	764.4	487.7	620.9
600	172	0.57	4.5	224.4	261.1	211.2	711.1	514.6	607.8
600	174.3	0.58	5.6	216.7	256.0	212.5	523.3	522.2	609.0
600	120	0.40	4.9	237.4	364.8	214.4	560.0	359.0	657.0
600	70	0.23	5.6	310.5	464.6	217.6	630.0	209.3	700.3
1200	405	0.68	4.4	133.9	215.0	134.4	1608.0	1212.6	1771.1
1200	270	0.45	5.5	306.0	403.2	380.0	1420.0	808.4	1381.7
1200	135	0.23	4.9	758.1	934.4	652.8	870.0	404.2	967.6
1200	411	0.69	5.2	128.7	388.5	114.6	1615.6	1230.5	1772.6
1200	274	0.46	4.5	255.2	659.2	372.5	1182.2	820.8	1395.5
1200	137	0.23	5.8	758.3	934.4	652.8	865.6	410.2	965.9
1200	398.7	0.66	5.7	132.7	412.8	126.7	1684.4	1193.8	1760.9
1200	265.8	0.44	5.7	515.6	678.4	390.4	1064.4	795.8	1366.7
1200	132.9	0.22	4.6	904.0	940.8	668.2	964.4	397.4	968.3
1200	405	0.68	5.8	210.0	400.0	120.3	1725.0	1209.6	1765.3
1200	270	0.45	4.8	392.7	669.4	382.7	1180.0	808.3	1375.4
1200	135	0.23	5.7	790.0	938.9	659.2	905.0	404.2	959.8
1200	172	0.29	5.7	551.5	865.3	581.8	1042.2	514.6	1077.8
1200	174.3	0.29	5.7	719.2	860.8	569.6	666.7	522.2	579.3
1200	25	0.04	4.8	840.2	1158.4	869.1	630.0	74.9	649.6

The correlation between the measured and MINTEQ predicted residual dissolved copper is depicted in Figure 5-16 (Series 2). Additional MINTEQ simulation was carried allowing only CuS to precipitate and the predicted residual dissolved copper concentrations are presented as Series 1. The difference between Series 1 and 2 MINTEQ predictions represents additional dissolved copper predicted to be precipitated as $Cu_3(PO_4)_2$. The goodness of fit of Series 2 to the perfect correlation (represented by the dotted line) shows that the measured residual dissolved concentrations are better explained by additional amounts of copper precipitating as $Cu_3(PO_4)_2$.

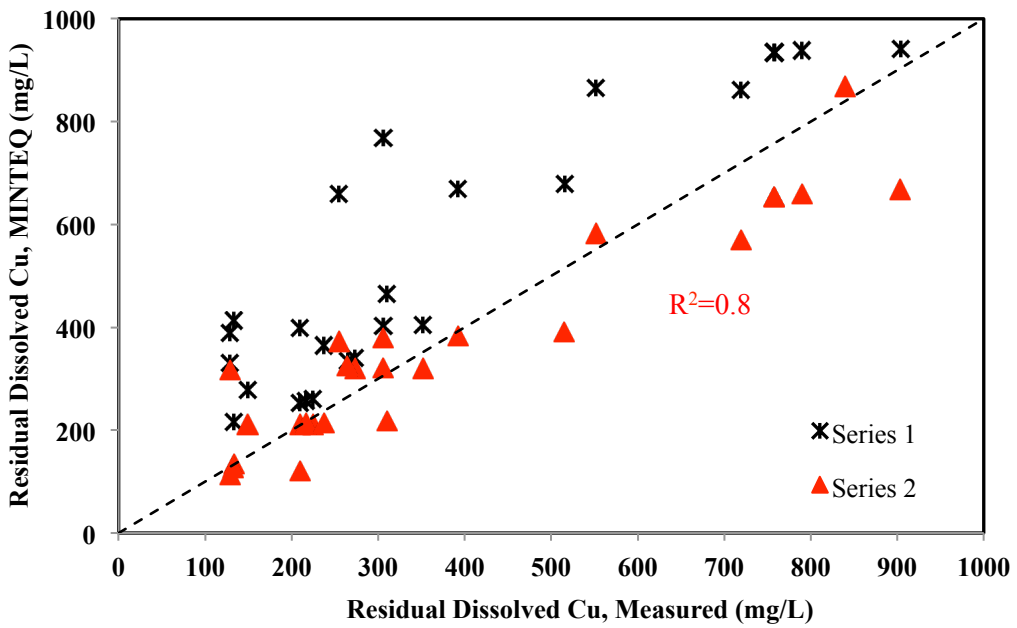


Figure 5-16 Correlation between measured residual dissolved copper and MINTEQ results in two different precipitation scenarios; series 1) formation of cus and series 2) precipitation of CuS and $Cu_3(PO_4)_2$

The correlation between the measured fixed solids and MINTEQ predicted copper precipitates is presented in Figure 5-17 (Series 2). Mass of predicted copper precipitates from the additional MINTEQ simulation allowing only CuS to precipitate are presented as Series 1. The difference between Series 1 and 2 MINTEQ predictions represents additional copper precipitates predicted to be $Cu_3(PO_4)_2$. The conclusions are similar to those from comparison of residual dissolved copper concentration [Figure 5-16]. The

goodness of fit of Series 2 with dotted line shows that the form and amount of copper precipitated was reasonably well-predicted by MINTEQ and that the higher amounts of measured fixed solids can be explained by additional precipitation of copper as $Cu_3(PO_4)_2$ under the conditions of these (Group 3) experiments.

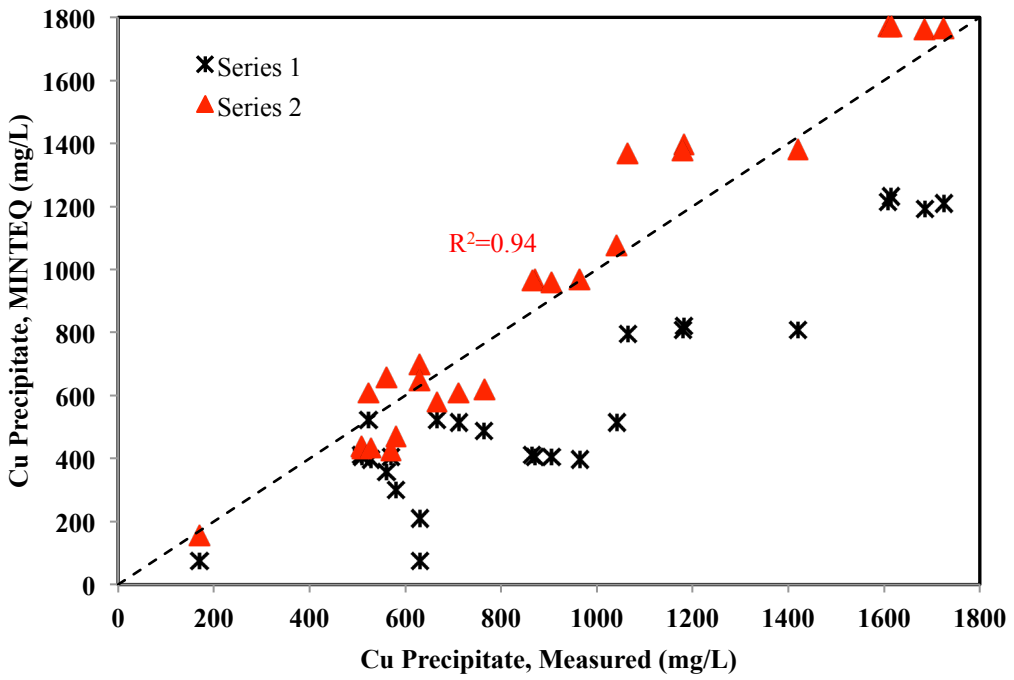


Figure 5-17 Correlation between measured concentration of precipitates and prediction by MINTEQ, series 1) CuS was selected in MINTEQ input series 2) both CuS and $Cu_3(PO_4)_2$ were selected to precipitate

SEM/EDS analysis was also carried out on the precipitates samples of this group. The results has been presented in Appendix E9. The quantification of residual phosphate in the supernatant after the separation of precipitates has been also included in the appendix.

For the majority of the samples the atomic Cu/S ratios were higher than 1.4, impling that the precipitation of the copper in other forms than CuS. The elemental quantification of precipitates samples of this class by SEM/EDAX showed no phosphorous unless for those samples for which the amount of copper sulfide was much lower than copper phosphate. The phosphorous was not identified in the elemental composition of different segments of the samples during the mapping by SEM (Appendix E12). One possibility of

this can be attributed to the heterogenisity of the samples due to the specific sample preparation for SEM analysis in this study. However, residual phosphate measurements showed significant decreases in the level of phosphate in comparison to the initial phosphate concentration indicating the of the precipitation of part of copper in the form of Cu_3 $(PO_4)_2$ in addition to CuS (Appendix E9). Based on data given in Table 5-11, it was also realized that the possible formation of Cu_3 $(PO_4)_2$, occurs at the pH levels of lower than 5.8 (Table 5-8).

5.3.4 Group 4 - Formation of CuS, $Cu_3(PO_4)_2$ and CuO at S/Cu < 1

For this Group, copper oxide, CuO was mentioned to precipitate in addition to CuS and $Cu_3(PO_4)_2$ in MINTEQ simulations. Results from fixed solids and dissolved copper mesurements and corresponding MINTEQ simulations for this Group are summarized in Table 5-12.

Three MINTEQ runs were carried out by the selection of CuS, [CuS+$Cu_3(PO_4)_2$] and [CuS+$Cu_3(PO_4)_2$ + CuO] in MINTEQ input. Three sets of data points were then created by correlation of analytical measurements and MINTEQ results for both residual dissolved copper and precipitates concentrations.

Table 5-12 Results of measurments and MINTEQ predictions for the experiments with S/Cu<1 and formation of CuS, $Cu_3(PO_4)_2$ and CuO

Cu (mg/L)	S^{2-} (mg/L)	S/Cu	pH	Residual dissolved copper Concentration (mg/L)			
				Measured	MINTEQ if CuS↓	MINTEQ if CuS+Cu3(PO4)2↓	MINTEQ if CuS+Cu3(PO4)2+CuO↓
600	25	0.08	5.4	280.8	554.2	265.0	256.0
1200	300	0.50	5.4	254.8	609.9	319.4	217.6
1200	200	0.33	5.2	290.8	809.6	518.4	313.3
1200	100	0.17	5.2	739.7	1009.3	718.7	649.6
1200	230	0.38	5.2	363.6	748.8	459.5	451.2
1200	163	0.27	5.4	369.5	883.2	592.6	553.6
1200	120	0.20	5.0	392.7	969.0	678.4	313.3
1200	70	0.12	5.5	730.6	1068.8	778.2	632.3
				Precipitates Concentration (mg/L)			
				Measured	MINTEQ if CuS↓	MINTEQ if CuS+Cu3(PO4)2↓	MINTEQ if CuS+Cu3(PO4)2+CuO↓
600	25	0.08	5.4	520.0	74.9	649.6	661.4
1200	300	0.50	5.4	1608.0	898.6	1473.3	1600.6
1200	200	0.33	5.2	1296.7	599.0	1173.7	1431.3
1200	100	0.17	5.2	960.0	299.5	874.2	960.2
1200	230	0.38	5.2	1438.0	688.3	1263.0	1273.2
1200	163	0.27	5.4	1102.2	487.7	1062.4	1111.1
1200	120	0.20	5.0	1340.0	359.0	933.7	1390.9
1200	70	0.12	5.5	806.0	209.3	784.0	966.4

The correlation between the measured and MINTEQ predicted residual dissolved copper is depicted in Figure 5-18. The difference between Series 1, that represents the CuS precipitation with Series 2 for which [$CuS+Cu_3(PO_4)_2$] was selected to precipitate, represents additional dissolved copper predicted to be precipitated as $Cu_3(PO_4)_2$. However, the best fit to the perfect correlation (represented by the dotted line) was obtained for Series 3 for which the measured residual dissolved copper concentrations are better explained by additional amounts of copper precipitating as CuO.

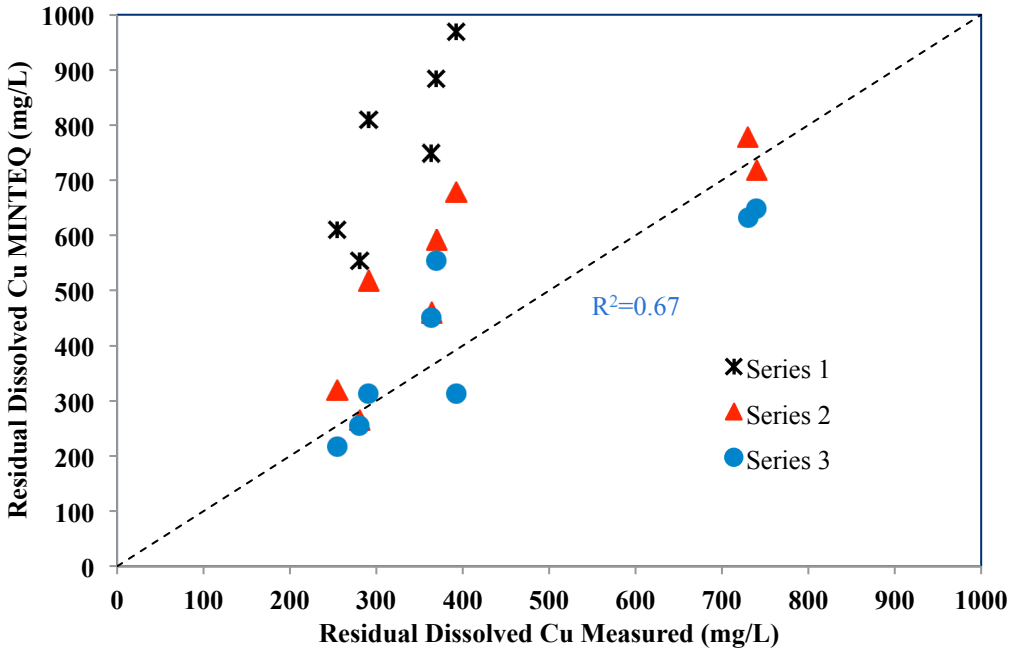


Figure 5-18 The relationship of the correlated data points of analytical measurement and MINTEQ for residual dissolved copper concentrations to the ideal situation (dotted line), series 1) CuS was selected to precipitate in MINTEQ input, series 2) [$CuS+Cu_3(PO_4)_2$] was selected to precipitate, and series 3) [$CuS+Cu_3(PO_4)_2+CuO$] was selected

The correlation between the measured fixed solids and MINTEQ predicted copper precipitates is presented in Figure 5-19 (Series 3). Mass of predicted copper precipitates from the additional MINTEQ simulations allowing only CuS and [$CuS+Cu_3(PO_4)_2$] to precipitate are presented as Series 1 and Series 2, respectively. The differences between Series 3 with Series 1 and 2 in MINTEQ predictions imply on additional copper precipitates in the forms other than CuS. The conclusions are similar to those from comparison of residual dissolved copper concentration [Figure 5-18]. The better

goodness of fit of Series 3 with dotted line shows that the form and amount of copper precipitated was reasonably well-predicted by MINTEQ and that the higher amounts of measured fixed solids can be explained by additional precipitation of copper as both CuO and $Cu_3(PO_4)_2$ under the conditions of these (Group 4) experiments.

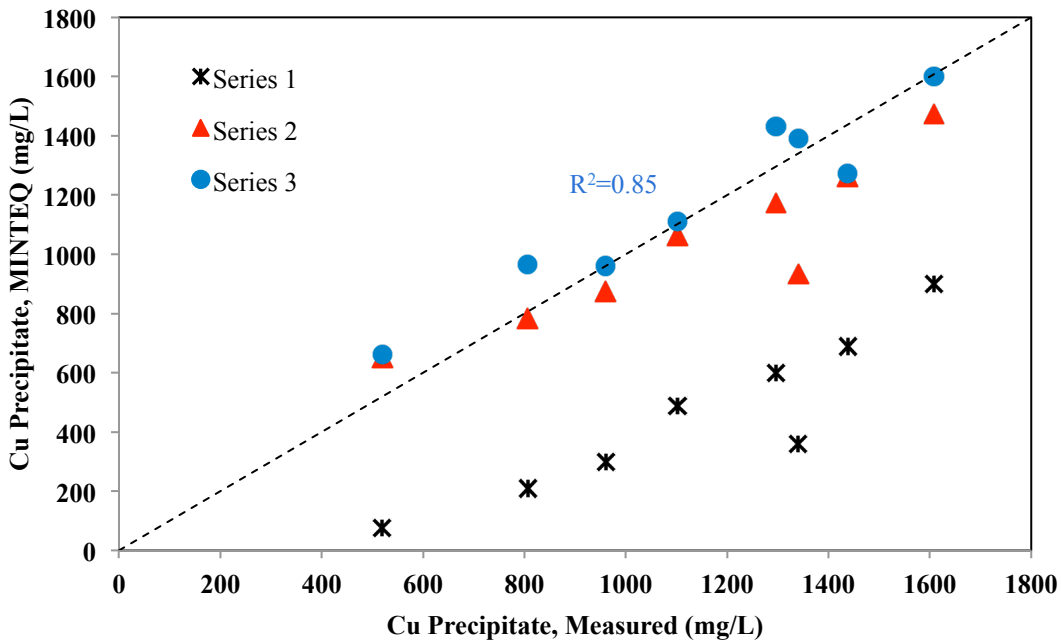


Figure 5-19 The relationship of the correlated data points of analytical measurement MINTEQ for copper precipitates concentrations to the ideal situation (dotted line), series 1) CuS was selected to precipitate in MINTEQ input, series 2) [CuS+$Cu_3(PO_4)_2$] was selected to precipitate, and series 3) [CuS+$Cu_3(PO_4)_2$+CuO] was selected

Formation of the species of copper phosphate and copper oxide could be identified from the results of the elemental quantitative analysis by SEM/EDAX and also the measurement of the residual phosphate of supernatant. The results of the analysis have been presented in Appendix E9.

The precipitates' atomic Cu/S ratios of more than 1.5 impling on the formation of other copper precipitates phases than CuS. The residual phosphate concentration of supernatant samples were at the range of 140-170 mg/L, compared to the initial level of 280 mg/L, suggesting the report of considerable amount of phosphate into the precipitates. Also, phosphorus were detected in the elemental compositions of some of the samples. These evidences confirm the formation of copper phosphate. More intense peaks of oxygen were observed in SEM/EDAX spectra of the precipitates samples of this class in comparison to the peaks of oxygen detected in the former groups of experiments (Appendix E13). It can be seen in the Figures E13-2 to E13-7 of Appendix E13 that the peaks of oxygen are more intense in the precipitates with the higher concentration of CuO compared to those with lower predicted CuO concentration. These provide enough evidences of the presence of oxides in the precipitates.

Formation of the other copper precipitates species may also be attributed to the pH of the experiments after mixing the copper solution and reactors' effluent. According to data given in Table 5-11 and 5-12, the formation of copper oxide did not occur at the pH levels less than 5. This confirms the finding of (Ko & Lee, 2010) which stated that cupric oxide will be dissolved in the acidic environment at the pH less than 5.

5.4 Comparison of Copper and Solids Inventory inside Single Stage and Two Stage Reactors

As discussed in Chapter 4, the formation of copper precipitates during the single stage precipitation of the copper in semi-continuously operated stirred tank reactors, was found to pose an adverse effect on the efficiencey of the process of anaerobic sulfate reduction. The application of high rate hybrid reactors was aimed to eliminate the negative effect of copper precipitates in single stage metal removal process with a higher range of metal loading. In such reactor configuration, sulfate reduction and copper precipitation are carried out in different sections. Thus, the precipitates do not affect the sulfate reduction process.

To better understand the performance of the single stage process and its comparison with the two stage process, a solids inventory was conducted for both HR1 and HR2 at the end of the experimental run. To conduct the inventory, the reactor volume below the top of the media section was divided into three sections (top – 1, middle – 2, and bottom – 3) for both the reactors, and the solids accumulated were removed and collected as described in Section 3.1.2.3. The concentrations of solids in both reactors and concentration of copper in different sections of single stage reactor was then measured. Results of solids and copper concentration measurments are presented in Appendix E5.

The inventory of solids at different portions of the reactors and copper distribution at in the single stage reactor was also determined. The results have been presented in Table 5-13. The concentrations of solids and copper were measured at different sections and the corresponding mass was determined using the volume of each section. Data given in Table 5-13 was used to evaluate the performance of single stage process in UAHR.

5.4.1 Distribution of Copper Precipitates Inside Single Stage Reactor

Upflow anaerobic hybrid reactor was designed with the expectation that the segregation of biomass and copper precipitates inside the reactor can overcome the inhibition of biomass with the accumulated precipitates. Thus, the evaluation of copper inventory inside the reactor is of great significant. The results given in Table 5-13 revealed that more than 99% of the total copper added with the influent of the reactor is settled at the

bottom section. This indicates that the objective of the application of UAHR has been satisfactory.

Table 5-13 Distribution of solids in both reactors and copper distribution in HR1

	Section	Vol (mL)	TSS (mg)	TSS Distribution (%)	VSS (mg)	VSS Distribution (%)	FSS (mg)	FSS Distribution (%)	Cu (mg)	Cu Distribution (%)
HR2	1	3040	9120	18.48	3040	13.76	6080	22.30	-	-
	2	1610	2147	4.35	1073	4.86	1073	3.94	-	-
	3	2900	38087	77.17	17980	81.38	20107	73.76	-	-
HR1	1	3040	10944	6.13	4053	16.39	6891	4.48	415	0.5
	2	1610	2576	1.44	1073	4.34	1503	0.98	253	0.3
	3	2900	164894	92.42	19604	79.27	145290	94.54	84390	99.2

As discussed in Section 5.2.1.2, in the single stage reactor copper precipitated in the form of CuS. During the entire experimental run, the upflow velocity was controlled by the rate of recirculation and was maintained at 0.013 cm/s. According to the Stokes Law, with an assumed density of 4.76 g/cm^3, CuS particles as small as 7µm or greater in diameter will have a settling velocity greater than the upflow velocity. Details of the calculation has been given in Appendix 1.

Formation of the large agglomerated particles of CuS has been well documented in the literatures. Villa Gómez et al. (2013) stated that CuS particles have high saturation indices over the broad range of pH. The particles with the high saturation index have a better tendency to agglomerate due to their enhanced nucleation (Al-Tarazi, Heesink, & Versteeg, 2004; Mokone, Van Hille, & Lewis, 2010). Thus, the small particles of CuS formed during the single stage copper precipitation will be agglomerated forming the large particles that are able to settle down at the bottom section counted for more than 99% of the total copper.

5.4.2 Distribution of Volatile Suspended Solids (VSS) Inside the Reactors

Volatile Suspended Solids (VSS) are an indicator of the concentration of biomass. Application of hybrid reactors for the single stage metal removal, can pose an advantage over the suspended growth culture if the active biomass (i.e., VSS) could be developed in a separate zone of precipitates accumulation zone. Distribution of VSS inside the single stage reactor revealed that the bottom portion of the reactor contains of 79% of total VSS (Table 5-13). However, this portion of biomass may not be involved in the process of sulfate reduction. During the operation of CSTRs (Chapter 4), it was shown that 300 mg/L of copper precipitates adversly affects the fuction of the sulfate reducers in the biomass. Concentration of CuS at the bottoom section of the reactors was calculated to be approximately 15 times of the inhibitory precipitates concentration of 300 mg/L. Therefore, no considerable sulfate reduction activity could be expected from the biomass accumulated at the bottom section.

The total amount of VSS measured in the single stage reactor was 26.5 gm of which 22 gm was inoculated to the reactor during the startup phase. Section 4 alone contained approximately 21.5 gm of the total 26.5 gm VSS. The main portion of inoculum source

during the startup phase was obtained from the anaerobic digester sludge for which the majority of VSS may be related to the presence of significant amount of non-biodegradable organics. These organics do not contain the significant amount of active SRB biomass. This could be another reason that the VSS accumulated at the bottom section of the reactor does not involve in the process of sulfate reduction. Thus, the reduction of sulfate may be attributed to the biomass developed in other sections of the reactor.

As given in Table 5-13, about 5200 mg of VSS was measured in sections 2 and 3 out of which 80% was found to be at section 2 where the support media (pall rings) are located. This accounts for 4050 mg of VSS. By assuming that the entire sulfate reduction happens in section 2, at the optimum conditions, the rate of sulfate reduction by the unit mass of the VSS was calculated as 0.39 mg SO_4^{2-}/mg VSS.d, higher than that of 0.15 mg SO_4^{2-} /mg VSS.d for the optimal operation of SCSTRs. The calculations have been given in Appendix 1. Therefore, it can be concluded that the single stage reactor applied in this study was operating successfully to overcome the inhibitory effect of copper precipitates on the anaerobic sulfate reduction process.

Chapter 6
Conclusions and Recommendations

6.1 Conclusions

The study was conducted in two distinctly separate parts with their respective results presented in Chapters 4 and 5 respectively. The conclusions from each part are summarized and presented below.

6.1.1 Effect of Copper Precipitates on Sulfate Reduction in Semi-continuous Stirred Tank Reactors

The aim of this study was to investigate the effect of metal precipitates on anaerobic biological sulfate reduction in the single-stage process. The study was carried out using semi-continuous stirred tank reactors (SCSTRs) operated at an HRT of 50 days at 37 ± 2 ^{o}C using synthetic wastewater containing various concentrations of copper as a test case. Near the end of the study (Day 98), a batch study was conducted to determine the rate of sulfate reduction. Based on the results of the study, the following conclusions can be drawn:

1. Anaerobic biological sulfate reduction process was significantly impacted by copper precipitates. At an HRT of 50 days, reduction in sulfate concentration was ~55% at copper concentrations of 0 and 200 mg/L, which declined to ~38.7% at copper concentration of 400 mg/L. No measurable dissolved copper concentrations were detectable at copper concentration of 200 and 400 mg/L.
2. Reduction in sulfate concentration at copper concentration of 600 mg/L was similar to that at copper concentration of 400 mg/L till around Day 70 when no detectable residual copper was measured in the effluent. After which its performance became unstable and a progressive failure of the sulfate reduction process was observed. Residual dissolved copper concentration was concomitantly detected in the reactor effluent which may be the cause for the failure of the sulfate reduction process.
3. The rate of sulfate reduction was progressively affected by increase in copper precipitates. Maximum sulfate reduction rates from batch kinetic study were

measured to be ~105 mg/L/day at Cu = 0 mg/L which declined by ~42% to ~61 mg/L/day with precipitates from Cu = 200 mg/L and by ~53% to ~50 mg/L/day with precipitates from Cu = 400 mg/L.

4. The growth of microorganisms was progressively affected by increase in copper precipitates as indicated by the reduction in volatile solids content by ~21% at Cu = 200 mg/L and by ~37% at Cu = 400 mg/L as compared to the control (Cu = 0 mg/L). This suggests that the progressive reduction in the rate of sulfate reduction with increase in copper precipitates may at least be partially explained by effect of copper precipitates on the growth of SRB.
5. Increase in copper precipitates resulted in a gradual shift in the microbial community including those expected to participate in the sulfate reduction pathway (*Petrimonas sulfuriphila* sp.; *Dethiosulfovibrio salsuginis* sp.). Microbial community with lower copper precipitates (Cu = 200 mg/L) was more similar to the control (Cu = 0 mg/L) than that with higher copper precipitates (Cu = 400 mg/L).

6.1.2 Sulfate Reduction and Copper Precipitation in High Rate Reactors: Comparison and Single and Two-Stage Processes

The aim of this study was to compare anaerobic biological sulfate reduction and metal precipitation in single-stage and two-stage processes using Upflow Anaerobic Hybrid reactors at 37 ± 2 ^{o}C. Based on the results of the study, the following conclusions can be drawn:

1. Similar rates of sulfate reduction were observed between the single-stage and two-stage processes for the entire duration of the study. Sulfate reduction was >99% for HRTs varying between 10 – 40 days declining to ~89 – 92% at HRT of 5 days.
2. Process operating conditions were successful in separating copper precipitates from biomass (volatile solids) accumulated in support media of the UAHR in the single-stage process. Greater than 99% of the copper precipitates were located in the bottom third of the reactor. This may help explain the lack of inhibition of

biological sulfate reduction in the single-stage process as compared to the two-stage process in the current study.

3. At HRT of 5 days, the sulfide produced was estimated to be able to precipitate 1780 m/L copper corresponding to a removal rate of 356 mg/L/day for the single-stage process. At the same HRT, the sulfide was estimated to precipitate 1015 mg/L copper corresponding to a removal rate of 203 mg/L/day. Thus maximum copper removal rate was ~75% higher in the singe-stage as compared to the two-stage process.
4. In the single-stage process, S^{2-}/ Cu ratio was >1 for the entire study and only copper (II) sulfide (CuS) was observed to precipitate inside the reactor.
5. In batch chemical precipitation experiments for the second-stage of the two-stage process, only copper (II) sulfide (CuS) was observed to precipitate when S^{2-}/ Cu ratio was >1.
6. In the two-stage process, about 56 – 59% of sulfate reduced was recovered as sulfide. In the single-stage process, the sulfide recovered is estimated to increase from 56 to 95% with reducing sulfide concentrations as the concentration of copper in the feed was increased. This is postulated to be the reason for the ~75% higher copper removal rate observed in the single-stage process as compared to the two-stage process.
7. Equilibrium calculations conducted using MINTEQ were in good agreement with both the residual dissolved and precipitated copper concentrations for all precipitation experiments. At S^{2-}/ Cu molar ratio >1, only CuS was predicted to precipitate. For S^{2-}/ Cu molar ratio <1, additional amounts of copper were precipitated as copper phosphate and copper oxide sometimes, depending on the experimental conditions.

6.2 Future Recommendations

Following suggestions are recommended as the possible research potential:

1. Investigation on the effect of the precipitates formed by the mixture of heavy metals in SCSTRs.

2. Precipitates accumulation effect on the kinetic of biological anaerobic sulfate reduction.
3. Conducting a systematic microbial analysis at different stages of the process to better understand the effect of metal loading change and precipitates accumulation on the microbial community and different species distribution.
4. Using an isolate of pure culture of sulfate reducers instead of mixed culture for the screening of the inhibitory effects of precipitates on dissimilatory sulfate reduction.
5. Investigation on the efficiency of sulfate reduction and metal removal processes in UAHR using a real wastewater.
6. More detailed study on the loss of sulfide during the conversion of sulfate to sulfide is recommended. The presence of alternate pathway during the dissimilatory sulfate reduction to sulfide can be investigated by monitoring of sulfide, thiosulfate and trithionate generation during the process. This can be also evaluated by monitoring the microorganisms reported to be involved in the alternate pathway.
7. More detailed study on the possible formation of different metal precipitates phases in presence of dissolved residual metal after sulfide precipitation in the single stage process is suggested.
8. Determination of microbial profile of single stage and two stage metal precipitation UAHR can be helpful for understanding the mechanism of metal precipitates inhibitory effect.

APPENDIX 1

Calculations

I. Feeding Regime Required for Semi-continuous Operation of the Reactors:

Assumptions:

- Hydaraulic Retention Time (Days): HRT
- Reactor Working Volume (L): V
- Amount of Feed to be Replaced; FV (L): Assumed to be maximum 20% of Reactor Working Volume (0.2V)
- Feeding Interval (Days): FI

$$FI = \frac{HRT}{(\frac{V}{FV})} = \frac{HRT}{5}$$

Therefore, if a reactor with a working volume of 4L is intended to be operated semi-continuous at HRT of 40Days, 20% of its volume, i.e. 800mL, has to be replaced with fresh medium every 8 Days.

II. Calculation of the Size of Particles That Settle down in UAHR

According to the Stokes Law, the diameter of particles with the settling velocity of Vs in any medium is calculated as follows:

$$D = \sqrt{\frac{18\mu V_s}{g(\rho_p - \rho_{fm})}}$$

Where:

- D is the particle diameter (m)
- μ is the viscosity of the medium (kg/m.s)
- g is the gravity acceleration (m/s^2)
- ρ_p is the density of particle (kg/m^3)
- ρ_m is the density of medium (kg/m^3)
- Vs is the settling velocity (m/s)

Considering the particle density of 4.76 g/cm^3, water as medium with the density of 1 g/cm^3, viscosity of 0.798×10^{-3} N.s/m^2 for water at temperature of 30ºC and gravity acceleration of 9.8 kg/m^2, it is concluded that the particles with a diameter of more than 7.1 µm will settle down in the medium upflow velocity of 0.013 cm/s.

III. Calculation of Rate of Sulfate Reduction per Unit Mass of Biomass for Semi-continuous Operation of the Reactor

Assumptions:

- Sulfate Reduction Efficiency (%): SR
- Influent Sulfate (mg/L): S
- Reactor Volume (L): V
- HRT (Days): HRT
- VSS Mass (mg) : M
- Sulfate Reduction per Unit Mass of biomass (mg/mg.d): R

$$R = \left(\frac{SR * S}{HRT}\right)/M$$

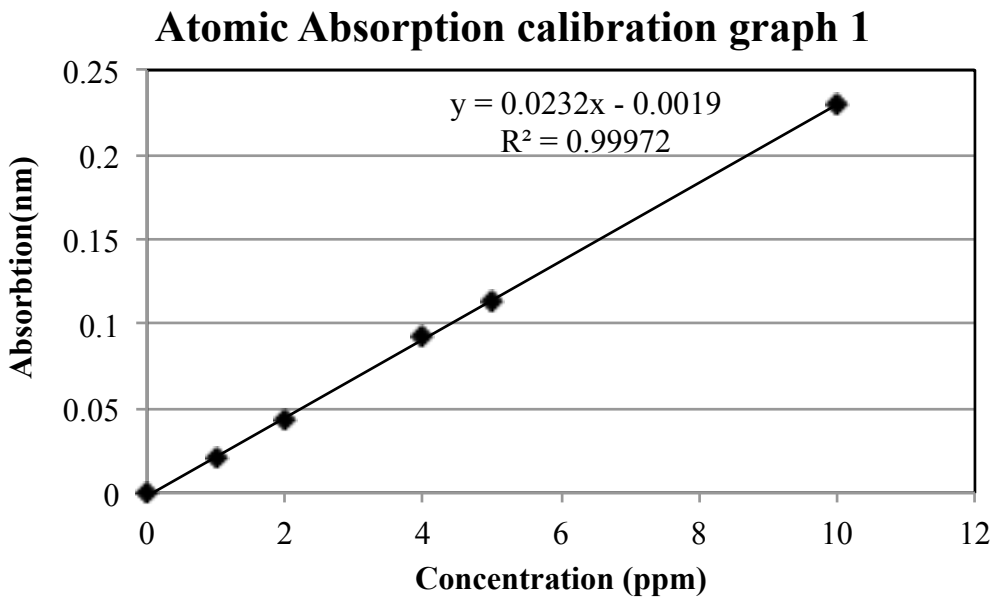
For SCSTR receiving no copper:

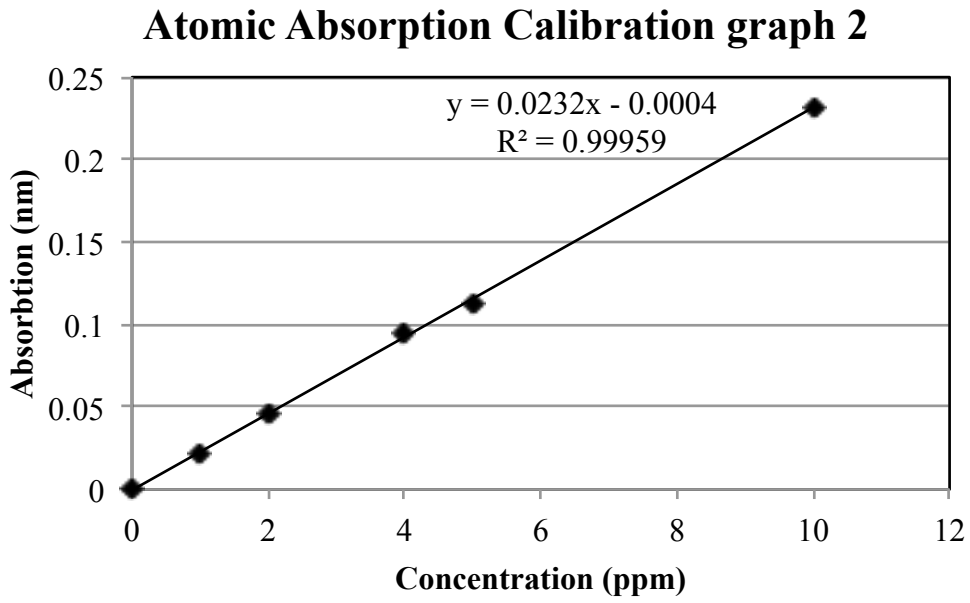
- SR= 55%
- S= 3040 mg/L
- V= 0.6 L
- HRT= 50 days
- M= 131.8 mg

R is calculated as 0.15 mg SO_4^{2-}/ mg VSS.d

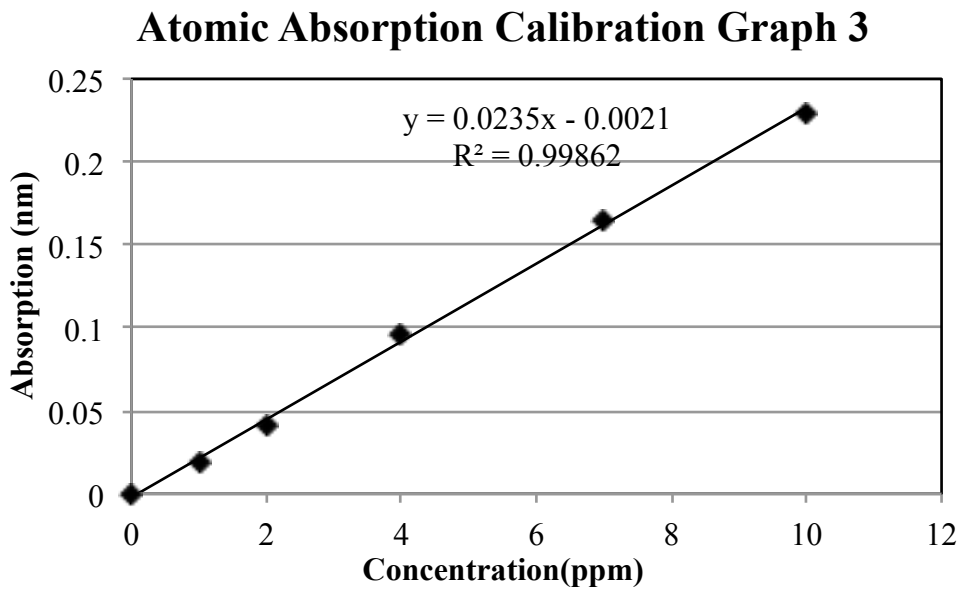
APPENDIX 2

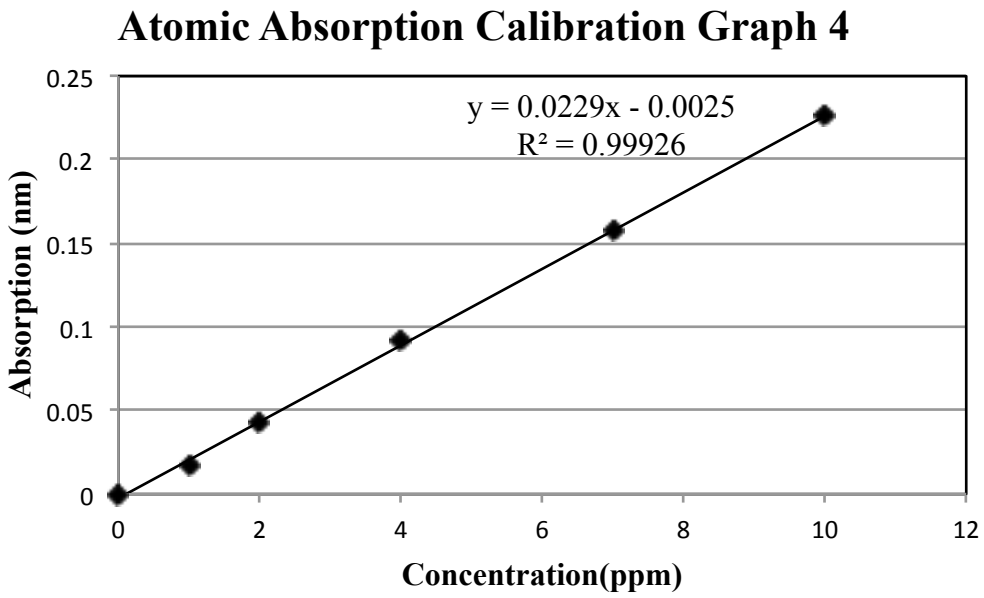
Calibration curves for atomic absorption





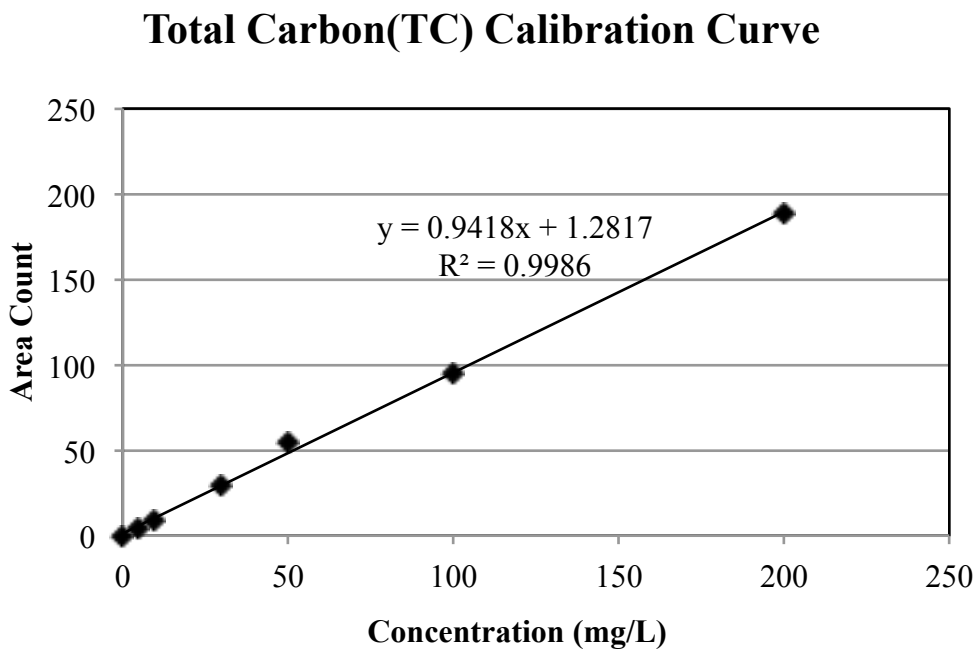
Cont.

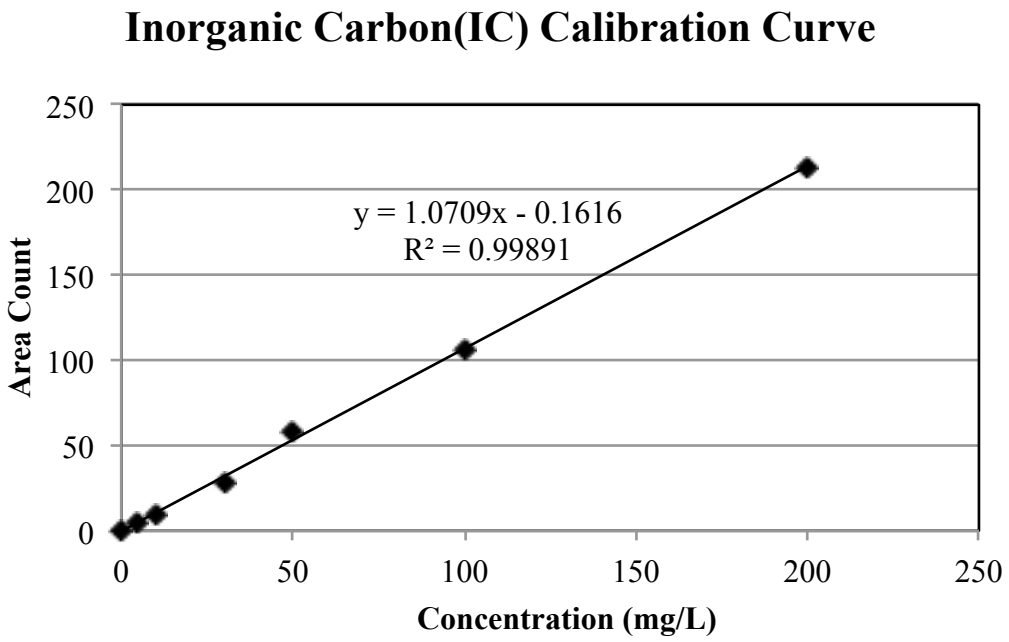

Atomic Absorption Calibration Graph 3
y = 0.0235x - 0.0021
R² = 0.99862
Absorption (nm)
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
10
12
Concentration(ppm)


Atomic Absorption Calibration Graph 4
y = 0.0229x - 0.0025
R² = 0.99926
Absorption (nm)
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
10
12
Concentration(ppm)

APPENDIX 3

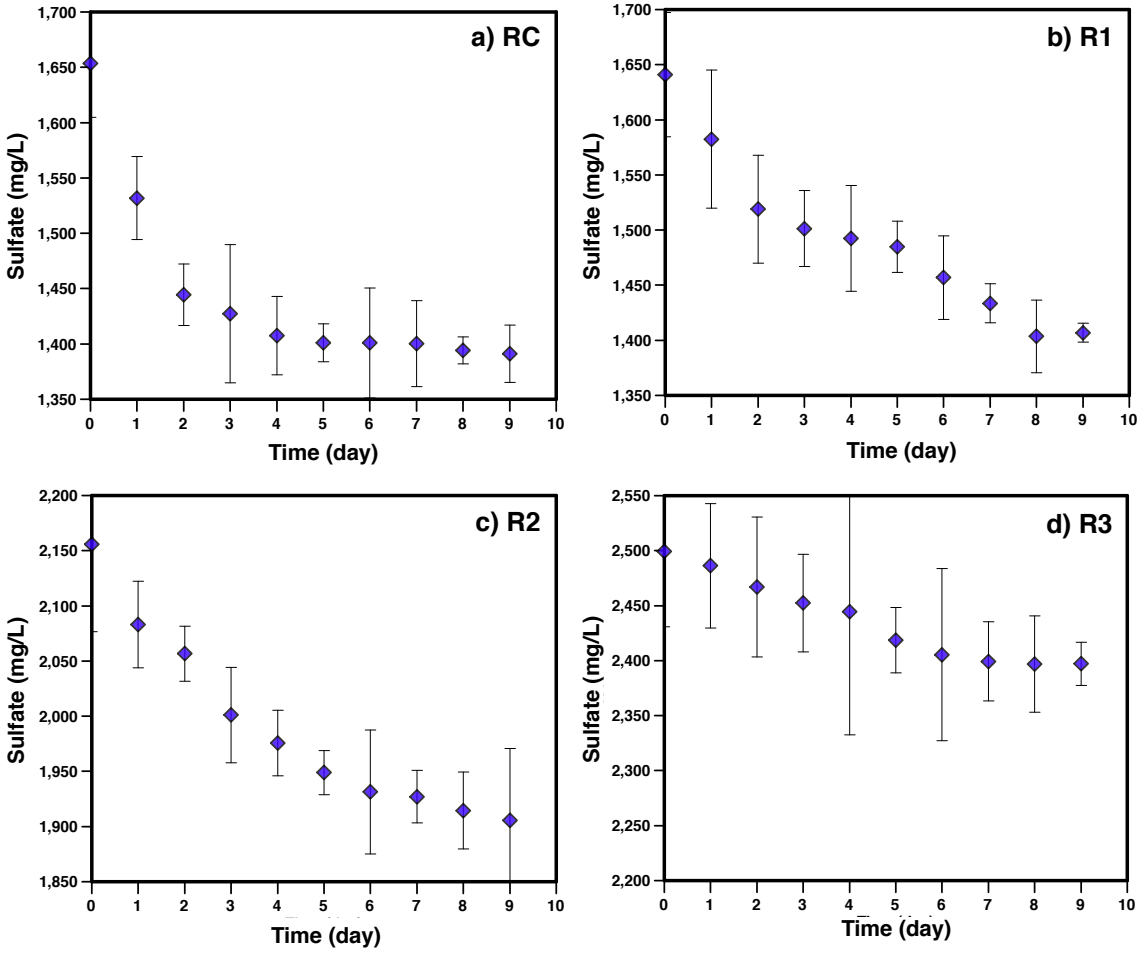
Calibration curves for TC and IC





APPENDIX 4

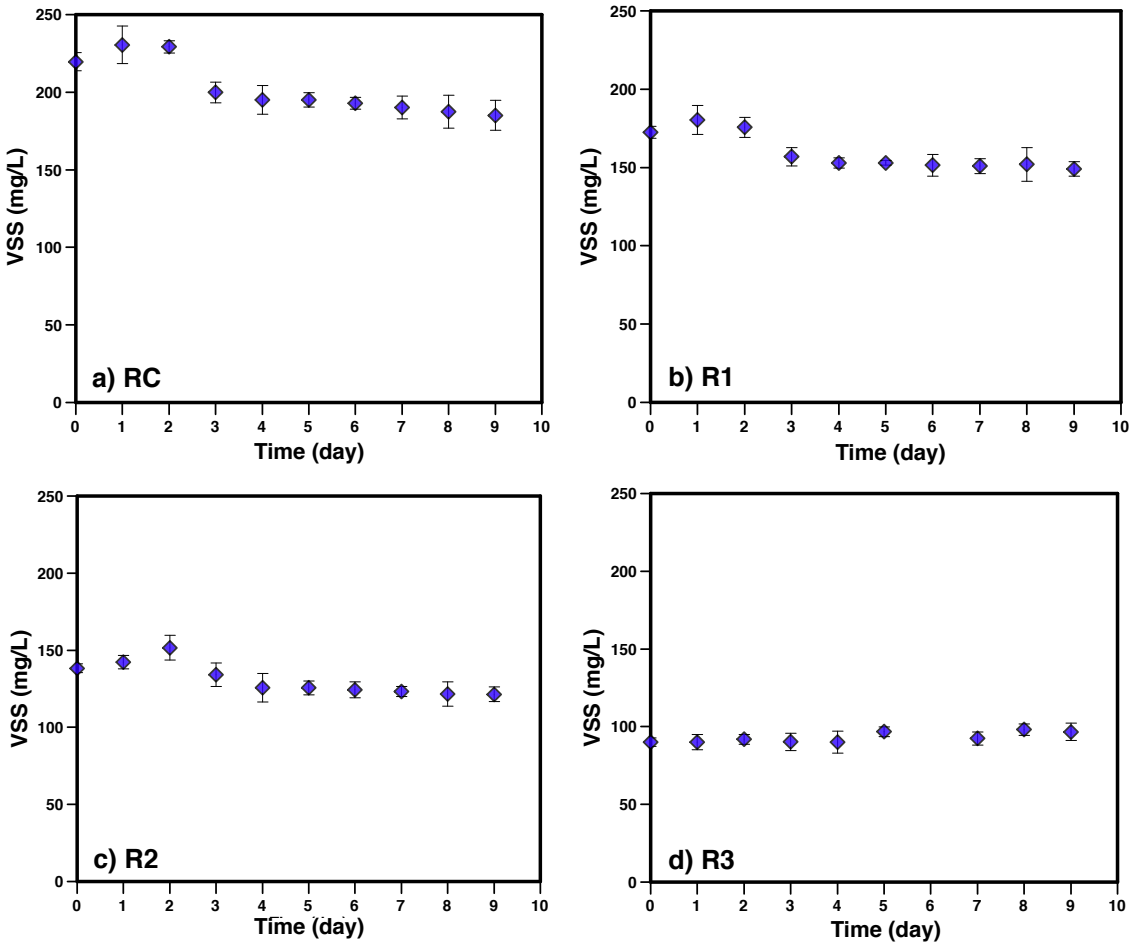
SCSTRs Batch Operation Results for Sulfate



Variation in sulfate concentration as function of time for reactors during the batch operation ((a) RC; (b) R1; (c) R2; and (d) R3). Each data point represents the average of the measurements for two samples and error bars represent standard error between two replicates.

Appendix 4- Count'd

SCSTRs Batch Operation Results for VSS



Variation in VSS for reactors as function of time during the batch operation ((a) RC; (b) R1: (c) R2; and (d) R3). Each data points represents the average of the measurements for two samples and error bars represent standard error between two replicates. For some points error bars are smaller than symbols, therefore not shown.

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