

Effect of *Bidens pilosa* L in sulfate removal from industrial wastewater in a hydroponic
system

By

Qhamukile Nkosingiphile Mthembu

A Dissertation Submitted for the Requirement for the Degree of Masters of Science
(Microbiology)

In the

Department of Biochemistry and Microbiology

Faculty of Science and Agriculture

University of Zululand



2018

KwaDlangezwa, South Africa

Supervisors: Dr Mathews Simon Mthembu

Co-supervisor: Prof Albertus Kotze Basson

**Effect of *Bidens pilosa* L in sulfate removal from industrial wastewater in a
hydroponic system**

This work is submitted in complete fulfilment for the degree of Masters (Microbiology) in
the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture at the
University of Zululand, KwaDlangezwa, South Africa

By

Qhamukile Nkosingiphile Mthembu

2018

Supervisor: Dr Mathews Simon Mthembu

Co-supervisor: Prof Albertus Kotze Basson

DECLARATION

I declare that the thesis herewith submitted for the Masters: Microbiology at the University of Zululand is my original work and has not been previously submitted for a Degree at any other University. I also declare that all the information cited or quoted is supported by a list of controls

Qhamukile Nkosingiphile Mthembu

I hereby approve the final submission of the following thesis.

Dr. M.S. Mthembu

Prof. A.K Basson

This _____ day _____ of 2018, at the University of Zululand.

TABLE OF CONTENTS

DECLARATION.....	i
LIST OF FIGURES.....	v
LIST OF TABLES	vi
ABBREVIATIONS.....	vii
ABSTRACT.....	ix
DEDICATION	xi
ACKNOWLEDGEMENTS	xii
PREFACE	xiii
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW	1
1.1 Introduction.....	1
1.2 Aims and objectives	4
1.2.1 Aim	4
1.2.2 Objectives.....	4
1.3 Literature review	5
1.3.1. Introduction	5
1.3.2 Sources of sulfate in the environment.....	5
1.3.3 Water pollution.....	6
1.3.4 Detrimental effects of sulfate	7
1.3.5 Sulfate-reducing bacteria and their role in the treatment of sulfate contaminated water ..	8
1.3.6 Wetlands as possible systems for remediation of sulfate contaminated water	10
1.3.7 Hydroponics	11
1.3.8 Mechanisms of sulfate removal from wastewater using wetland technology.....	12
1.3.9 <i>Bidens pilosa</i> as a hyperaccumulator.....	18
1.3.10 Macrophytes and bacterial interaction in sulfate removal from wastewater.....	20
1.3.11 Factors affecting sulfate removal from industrial wastewater.....	21
1.4 Conclusion	23
1.5 References	24
CHAPTER 2: SULFATE REMOVAL AND THE ROLE OF MACROPHYTES IN SULFATE REMOVAL FROM INDUSTRIAL WASTEWATER IN A HYDROPONIC SYSTEM	30
2.1 Introduction.....	30
2.2 Aim, hypothesis and objectives	33
2.2.1 Aim	33
2.2.2 Hypothesis.....	33
2.2.3 Objectives.....	33
2.3 Methodology	34

2.3.1 Hydroponic system construction	34
2.3.2 Macrophytes cultivation and wastewater collection	35
2.3.3 Sample collection	36
2.3.4 Determination of sulfate concentration in wastewater	37
2.3.5 Harvesting the macrophytes and sample preparation	37
2.3.6 Determination of sulfate concentration in plants	37
2.3.7 Statistical data analysis	38
2.4 Results and discussion	38
2.4.1 Performance of the hydroponic system	38
2.4.2 Sulfate removal in the hydroponic system and the mechanisms of removal	39
2.5 Conclusion	45
2.6 Recommendations	46
2.7 References	47
CHAPTER 3: PHYSICOCHEMICAL PARAMETERS AND THEIR EFFECT ON SULFATE REMOVAL FROM INDUSTRIAL WASTEWATER IN A HYDROPONIC SYSTEM	50
3.1 Introduction	50
3.2 Aim, hypothesis and objectives	51
3.2.1 Aim	51
3.2.2 Hypothesis.....	51
3.2.3 Objectives.....	51
3.3 Methodology	52
3.3.1 Determination of the physicochemical parameters	52
3.3.2 Statistical data analysis	53
3.4 Results and discussion	53
3.4.1 The analysis of physicochemical parameters.....	54
3.4.2 The effect of physicochemical parameters on sulfate removal.....	59
3.5 Conclusion	65
3.6 Recommendations	66
3.7 References	67
CHAPTER 4: DETERMINATION OF THE MICROBIAL POPULATION SHIFT AND DYNAMICS OF SULFATE REDUCING BACTERIA IN A HYDROPONIC SYSTEM	70
4.1 Introduction	70
4.2 Aim, Hypothesis and Objectives	72
4.2.1 Aim	72
4.2.2 Hypothesis.....	72
4.2.3 Objectives.....	72

4.3 Methodology	72
4.3.1 Sample collection	72
4.3.2 The growth of sulfate-reducing bacteria	73
4.3.3 DNA extraction	74
4.3.4 Gel electrophoresis	74
4.3.5 Real-time/quantitative polymerase chain reaction	75
4.3.7 Real time/qPCR products visualization	77
4.3.8 Statistical data analysis	77
4.4 Results and discussion	77
4.5 Conclusion	85
4.6 Recommendations	86
4.7 References	87
CHAPTER 5: DISCUSSION, CONCLUSION AND RECOMMENDATIONS.....	90
5.1 Introduction.....	90
5.2 Sulfate removal and the mechanisms of removal from the hydroponic system.....	90
5.3 Effects of physicochemical parameters on sulfate removal	91
5.4 Population shift and dynamics of sulfate-reducing bacteria.....	92
5.5 Conclusion	93
5.6 Recommendations	93
5.7 References	94
APPENDICES	94

LIST OF FIGURES	Page
Figure 1: Interactions of sulfate-reducing bacteria.	9
Figure 2: Sulfate degradation by sulfate-reducing bacteria and sulfate assimilation by plants	10
Figure 3: A diagram showing vertical flow hydroponic, illustrating the direction of movement of water in the system (Vymazal, 2005).	11
Figure 4: Sulfur lifecycle in the atmosphere, vegetation and underground.	15
Figure 5: Sulfate assimilation and protein synthesis in plants.	18
Figure 6: Blackjack plants (<i>Bidens pilosa</i> L) used in the study for accumulation of sulfate.	19
Figure 7: A constructed hydroponic system that was used in the study for removal of sulfate from industrial wastewater.	36
Figure 8: Sulfate removal efficiency (%) in the control and planted section over sampling periods.	40
Figure 9: Sulfate concentration in <i>Bidens pilosa</i> L harvested before and after treatment.	44
Figure 10: The pH obtained in the system over sampling periods.	54
Figure 11: Temperature obtained in the system over sampling periods.	56
Figure 12: Different concentrations of dissolved oxygen obtained in the system over sampling periods.	57
Figure 13: Chemical oxygen demand (COD) in the system over sampling periods.	58
Figure 14: The effect of pH on sulfate removal in the system.	60
Figure 15: The effect of temperature on sulfate removal in the system.	61
Figure 16: The effect of dissolved oxygen in sulfate removal in the system.	63
Figure 17: The effect of COD on sulfate removal in the system.	64
Figure 18: Images showing inoculums of mine water in three different carbon sources (glycerol, lactate and ethanol) and the black precipitates of iron sulfide precipitation by sulfate-reducing microorganisms.	78
Figure 19: An image of gel electrophoresis showing DNA bands of sulfate-reducing microorganisms. The first section of the gel presents DNA extracted in the control section. The second section of the gel presents the DNA extracted in the planted section (Lane M represents a DNA marker, Lane 1-4 DNA in the samples collected after 0, 48, 96 and 144 hours respectively).	79
Figure 20: A 3D view of extracted DNA of sulfate-reducing bacteria in the control and planted sections of the hydroponic system.	80
Figure 21: PCR products in the control section. (Lane M represents a DNA marker, Lane 1-4 DNA in the samples collected after 0, 48, 96 and 144 hours respectively).	81
Figure 22: PCR products in the planted section. (Lane M represents a DNA marker, Lane 1-4 DNA in the samples collected after 0, 48, 96 and 144 hours respectively).	83
Figure 23: Microbial genera of sulfate-reducing bacteria in the hydroponic system.	84

LIST OF TABLES	Page
Table 1: Sulfate concentrations in wastewater over retention time (control section).	42
Table 2: Sulfate concentrations in wastewater over retention time (planted section).	43
Table 3: The ingredients used for the growth of sulfate-reducing bacteria.	73
Table 4: The PCR primers for the identification of sulfate-reducing bacteria that were used in the study.	76
Table 5: qPCR constituents that were used in 16S rDNA amplification.	76
Table 6: The conditions of the hot-start cycling for DNA amplification.	76

ABBREVIATIONS

Al: Aluminium

APS: Adenosine-5-phosphosulfate

ATPS: Adenosine triphosphate sulfurylase

Ba²⁺: Barium cation

Ca²⁺: Calcium cation

Cd: Cadmium

COD: Chemical oxygen demand

Cu: Copper

DNA: Deoxyribonucleic acid

DO: Dissolved oxygen

EDTA: Ethylene diamine triacetic acid

Fe: Iron

H⁺: Hydrogen ion

HCO₃: Bicarbonate

H₂S: Hydrogen sulfide

K⁺: Potassium cation

Li⁺: Lithium cation

Mg²⁺: Magnesium cation

Na⁺: Sodium cation

NH₄⁺: Ammonium cation

Ni: Nickel

PAPS: Phosphoadenosine-5-phosphosulfate

qPCR: Quantitative polymerase chain reaction

ROS: Reactive oxygen species

rRNA: Ribosomal ribonucleic acid

Rb⁺: Rubidium cation

S: Sulfur

SRB: Sulfate-reducing bacteria

Sr²⁺: Strontium cation

SULTR: Sulfate transporter

TMA: Trimethylamine

TMAO: Trimethylamine oxide

Zn: Zinc

ABSTRACT

Water contamination from human activities such as discarding sulfate-rich wastes into natural water resources leads to the introduction of sulfate and other toxic substances like heavy metals. This poses as a threat to human health and the environment since consumption of sulfate concentration greater than 250 mg/l causes diarrhoea and dehydration. Accumulation of sulfate in water also leads to the death of aquatic species and when sulfur is produced from sulfate, it may react with oxygen in the atmosphere and form sulfur dioxide which causes acid rain when reacted with nitrogenous gases. Acid rain is detrimental to the environment. Ion exchange chromatography is currently used in sulfate removal but it is expensive and energy consuming. This has necessitated the development of an environmentally friendly, cost-effective, and simple wastewater technique for sulfate removal using wetland technologies. In order to remove sulfate from wastewater, two hydroponic systems were constructed, and the first one was cultivated with *Bidens pilosa L* and the other one was left unplanted (control section). Wastewater collected from Tende Coal Mine was introduced into both sections and the initial sample was collected. After every 24 hours the samples were collected at different hydraulic retention time, for 2 weeks. In all samples physicochemical parameters were determined using a pH meter. Sulfate concentration was determined using sulfate test kits and a spectrophotometer. The qPCR was used to identify the microorganisms responsible for the removal of sulfate in the system.

Sulfate removal in the planted section was higher than in the control section. It was 2.9%, 4.9% after 24 hours, 6.5%, 11% after 48 hours, 12%, 17% after 72 hours, 16.3%, 25.4% after 96 hours, 18.2%, 34.8% after 120 hours, 26.9%, 44.6% after 144 hours, 34.7%, 55.1% after 168 hours, 42%, 63.7% after 192 hours, 47.5%, 71.5% after 216 hours, 53.2%, 73.3% after 240

hours, 54.7%, 74% after 264 hours and 56%, 76.3 % after 288 hours over 2 weeks in the control and planted sections respectively. Sulfate concentration in the macrophytes was found to be 110 mg/l before treatment, and 353 mg/l after treatment. There was a significant difference between sulfate removal in the planted and control section and also in macrophytes before and after treatment, indicated by $p=0.0001$. This indicated that the hydroponic system was able to remove sulfate from wastewater using the combination of the mechanisms of plant uptake and microbial degradation. Sulfate removal was also indicated by final concentration of sulfate, which was 169 mg/l in the planted section which was below the acceptable amounts of sulfate in water (by World Health Organization) while it was 309 mg/l in the control section. Temperature had a moderate negative correlation on sulfate removal ($-0.38 \leq r \leq -0.42$) while COD had a very strong negative correlation ($-0.94 \leq r \leq -0.97$). The dissolved oxygen indicated weak positive correlation ($0.29 \leq r \leq 0.37$), and pH indicated a strong positive correlation ($0.80 \leq r \leq 0.79$) in the planted and control section respectively. These correlations indicated that physical and chemical parameters were had an effect on sulfate removal. Microbial population of sulfate-reducing bacteria (*Desulfobacter*, *Desulfovibrio*) was present in both systems. *Desulfococcus* was present in the control section but absent in planted section due to its sensitiveness to oxygen. These findings shown that the hydroponic system had an ability to remove sulfate from industrial wastewater using macrophytes and sulfate reducing bacteria but the removal was dependent on physicochemical parameters.

DEDICATION

This work is dedicated to my lovely mom, Bonangani Sphiwe Mthembu and my late grandmother, Bettina Khanyile. *Mother*, thank you so much for always being there for me, your unconditional love, constant support and prayers are much appreciated. I love you dear mother, you are the best.

ACKNOWLEDGEMENTS

I would like to thank *UNyazi LweZulu* for keeping me in this world and giving me the strength and power to stay strong through hardships of this journey. *Ndlamhlathi kaMayekisa* thank you for your kindness.

To my supervisors Dr Mathews Simon Mthembu and Prof Albertus Kortze Basson. I am forever going to be grateful for an opportunity that you provided me with, in order for me to develop myself academically. I appreciate all the assistance, advice and support you gave me. Thank you so much for having faith in me.

I would also like to thank my family for always being there for me. I also pass great thanks to all my lab mates (Dr. Mthembu's postgraduate students). They have been very supportive right from the beginning up to the end. Thank you so much family, not forgetting the University of Zululand's staff and the departments that contributed to making my study a success (Department of Biochemistry and Microbiology and Department of Agriculture).

I would also like to thank the National Research Foundation (NRF) for funding my studies. This work would have not been a success without it.

PREFACE

Papers Presented at National Conferences

- **Mthembu, Q.N.**, Ndulini, S.F. and Mthembu, M.S. The effects of physical and chemical parameters in sulfate removal from industrial wastewater in a hydroponic system. *Science and Agriculture Symposium*. 08 November 2018. University of Zululand, Science Centre, South Africa.

Papers Presented at International Conference

- **Mthembu, Q.N.**, Ndulini, S.F. and Mthembu, M.S. The effect of physical and chemical parameters in sulfate removal from industrial wastewater in a hydroponic system. *19th WaterNet/WAFSA/GWP-SA Symposium*. 31st October – 02nd November 2018, Livingstone, Zambia.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Economic growth transforms the world and lifts millions of citizens out of poverty. However, it is usually being challenged by environmental degradation such as deterioration of water quality as a result of urbanization and industrialization (Ahmadpour *et al.*, 2014). According to Ebenstein (2012), total domestic pollution is composed of 75% industrial pollution wastes yet in toxic terms of industrial pollution is much more than 75%. Industrial wastes include toxic pollutants such as heavy metals (e.g. mercury and chromium) and sulfate. Sulfate has also become a major problematic industrial wastewater pollutant nowadays, and has received much attention in industrial wastewater research (Ntuli *et al.*, 2016). Water contamination by sulfate threatens human beings since it is estimated that currently, 1.1 billion people do not have access to safe and clean water, and 70 million work days are lost to water-related diseases (Kulkarni *et al.*, 2018).

Human health and the environment are both negatively affected by industrial wastes (Shakir *et al.*, 2017). Also, population growth, climate change and water scarcity bring challenges that affect the world's economies and societies (Liu *et al.*, 2017). Recent studies on the impact of climate change on water scarcity show that about 2 billion people are experiencing water scarcity in several areas worldwide (Liu, 2017). Water scarcity has a negative influence on food production in agriculture since 70% of water withdrawn globally is used for crop irrigation. That shows that water availability is not only essential for human consumption but is also required in food production since the world population has doubled between 1970 and 2015, resulting in the high demand for food supply (Koch *et al.*, 2018).

Crop, cereal and sea food production is growing rapidly but not as fast as the livestock sectors are growing in almost all countries (Saeed *et al.*, 2018). With that, population growth puts pressure on agriculture resulting in the use of inorganic compounds to increase the yield of food products (Liu *et al.*, 2018). Manufacturing firms use inorganic compounds for the production of fertilizers that enhance the growth of crops. This practice of using fertilizers indirectly contributes to water pollution through surface runoff (Raper *et al.*, 2018). Contaminated water must be treated for reuse to eliminate toxic pollutants and conventional methods are currently used for wastewater treatment.

Furthermore, conventional wastewater treatment techniques such as conventional activated sludge plants and membrane bioreactors are currently used for domestic and industrial wastewater treatment respectively. However, the problems associated with these conventional methods include high levels of energy consumption, huge capital injection & maintenance, and the complexity associated with the systems. Constructed wetlands have been reported to be the alternative wastewater treatment technique with an ability to remove sulfate from industrial wastewater (O'Sullivan, 1999).

Sulfate is widely distributed in natural resources such as water bodies due to natural and anthropogenic (mankind related) activities. Activities or human practices such as mining, sludge and discarding of industrial effluent, power and energy transmission and fuel production can lead to the introduction of sulfate and other toxic substances such as heavy metals into water resources (Ramla, 2015). Industrial wastes can be washed off as fertilizers from agricultural lands as surface runoff and introduced to water resources during rainfall. Industrial wastes dumped in water bodies are composed of toxic substances that have a negative impact on the environment and human health (Adebisi *et al.*, 2011). In the same

vein, sulfate and heavy metals are major pollutants derived from industrial and mining wastes and contribute to acid mine drainage. Acid mine drainage is defined as acid water production during the exposure of sulfide minerals to water and air through chemical reaction to produce sulfuric acid. Acid mine drainage and mineral processing occurs at about 70% of world's mine sites resulting in the production of metal and sulfate contaminated water.

Sulfate contamination is quite prevalent in mining areas, and has received much attention in mine water (Ntuli *et al.*, 2016). Sulfate oxidation is associated with many mining ore bodies, extracted or processed ore. Products of this reaction enter water bodies and result in the reduction of water quality and an increase in acidity, salts and sulfate in wastewater (Bowell, 2004). Importantly, accumulation of salts such as calcium sulfate in water creates environmental problems if discharged, and also limits cycles of water reuse. Sulfate-rich water leads to pathological disturbances such as hypertension, heart failure, diabetes, sepsis, inflammation, erectile dysfunction, asthma and neurodegenerative diseases (Wang *et al.*, 2011). Sulfate and heavy metals in industrial wastewater can be absorbed and accumulate within plants and marine organisms (Driscoll *et al.*, 2007).

Since plants and marine organisms are important food sources for human beings; sulfate and heavy metals can easily enter the food chain. The accumulation of these contaminants endangers both marine organisms and seafood consumers because some of these contaminants are carcinogenic and may lead to the death of aquatic organisms. Plants can also obtain sulfate from wastewater through irrigation if they are able to withstand phytotoxicity, defined as the inhibition of growth in plants due to accumulation of toxic substances within their cells. This necessitates the use of environmentally friendly

wastewater treatment technologies like wetlands for removal of sulfate from wastewater before disposal and reuse.

Constructed wetlands are defined as engineered systems designed to use natural processes involving wetland vegetation and their related microbial population to treat wastewater (Vymazal, 2004). The wetland system is energetically sustainable because it uses only natural energy to reduce pollutants. The constructed wetland system is much better compared to conventional wastewater treatment systems because it requires low construction and operational costs (Wang, 2017). In this study, a hydroponic system, that is a constructed wetland, was used to remove sulfate from industrial wastewater. It was hypothesized that *Bidens pilosa L* does have a potential ability to remove sulfate from industrial wastewater in a hydroponic system.

1.2 Aims and objectives

1.2.1 Aim

The aim of the study was to remove sulfate from industrial wastewater and to establish the macrophytes' (*Bidens pilosa L*) and sulfate-reducing bacteria's (SRB) ability to remove sulfate from wastewater.

1.2.2 Objectives

The objectives of the study were:

1. To establish the mechanism of sulfate removal by the hydroponic system.
2. To determine physical and chemical parameters in industrial wastewater circulating in the hydroponic system.
3. To determine sulfate removal efficiency from wastewater in a hydroponic system.
4. To determine the population dynamics of SRB in the hydroponic system.

1. 3 Literature review

1.3.1. Introduction

Anthropogenic activities and natural processes such as sea level rise, agricultural practices, acid rain, and industrial runoff are the main factors contributing to the introduction of sulfate to water resources. The presence of sulfate in water promotes methylation of mercury, which is the most toxic metal. Mercury methylation in sulfate-rich water endangers the environment (plants and aquatic organisms) and human health. Mercury is known to be a bioaccumulative metal that may accumulate in plants when irrigated with contaminated water. It may also accumulate in aquatic organisms such as fish, oysters, crabs etc. Wastewater treatment techniques play a vital role in the removal of sulfate from industrial wastewater.

Hydroponic systems have been recognized as one of the ideal wastewater treatment alternatives that rely on biological, biochemical processes and climatic conditions. Uptake of contaminants uptake by plants can be affected by climatic conditions in both direct and indirect ways. The direct influence refers to temporal changes in wetlands performance, depending on the physiological characteristics of the plants, governed by solar radiation and temperature. The indirect influence means that the biological wastewater treatment processes rely on physical conditions such as low temperature. Low temperature restrains microbial activities, and thereby decreasing bacterial growth, resulting in low purification efficiency (Garret *et al.*, 2008).

1.3.2 Sources of sulfate in the environment

Sulfate occurs both in natural and anthropogenic (originating from human activity) water systems. Primary natural sources of sulfate include sulfate mineral dissolution, sulfate mineral

oxidation and atmospheric deposition. Sulfate is widely distributed in nature and may be present in natural waters at concentrations ranging from a few to several milligrams per litre (Miao, 2013).

Anthropogenic sources include: phosphate refineries, power plants, coal mines and metallurgical refineries. Since sulfate containing salts are natural substances in the environment, sulfate is expected not to be more toxic compared to other compounds contaminating industrial wastewater. Processes like phytoremediation are currently used for detoxification of industrial wastewater (Saha *et al.*, 2017). Sulfate is therefore a source of water pollution which needs to be removed from wastewater.

1.3.3 Water pollution

Water pollution is a process whereby water resources (dams, rivers, oceans and groundwater) get contaminated. A lot of factors contribute to water contamination, mostly by human activities. Pollutants may enter the water bodies through surface runoff during rainfall. Contaminated surface water also infiltrates through the soil, contaminating groundwater. Water pollution is caused by different types of contaminants such as chemicals, pathogens and physical changes such as elevated temperatures. The bacterial community also contributes to water contamination. Bacterial population within contaminated water consists of both harmful and beneficial bacteria (Azizullah *et al.*, 2011).

Pathogens are microorganisms that cause disease. Coliform bacteria are beneficial and usually not the actual cause of diseases in polluted water, therefore can be used as indicators for water quality. Other factors that contribute to water pollution include organic and inorganic substances. While organic contaminants from industries that contribute to water contamination include detergents, food processing waste, petroleum hydrocarbons,

insecticides and herbicides etc.; inorganic water pollutants include fertilizers, sulfate, heavy metals and acidity caused by industrial deposition. Introduction of sulfate to water resources lead to reduction of water quality and serious complications such as death of young livestock through consumption of sulfate-rich water.

1.3.4 Detrimental effects of sulfate

Sulfate is a common wastewater contaminant that is not usually a threat to health, but is challenging wastewater reuse since it can cause diarrhoea when consumed in high concentrations. Reduction of sulfate may produce hydrogen sulfide (H_2S) and organic sulfur (S) compounds. Sulfate is known to trigger problems related to odour, colour and taste in wastewater and rivers from which the effluent of contaminated water is discarded. Hydrogen sulfide has an ability to corrode water pipes during transportation of reused water. Corrosion of water pipes during water transportation leads to production of rust or metal ions which pollute water, change water colour and flow rate. It affects many industries such as: oil production, power generation and transportation of water, since corrosion of water pipes in industries impact water distribution, thus affecting economy. It also causes phytotoxicity to plant irrigated with H_2S containing water (Chen *et al.*, 2016).

Consumption of sulfate-rich wastewater in high concentrations can also lead to dehydration, nausea, gastrointestinal effects and death in some cases, and is of special concern to infants. It is poisonous to fish and contributes to acid rain which is harmful to the environment (Fu *et al.*, 2011). When sulfate is reduced to hydrogen sulfide, it becomes poisonous and flammable. Sulfate-reducing bacteria are the microorganisms known to have the ability to eradicate sulfate from wastewater. Oxidation of sulfate to hydrogen sulfide is thereby carried out by sulfate-reducing bacteria.

1.3.5 Sulfate-reducing bacteria and their role in the treatment of sulfate contaminated water

Activities involving food processing, paper industry, dye and detergent manufacture have been confirmed to contribute to high sulfate concentrations in wastewater (Kaksonen *et al.*, 2004). Sulfate is known to cause much damage if consumed by humans in high concentrations (>250 mg/l). It is therefore essential to remove sulfate from industrial wastewater before water is discharged to water bodies. Sulfate-reducing bacteria have an ability to degrade sulfate from wastewater. They are anaerobic microbes that tolerate salinity and terrestrial conditions, and they obtain energy by oxidizing compounds or molecular hydrogen while reducing sulfate to hydrogen sulfide. These microorganisms obtain energy from oxidizing organic compounds as the carbon source both autotrophic and mixotrophic (which means that they use both organic and inorganic carbon source) (Hao *et al.*, 1996). These bacteria also reduce inorganic sulfur compounds such as sulfite and elementary sulfur. They also have the ability to reduce nitrate, nitrite, iron and some other metals. Growth of these bacteria therefore depends on the presence of sulfate and carbon concentration which increases the pH. Sulfate-reducing bacteria survive in the environments such as plumbing systems, water softeners and water heaters and usually flourish onto the hot waterside of water distribution systems. They naturally occur in surface waters, including seawater.

Moreover, the accumulation of these bacteria in water leads to pitting of steel and build-up of hydrogen sulfide which increases corrosiveness of water, thus increasing sulfide production (Qian *et al.*, 2016). Sulfate-reducing bacteria have the ability to cause both internal and external corrosion of wastewater and petroleum pipeline and natural gas. They can be used in the removal of sulfate from industrial wastewater (van de Brand *et al.*, 2015). Sulfate-reducing bacteria and macrophytes have a symbiotic relationship they use in wetlands for sulfate removal.

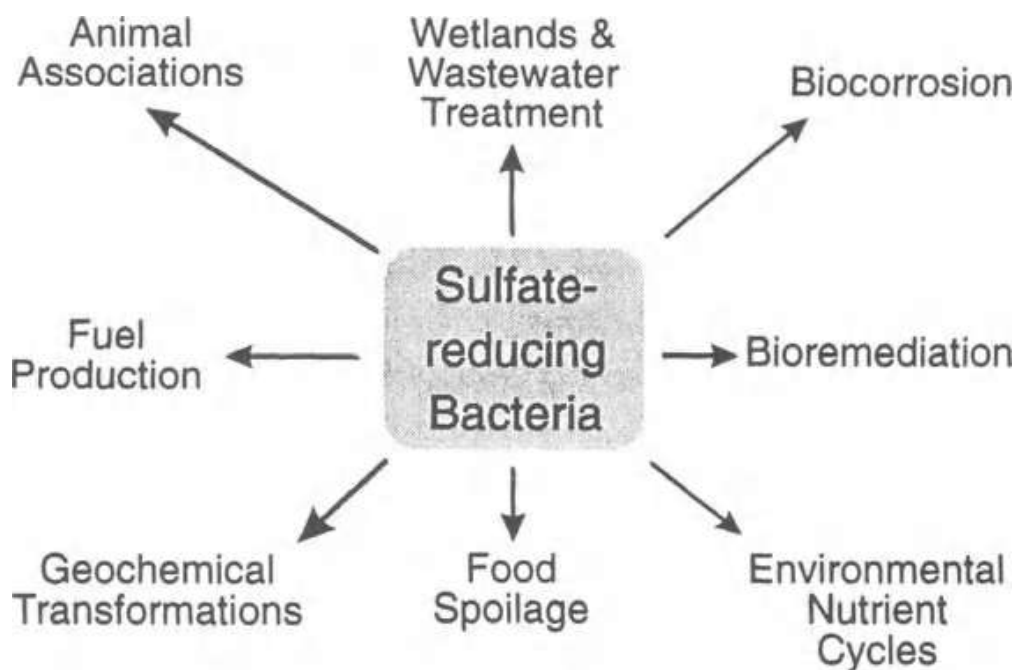


Figure 1: Interactions of sulfate-reducing bacteria (Barton and Tomei, 2002).

Figure 1 illustrates how sulfate-reducing bacteria interact with living and non-living organisms and their use in the industries. Sulfate-reducing bacteria may be found in the cattle rumen and some other bovine species. They may also be found in the human gut and faecal matter (Barton and Tomei, 2002). However, these bacteria may also cause corrosion of metals and food spoilage e.g. fish. Though most fish contain trimethylamine oxide (TMAO); sulfate-reducing bacteria (*Vibrio* species) have the capability to oxidize TMAO to trimethylamine (TMA) in anaerobic respiration. TMA also leads to formation of ammonia-like bad odours in fish thus causing fish spoilage. These bacteria may also cause food spoilage in improperly canned foods via production of rotten odours (Barton and Tomei *et al.*, 2002). In industries, sulfate-reducing bacteria are used for bio-remediation and fuel production in a coupled reaction where sulfate is bio-remediated while methylation of mercury is also occurring. This results in the production of methane. These bacteria are also involved in geochemical

transformations and environmental nutrients recycling e.g. completing sulfur cycle illustrated in Figure 2.

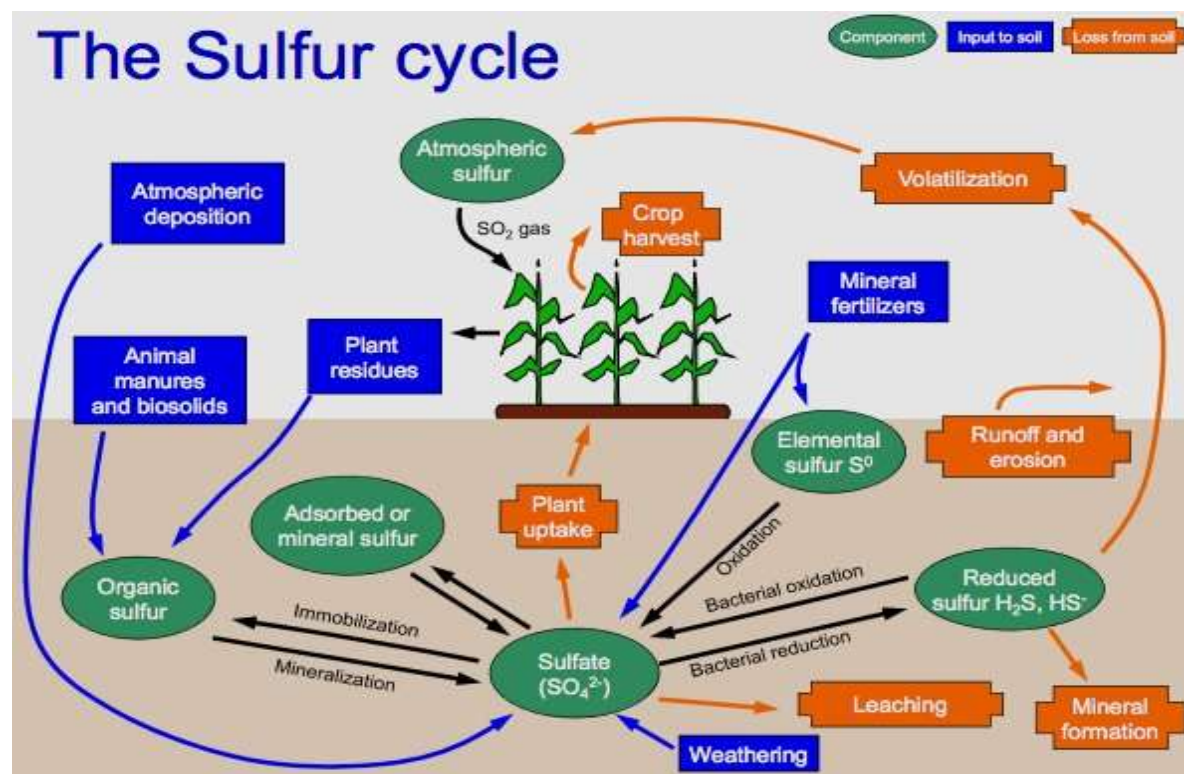


Figure 2: Sulfur lifecycle in the atmosphere, vegetation and underground (Zhao *et al.*, 2014).

1.3.6 Wetlands as possible systems for remediation of sulfate contaminated water

A wetland may be described as a piece of water logged and shallow water table. This can be either seasonal or permanent. A characteristic that differentiates wetlands from other land forms or water resources is vegetation of aquatic macrophytes. Wetlands play various roles in the environment, and these roles include: wastewater treatment, flood control, storm protection etc. Also, wetlands may be classified into natural and constructed wetlands.

Constructed wetlands are engineered systems that are designed to make use of biological or natural processes using vegetation and their associated microbial population to treat or decontaminate wastewater (Vymazal, 2005). They provide habitat for wetland organisms and

promote water reuse and recycling. It should also be noted that hydroponic systems have been previously used in the treatment of various kinds of wastewater, including sewage and agricultural wastewater.

1.3.7 Hydroponics

Hydroponics are defined as methods of growing plants using nutrient rich (wastewater in most cases) medium in a soilless environment. Using that system, aquatic plants may be grown with their root system suspended in a nutrient solution (Xydis *et al.*, 2017). Various wetland systems incorporate different types of plants for removal of nutrients and microorganisms from wastewater. Nutrient rich solution is supplied to the planter box by means of a pipe and an electric pump (Figure 3). In that way, nutrients are dispersed throughout the system (Brix, 1997). Organic substances within wastewater serve as natural fertilizers to the plants being grown in the hydroponic system, therefore, can be used when growing plants instead of using chemical fertilizers.

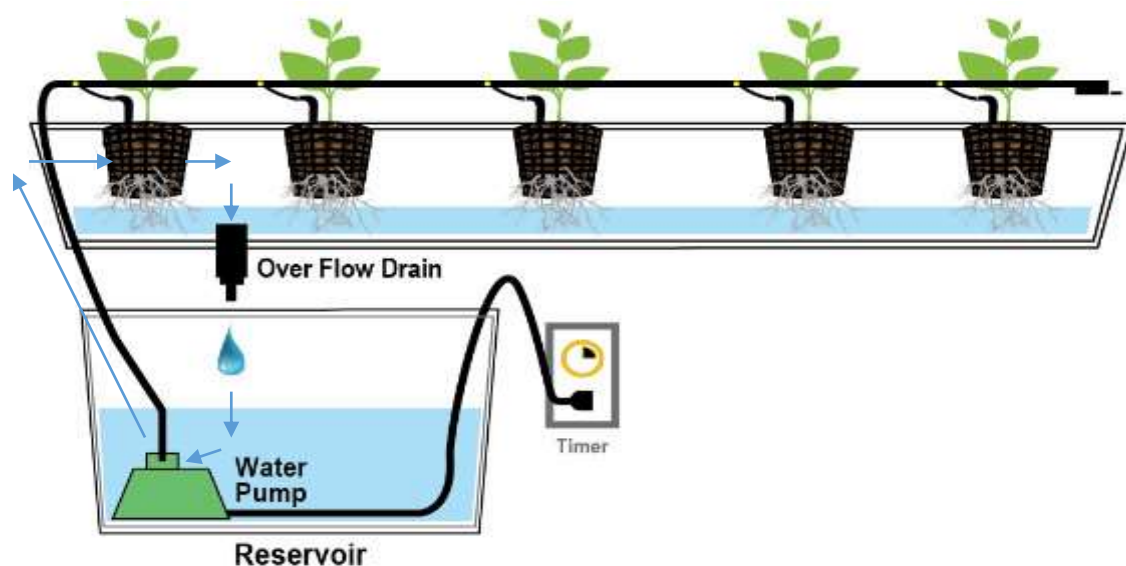


Figure 3: A diagram showing vertical flow hydroponic, illustrating the direction of movement of water in the system (Vymazal, 2005).

Treatment of wastewater using hydroponics is better compared to other wastewater treatment techniques because there are more benefits in plants growing without soil. Crop production yield is greater compared to traditional planting in soil. This is due to the fact that hydroponically grown plants dip their root systems into the nutrient rich solutions and access nutrients more easily than the plants that are grown on soil. Plants need smaller root systems so that they can transfer more energy into shoot (leaves and stem) growth. With smaller roots, plants can be grown in the same area and the output would be clean water and high yield of plants than the ones planted on the ground. Hydroponic plants grow faster than those planted in soil because it takes longer for a shoot of a germinated seed to emerge from soil and roots to penetrate deeper into the soil. Furthermore, hydroponics may be used to remove sulfate from industrial wastewater even though it may expose macrophytes seedlings to toxicity (Pastor *et al.*, 2017). Phytoremediation of toxic substances is influenced by factors such as oxygen supply and nutrition to adapt plants to hydroponics (Huang *et al.*, 2016). Hydroponics use both plants and microorganisms using different mechanisms to remove sulfate from industrial wastewater, referred to as biological processes. Some other mechanisms use physical processes.

1.3.8 Mechanisms of sulfate removal from wastewater using wetland technology

Wetland technology depends on several basic processes for the removal of sulfate and heavy metals from wastewater. The amount of sulfate ions removed is determined by a combination of interacting processes of settling, sedimentation, sorption, phytoaccumulation, biodegradation, microbial activity and plant uptake (Sheoran and Sheoran, 2006). Sulfate removal mechanisms use three different processes namely: physical, chemical and biological processes.

➤ Physical processes

Settling and sedimentation are the physical processes responsible for the removal of sulfate from industrial wastewater (Khan *et al.*, 2009). A number of dynamic transformations may take place in a wetland due to the presence of sulfate and hydrous oxides, whether the water is motionless or mobile. Sulfate may be transformed from water to the soil substitute, then denser particles settle out of water in calm waters. Sedimentation rate can be expressed in mass accumulation. Mats of macrophytes in wetlands serve as sedimentation traps. Efficiency of suspended solids removal is equivalent to settling velocity and the length of the wetland.

➤ Chemical processes

Mechanisms of sulfate removal in wetland technologies via chemical processes include: adsorption, precipitation of sulfate and metal sulfides.

Adsorption

Sulfate is adsorbed to the soil substitute by cat-ion exchange or chemisorption. Cat-ion exchange involves the physical attachment of positively charged ions to the surface of organic matter via electrostatic attraction. The capacity of the substrate for the retention of the ions increases with an increase in organic matter content. Adsorption depends on the physical and chemical environment of the medium, and properties of the metals concerned. More than 50% of acid mine drainage can be adsorbed onto particulate matter in the wetland, hence removed from the water component (Minh *et al.*, 1997).

Sulfate precipitation

Another common way to remove sulfate from wastewater is to remove it as a solid, insoluble sulfate salt. Chemical precipitation for sulfate removal is used widely in both mining and

industrial applications. The minimum achievable sulfate concentration depends on the specific salt formed. For example, lime (calcium hydroxide) can be added to water in order to remove sulfate as gypsum (calcium sulfate). However, this method can only reduce the sulfate concentration to a limit of 1,500 mg/l (Bowell, 2004). This is significantly higher than the sulfate concentrations usually found in wastewater. Sulfate salts, such as barium sulfate, are less soluble in water, so can be used to remove sulfate to lower concentrations around 100 mg/l and 50 mg/l. Metal salts are not effective at precipitating sulfate but can be used to remove sulfide from solution and provide another means to remove sulfur from an aqueous system.

Metal sulfides precipitation

Wetlands with proper substrate promote the growth of sulfate-reducing bacteria. In acid mine water which is rich in sulfate, these bacteria generate hydrogen sulfide. Most of heavy metals react with hydrogen sulfide and thereby producing highly insoluble metal sulfides. Bacterial sulfate removal also results in the precipitation of dissolved metals such as metal sulfide solids. Precipitation of metal sulfide in an organic substrate improves water quality by decreasing mineral acidity without the cause of parallel increase in proton acidity. Protons released by hydrogen sulfide dissociation can be neutralized by an equal release of HCO_3 during sulfate removal (Lewis, 2010). The substrate also plays an important role in acid mine drainage treatment and positively influences sulfate removal in the wetland technology.

➤ Biological processes

Biological sulfate removal processes include: microbial sulfate oxidation of sulfate and plant uptake.

Microbial oxidation of sulfate

The mechanism of sulfate removal by sulfate reduction through sulfate-reducing bacteria involves two stages: The first stage is when sulfate-reducing bacteria oxidize simple organic compounds (e.g. lactate, acetate, butyrate etc.) by utilizing sulfate as the electron acceptor and generating hydrogen sulfide and bicarbonate ion under anaerobic conditions (Zhang *et al.*, 2014); while the second stage involves the reaction of biologically produced hydrogen sulfide with dissolved metals such as Zn, Cu and Ni to form insoluble metal precipitates (Al-Abed *et al.*, 2017). These metals are responsible for reacting with hydrogen sulfide (produced by sulphate reducing bacteria and phototrophic bacteria from sulfate removal) to produce metal sulfides. Precipitation of metal sulfides decreases acidity and sulfate concentration.

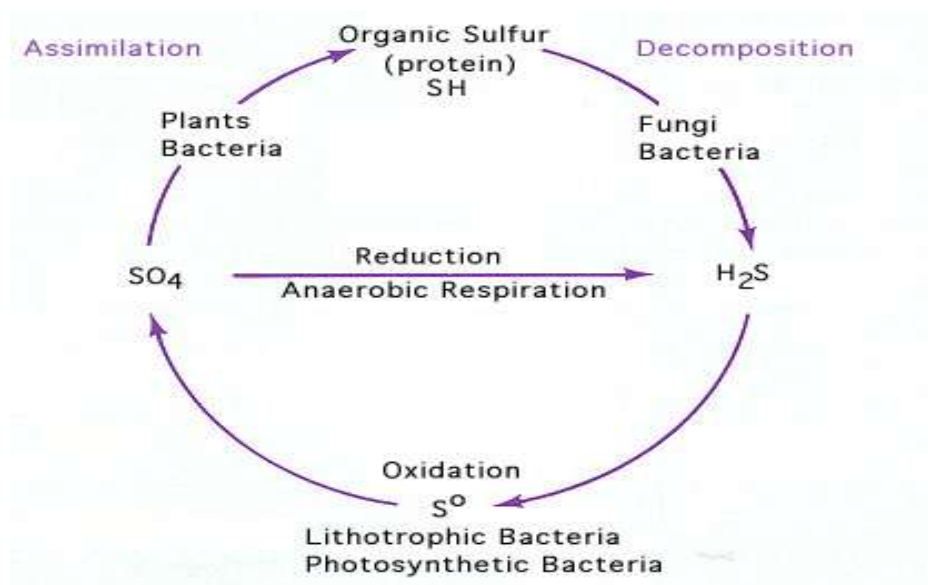


Figure 4: Sulfate degradation by sulfate-reducing bacteria and sulfate assimilation by plants (Zhao *et al.*, 2014).

Figure 4 illustrates sulfate degradation to hydrogen sulfide by sulfate-reducing bacteria (SRB), which is coupled with the stimulation of the anaerobic microbial respiration of sulfate to H₂S.

Hydrogen sulfide oxidation into elemental sulfur is carried out by lithotrophic (organism that obtain its reducing agent from catabolism of organic compounds). These bacteria are responsible for the conversion of sulfur back to sulfate which re-enters the reduction and anaerobic respiration cycle. Some of the sulfate is assimilated by plants and bacteria for the production of proteins (organic sulfur). Fungi and bacteria are the microorganisms responsible for the decomposition of the organic protein back to hydrogen sulfide. Hydrogen sulfide re-enters the cycle to be oxidized back to sulfur and later to sulfate to keep the cycle of sulfate oxidation going (Zhao *et al.*, 2014).

Sulfate is available in the atmosphere as atmospheric sulfur, which undergoes atmospheric deposition as sulfate and gets distributed underground where it accumulates in plants via roots and back to the atmosphere via volatilization of degraded form of sulfate (hydrogen sulfide) from bacterial degradation by sulfate-reducing bacteria. It can be introduced to ground water via surface runoff from mineral fertilizers that later seeps into the soil. It may also leach from industries and reach underground environment which is suitable enough for mineral formation.

Sulfate uptake by plants

Growing plants require sulfate for the synthesis of amino acids, sulfolipids and other sulfur-related compounds. Sulfate demand depends on the tissues, organs and development stage of the plant. Sulfate assimilation and distribution is regulated in response to plant demand and the changing environment (Kaksonen *et al.*, 2004). Sulfate is absorbed by the plants in the root cells transported to the aerial parts of the plants via vascular system and enter metabolic processes. Sulfate may also be redistributed during development from mature leaves to roots, younger leaves or seeds. During sulfate starvation, sulfate transporters/

enzymes responsible for sulfate transportation within the plant leaves' namely: *SULTR2.2* and *SULTR 1.3* play a major role in sulfate distribution. Furthermore, while the main reservoir of sulfate in plants is in the vacuoles of mature leaves; sulfate transportation from plants' roots into the shoot is catalysed by the enzymes, *ATP sulfurylase* and *APS reductase*. Sulfate absorption gets reduced immediately after the process of amino acids synthesis is initiated by the enzyme *o-acetylserine (thiol) lyase* (Figure 5). The expression of the genes encoding for sulfate transporters and enzymes for sulfur assimilation are controlled mainly at the transcriptional level in response to sulfur status (Leustek and Saito, 1999). Sulfate accumulated by plants' roots is either transported to the vacuole or synthesized into amino acids, which are the subunits of proteins (Kopriva *et al.*, 2012). Meanwhile, macrophytes must have an ability to survive the toxic effects of the effluent and its variability (McIntyre, 2003). Macrophytes with an ability to grow in areas that are contaminated with toxic substances are referred to as hyperaccumulators (Rene *et al.*, 2017).

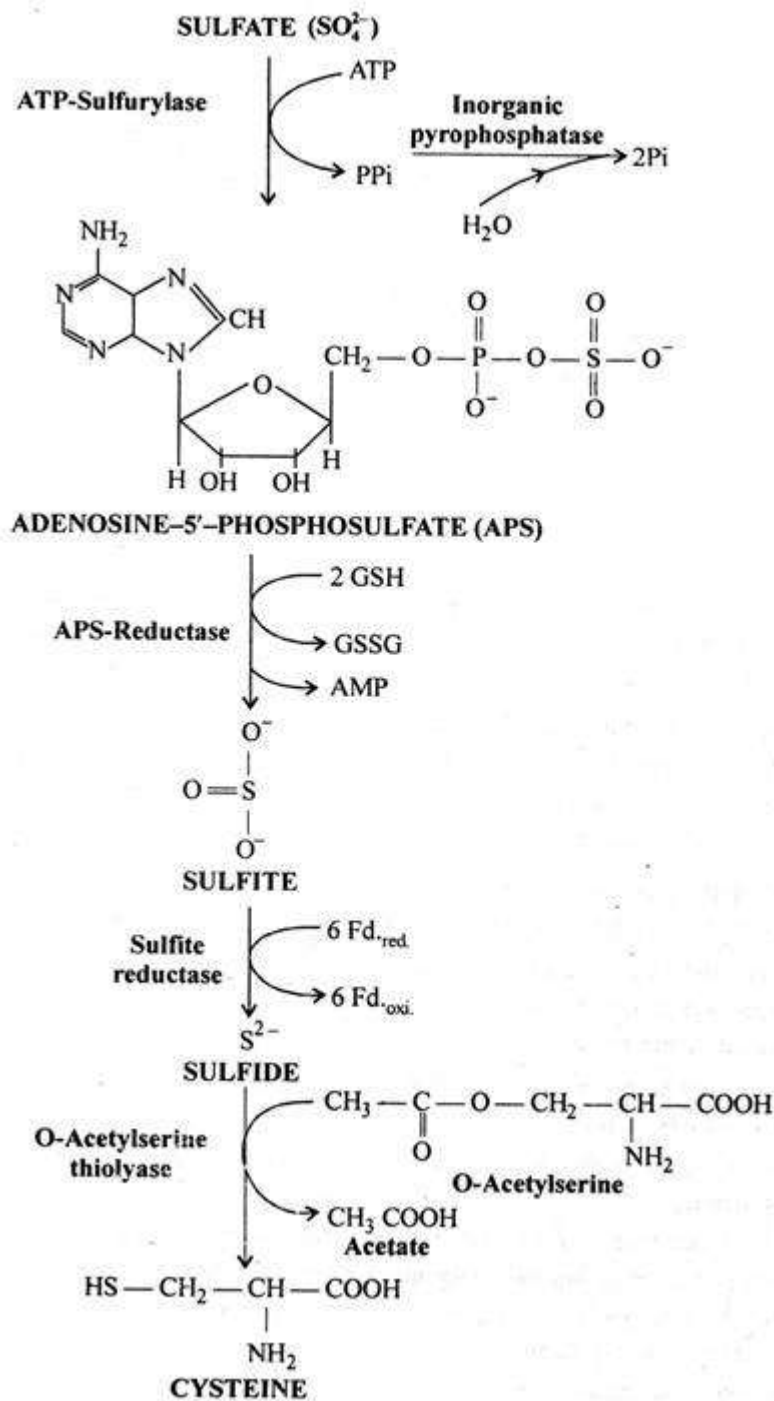


Figure 5: Sulfate assimilation and protein synthesis in plants (Kopriva *et al.*, 2012).

1.3.9 *Bidens pilosa* as a hyperaccumulator

At present, researchers are focusing on wastewater and contaminated soil treatment using hyperaccumulators for decontamination of sulfate and heavy metals (Ndulini *et al.*, 2018).

Hyperaccumulators are defined as herbaceous woody plants with an ability to accumulate extremely high concentrations of sulfate and heavy metals in their tissues. Furthermore, they show no symptoms of toxic substance accumulation and growth inhibition. Hyperaccumulators in another view produce enzyme *superoxide dimutases* and *peroxidases* which play an important role in scavenging reactive oxygen species (ROS) (Liu *et al.*, 2017). *Bidens pilosa* L (Figure 6) is known to be a macrophyte with an ability to naturally tolerate high concentration of Cadmium (Cd), and is widely distributed worldwide. Based on the above, irrigation of crops using metals and sulfate-rich water may result in a decrease in crop production and may be harmful to human health via the food chain, causing fatal diseases (Sun *et al.*, 2009). Hyperaccumulators are used in various processes, namely: phytoremediation, phytoextraction, phytofiltration, phytovolatilization and phytostabilization (Leguizamo *et al.*, 2017). For a proper functioning of the wetland system, there must be a relationship between macrophytes and microorganisms responsible for the degradation of pollutants.



Figure 6: Blackjack plants (*Bidens pilosa* L) used in the study for accumulation of sulfate (Sun *et al.*, 2009).

1.3.10 Macrophytes and bacterial interaction in sulfate removal from wastewater

Slow leaching of sulfate and metal ions from soil and rocks leads to natural occurrence of metal ions at low levels in aquatic systems. However, these metals have no effect on aquatic biota. It should also be stated that excessive metal ions in water resources are due to industrial, agricultural and municipal waste, just as sulfate degradation assists in the removal of excessive sulfate and metals from water. In another words, degradation of sulfate in water is influenced by several factors including pH, temperature, redox potential, metal carbonates and plant-microbe interaction. Sulfate removal processes are associated with iron oxidation in mine waters, where by sulfate-reducing bacteria reduce sulfate to sulfides, thereby lowering the pH, which is required by microbial cells for adsorption of metal ions.

Adsorption of toxic metals (such as zinc, nickel, copper etc.) and sulfate by macrophytes is enhanced by association with the bacterium (sulfate-reducing bacterium) e.g. *Desulfovibrio vulgaris*. Mycorrhizae (the role of the microbes in the plants' rhizosphere) also forms association with the endophytes of aquatic plants. This enhances nutrient uptake in plants especially phosphorus. Mycorrhizal associations protect plants from toxic pollutants (sulfates and heavy metals).

Furthermore, plants are involved in the input of oxygen into the root zone, uptake of nutrients and degradation of sulfate and toxic metals. The rhizosphere of the plants in the wetland consist of endorhizosphere and exorhizosphere (Stottmeister *et al.*, 2003). They meet in a zone referred to as a rhizoplane, where microorganisms are expected to interact with the plant. This is the most active region of the plant where biochemical and biological processes for wastewater treatment occur. Once microorganisms are established on aquatic plant roots, they form symbiotic relationships. This relationship results in an increase in the degradation

rate of removal of sulfate and heavy metals from wastewater surrounding the plant root system. Degradation of sulfate from industrial wastewater is influenced by various factors such as pH, temperature, hydrogen sulfide, sulfate concentration, retention time and hydrous oxides.

1.3.11 Factors affecting sulfate removal from industrial wastewater

Mechanisms of sulfate removal by soil retention are complex. They include coordination of hydrous oxide, exchange on the edges of silicate clays, incorporation in mineral structure and molecular adsorption. Some of the factors that may affect sulfate removal include the nature of clay soil minerals, dissolved oxygen, chemical oxygen demand (COD), pH, sulfate concentration, temperature and retention time.

➤ Potential of hydrogen and hydrogen sulfide (H₂S)

Adsorption of sulfate in soil systems is favoured by strongly acidic conditions. At pH values above six, it becomes almost insignificant. Sulfate-reducing bacteria degrades sulfate perfectly in the environment with pH ranging between 6 and 8. These bacteria are the commonly known acidophilic bacteria with an ability to withstand low pH levels, with optimum pH of 0.7 (Koschorreck, 2008). Free sulfide reacts with metal ions, functional groups, metabolic coenzymes and amino acids; thus may be toxic to all bacteria (Sánchez-Andrea *et al.*, 2014). Hydrogen sulfide may have a negative impact on bacteria through precipitation of essential trace elements within wastewater. At low pH levels sulfate-reducing bacteria produce hydrogen sulfide from degradation of sulfate, the process which has an ability to inhibit or reduce performance of these organisms.

➤ Sulfate concentration and temperature

The amount of sulfate adsorbed is dependent on concentration and the ambient temperature. As adsorbed sulfate is in kinetic equilibrium with sulfate in solution; temperature has a relatively small effect on sulfate adsorption by soils. Specifically, microbial community of sulfate-reducing bacteria decreases with the decrease in temperature of wastewater. This leads to a decrease in sulfate-reducing bacteria since most of them are thermophilic (Koschorreck, 2008). Oxygen transfer in the roots of macrophytes in wetlands enhances the degradation of sulfate and other organic matter. At low temperatures, wetlands perform poorly due to low metabolic rates. A good example is the destabilization of macromolecules within the roots of the macrophytes such as protein denaturation.

➤ Cat-ions and hydrous oxides

Hydrous oxides of Al and Fe have tendencies to retain sulfate. These compounds are probably responsible for most of sulfate adsorption in many areas contaminated with sulfate and heavy metals. The amount of sulfate retained is affected by the associated cat-ions of the salt or by the exchangeable cat-ions (Lopes, 2007). This effect follows the lyotropic (forms liquid crystal during addition of solvents) series like: H^+ , Sr^{2+} , Ba^{2+} , Ca^{2+} , Mg^{2+} , Rb^+ , K^+ , NH_4^+ , Na^+ , and Li^+ . Both sulfate and the cat-ion from a salt may be retained but persistence of adsorption of anion and cat-ion tends to differ.

➤ Hydraulic retention time

Hydraulic retention time is the measure of the average length of time wastewater spends in the water tank. Infiltration rate of industrial wastewater circulating within the system contributes to the decrease in sulfate concentration with an increase in retention time.

Maintaining hydraulic retention time improves treatment performance. That suggests that if the retention time is too short, some functions of microbes may not be supported, but with long contact time, that may increase the chances of finding positive results (Smith *et al.*, 2014). Sulfate retention therefore increases with the longer hydraulic retention time with the adsorbing substances.

➤ Chemical oxygen demand (COD) and dissolved oxygen (DO)

The level of dissolved oxygen is an essential parameter in wetlands for the evaluation of activities of sulfate removal. Sulfate-reducing bacteria (SRB) are anaerobic bacteria that degrade sulfate in wetlands. Excessive amounts of hydrogen sulfide and oxygen inhibits the growth of these bacteria (Subtil *et al.*, 2012). However, it has been established that a significant amount of oxygen is transported from the atmosphere to the shoots and into the rhizosphere of the macrophytes during photosynthesis (Kjeldsen *et al.*, 2017). Basically, chemical oxygen demand (COD) is another factor that affects sulfate removal. COD is the amount of oxygen that is used in microbial degradation processes of sulfate removal. The competition for substrates between SRB and other anaerobic bacteria depends on the ratio of sulfate and COD concentration in wastewater (Barber and Stucky, 2000).

1.4 Conclusion

It has been established that sulfate contaminated water leads to serious implications such as life threatening diseases, death of livestock and infants when consumed in high concentrations. It also causes environmental related complications. These complications validate how essential the removal of sulfate from wastewater is. Hydroponics can be used as the suitable alternative for sulfate removal with low maintenance without the use of chemicals that are harmful to the environment. Furthermore, sulfate-reducing bacteria play

a major role in the degradation of sulfate. Sulfate-reducing bacteria can be widely used in the treatment of wastewater because of their advantages such as low processing cost. However, the presence of hydrogen sulfide affects the performance of these microorganisms. The increase in the retention results to the reduction of sulfate to the acceptable levels.

1.5 References

Adebisi, S.A. and Fayemiwo, K.A. (2011). Physicochemical properties of industrial effluents in Ibadan, Nigeria. *Electronic Journal of Environmental and Agricultural and Food Chemistry* **10**: 2026-2031.

Ahmadpour, P., Ahmadpour, F., Sadeghi, S., Tayefeh, F.H., Soleimani, M. and Abdu, A.B. (2014). Evaluation of four plant species for phytoremediation of copper-contaminated soil. *Soil Remediation and Plants: Prospects and Challenges* **1**: 147.

Al-Abed, S.R., Pinto, P.X., McKernan, J., Feld-Cook, E. and Lomnicki, S.M. (2017). Mechanisms and effectivity of sulfate-reducing bioreactors using a chitinous substrate in treating mining influenced water. *Chemical Engineering Journal* **323**: 270-277.

Azizullah, A., Khattak, M.N.K., Richter, P. and Häder, D.P. (2011). Water pollution in Pakistan and its impact on public health—a review. *Environment International* **37**(2): 479-497.

Barber, W.P. and Stuckey, D.C. (2000). Effect of sulfate removal on chemical oxygen demand removal in an anaerobic baffled reactor. *Water Environment Research* **72**(5): 593-601.

Barton, L.L. and Tomei, F.A. (2002). Characteristics and activities of sulfate-reducing bacteria. *Sulfate-Reducing Bacteria*: 1-32.

Bowell, R.J. (2004). A review of sulfate removal options for mine waters. *Proceedings of Mine Water*: 75-88.

Brix, H. (1997). Do macrophytes play a role in constructed treatment wetlands? *Water Science and Technology* **35**(5): 11-17.

Chen, Y., Wen, Y., Zhou, Q., Huang, J., Vymazal, J. and Kuschik, P. (2016). Sulfate removal and sulfur transformation in constructed wetlands: The roles of filling material and plant biomass. *Water Research* **102**: 572-581.

Driscoll, C.T., Han, Y.J., Chen, C.Y., Evers, D.C., Lambert, K.F., Holsen, T.M., Kamman, N.C. and Munson, R.K. (2007). Mercury contamination in forest and freshwater ecosystems in the northeastern United States. *BioScience* **57**(1): 17-28.

Ebenstein, A. (2012). The consequences of industrialization: evidence from water pollution and digestive cancers in China. *Review of Economics and Statistics* **94**(1): 186-201.

Fu, F. and Wang, Q. (2011). Removal of heavy metal ions from wastewaters: a review. *Journal of Environmental Management* **92**(3): 407-418.

Garrett, T.R., Bhakoo, M. and Zhang, Z. (2008). Bacterial adhesion and biofilms on surfaces. *Progress in Natural Science* **18**(9): 1049-1056.

Hao, O.J., Chen, J.M., Huang, L. and Buglass, R.L. (1996). Sulfate-reducing bacteria. *Critical Reviews in Environmental Science and Technology* **26**(2): 155-187.

Huang, Y., Miyauchi, K., Inoue, C. and Endo, G. (2016). Development of suitable hydroponics system for phytoremediation of arsenic-contaminated water using an arsenic hyperaccumulator plant *Pteris vittata*. *Bioscience, Biotechnology, and Biochemistry* **80**(3): 614-618.

Kaksonen, A.H. and Puhakka, J.A. (2004). Sulfate removal based bioprocesses for the treatment of acid mine drainage and the recovery of metals. *Engineering in Life Sciences* **7**(6): 541-564.

Khan, S., Ahmad, I., Shah, M.T., Rehman, S. and Khaliq, A., 2009. Use of constructed wetland for the removal of heavy metals from industrial wastewater. *Journal of environmental management* **90**(11): 3451-3457.

Kjeldsen, K.U., Joulain, C. and Ingvorsen, K. (2004). Oxygen tolerance of sulfate-reducing bacteria in activated sludge. *Environmental science and Technology* **38**(7): 2038-2043.

Koch, J., Wimmer, F. and Schaldach, R. (2018). Analyzing the relationship between urbanization, food supply and demand, and irrigation requirements in Jordan. *Science of the Total Environment* **636**: 1500-1509.

Kopriva, S., Mugford, S.G., Baraniecka, P., Lee, B.R., Matthewman, C.A. and Koprivova, A. (2012). Control of sulfur partitioning between primary and secondary metabolism in Arabidopsis. *Frontiers in Plant Science* **3**: 163.

Koschorreck, M. (2008). Microbial sulphate reduction at a low pH. *Federation of European Microbiology Societies for Microbiology Ecology* **64**(3): 329-342.

Kulkarni, P., Olson, N.D., Paulson, J.N., Pop, M., Maddox, C., Claye, E., Goldstein, R.E.R., Sharma, M., Gibbs, S.G., Mongodin, E.F. and Sapkota, A.R. (2018). Conventional wastewater treatment and reuse site practices modify bacterial community structure but do not eliminate some opportunistic pathogens in reclaimed water. *Science of the Total Environment* **639**: 1126-1137.

Leguizamo, M.A.O., Gómez, W.D.F. and Sarmiento, M.C.G. (2017). Native herbaceous plant species with potential use in phytoremediation of heavy metals, spotlight on wetlands—a review. *Chemosphere* **168**: 1230-1247.

Leustek, T. and Saito, K. (1999). Sulfate transport and assimilation in plants. *Plant Physiology* **120**(3): 637-644.

Lewis, A.E., 2010. Review of metal sulphide precipitation. *Hydrometallurgy* **104**(2): 222-234.

Liu, L., Ma, Q., Lin, L., Tang, Y., Wang, J., Lv, X., Liao, M.A., Xia, H., Chen, S., Li, J. and Wang, X. (2017). Effects of exogenous abscisic acid on cadmium accumulation in two ecotypes of hyperaccumulator *Bidens pilosa*. *Environmental Progress and Sustainable Energy* **00**(10): 1002.

Liu, J., Wang, Y., Yu, Z., Cao, X., Tian, L., Sun, S. and Wu, P. (2017). A comprehensive analysis of blue water scarcity from the production, consumption, and water transfer perspectives. *Ecological Indicators* **72**: 870-880.

- Liu, W., Yang, H., Liu, Y., Kummu, M., Hoekstra, A.Y., Liu, J. and Schulin, R. (2018). Water resources conservation and nitrogen pollution reduction under global food trade and agricultural intensification. *Science of the Total Environment* **633**: 1591-1601.
- Lopes C. (2007). Sulfate removal at low pH in organic wastewaters. *Socio-Economic Environmental and National Sciences of the Environment*.
- McIntyre, T. (2003). Phytoremediation of heavy metals from soils. *In Phytoremediation*: 97-123.
- Miao, Z., Carroll, K.C. and Brusseau, M.L. (2013). Characterization and quantification of groundwater sulfate sources at a mining site in an arid climate: The Monument Valley site in Arizona, USA. *Journal of Hydrology* **504**: 207-215.
- Minh, L.Q., Tuong, T.P., van Mensvoort, M.E.F. and Bouma, J. (1997). Contamination of surface water as affected by land use in acid sulfate soils in the Mekong River Delta, Vietnam. *Agriculture, Ecosystems and Environment* **61**(1): 19-27.
- Ndulini, S.F., Sithole, G.M. and Mthembu, M.S. (2018). Investigation of nutrients and faecal coliforms removal in wastewater using a hydroponic system. *Physics and Chemistry of the Earth, Parts A/B/C*.
- Ntuli, F., Falayi, T. and Thwanane, U. (2016). Removal of sulphates from acid mine drainage using desilicated fly ash slag. *Transactions on Ecology and the Environment* **202**: 383-390.
- O'Sullivan, A.D., McCabe, O.M., Murray, D.A. and Otte, M.L. (1999). Wetlands for rehabilitation of metal mine wastes. In *Biology and Environment: Proceedings of the Royal Irish Academy*: 11-17.
- Pastor, J., Dewey, B., Johnson, N.W., Swain, E.B., Monson, P., Peters, E.B. and Myrbo, A. (2017). Effects of sulfate and sulfide on the life cycle of *Zizania palustris* in hydroponic and mesocosm experiments. *Ecological Applications* **27**(1): 321-336.
- Qian, J., Wei, L., Liu, R., Jiang, F., Hao, X. and Chen, G.H. (2016). An exploratory study on the pathways of Cr (VI) reduction in sulfate-reducing up-flow anaerobic sludge bed (UASB) reactor. *Scientific Reports* **6**: 23694.

Ramla, B. and Sheridan, C. (2015). The potential utilisation of indigenous South African grasses for acid mine drainage remediation. *Water South Africa* **41**(2): 247-252.

Raper, E., Stephenson, T., Anderson, D.R., Fisher, R. and Soares, A. (2018). Industrial wastewater treatment through bioaugmentation. *Process Safety and Environmental Protection* **41**: 16-20.

Rene, E.R., Sahinkaya, E., Lewis, A. and Lens, P.N. (2017). Sustainable Heavy Metal Remediation. *Principles and Processes* **8**: 19.

Saeed, T., Muntaha, S., Rashid, M., Sun, G. and Hasnat, A. (2018). Industrial wastewater treatment in constructed wetlands packed with construction materials and agricultural by-products. *Journal of Cleaner Production* **189**: 442-453.

Sánchez-Andrea, I., Sanz, J.L., Bijmans, M.F. and Stams, A.J. (2014). Sulfate removal at low pH to remediate acid mine drainage. *Journal of Hazardous Materials* **269**: 98-109.

Saha, P., Shinde, O. and Sarkar, S. (2017). Phytoremediation of industrial mines wastewater using water hyacinth. *International Journal of Phytoremediation* **19**(1): 87-96.

Shakir, E., Zahraw, Z. and Al-Obaidy, A.H.M. (2017). Environmental and health risks associated with reuse of wastewater for irrigation. *Egyptian Journal of Petroleum* **26**(1): 95-102.

Sheoran, A.S. and Sheoran, V. (2006). Heavy metal removal mechanism of acid mine drainage in wetlands: a critical review. *Minerals Engineering* **19**(2): 105-116.

Smith, R., Elger, S. and Mleziva, S. (2014). Wastewater: Solids retention time control in wastewater treatment. *Filtration and Separation* **51**(3): 12-17.

Stottmeister, U., Wießner, A., Kusch, P., Kappelmeyer, U., Kästner, M., Bederski, O., Müller, R.A. and Moormann, H. (2003). Effects of plants and microorganisms in constructed wetlands for wastewater treatment. *Biotechnology Advances* **22**(1): 93-117.

Subtil, E.L., Cassini, S.T.A. and Gonçalves, R.F. (2012). Sulfate and dissolved sulfide variation under low COD/Sulfate ratio in Up-flow Anaerobic Sludge Blanket (UASB) treating domestic wastewater. *Revista Ambiente and Água* **7**(1): 130-139.

- Sun, Y., Zhou, Q., Wang, L. and Liu, W. (2009). Cadmium tolerance and accumulation characteristics of *Bidens pilosa* L. as a potential Cd-hyperaccumulator. *Journal of Hazardous Materials* **161**(2): 808-814.
- Van den Brand, T.P., Roest, K., Chen, G.H., Brdjanovic, D. and van Loosdrecht, M.C.M. (2015). Potential for beneficial application of sulfate-reducing bacteria in sulfate-containing domestic wastewater treatment. *World Journal of Microbiology and Biotechnology* **31**(11): 1675-1681.
- Vymazal, J. (2005). Horizontal sub-surface flow and hybrid constructed wetlands systems for wastewater treatment. *Ecological Engineering* **25**(5): 478-490.
- Wang, M., Zhang, D.Q., Dong, J.W. and Tan, S.K. (2017). Constructed wetlands for wastewater treatment in cold climate—A review. *Journal of Environmental Sciences*.
- Xydis, G.A., Liaros, S. and Botsis, K. (2017). Energy demand analysis via small scale hydroponic systems in suburban areas—An integrated energy-food nexus solution. *Science of the Total Environment* **593**: 610-617.
- Zhang, M. and Wang, H. (2014). Organic wastes as carbon sources to promote sulfate-reducing bacterial activity for biological remediation of acid mine drainage. *Minerals Engineering* **69**: 81-90.
- Zhao, C., Xie, H., Mu, Y., Xu, X., Zhang, J., Liu, C., Liang, S., Ngo, H.H., Guo, W., Xu, J. and Wang, Q. (2014). Bioremediation of endosulfan in laboratory-scale constructed wetlands: effect of bioaugmentation and biostimulation. *Environmental Science and Pollution Research* **21**(22): 12827-12835.

CHAPTER 2: SULFATE REMOVAL AND THE ROLE OF MACROPHYTES IN SULFATE REMOVAL FROM INDUSTRIAL WASTEWATER IN A HYDROPONIC SYSTEM

2.1 Introduction

Nowadays, sulfate has become a major problem as an industrial wastewater pollutant, and has received much attention in various wastewater research (Ntuli *et al.*, 2016). The discharge of inadequately treated industrial wastewater usually results in the contamination of water bodies by sulfate. High sulfate concentrations in the water bodies lead to various environmental problems such as water mineralization, release of hydrogen sulfide to the atmosphere, and disruption of the food chain and the natural sulfur cycle. The oxidation of sulfate in the atmosphere contributes to acid rain through volatilization of the reduced products. Acid rain negatively affects aquatic species and poison fish (Basiglini *et al.*, 2018). Furthermore, sulfate attack is another negative implication of high concentrations of sulfate in the environment which leads to sulfate infiltration and accumulation in ground water, and acidic ground water promotes leaching of heavy metals.

However, it is documented that sulfate attack leads to the formation of corrosive products with an ability to induce cracking of structures in industries and wastewater pipeline (Zhang *et al.*, 2017). In addition, human consumption of sulfate at high concentration results in laxative effects. According to the World Health Organization (2011), sulfate concentration greater than 250 mg/l can lead to diarrhoea and dehydration (Mohammadi *et al.*, 2018). The implications of sulfate accumulation necessitate the removal of sulfate from industrial wastewater.

The necessity of wastewater treatment to remove sulfate from wastewater is not only for increasing accessibility to clean water for human consumption and water reuse; but also to

improve water quality and preserve the environment and human health. Sulfate removal from wastewater is also essential for the safe disposal of wastewater to the environment after treatment, thus complying with the disposal regulations (Salgot and Folch, 2018). Moreover, water availability is not only essential for human consumption but is also required for food production in agriculture since the world population has doubled between 1970 and 2015, resulting in the high demand of food supply (Koch *et al.*, 2018).

Population growth puts pressure on agriculture, leading to the high demand of food production (Mateo-Sagasta *et al.*, 2017). Therefore, the removal of sulfate from wastewater may also allow water reuse for agricultural purposes. Traditional wastewater treatment methods that are currently used for sulfate removal combine physical and biological treatment of industrial wastewater. Physical treatment includes membrane filtration, irradiation, coagulation and ion-exchange. Biological treatment includes decolourization by microbial cultures. These methods are used to remediate contaminated water in order to preserve the environment and human health.

The traditional wastewater treatment techniques mentioned above have drawbacks such as expensive operational and maintenance costs, and the use of the chemicals to treat wastewater. This poses a threat to the ecosystem because some of the chemicals and dyes that are used in the chemical precipitation of sulfate from water are harmful to the environment, and are carcinogenic to human beings (Kulkarni *et al.*, 2018). The composition of chemicals within the dyes may lead to the accumulation of these chemicals in aquatic edible animals and endanger the ecosystem. The drawbacks of these traditional methods necessitate the development of environmentally friendly and cheap methods such as constructed wetlands.

A hydroponic system is a wetland system that uses microorganisms, substrate and macrophytes to remove sulfate from wastewater. Sulfate removal mechanisms are a combination of biochemical transformation and adsorption that makes use of the physical, chemical and biological processes to degrade sulfate (Riggio *et al.*, 2018). These processes include chemical precipitation, adsorption, microbial degradation and plant uptake. Pollutants assimilation by plants roots have been reported to be the most effective method of pollutants removal, termed phytoremediation (Fernando *et al.*, 2018).

In addition, sulfate assimilation pathway is activated by the reduction of *ATP sulfurylase (ATPS)* to *5' adenosine 5 phosphosulfate (APS)*. The assimilation of sulfate can be further processed through the reduction of APS to sulfide by the enzyme *APS reductase* or the phosphorylation of APS to *3' phosphoadenosine 5'-phosphosulfate (PAPS)*. This is the branching point of sulfate assimilation (Koprivova *et al.*, 2014). The reduction of sulfite to sulfide is catalysed by *sulfide reductase*, which is followed by the synthesis of amino acid cysteine from the amino acid skeleton (*O-acetylserine*). Cysteine is further oxidized to glutathione. This amino acid serves as a reduced sulfur for all metabolites, and *PAPS* serves as a donor for activated sulfate for sulfation (which is defined as the conversion of peptide molecules into sulfate) (Leustek and Saito, 1999). The pathway of sulfate assimilation is regulated by the demand of sulfur, availability of sulfur within plants, environmental factors (such as carbon availability) and phytohormones. However, sulfate transporters are grouped according to their affinity in sulfate translocation. Group 1 transporters, with high affinity are responsible for the assimilation of sulfate from the soil by the roots. Group 2 (located in the xylem parenchyma and phloem cells and has low affinity) is responsible for the translocation of sulfate within the leaves. Group 4 is responsible for sulfate efflux from the vacuole. Group 3 and 5 increase root to shoot sulfate translocation (Kopriva, 2006). These sulfate transporters

play a major role in maintaining and regulating sulfate transportation within the macrophytes that are used in wetlands.

The hydroponic system's macrophytes (*Bidens pilosa L*) have the ability to assimilate sulfate via the roots while sulfate-reducing bacteria undergo microbial degradation of sulfate to hydrogen sulfide. Sulfate-reducing bacteria also contribute to sulfate oxidation into hydrogen sulfide. This process is termed biosulfidogenesis and is coupled with metal precipitation and proton consuming reaction (Sahinkaya *et al.*, 2018). Sulfate removal by these mechanisms was established using the hydroponic system.

2.2 Aim, hypothesis and objectives

2.2.1 Aim

To remove sulfate from industrial wastewater using *Bidens pilosa L* as well as to establish the role of the macrophytes in sulfate removal from the hydroponic system.

2.2.2 Hypothesis

Sulfate concentration will be higher in the macrophytes harvested after treatment (in the hydroponic system) compared to the macrophytes harvested before treatment.

2.2.3 Objectives

- To access sulfate concentration in wastewater before and after treatment in a hydroponic system.
- To investigate sulfate concentration in macrophytes before and after exposure (treatment) to sulfate contaminated industrial effluents.

2.3 Methodology

To determine sulfate removal in a hydroponic system, as well as to establish the role of macrophytes in sulfate removal in wastewater of the hydroponic system, two hydroponic systems were constructed (planted and control section). The control section was left unplanted, and *Bidens pilosa* L was planted in the planted section and a few plants were harvested before treatment. Industrial wastewater collected from Tendele Coal Mine was introduced to both sections. *Bidens pilosa* L was harvested after 2 weeks of treatment. Sample collection and sulfate concentration measurement were conducted at different hydraulic retention times (after every 24 hours for 2 weeks) and sulfate removal was compared in the two systems. Sulfate concentration was also compared in macrophytes before and after treatment.

2.3.1 Hydroponic system construction

Water tank and planter box made of fine glass was used for the construction of the hydroponic system, and for the removal of sulfate from industrial wastewater. The planter box was placed on top of the four-legged steel stand. The planter box had 32 mm predrilled-hole which was used for the insertion of 25 mm stand pipe in order to allow water from the planter box back to the water tank. A stand pipe was secured with siphon. A large gravel pipe was placed over the siphon pipe in order to avoid blockage of channels for water intake by the growth medium (Figure 7).

The sand and gravel were used as growth media. They served as filtration bed for microorganisms and nutrients during wastewater circulation through the system. The base of the planter box was filled with sand surrounding the siphon pipe. The sand was covered with gravel until it reached the top of the planter box. The circulation of wastewater was

accomplished with the use of submersible electric pump with the flow rate of 1400 litres per hour, which was plugged to the nearest power socket and immersed into the water tank.

2.3.2 Macrophytes cultivation and wastewater collection

Bidens pilosa L seeds were collected from the Department of Agriculture (University of Zululand) and were taken to Vulindlela Wastewater Treatment Plant and planted in the planter box. The second hydroponic system (the control system) had no plants cultivated in its planter box. This served as a control, in comparing the planted and unplanted section. The water tank in the hydroponic pond system was filled with 75 l of wastewater from the clarifier tank. Since sewage water consisted of harmful contaminants, protective clothing (gloves, lab coat and closed shoes) were worn when collecting water samples from the clarifier tank. Sewage wastewater was allowed to circulate within the system for one week. This was done in order to facilitate the rapid growth of the macrophytes within the hydroponic system through provision of nutrients they required for growth from the sewage wastewater. Furthermore, a shelter was built around the two hydroponic systems in order to eliminate interferences that may have affected this project such as rain and consumption of *Bidens pilosa L* by nearby herbivorous animals. Industrial wastewater was collected from Tendele Coal Mine Mtubatuba, and transported to the Vulindlela Wastewater Treatment Plant. The wastewater was filled into water tanks of the two systems and allowed to circulate within the systems for 2 weeks.



Figure 7: A constructed hydroponic system that was used in the study for removal of sulfate from industrial wastewater (Ndulini *et al.*, 2018).

2.3.3 Sample collection

The initial samples were collected using 500 ml Schott bottle right after filling the tanks with wastewater and labelled 0 hours. The same procedure was carried out every 24 hours for two weeks, and labelled as such. All samples were collected using sterile 500 ml Schott bottles according to the Standard Water sampling procedures. For each sample collected, a pH meter was used to measure pH and temperature of water on site and the samples were stored on ice and taken to University of Zululand Microbiology Laboratory for further analysis. Industrial wastewater is composed of toxic substances such as heavy metals and sulfate. The protective clothing (closed shoes, a lab coat and gloves) were worn when collecting water samples.

2.3.4 Determination of sulfate concentration in wastewater

For each sample, one ml of the water sample was pipetted into the reaction cell and mixed. One level of green micro spoon of reagent $\text{SO}_4\text{-1 K}$ was added into the reaction cell and the cell was tightly closed. The cell was vigorously shaken until the reagent was completely dissolved. After two minutes (reaction time), the cell was placed into the cell compartment and the mark on the cell was aligned with the one on the spectrophotometer in order to read the concentration of sulfate. The results were recorded for all the samples.

2.3.5 Harvesting the macrophytes and sample preparation

Bidens pilosa L was harvested before and after treatment, washed (to remove soil and dust deposits), oven-dried for two days, and ground into powder (Ahmadpour *et al.*, 2014). One gram of *Bidens pilosa L* powder for both before and after the exposure to sulfate contaminated water (treatment) was mixed with 5 ml distilled water in two separate test tubes. The mixtures in the test tubes were boiled in the glass beaker with water boiling at 40°C for 5 minutes in order to extract sulfate from the plants into the water. The mixture was sieved and used for the measurement of sulfate.

2.3.6 Determination of sulfate concentration in plants

For each sample, one ml of the plant extract was pipetted into the reaction cell and mixed. One level of green micro spoon of reagent $\text{SO}_4\text{-1 K}$ was added into the reaction cell and the cell was tightly closed. The cell was vigorously shaken until the reagent was completely dissolved. After two minutes, the cell was placed into the cell compartment and the mark on the cell was aligned with the one on the spectrophotometer in order to read the

concentration of sulfate. The results for sulfate in plants before and after treatment were recorded.

2.3.7 Statistical data analysis

SPSS-Paired sample t -test is a data analysis method used to determine the statistical difference between two measurements or time points. It uses H_0 (null hypothesis) which states there is no difference if $p < 0.05$. Then H_0 is rejected. But if $p > 0.05$, H_0 is accepted. The other hypothesis that is used by this test is the H_i (alternative hypothesis) which states that there is significant difference at a default significant level of 5% or 0.05. Paired sample t -test was used to analyze data in order to compare sulfate concentration within the plants harvested before and after exposure to sulfate contaminated water (treatment) and sulfate removal in the control and planted sections.

2.4 Results and discussion

The results for sulfate removal in a system are presented in Figure 8. Figure 9 shows different sulfate concentration assimilated by *Bidens pilosa* L before and after treatment (before and after exposure to sulfate contaminated water). Tables 1 and 2 show the decreasing sulfate concentrations in the planted and control sections with the increase in retention time of water circulation.

2.4.1 Performance of the hydroponic system

The hydroponic system was operated and checked every 24 hours in order to avoid technical errors like power failure. This was also done to ensure that wastewater circulating within the systems was enough to prevent the burst of electric pump. Cultivated hyperaccumulators

(*Bidens pilosa* L) were not negatively affected by toxic components of wastewater since they did not show any symptoms of growth inhibition.

2.4.2 Sulfate removal in the hydroponic system and the mechanisms of removal

Figure 8 shows sulfate removal efficiency over retention time (in hours) in the control and planted sections. Sulfate removal was increasing with the increasing hydraulic retention time both in the control and in the planted sections. There was a rapid increase of sulfate removal in the control section after 120 hours but it was increasing faster in the planted section compared to the control section. Sulfate removal in the control section was due to microbial degradation of sulfate by microorganisms that utilized sulfate. The rapid increase of sulfate removal in the planted section was after 96 hours. The plant uptake mechanism by *Bidens pilosa* L and microbial degradation of sulfate led to the rapid increase of sulfate removal in the planted section. According to Zhao *et al.* (2014) plant cells activity increased as the plants grew, leading to the increase in sulfate uptake by *Bidens pilosa* L. This is confirmed by Liu *et al.* (2017) who found that *Bidens pilosa* L had a potential to accumulate pollutants. The results of sulfate removal (Figure 8) proved that the aim of the study (which was to establish the effect of *Bidens pilosa* L in sulfate removal from industrial wastewater) was achieved. *Bidens pilosa* L positively influenced sulfate removal from wastewater. The highest removal efficiency was obtained after 288 hours in both systems (76% in the planted section) and (56% in the control section).

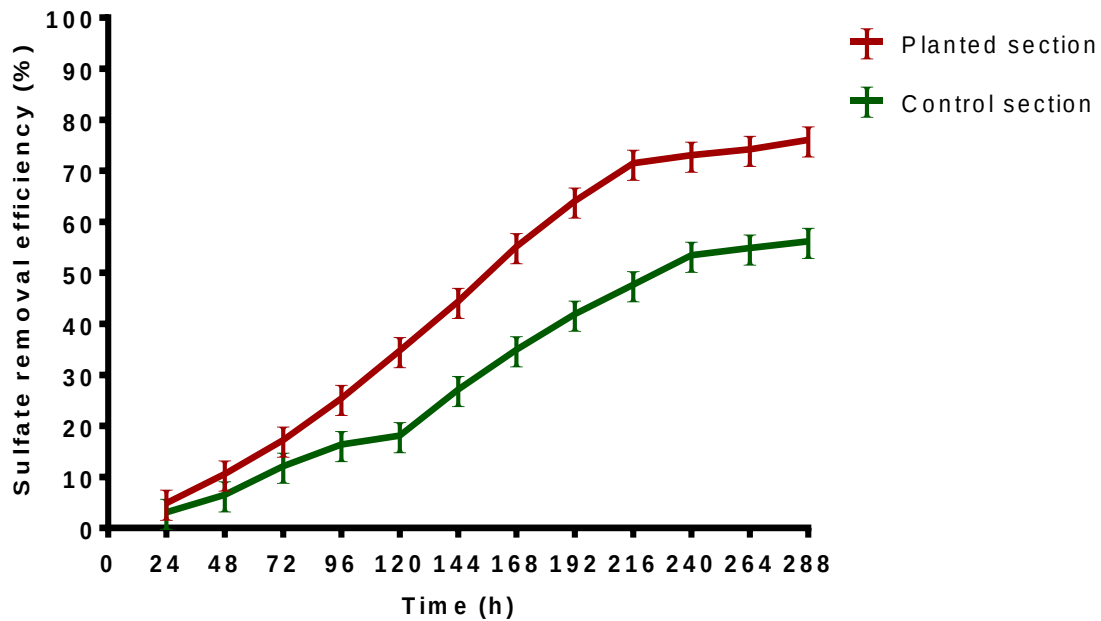


Figure 8: Sulfate removal efficiency (%) in the control and planted section over sampling periods.

After 288 hours, sulfate removal would be expected to stabilize due to the age of plants. It would also be expected that sulfate would be recycled back into water from plants and assimilated by plants again leading to the eventual wave of the sulfate removal. Similar findings were reported by (Scholz and Lee, 2005) who demonstrated that the results of sulfate removals were higher in planted section compared to the control section. The high removal percentages of sulfate in the planted section of the study that was conducted by (Scholz and Lee, 2005) was due to degradation of sulfate by microorganisms and macrophytes, which assisted in the reduction of sulfate from industrial wastewater through sulfate accumulation via the roots. Although other factors might have contributed in sulfate removal, Zhao *et al.* (2014) has reported that vegetation played a major role in global recycling of persistent organic pollutants and their uptake from the environment into plant roots is a significant pathway. The results in Figure 8 support the findings by Zhao *et al.* (2014).

The results in Figure 8 support the fact that substrate (gravel and sand) and microorganisms all played a role in sulfate removal in both systems. The substrate served as a filter bed for sulfate salts within water since there was a removal of sulfate in the control section regardless of the absence of macrophytes but *Bidens pilosa* L also played a significant role in sulfate removal in the planted section.

In addition, sulfate removal in the hydroponic system after 240 hours seemed to reach a stationary phase (Figure 8). Aging of *Bidens pilosa* L macrophytes may have attributed to the static motion of sulfate assimilation. According to Kowalska, (2005), sulfate assimilation is dependent on the stage of plant growth. Young leaves have the most active sulfate assimilative reduction into organic compounds, the most intense sulfate assimilation is at the stage of maximal leaf growth. In the aging plants, organic sulfur decreases in leaves with the decrease in non-organic compounds which leads to the weakening of sulfate degradation processes and sulfate translocation to generative organs (Buchner *et al.*, 2004). The competition between plants and microorganisms for sulfate might have led to the depletion of sulfate in water, which might also have been the reasonable explanation of the static mode of sulfate removal after 240 hours.

However, contradictory results were reported by Shamshad *et al.* (2016) where the steady state in sulfate removal was reached within 7 days due to acidic pH of wastewater resulting from high concentrations of sulfate. Acidic environment may have led to the inhibition of microalgae that was used in Shamshad's *et al.* (2016) study since microalgae prefers environments with neutral or weakly alkaline pH. This proves that failure to choose macrophytes of choice that are able to withstand harsh or unfavourable conditions yields negative results. However, *Bidens pilosa* L used in this study was able to withstand harsh

condition. The macrophytes of choice removed sulfate without showing any symptoms of growth inhibition.

Table 1: Sulfate concentrations in wastewater over retention time (control section).

Time (h)	pH	Temperature (°C)	Sulfate concentration (mg/l)
0	5.03	22.0	705
24	5.35	21.7	684
48	6.15	23.6	659
72	6.17	24.1	620
96	6.22	26.2	590
120	6.34	25.1	577
144	6.1	23.4	515
168	6.6	22.9	460
192	6.9	22	409
216	7.4	23.1	370
240	6.5	24.6	330
264	6.4	21	316
288	5.8	20.8	309

The significance or the ability of macrophytes (*Bidens pilosa L*) in the wetland system to remove sulfate is supported by the sulfate concentrations shown in Tables 1 and 2. Even though microorganisms may have degraded sulfate in both systems, the presence of *Bidens pilosa L* was the cause of high sulfate removal in the planted section compared to the control section, since the final concentration in the planted section was 169 mg/l (<250 mg/l, the acceptable sulfate concentration in water) while in the control section it was 309 mg/l, which was way higher than 250 mg/l. This indicated that microorganisms played a role in sulfate degradation in the control section, but it was not as effective as in the planted section due to

the presence of *Bidens pilosa* L. Positive results in the study by Shamshad *et al.* (2016) were only achieved in the bioreactor with consortium of bacteria containing cyanobacteria and algae, until pH dropped to 4.4 and the steady stage was reached again. Shamshad *et al.* (2016) results proved that pH below 5 is unfavourable to the sulfate-degrading microorganisms.

Table 2: Sulfate concentrations in wastewater over retention time (planted section).

Time (h)	pH	Temperature (°C)	Sulfate concentration (mg/l)
0	5.03	22.0	705
24	6.61	26	670
48	6.3	24.2	630
72	6.5	25	585
96	6.0	21	526
120	7.2	25	460
144	7.0	23.8	390
168	6.8	22.4	316
192	6.5	21.6	256
216	6.8	23	201
240	6.0	20.9	190
264	5.42	24.7	184
288	5.85	21.6	169

The paired *t*-test was carried out in order to compare removal efficiency over hydraulic retention time between the control and planted sections. (*P* value=0.0001), $p < 0.05$ and it was concluded that there was a significant difference between the removal of sulfate in the control and the planted section. Similar findings were reported by Kopriva *et al.* (2012).

2.4.2.1 Sulfate uptake by *Bidens pilosa* L

Figure 9 presents the results of sulfate concentration accumulated by *Bidens pilosa* L. Sulfate was present in all macrophytes but was in low concentrations in *Bidens pilosa* L harvested before treatment. Sulfate concentration in plants before treatment was 110 mg/l and this proved that sulfate was naturally present in the macrophytes in a form of sulfur (but not in excessive amounts) which was an essential element for plant growth. Sulfate concentrations increased in plants after treatment (exposure to sulfate contaminated water) and it was (353 mg/l). The increase in sulfate concentration in plants after treatment indicated that *Bidens pilosa* L had accumulated toxic sulfate, and assimilated this compound without any symptoms of stress. The high levels of sulfate present in *Bidens pilosa* L harvested after treatment was due to the assimilation of sulfate by the macrophytes via the roots. Kowalska (2005) stated that high content of sulfate in plants indicates intake of sulfate.

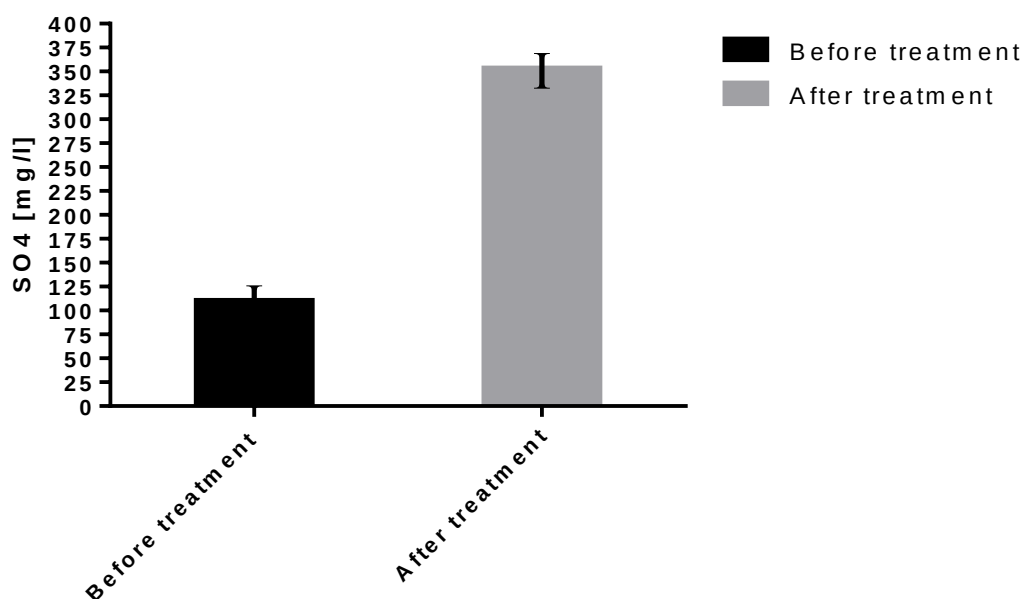


Figure 9: Sulfate concentration in *Bidens pilosa* L harvested before and after treatment.

According to Sun (2009), excessive sulfate concentration in the root zone indicates excessive uptake by the plants roots which eventually leads to the increase in the synthesis of glutathione. The increase in glutathione synthesis tends to signal the decrease of sulfate intake. The paired *t*-test was also carried out in order to establish if there was a significant difference between the concentrations of sulfate in samples harvested before and after treatment. Since $p < 0.05$ was less than the significant level, the null hypothesis was rejected, which means there was a significant difference between the concentrations of sulfate within plants that were harvested before and after treatment (Figure 9). Similar results were reported by Guittonny-Philippe *et al.* (2015). *Bidens pilosa* L and other macrophytes used in Guittonny's study were tolerant to toxic sulfate and metals that they were exposed to. The increased sulfate concentration in macrophytes after treatment proved that the introduction of macrophytes in a wetland system had a positive effect on sulfate removal from mine water as documented by Zhao *et al.* (2014).

2.5 Conclusion

Based on the obtained results, *Bidens pilosa* L showed a potential to remove sulfate. As much as sulfate-rich industrial wastewater was remediated in the control section, it was removed better in the planted section of the system. *Bidens pilosa* L had an ability to grow in contaminated water and withstood harsh conditions while accumulating sulfate via roots without the inhibition of growth. Sulfate was naturally present in *Bidens pilosa* L used in the study but in small quantities as it was required in plants as a secondary element (sulfur) for plant growth and the synthesis of amino acids. It was 110 mg/l before treatment but increased to 353 mg/l after treatment. This proved that *Bidens pilosa* L had the ability to take up sulfate with the prolonged hydraulic retention time. Thus, the hypothesis was accepted.

In the control section, there was sulfate removal in the absence of macrophytes but *Bidens pilosa* L seemed to be more effective in sulfate removal. The effectiveness of the presence of *Bidens pilosa* L was indicated by the reduction of sulfate concentration up to 169 mg/l, which was less than the acceptable levels of sulfate in water (250 mg/l) in the planted section. The final concentration of sulfate in wastewater treated in the control section was above the limits permissible limits by WHO. Therefore, it can be concluded that a hydroponic system cultivated with *Bidens pilosa* L can be used as an alternative technique to remove sulfate from industrial wastewater for safe disposal of sulfate contaminated water to the environment.

2.6 Recommendations

Introduction of various macrophytes is recommended for further studies. This is to compare their ability to remove sulfate from industrial wastewater. It is also recommended that the control section is provided with lactate as the nutrient for sulfate-reducing bacteria to serve as the positive control to compare sulfate removal by different macrophytes and SRB when provided with nutrients. Further investigation of sulfate removal mechanisms is also recommended. Pilot scale trials for small communities where conventional wastewater treatment might be costly is recommended.

2.7 References

Ahmadpour, P., Ahmadpour, F., Sadeghi, S., Tayefeh, F.H., Soleimani, M. and Abdu, A.B. (2014). Evaluation of four plant species for phytoremediation of copper-contaminated soil. *Soil Remediation and Plants: Prospects and Challenges* **1**: 147.

Basiglini, E., Pintore, M. and Forni, C. (2018). Effects of treated industrial wastewaters and temperatures on growth and enzymatic activities of duckweed (*Lemna minor* L.). *Ecotoxicology and Environmental Safety* **153**: 54-59.

Buchner, P., Takahashi, H. and Hawkesford, M.J. (2004). Plant sulphate transporters: co-ordination of uptake, intracellular and long-distance transport. *Journal of Experimental Botany* **55**(404): 1765-1773.

Fernando, W.A.M., Ilankoon, I.M.S.K., Syed, T.H. and Yellishetty, M. (2018). Challenges and opportunities in the removal of sulphate ions in contaminated mine water: A review. *Minerals Engineering* **117**: 74-90.

Guitttonny-Philippe, A., Petit, M.E., Masotti, V., Monnier, Y., Malleret, L., Coulomb, B., Combroux, I., Baumberger, T., Viglione, J. and Laffont-Schwob, I. (2015). Selection of wild macrophytes for use in constructed wetlands for phytoremediation of contaminant mixtures. *Journal of Environmental Management* **147**: 108-123.

http://www.who.int/about/licensing/copyright_form/en/index.html

Koch, J., Wimmer, F. and Schaldach, R. (2018). Analyzing the relationship between urbanization, food supply and demand, and irrigation requirements in Jordan. *Science of the Total Environment* **636**: 1500-1509.

Kopriva, S. (2006). Regulation of sulfate assimilation in Arabidopsis and beyond. *Annals of Botany* **97**(4): 479-495.

Kopriva, S., Mugford, S.G., Baraniecka, P., Lee, B.R., Matthewman, C.A. and Koprivova, A. (2012). Control of sulfur partitioning between primary and secondary metabolism in Arabidopsis. *Frontiers in Plant Science* **3**: 163.

Koprivova, A. and Kopriva, S. (2014). Molecular mechanisms of regulation of sulfate assimilation: first steps on a long road. *Frontiers in Plant Science* **5**: 589.

Kowalska, I. (2005). Effects of sulphate level in the nutrient solution on plant growth and sulphur content in tomato plants. *Folia Hort* **17**(1): 91-100.

Kulkarni, P., Olson, N.D., Paulson, J.N., Pop, M., Maddox, C., Claye, E., Goldstein, R.E.R., Sharma, M., Gibbs, S.G., Mongodin, E.F. and Sapkota, A.R. (2018). Conventional wastewater treatment and reuse site practices modify bacterial community structure but do not eliminate some opportunistic pathogens in reclaimed water. *Science of the Total Environment* **639**: 1126-1137.

Leustek, T. and Saito, K. (1999). Sulfate transport and assimilation in plants. *Plant Physiology* **120**(3): 637-644.

Liu, L., Ma, Q., Lin, L., Tang, Y., Wang, J., Lv, X., Liao, M.A., Xia, H., Chen, S., Li, J. and Wang, X. (2017). Effects of exogenous abscisic acid on cadmium accumulation in two ecotypes of hyperaccumulator *Bidens pilosa*. *Environmental Progress and Sustainable Energy* **00**(10): 1002.

Mateo-Sagasta, J., Zadeh, S.M., Turrall, H. and Burke, J. (2017). Water pollution from agriculture: a global review. Executive summary. Rome, Italy: FAO Colombo, Sri Lanka: *International Water Management Institute (IWMI). Consultative Group for International Agricultural Research (CGIAR): Research Program on Water, Land and Ecosystems (WLE)*.

Mohammadi, M., Mowla, D., Esmaeilzadeh, F. and Ghasemi, Y. (2018). Cultivation of microalgae in a power plant wastewater for sulfate removal and biomass production: A batch study. *Journal of Environmental Chemical Engineering* **6**(2): 2812-2820.

Ndulini, S.F., Sithole, G.M. and Mthembu, M.S. (2018). Investigation of nutrients and faecal coliforms removal in wastewater using a hydroponic system. *Physics and Chemistry of the Earth* **10**: 10.

Ntuli, F., Falayi, T. and Thwanane, U. (2016). Removal of sulphates from acid mine drainage using desilicated fly ash slag. *Transactions on Ecology and the Environment* **202**: 383-390.

- Riggio, V.A., Ruffino, B., Campo, G., Comino, E., Comoglio, C. and Zanetti, M. (2018). Constructed wetlands for the reuse of industrial wastewater: A case-study. *Journal of Cleaner Production* **171**: 723-732.
- Sahinkaya, E., Yurtsever, A., Isler, E., Coban, I. and Aktaş, Ö. (2018). Sulfate removal and filtration performances of an anaerobic membrane bioreactor (AnMBR). *Chemical Engineering Journal* **349**: 47-55.
- Salgot, M., and Folch, M. (2018). Wastewater treatment and reuse. *Current Opinion in Environmental Science and Health* **2**: 64-74.
- Scholz, M. and Lee, B.H. (2005). Constructed wetlands: a review. *International Journal of Environmental Studies* **62**(4): 421-447.
- Shamshad, I., Khan, S., Waqas, M., Asma, M., Nawab, J., Gul, N., Raiz, A. and Li, G. (2016). Heavy metal uptake capacity of fresh water algae (*Oedogonium westti*) from aqueous solution: A mesocosm research. *International Journal of Phytoremediation* **18**(4): 393-398.
- Sun, Y., Zhou, Q., Wang, L. and Liu, W. (2009). Cadmium tolerance and accumulation characteristics of *Bidens pilosa* L. as a potential Cd-hyperaccumulator. *Journal of Hazardous Materials* **161**(2): 808-814.
- Zhang, H.Q., Yang, Y.Y. and Yi, Y.C. (2017). Effect of sulfate erosion on strength and leaching characteristic of stabilized heavy metal contaminated red clay. *Transactions of Nonferrous Metals Society of China* **27**(3): 666-675.
- Zhao, C., Xie, H., Mu, Y., Xu, X., Zhang, J., Liu, C., Liang, S., Ngo, H.H., Guo, W., Xu, J. and Wang, Q. (2014). Bioremediation of endosulfan in laboratory-scale constructed wetlands: effect of bioaugmentation and biostimulation. *Environmental Science and Pollution Research* **21**(22): 12827-12835.

CHAPTER 3: PHYSICOCHEMICAL PARAMETERS AND THEIR EFFECT ON SULFATE REMOVAL FROM INDUSTRIAL WASTEWATER IN A HYDROPONIC SYSTEM

3.1 Introduction

Analyses of physical and chemical parameters play a major role in estimation of pollution load and source, and the damage caused by the introduction of pollutants into water (Patil *et al.*, 2012). Therefore, it is crucial to analyse the quality of water regularly for sustainable personal, domestic, industrial and agricultural uses, while at the same time reducing pollutants to acceptable levels, and thus meeting discharge regulations by authorities and environmental legislations. The determination of physical and chemical parameters can demonstrate the effectiveness of constructed wetlands (Fang *et al.*, 2018). Analysis of selected physicochemical parameters in a hydroponic system assists in determining the factors affecting the mechanisms of sulfate removal in sulfate-rich water-treatment technologies.

Sulfate adsorption associated with metal sulfides precipitation and microbial degradation of sulfate are pH and temperature dependent (Costabile *et al.*, 2011). Microbial degradation and metal sulfides precipitation by sulfate-reducing bacteria are favourable under acidic conditions. The amount of oxygen used in microbial degradation processes of sulfate removal (COD) decreases with sulfate removal in wastewater and the presence of dissolved oxygen in wetlands enhance sulfate removal. Several scientific reporters have demonstrated that sulfate adsorption increases with decreasing soil pH (Zhao *et al.*, 2009; Dakiky *et al.*, 2002; Lozano *et al.*, 2018).

Additionally, plant uptake mechanism is another sulfate removal mechanism that is dependent on pH since macrophytes may be sensitive to acidic environments of sulfate-rich water. Thus, pH determination in wetlands assists in the selection of the macrophytes to be

cultivated for treatment of sulfate-rich waters. In some cases, oxygen production by the plant roots may lead to the alteration of pH (Sadik *et al.*, 2015). In other wastewater treatment methods like chemical precipitation, pH and temperature can be manipulated to favour the mechanisms of sulfate removal. For instance, the addition of dyes and salts in sulfate-rich water with the intentions of adjusting pH (Al-Zuhair *et al.*, 2008). Some equipment used in sulfate removal from wastewater such as ion exchange are designed with physical parameters adjustments. This aids in adjusting pH and temperature whenever they are found not to be favourable for sulfate removal mechanisms. The pH may also be adjusted to prevent corrosion of pipeline in industries (Geldenhuys, 2003). The main objective of this chapter was to determine the effect of physical and chemical parameters on sulfate removal from wastewater in a hydroponic system.

3.2 Aim, hypothesis and objectives

3.2.1 Aim

The aim of this chapter was to determine the physicochemical parameters and their effect on the removal of sulfate from industrial wastewater.

3.2.2 Hypothesis

Physical parameters have an effect on sulfate removal and are dependent on hydraulic retention time. Physical parameters also affect the biological processes of sulfate removal by sulfate-reducing bacteria and *Bidens pilosa* L.

3.2.3 Objectives

- To determine the physicochemical parameters and COD and correlate them to sulfate removal in wastewater in the hydroponic system.

- To determine the optimum pH and temperature favourable for the mechanisms of sulfate removal in the system.

3.3 Methodology

For the investigation of the effect of physical parameters on sulfate removal in a hydroponic system, wastewater samples were collected every 24 hours for 2 weeks and labelled with the different sampling time. While the pH and temperature were determined; the sulfate concentration was also determined in all water samples explained in chapter 2 (section 2.3.4) which correlated with the physical parameters.

3.3.1 Determination of the physicochemical parameters

- Measurement of temperature, pH and dissolved oxygen

Temperature, pH and dissolved oxygen were determined using InoLab IDS Multi 9310 from Merck. Measurements were conducted in triplicate of the different samples in order to have statistically accurate data. Water samples were also taken to the laboratory for sulfate determination in the wastewater as described in chapter 2 (section 2.3.4). This was done for all the samples collected at different hydraulic retention times (after every 24 hours for 2 weeks).

- Chemical oxygen demand (COD) determination

A COD reaction cell was swirled to suspend the bottom sediment and 2ml of water sample was carefully pipetted into the reaction cell and the screw cap tightly closed and the mixture was mixed vigorously. The reaction cell was then heated in the thermoreactor at 148 °C for 2 hours. After 2 hours the reaction cell was removed from the thermoreactor and placed in the test-tube rack to cool down at room temperature. After 10 minutes (reaction time) the

reaction cell was swirled and placed back into the rack for complete cooling. The cell was placed into the cell compartment and the mark on the cell was aligned with the one on the spectrophotometer and COD concentration was read.

3.3.2 Statistical data analysis

SPSS-Paired Samples *t*-test was the data analysis method employed to determine the statistical difference between the two measurements: time points or matched pair. It uses H_0 (null hypothesis, which means there is no difference). If $p < 0.05$ H_0 is rejected. However, if $p > 0.05$, H_0 is accepted. The other hypothesis that is used by this test is the H_i (alternative hypothesis), which means that there is the significant difference at a level of 5% or 0.05. Paired samples *t*-test was used to analyze data in order to compare and test for a statistical difference between the physicochemical parameters (pH, dissolved oxygen, COD and temperature). R square (*r*) was used for the determination and the analysis of the correlation between the physical parameters and sulfate removal from the hydroponic system.

3.4 Results and discussion

The results of the physical parameters (temperature, pH, dissolved oxygen and chemical oxygen demand) over the sampling periods are presented in Figures 10, 11, 12 and 13. The physical parameters were presented in line graphs with error bars indicating the variation in temperature and pH in the planted and control section. Even though other parameters may affect sulfate removal, it is documented that sulfate removal mechanisms are mostly affected by pH, temperature chemical oxygen demand (COD) and dissolved oxygen Vela *et al.* (2002).

3.4.1 The analysis of physicochemical parameters

The determination of physicochemical parameters is essential in wetland technologies for wastewater treatment because they affect biological processes of sulfate removal, and the activity of microbial communities. The physical parameters were not controlled, and this may have contributed to the fluctuations and changes in pH and temperature. The fluctuations in temperature and decrease in DO resulted in the decrease of sulfate removal. The obtained findings are acceptable and within the limits of constructed wetlands, since Albalawneh *et al.* (2016) reported that wetlands are up 98% efficient in COD removal and 98.5% in sulfate removal.

3.4.1.1 The potential Hydrogen

The results of pH in the control and planted sections ranging between 5 and 7.4, and with their fluctuating pattern at different hydraulic retention times are presented in Figure 10.

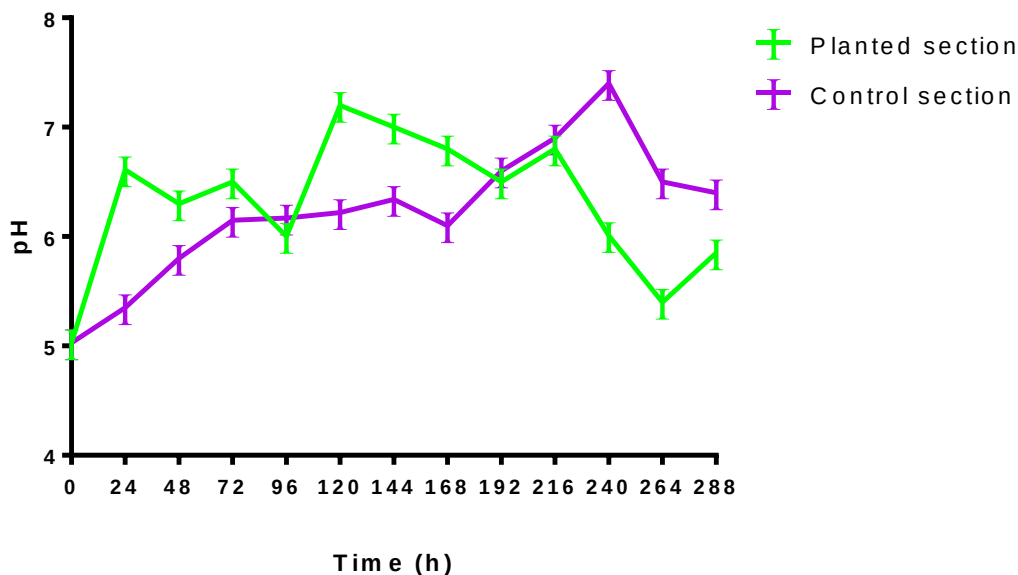


Figure 10: The pH obtained in the system over time.

After 96 hours in the control section, the pH there was greater than that of the planted section. There was also an increase in pH between 192th hour and 216th hour in the control section, resulting to the overlapping of the pH in planted section. The fluctuations in pH were because biological processes of water treatment are generally accompanied by a change in pH due to production of oxygen by plants' roots in the system as reported by Shigeyuki *et al.* (2013). According to Kiran *et al.* (2017), different biological, chemical and physicochemical processes such as sulfate-reducing bacteria- based sulfate oxidation leads to alteration in pH. Microbial degradation of sulfate and solubilisation of some acids also lead to the production of alkalinity which caused the increase of the pH, which was then stabilized around the neutral point. This indicated the removal of sulfate in water since pH of clean water is the neutral level. Similar findings were reported by Koschorreck (2008). The results in Figure 10 support the findings by Kiran *et al.* (2017) since pH was stabilized around the neutral pH after 120th hour in the planted section and after 240th hour in the control section as a result of microbial degradation of sulfate. SPSS Paired sample *t*-test was carried out and $p < 0.05$. It was concluded that there was a significant difference between the pH in the control and planted section.

3.4.1.2 Temperature

The natural processes of sulfate removal are temperature dependent and warm and terrestrial temperatures are favourable to the wetlands' mechanisms of sulfate removal.

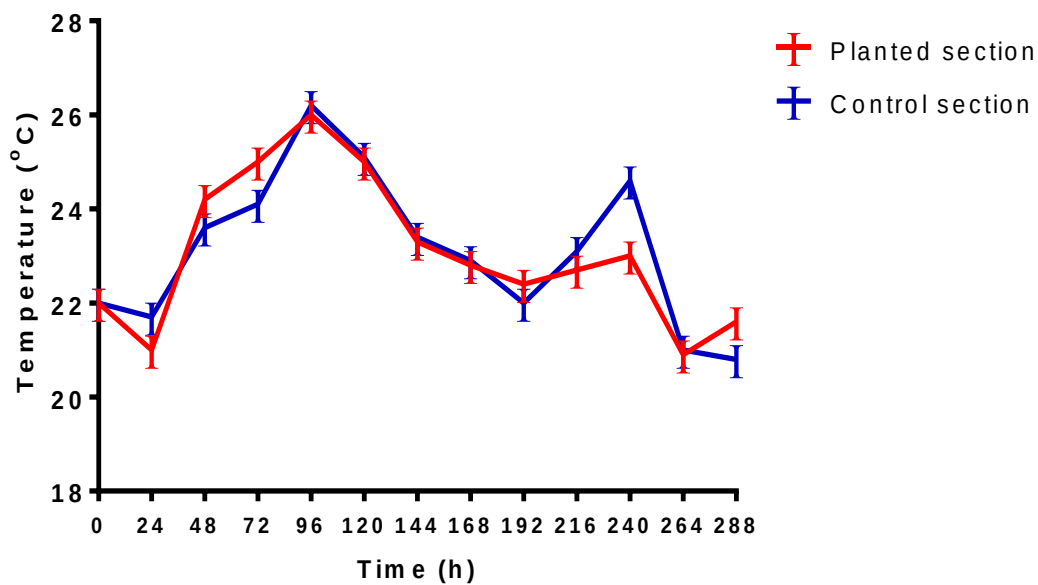


Figure 11: Temperature recorded in the system over time.

Figure 11 presents the results of temperature at different hydraulic retention times in the planted and control sections with the fluctuating pattern ranging between 20°C and 26.3°C. Similar results were reported by Allen *et al.* (2002) and Najib *et al.* (2017). For the control section, there was a significant increase of temperature after 240 hours. The environmental conditions were not controlled in the system. This attributed to the fluctuations of temperature over time. The change in pH between acidic and neutral points also contributed to the fluctuations in temperature since it is the essential physical parameter in biological processes of sulfate removal mechanisms in wetlands. The results in Figure 11 indicated that the microorganisms that were degrading sulfate in wastewater were mesophiles with the optimum temperature mentioned above. The paired *t*-test was carried out with $p=0.805$, which is greater than 0.05. The null hypothesis was accepted since $p>0.05$ and it was concluded that there was no significant difference between temperature in the control and planted section. Similar findings were reported by Guittonny-Philippe *et al.* (2015) whereby

there were no significant variations both in temperature and pH values in the planted and control sections.

3.4.1.3 Dissolved oxygen

In wetlands, oxygen is required by microorganisms that are responsible for degrading pollutants but sulfate-reducing microorganisms are dominated by anaerobic microbes.

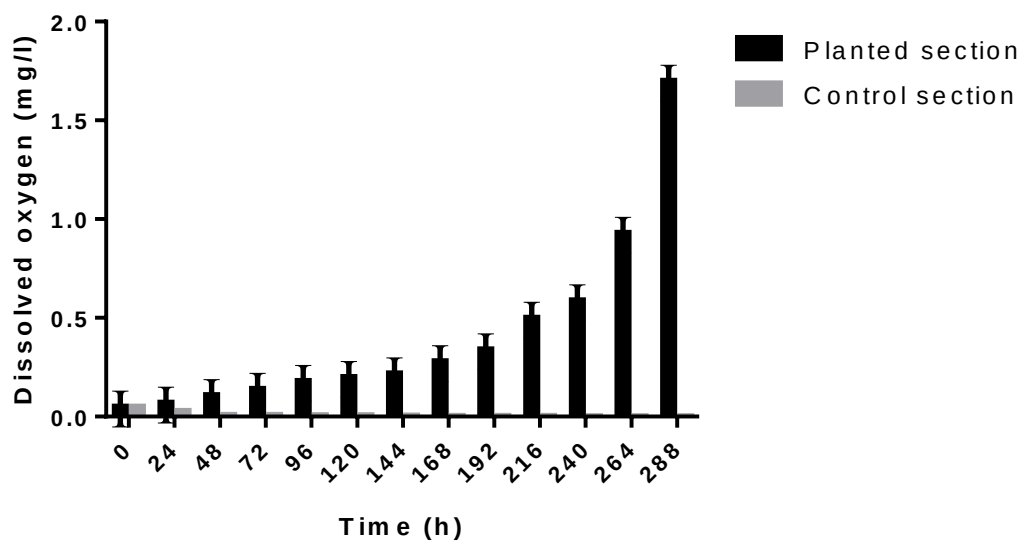


Figure 12: Different concentrations of dissolved oxygen obtained in the system over time. The amounts of dissolved oxygen in wastewater is presented in Figure 12. Dissolved oxygen was available in both planted and control sections. It increased with the increase in hydraulic retention time in the planted section, while decreasing in the control section. There was a drastic increase of dissolved oxygen in the planted section. It was 1.7 mg/l after 288th hour in the planted section while it was 0.003 mg/l in the control section. According to Rehman *et al.* (2017) a significant amount of oxygen is transported from the atmosphere to the rhizosphere of the macrophytes in order to facilitate biological sulfate removal processes. Similar results were reported by Kjeldsen *et al.* (2004), who found that there were low

amounts of dissolved oxygen in the unplanted section compared to the planted section. Rehman *et al.* (2017) also reported that dissolved oxygen in unplanted section ranges between 0.01 and 0.007 mg/l. The results in Figure 12 established that the absence of macrophytes in the control section led to the low amounts of oxygen which might have favoured sulfate removal in the control section since sulfate-reducing bacteria are obligate anaerobes. The statistical difference was analysed using SPSS-paired *t*-test and the significant difference (*p*) between dissolved oxygen in the planted and the control sections was found to be 0.0001. It was concluded that there was a significant difference between DO in the planted and control sections.

3.4.1.4 Chemical oxygen demand

COD is the amount of oxygen utilized in the chemical reactions of pollutants removal by microorganisms and chemical oxygen demand is also regarded as a pollutant, thus reduction of COD leads to the reduction of sulfate.

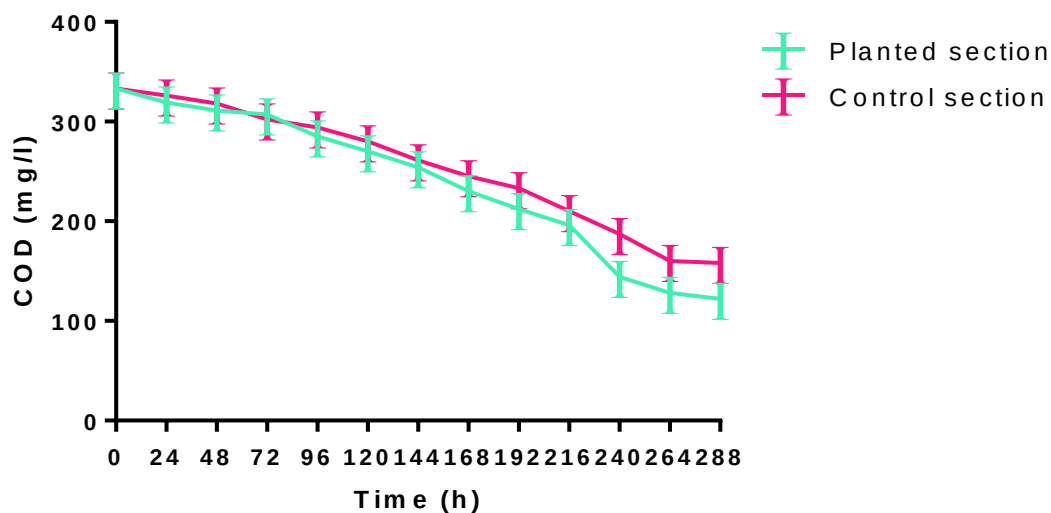


Figure 13: Chemical oxygen demand (COD) in the system over time.

Figure 13 presents the results of COD in the hydroponic system. COD was reduced rapidly in the planted section compared to the control section. The reduction of COD in the planted section increases drastically after 216 hours. It was 196 mg/l, and 261 mg/l in the control section. The final COD concentration was 122 mg/l in the planted section and 210 mg/l in the control section. Subtil *et al.* (2012) reported that the competition between sulfate-reducing and methanogenic microorganisms occurs in sulfate removal in wetlands and the competition for substrates between SRB and other anaerobic bacteria depends on the ratio of sulfate and COD concentration in wastewater (Barber and Stucky, 2000). The results in Figure 13 indicated that COD is required for microbial degradation of sulfate. This was indicated by the decrease in COD with the decrease in sulfate concentrations in water in both sections as it was utilized by the microorganisms. The SPSS-paired sample *t*-test indicated that there was a significant difference between COD in the planted and control sections since *p* value was 0001.

3.4.2 The effect of physicochemical parameters on sulfate removal

The linear and nonlinear regression model was used to determine the effect of physicochemical parameters on sulfate removal in both hydroponic systems. The choice of regression used depended on the presented data.

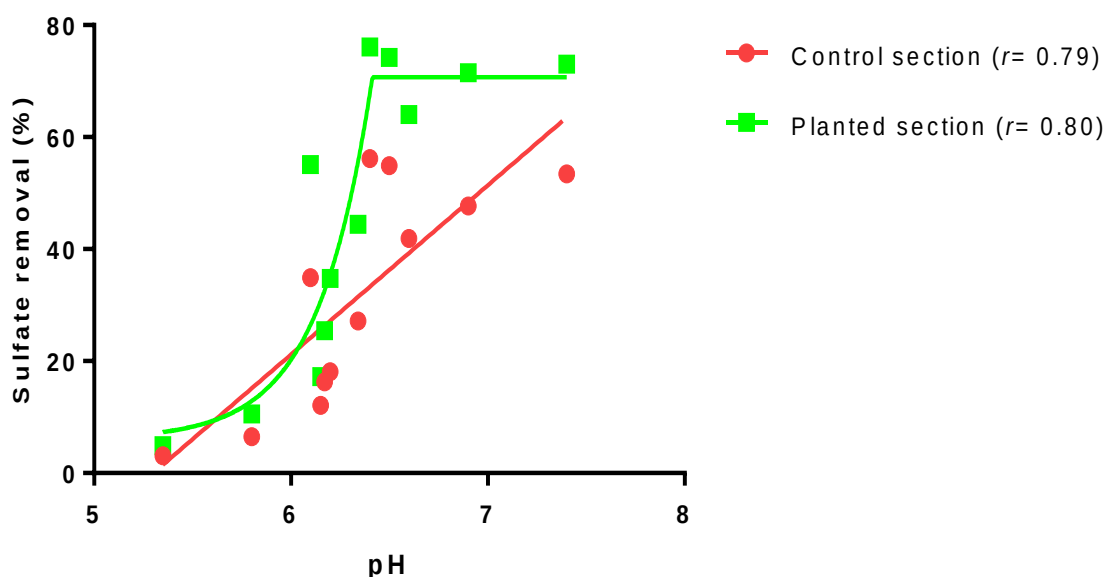


Figure 14: The effect of pH on sulfate removal in the system.

The line graphs in Figure 14 presents the correlations between sulfate removal and pH in the planted and control sections. The Pearson coefficient of correlation(r) was 0.80 in the planted section and 0.79 in the control section. The equation $y = 30.24X - 160.3$ presents the correlation of pH of sulfate removal in planted section and $y = 41.7X + 216.4$ in the control section. This means there was a strong positive linear correlation between pH and sulfate removal in both sections. This implied that sulfate removal increased with the increase in pH. Similar findings were reported by Oladejo *et al.* (2015). The observations were due to removal of sulfate by sulfate-reducing bacteria using carbon source, while at the same time, increasing the pH of the system. The results in Figure 14 showed that the pH conditions of wastewater that was introduced into the system were not too acidic and were favourable to both the macrophytes and microorganisms that were degrading sulfate and their mechanism of removal. The fact that the hydroponic system did not corrode after its exposure to acidic wastewater proved that the acidic conditions of the wastewater were not extreme. According

to Geldenhuys, (2003) acidic pH below 5.5 are toxic to aquatic plants and corrosive to water pipeline. The results presented in Figure 14 supports the findings by Geldenhuys, (2003).

Contradictory results were reported by Shigeyuki *et al.* (2013) where there was lower pH in the vegetated mesocosm compared to the control section due to the supply of oxygen by plant roots which led to the decrease in removal of sulfate with the decrease in pH. However, the supply of oxygen by macrophytes' roots may not have been the only cause of contradictory results in this study. According to Verma *et al.* (2015) high levels of metals in industrial wastewater may lead to the inhibition of enzymatic pathway in the plants, thus negatively influencing sulfate assimilation by plant roots and decreasing the levels of sulfate removal. The results reported by Verma *et al.* (2015) had moderate negative correlation between pH and sulfate removal. The results in Figure 14 established that sulfate-reducing microorganisms were efficient at pH between 5 and 8 and that the biological processes for sulfate removal were positively influenced by the increase in pH.

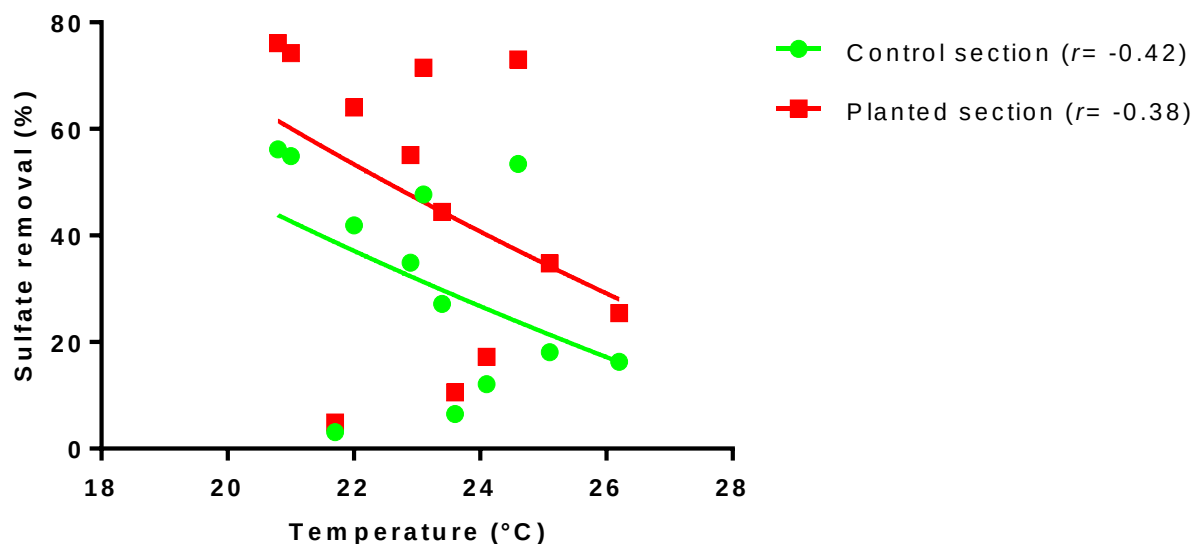


Figure 15: The effect of temperature on sulfate removal in the system.

Figure 15 presents the moderate negative linear correlation between temperature and sulfate removal both in the planted and control sections. The negative correlation was supported by the Pearson coefficient of correlation (r) that was found in the correlation analysis, -0.42 in the control section and -0.38 in the planted section. The relationship between temperature and sulfate removal in the planted section may also be represented by the formula $y = -7.704X + 224.4$ in the planted section, and $y = -5.068X + 148.6$ in the control section. Figure 15 pointed out that the relationship between temperature and sulfate removal was inversely proportional. According to Chao *et al.* (2014), temperature is the most important parameter that influences sulfate removal in wastewater, and has a significant correlation with any pollutant removal. Chao *et al.* (2014) also argued that microbial -related and plant-mediated degradation processes tended to be more effective in sulfate degradation during summer than in winter.

Since this study was conducted in winter, that may have been the cause of the inversely proportional relationship between sulfate removal and temperature. This is because sulfate-reducing bacteria preferred mesophilic temperatures and low temperature yield negative results in pollutants removal in wetlands. The optimum temperature required for the survival of the microorganisms responsible for microbial degradation of sulfate are mesophilic temperatures, ranging between 18°C and 40°C (Sawicka *et al.* 2012). The results in Figure 15 suggested that the optimum temperature for microbial degradation is indeed in the temperature range mentioned above. This study found the optimum temperature between 20°C and 26°C (mesophilic) to be favourable to the microbial degradation mechanism of sulfate removal in the hydroponic system. The desirable results would have been obtained if the study was conducted in summer. Similar results were also reported by Kadlec *et al.* (2001).

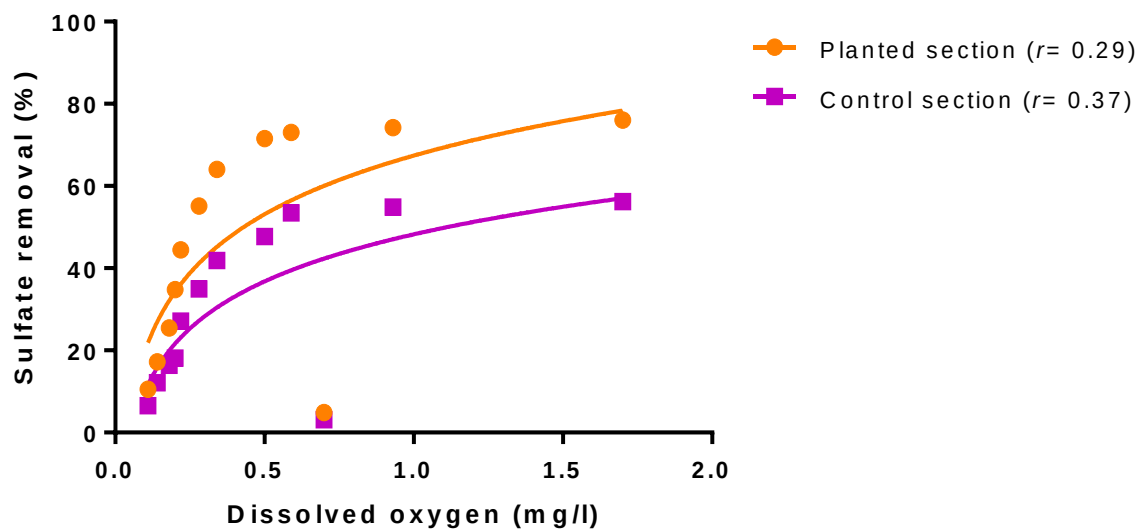


Figure 16: The effect of dissolved oxygen in sulfate removal in the system.

The dissolved oxygen in wetlands provides macrophytes with the oxygen that is required for sulfate degradation processes. The results in Figure 16 therefore presents the effect of DO on sulfate removal. The dissolved oxygen increased with the increase of sulfate removal. These results indicated that the presence of dissolved oxygen around sulfate-reducing microorganisms did not have a negative impact on sulfate removal. Even though sulfate-reducing microorganisms are known to be anaerobic microbes that degrade sulfate in wetlands technologies for sulfate-rich water treatment, these microorganisms can also survive in aerobic environments Sigalevich *et al.* (2000). The results in Figure 16 suggested that some of sulfate-reducing microorganisms can survive aerobic conditions. The correlation analysis was carried out and the Pearson (r) coefficient was 0.29 in the planted section and 0.37 in the control section. The relationship between dissolved oxygen and sulfate removal in the system can also be indicated by equation in the planted section $y = 20.18X + 47.5$ and $y = -13X + 38.09$ in the control section. These r values indicated that there was a weak positive correlation between sulfate removal and dissolved oxygen. According to Kjeldsen *et al.* (2004)

dissolved oxygen dissolves from the plant shoots and the atmosphere into the water during photosynthesis and is required by plants and microorganisms in degradation of pollutants. This may have been the reason why there was a positive correlation between dissolved oxygen and sulfate removal. Contradictory results were reported by Thongnueakhaeng and Chaiprasert. (2015) as they opined that dissolved oxygen inhibited the growth of sulfate-reducing bacteria.

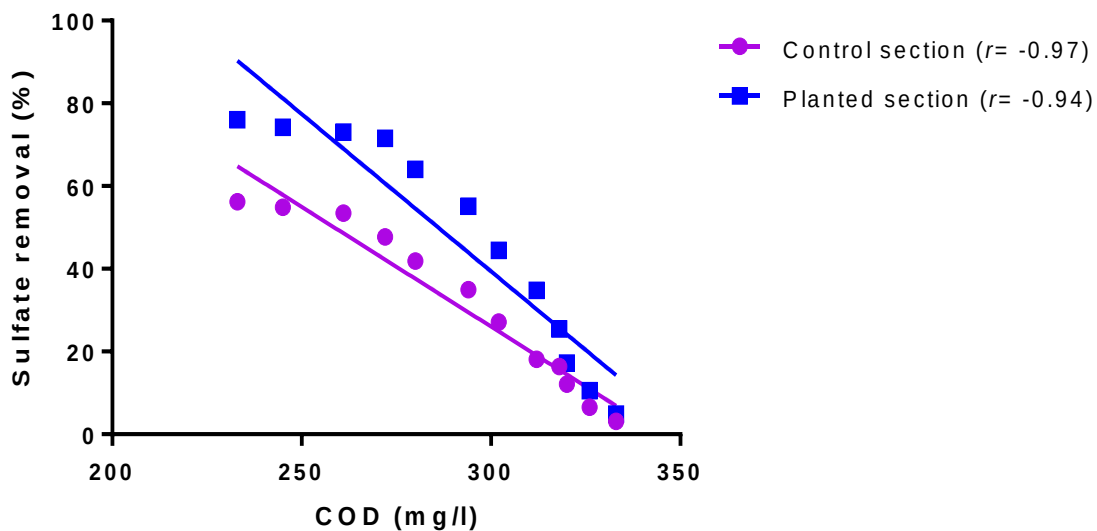


Figure 17: The effect of COD on sulfate removal in the system.

Figure 17 presents the effect of COD on sulfate removal in a hydroponic system. Sulfate removal was inversely proportional to the removal of COD. This means that concentration of sulfate was decreasing with the decrease in COD. Demirci and Saatci (2013) reported that sulfate-reducing bacteria are the competitive microorganisms. He also reported that the increase in hydrogen sulfide production (from sulfate oxidation processes) is harmful to methanogens (fermentative microorganisms) not to sulfate-reducing bacteria. The results in Figure 17 indicated that COD was being removed from water, while at the same time, utilized

by sulfate-reducing microorganisms. The inversely proportional relationship between COD and sulfate removal was also indicated by a very strong negative correlation from the correlation analysis whereby r was found to be -0.97 in the control section and -0.94 in the planted section. The relationship between COD and sulfate removal in the system can also be established by equation $y = -0.761X + 267.5$ in the planted section and $y = -0.579X - 199.7$ in the control section. Similar results were reported by Subtil *et al.* (2012). The decrease in COD may have resulted to the inversely proportional correlation between COD and sulfate removal, since COD decreases with the decrease in sulfate availability in wastewater treatment Subtil *et al.* (2012). The results in Figure 17 supported findings by Subtil *et al.* (2012) that depletion of sulfate due competition of sulfate and COD between sulfate-reducing microorganisms and methanogens leads to the inversely proportional relationship.

3.5 Conclusion

In this chapter, it was found that with the prolonged retention time, the relationship between (pH, DO) and sulfate removal was directly proportional while the relationship between temperature and sulfate removal was inversely proportional. The optimum temperature for sulfate removal mechanisms was found to be between 20°C and 27°C, and the optimum pH was between 5 and 8 and COD decreased with the decrease in sulfate availability. These physicochemical parameters were favourable to sulfate removal mechanisms in the hydroponic system. The pH conditions were found to be acidic but not extremely acidic since growth inhibition in plants and corrosion within the hydroponic system's structure had not been observed after treatment.

3.6 Recommendations

From the findings, it is recommended that physical and chemical parameters are optimized to yield better results of sulfate removal in future studies. Addition of natural salts in wastewater circulating within the system when necessary will aid in pH manipulation. Introduction of wastewater into an anaerobic control section is also recommended since sulfate-reducing bacteria are anaerobic microorganisms.

3.7 References

- Albalawneh, A., Chang, T.K., Chou, C.S. and Naoum, S. (2016). Efficiency of a Horizontal Sub-Surface Flow Constructed Wetland Treatment System in an Arid Area. *Water* **8**(2): 51.
- Allen, W.C., Hook, P.B., Biederman, J.A. and Stein, O.R. (2002). Temperature and wetland plant species effects on wastewater treatment and root zone oxidation. *Journal of Environmental Quality* **31**(3): 1010-1016.
- Al-Zuhair S, El-Naas, M.H., and Al-Hassani, H. (2008). Sulfate inhibition effect on sulfate-reducing bacteria. *Journal of Biochemical Technology* **1**(2): 39-44.
- Barber, W.P. and Stuckey, D.C. (2000). Effect of sulfate removal on chemical oxygen demand removal in an anaerobic baffled reactor. *Water Environment Research* **72**(5): 593-601.
- Chao, W., Zheng, S., Wang, P.F. and Jin Q.I. (2014). Effect of vegetation on the removal of contaminants: A review. *Journal of Hydrodynamics* **26**(4): 502.
- Costabile, A.L., Canto, C.S., Ratusznei, S.M., Rodrigues, J.A., Zaiat, M. and Foresti, E. (2011). Temperature and feed strategy effects on sulfate and organic matter removal in an AnSBB. *Journal of Environmental Management* **92**(7): 1714-1723.
- Dakiky, M., Khamis, M., Manassra, A. and Mer'Eb, M. (2002). Selective adsorption of chromium (VI) in industrial wastewater using low-cost abundantly available adsorbents. *Advances in Environmental Research* **6**(4): 533-540.
- Demirci, Y. and Saatci, Y. (2013). Influence of COD/SO₄ 2-Ratios on Performance of Anaerobic Hybrid Reactor and Up-Flow Anaerobic Sludge Bed Reactor. *Asian Journal of Chemistry* **25**(18): 1.
- Fang, P., Tang, Z.J., Chen, X.B., Huang, J.H., Tang, Z.X. and Cen, C.P. (2018). Removal of High-Concentration Sulfate Ions from the Sodium Alkali FGD Wastewater Using Ettringite Precipitation Method: Factor Assessment, Feasibility, and Prospect. *Journal of Chemistry* **10**: 1.
- Geldenhuys, P. (2003). An integrated limestone/lime process for partial sulphate removal. *Journal of the Southern African Institute of Mining and Metallurgy* **103**(6): 345-353.

Guitttonny-Philippe, A., Petit, M.E., Masotti, V., Monnier, Y., Malleret, L., Coulomb, B., Combroux, I., Baumberger, T., Viglione, J. and Laffont-Schwob, I. (2015). Selection of wild macrophytes for use in constructed wetlands for phytoremediation of contaminant mixtures. *Journal of Environmental Management* **147**: 108-123.

Kadlec, R.H. and Reddy, K.R. (2001). Temperature effects in treatment wetlands. *Water Environment Research* **73**(5): 543-557.

Kiran, M.G., Pakshirajan, K. and Das, G. (2017). An overview of sulfidogenic biological reactors for the simultaneous treatment of sulfate and heavy metal rich wastewater. *Chemical Engineering Science* **158**: 606-620.

Kjeldsen, K.U., Joulain, C. and Ingvorsen, K. (2004). Oxygen tolerance of sulfate-reducing bacteria in activated sludge. *Environmental Science and Technology* **38**(7): 2038-2043.

Koschorreck, M. (2008). Microbial sulphate reduction at a low pH. *Federation of European Microbiology Societies for Microbiology Ecology* **64**(3): 329-342.

Lozano, A., Fernández-Martínez, A., Ayora, C. and Poulain, A. (2018). Local structure and ageing of basaluminite at different pH values and sulphate concentrations. *Chemical Geology* **496**: 25-33.

Najib, T., Solgi, M., Farazmand, A., Heydarian, S.M. and Nasernejad, B. (2017). Optimization of sulfate removal by sulfate-reducing bacteria using response surface methodology and heavy metal removal in a sulfidogenic UASB reactor. *Journal of Environmental Chemical Engineering* **5**(4): 3256-3265.

Oladejo, O.S., Owoade, N.A., Kusamotu, K. and Taiwo, S.A. (2015). Kitchen Wastewater Treatment with Constructed Wetland Using Water Hyacinth. *International Journal of Scientific and Engineering Research* **6**(1): 834-839.

Patil, P.N., Sawant, D.V. and Deshmukh, R.N. (2012). Physico-chemical parameters for testing of water-A review. *International Journal of Environmental Sciences* **3**(3): 1194.

Rehman, F., Pervez, A., Mahmood, Q. and Nawab, B. (2017). Wastewater remediation by optimum dissolve oxygen enhanced by macrophytes in constructed wetlands. *Ecological Engineering* **102**: 112-126.

- Sadik, R., Lahkale, R., Hssaine, N., ElHatimi, W., Diouri, M. and Sabbar, E. (2015). Sulfate removal from wastewater by mixed oxide-LDH: Equilibrium, kinetic and thermodynamic studies. *Journal of Material Environment and Science* **6**(10): 2895-2905.
- Sawicka, J.E., Jørgensen, B.B. and Brüchert, V. (2012). Temperature characteristics of bacterial sulfate removal in continental shelf and slope sediments. *Biogeosciences* **9**(8): 3425-3435.
- Shigeyuki, A., Kazunori, N., Osamu, N. and Yoshio, A. (2013). Effect of temperature, pH, and vegetation in the removal of zinc from mine water in anerobic wetland mesocosm **69**: 94-95.
- Sigalevich, P., Baev, M.V., Teske, A. and Cohen, Y. (2000). Sulfate removal and possible aerobic metabolism of the sulfate-reducing bacterium *Desulfovibrio oxyclinae* in a chemostat coculture with *Marinobacter* sp. strain MB under exposure to increasing oxygen concentrations. *Applied and Environmental Microbiology* **66**(11): 5013-5018.
- Subtil, E.L., Cassini, S.T.A. and Gonçalves, R.F. (2012). Sulfate and dissolved sulfide variation under low COD/Sulfate ratio in Up-flow Anaerobic Sludge Blanket (UASB) treating domestic wastewater. *Revista Ambiente and Água* **7**(1): 130-139.
- Thongnueakhaeng, W. and Chaiprasert, P. (2015). Effect of Dissolved Oxygen Concentrations on Specific Microbial Activities and Their Metabolic Products in Simultaneous Sulfur and Nitrogen Removal. *International Journal of Environmental Science and Development* **6**(4): 235.
- Vela, F.J., Zaiat, M. and Foresti, E., (2002). Influence of the COD to sulphate ratio on the anaerobic organic matter degradation kinetics. *Water* **28**(2): 213-216.
- Verma, S., Suthar, S. (2015). Lead and cadmium removal from water using duckweed- *Lemna gibba* L: Impact of pH and initial metal load. *Alexandria Engineering Journal* **8**: 5.
- Zhao, F., Rahunen, N., Varcoe, J.R., Roberts, A.J., Avignone-Rossa, C., Thumser, A.E. and Slade, R.C. (2009). Factors affecting the performance of microbial fuel cells for sulfur pollutants removal. *Biosensors and Bioelectronics* **24**(7): 1931-1936.

CHAPTER 4: DETERMINATION OF THE MICROBIAL POPULATION SHIFT AND DYNAMICS OF SULFATE REDUCING BACTERIA IN A HYDROPONIC SYSTEM

4.1 Introduction

Sulfate contamination can lead to availability of toxic compounds in wetlands. A hydroponic system uses biological processes for sulfate removal such as microbial degradation of sulfate by sulfate-reducing bacteria (Niu *et al.*, 2018). These acidophilic microorganisms use sulfate as an electron acceptor, while at the same time, degrading toxic compounds of industrial waste. However, in crude oil production, these microorganisms cause severe problems such as souring of oil through H₂S production from sulfate and corrosion of the facilities. Despite their disadvantages, these microorganisms play an important role in wastewater treatment. Therefore, studying microbial communities is essential in sulfate-rich water treatment and fuel producing industries (Ben-Dov *et al.*, 2007).

Hydrogen sulfide from sulfate oxidation may accumulate and penetrate into sulfate-reducing bacteria' cell membranes, thus disrupting their metabolic activity. This contributes to population shift (Karna *et al.*, 2018). According to (D'Souza *et al.*, 2018), the metabolic activities of bacteria transform genetically due to the environment they live in, and thereby drastically influence the growth and metabolism of co-occurring microorganisms. D'Souza *et al.*, (2018) also reported that 17-42 % genetic material of bacterial cells can encode traits that are responsible for mediating ecological interactions. In sulfate limited systems, the degradation of sulfate can become very complex due to the availability of different carbon sources such as propionate, lactate, pyruvate, fumarate and ethanol. This allows the survival of different microbial populations of sulfate-reducing microorganisms such as fermentative and syntrophic sulfate reducers (depend on other microorganisms for the conversion of

complex organics into simpler compounds) interacting in a food web (Icgen and Harrison, 2006). Sulfate-reducing bacteria and methanogens catalyse mineralization of sulfate and other organic compounds but have limited substrate due to their syntrophic nature. However, recent studies have reported that some sulfate-reducing microorganisms can be found in large numbers in sulfate depleted environments. This is due to their ability to grow syntrophically with hydrogen consuming methanogens in the presence of lactate, fumarate and pyruvate, thus eliminating their need and utilization of sulfate (Girguis *et al.*, 2005). The alteration of microbial population due to utilization of different carbon sources necessitates determination population dynamics and shifts of sulfate-reducing bacteria using (polymerase chain reaction) qPCR.

The real time (real-time PCR) also known as quantitative polymerase chain reaction (qPCR) is one of the innovative methods of determining sulfate-reducing bacteria that are currently used (Pereyra *et al.*, 2010). In this method, sulfate-reducing bacteria are targeted with the functional gene surveys sequencing of two functionally converted and phylogenetically informative key genes, alpha and beta subunits of dissimilatory sulfide reductase (*dsr A* and *dsr B*) and adenosine-5-phosphosphate reductase alpha units (*apr A*) (Laue *et al.*, 2001). These genes allow selective detection and phylogenetic determination of sulfate-reducing bacteria amongst abundant background populations. Quantitative PCR involves 16 S r RNA at a later stage.

Furthermore, the 16 S rRNA hybridization allows quantification of sulfate-reducing bacteria with the use of designed primers and probes that allow determination of bacteria' genus or family. This molecular technique according to (Dar, 2007) has a potential to monitor alterations in microbial activity and population shifts. Having said that, the main objective of

this study was to determine diversity and abundance of dominant communities of sulfate-reducing bacteria before and after changes in sulfate availability.

4.2 Aim, Hypothesis and Objectives

4.2.1 Aim

The aim of this chapter was to determine the population shifts and dynamics of sulfate-reducing bacteria during the sulfate removal in a hydroponic system.

4.2.2 Hypothesis

There will be a large population of sulfate-reducing microorganisms in the planted section compared to the control (unplanted) section before treatment with a hydroponic system, and which will decrease with the decrease in sulfate availability.

4.2.3 Objectives

- To amplify the 16S rRNA gene of sulfate-reducing bacteria to confirm their presence in industrial wastewater.
- To determine of the concentrations of sulfate-reducing bacteria in water samples collected at different hydraulic retention times before and after treatment in a hydroponic system.

4.3 Methodology

4.3.1 Sample collection

The initial samples were collected using 500 ml Schott bottle right after filling the tanks with wastewater and labelled 0 hours. The same procedure was carried out after every 24 hours for 2 weeks and labelled. All the samples were collected using sterile 500 ml Schott bottles

according to the Standard Water sampling procedures. The samples were stored on ice and taken to University of Zululand Microbiology Laboratory for DNA extraction.

4.3.2 The growth of sulfate-reducing bacteria

The DNA of sulfate-reducing microorganism is difficult to isolate, therefore the samples collected at different hydraulic retention time were enriched with nutrients to facilitate the growth of sulfate-reducing bacteria and the media were then used for DNA extraction. The sulfate-reducing bacteria were grown using the ingredients and the method by (Ben-Dov *et al.*, 2007).

Table 3: The ingredients used for the growth of sulfate-reducing bacteria.

Ingredients	Mass (mg)
K ₂ HPO ₄	500
NH ₄ Cl	1000
Na SO ₄	500
CaCl ₂	100
Yeast extract	1000
Sodium lactate	4000
FeSO ₄	1200

The ingredients (Table 3) were measured, with different carbon sources per bottle, and pH was adjusted between 7.0 and 7.5. The mixtures were then autoclaved for 15 minutes at 121⁰C. A 40 ml of the media was poured in 250 ml Schott bottle and inoculated with 10 ml

mine water, incubated for 7 days at 37°C. The bottle was flushed with methane and tightly closed. The culture was monitored daily for the formation of black precipitation.

4.3.3 DNA extraction

For all the inocula, the DNA was extracted from wastewater using the ZR Fungal/Bacterial DNA miniprep kit, according to the manufacturer's protocol (Inqaba Biotech). A 200 µl of water sample was micro-pipetted into ZR bashing beads lysis tube and 750 µl was added and vortexed for 5 minutes. After being vortexed, the ZR bashing beads lysis tube was centrifuged at 10000 xg for 1 minute. A 400 µl of the supernatant was transferred into Zymo spin filter in a new collection tube and centrifuged at 7000 xg for a minute. A 1200 µl of binding buffer was added into a filtrate in a collection tube and centrifuged at 10000 xg for 1 minute. The flow through was discarded and this step was repeated. The DNA pre-wash buffer (200 µl) was pipetted into Zymo IIC column in a new collection tube and centrifuged at 10000 xg for 1 minute. The flow through was discarded from the collection tube, followed by the addition of 500 µl of wash buffer into Zymo IIC column and centrifuged at 10000 xg for a minute. The Zymo spin IIC column was transferred into a sterile 1.5 microcentrifuge tube and the DNA was eluted using 100 µl elution buffer, which was directly added to the matrix of the Zymo spin IIC column and then centrifuged at 10000 xg for 30 seconds. The eluted buffer was stored on ice and later used in the gel electrophoresis in order to confirm the presence of the DNA that was extracted.

4.3.4 Gel electrophoresis

The gel to be used in the gel electrophoresis was prepared, by dissolving 3 g of agarose gel in 300 ml of 1xTAE buffer composed of (4.84 ml of the base, 2 ml of 0.5 M EDTA and 1.142 ml glacialaceticacid) in a conical flask and then microwaved for 6 minutes and it was ensured

that the gel was completely mixed with the buffer. After boiling, the mixture was taken out of the microwave and 10 µl of ethidium bromide was added into the flask with the gel and mixed. The gel was then poured into the gel tray with 20 wells comb and cooled at room temperature. After cooling the gel, the well comb was removed and 5 µl of the gene ruler was loaded into the first well of the gel and 5 µl of the extracted DNA was mixed with 2 µl of loading dye and the mixture was loaded into the wells. The same amount of the gene marker was loaded on the empty well next to the ones loaded with DNA and loading dye mixture. The gel was then run at 100 voltages for 45 minutes and was visualized under IN genus sygen bio-imaging with high ultraviolet radiation illumination. After the confirmation of its presence, the extracted DNA was then used in qPCR.

4.3.5 Real-time/quantitative polymerase chain reaction

The amplification of the nucleic acids of sulfate-reducing bacteria was conducted using the primers in Table 4 and qPCR constituents in Table 5 (Daly *et al.*, 2000). These primers specifically targeted acidophilic mesophilic chemoheterotrophic group of sulfate-reducing bacteria. The qPCR mixture contained the constituents in Table 5. Universal primers were used to amplify a 16S rRNA gene fragment (*dsv435F* and *dsv1425R*) to measure the abundance of the total bacteria in the sample. Complex specific primers were used to selectively amplify genomic DNA sequences from each genus, yielding fragments. The qPCR was performed using the ABI 7500 Fast real-time PCR system (Applied Biosystems, Carlsbad, CA) with an initial step of denaturation for 4 min at 95°C. This was followed by the incubation at 54°C for 40s, 72°C for 40s and 120s. Melting curves were determined following qPCR by 1 cycle of 20 min at 72°C.

Table 4: The PCR primers for identification of sulfate-reducing bacteria that were used in the study (Daly *et al.*, 2000).

Organism	Primer	Sequence 5'-3'
<i>Desulfobulbus</i>	DBB 121	CGC GTA GAT AAC CTG TCY TCA TG
	DBB1237	GTA GKA CGT GTG TAG CCC TGG TC
<i>Desulfobacterium</i>	DBN 169	CTA ATR CCG GAT RAA GTC AG
	DBM1006	ATT CTC ARG ATG TCA AGT CTG
<i>Desulfobacter</i>	DSB 127	GAT AAT CTG CCT TCA AGC CTG G
	DSM1273	CYY YYY GCR RAG TCG STG CCC T
<i>Desulfococcus</i>	DCC 305	GAT CAG CCA CAC TGG RAC TGA CA
	DCC1165	GGG GCA GTA TCT TYA GAG TYC
<i>Desulfovibrio</i>	DSV 230	GRG YCY GCG TYY CAT TAG C
	DSV 838	SYC CGR CAY CTA GYR TYC ATC

Table 5: qPCR constituents that were used in 16S rDNA amplification.

qPCR constituents	Volume
Absolute QPCR SYBR Green Rox Mix	10µl
Forward primer	150 nM
Reverse primer	150 nM
DNA template	5.0 µl
1 U Taq Polymerase	0.7 µl
Super dNTP	2 µl
Distilled water	2 µl
Total volume	20 µl

Table 6: The conditions of the hot-start cycling for DNA amplification.

Process	Temperature	Time
Initial denaturation	95°C	4 min
Incubation	54 °C	40 s
	72°C	40 s
	72°C	120 s
Elongation	72°C	20 min

4.3.7 Real time/qPCR products visualization

PCR products were electrophoresed through a 1% (w/v) agarose gel in 1×TAE containing ethidium bromide (0.2 µg/ml). The DNA bands were visualized by UV illumination. The marker pBR322 DNA/ Alw441/ Mva1 (MBI Fermentas) was included to enable estimation of the molecular mass of the DNA bands amplified. The 16S rRNA for gene sequence analysis was performed. The 16S rRNA uses a database that allows determination of phylogenetic affiliation or relationship of microorganisms. The NCBI GenBank database is the database that was used in this study for the sequence analysis.

4.3.8 Statistical data analysis

The ABI prism 7000 sequence detection system and SDS software were used to analyse the data. The ABI prism and SDS software carry out quantitative analysis and qualitative detection using end point and dissociation curve analysis. The SDS software detects accumulated PCR products. The images from gel electrophoresis were viewed under three dimensional (3D) view in order to observe the peaks of the DNA quantity in order to evaluate microbial population dynamics.

4.4 Results and discussion

The images (Figure 18) present the different media that was used to grow sulfate-reducing bacteria. The growth of the SRBs was indicated by the formation of the black precipitates after 7 days of incubation.

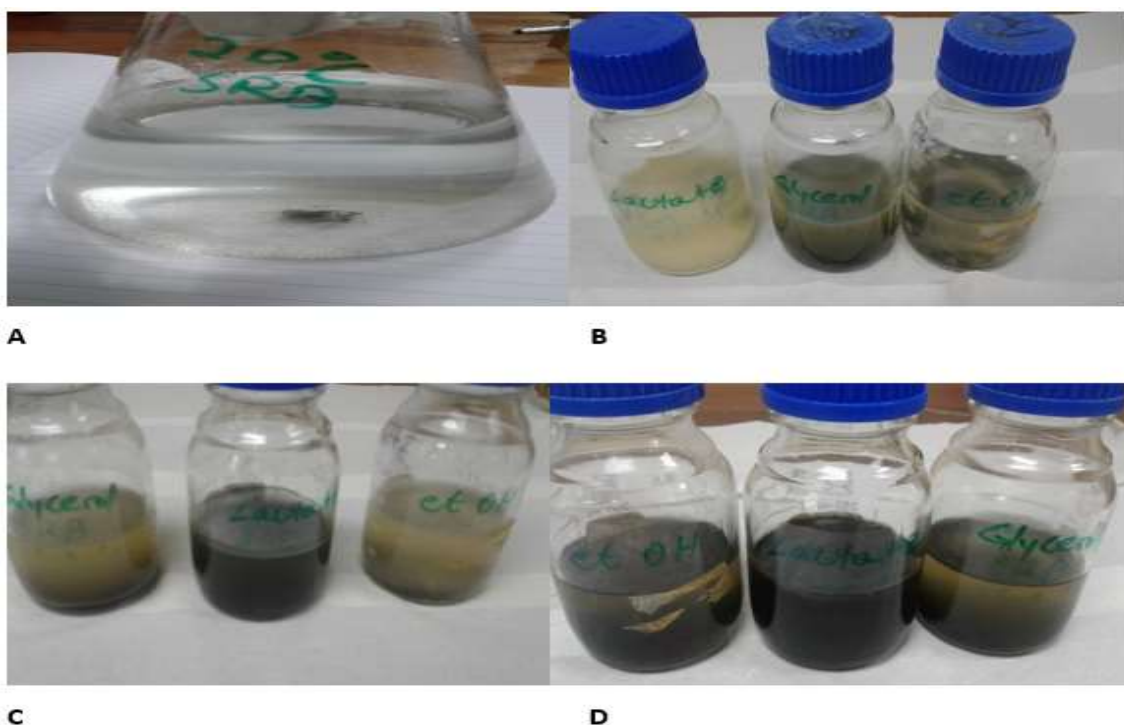


Figure 18: Images showing inocula of mine water in three different carbon sources (glycerol, lactate and ethanol) and the black precipitates of iron sulfide precipitation by sulfate-reducing microorganisms.

Figure 18 A, B, C and D present the iron precipitation by sulfate-reducing microorganisms. This was indicated by the formation of black precipitates in the Schott bottles with different enriched media (glycerol, lactate and ethanol) which served as the carbon sources for provision of nutrients for sulfate-reducing bacteria. Image A was mine water, while B, C, and D represented water sample inoculated in three different carbon sources and incubated at 37°C (after 2 days), (after 4 days), and (after 7 days) respectively. According to Meier *et al.* (2012), in the presence of sulfate, sulfate-reducing microorganisms oxidize sulfate with the precipitation of iron and other metals. The findings in Figure 18 supported the findings by Meier *et al.* (2012). Figure 18 established the presence of sulfate-reducing microorganisms. According to Gramp *et al.* (2010), nutrients availability and temperature affect iron precipitation by sulfate-reducing bacteria. The findings in Figure 18 showed a strong black precipitates in the medium enriched with lactate. This indicated that inoculated microorganisms preferably fed on lactate because sulfate-reducing microorganisms readily

utilize lactate. Similar findings were reported by Liu *et al.* (2018), who indicated that sulfate-reducing bacteria grew well in the inoculums enriched with lactate, ethanol and metals arsenic. Figure 19 presents the DNA bands of sulfate-reducing bacteria in the control and planted sections.

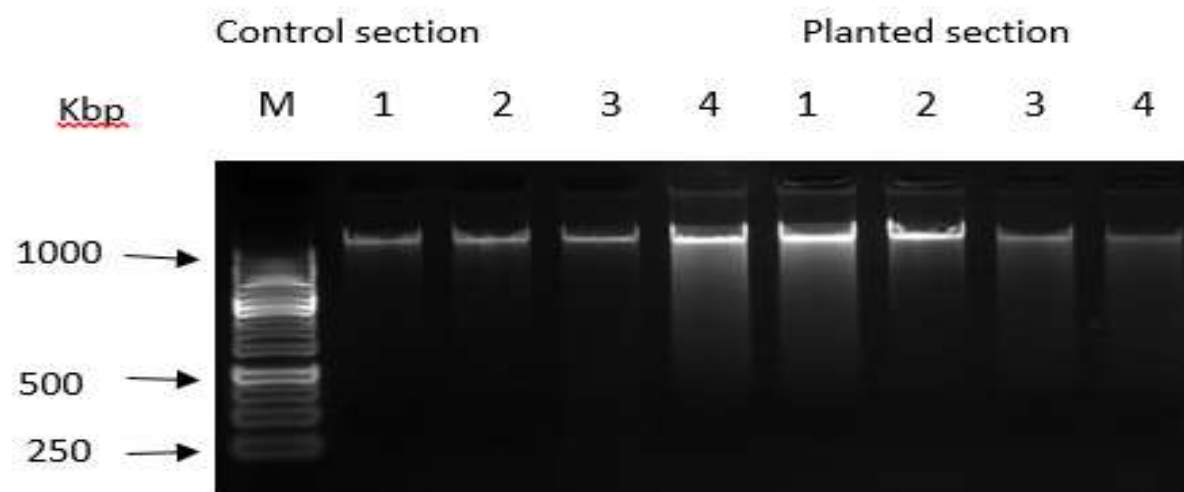


Figure 19: An image of gel electrophoresis showing DNA bands of sulfate-reducing microorganisms. The first section of the gel presents DNA extracted in the control section. The second section of the gel presents the DNA extracted in the planted section (Lane M represents a DNA marker, Lane 1-4 DNA in the samples collected after 0, 48, 96 and 144 hours respectively).

Microbial population of sulfate-reducing microorganisms was indicated by the DNA bands. These microorganisms were present both in the planted and control section sections, but the abundant microbial populations of sulfate-reducing bacteria was in the control section. There was a decrease in the bacterial DNA concentration in lane 2 of both sections after 48 hours. After 96 hours, there was still a drastic decrease in the microbial communities of sulfate-reducing bacteria in the planted section while the DNA in control section after 96 hours was higher than in the planted section. This indicated that the reduction in sulfate and the presence of macrophytes led to the reduction of sulfate-reducing microorganisms as the competition of sulfate was high and there was oxygen which is known to inhibit the growth

of sulfate-reducing bacteria. The 3D of extracted DNA of sulfate-reducing bacteria in the hydroponic stem is shown in Figure 20.

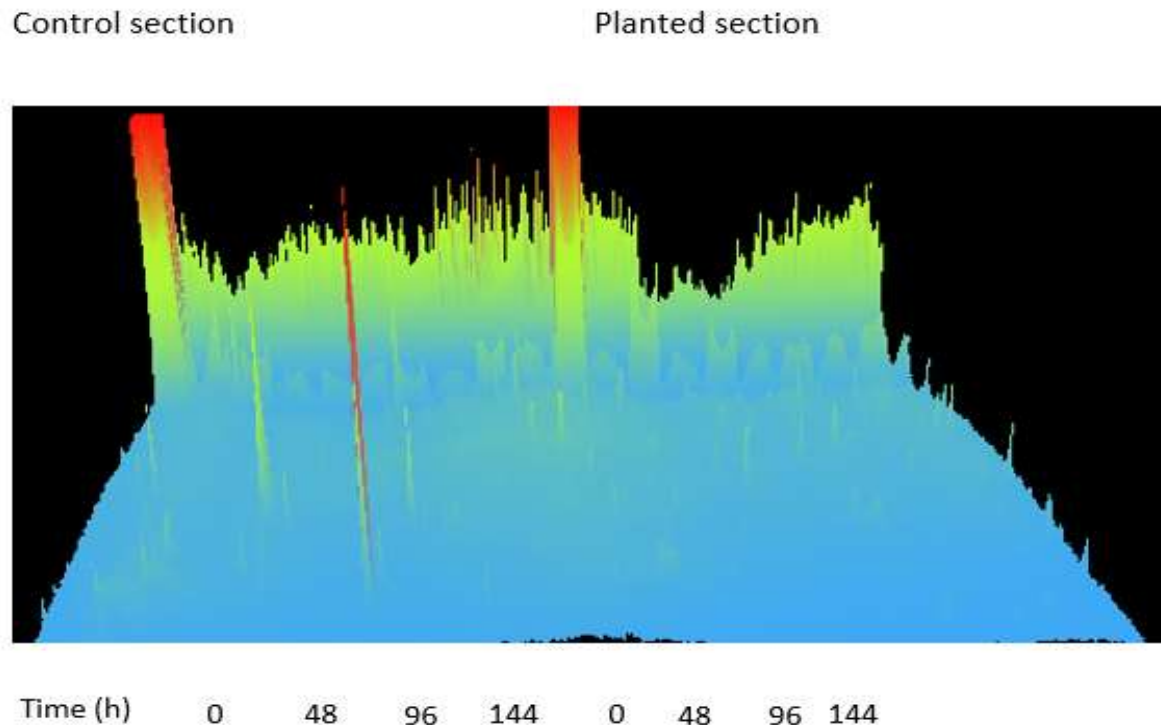


Figure 20: A 3D view of extracted DNA of sulfate-reducing bacteria in the control and planted sections of the hydroponic system.

Figure 20 presents the three dimensional (3D) view of the DNA quantity. These findings pointed out the peaks of DNA concentration at different hydraulic retention time. The DNA quantity was high at zero hours in both sections but there was a drastic decrease of microbial population in the planted section after 48-96 hours. This might have been due to the presence of oxygen produced by macrophytes shoots into the rhizosphere (where microorganisms-microbe relationship occurs) during photosynthesis. Sigalevich *et al.* (2010) reported that sulfate-reducing bacteria are obligate anaerobes and some have an ability to survive aerobic conditions.

It was also observed that sulfate removal within the system may also have led to the decline in microbial communities. The presence macrophytes and their assimilation of sulfate as well as the competition of sulfate amongst microbes may have contributed to the decline in population dynamics of sulfate-reducing bacteria (SRB) through reduction of sulfate availability. The microbial communities also decreased in the control section after 48 hours but not as compared to the planted section. The ability of SRB to outcompete other microbes that utilize sulfate while using a variety of carbon sources may have contributed to high DNA concentrations in the final samples that were used for DNA extraction (after 144 hours). According to Achá *et al.* (2005), sulfate-reducing microorganisms degrade sulfate symbiotically with the macrophytes and have a potential to outcompete methanogens (utilize sulfate) while using other carbon sources as the source of nutrients and energy. As shown in Figure 20 above, findings maintained that the SRB were able to survive and outcompete their competitors but they were in high abundance in the control section compared to the planted section. Figure 21 shows the PCR products in the control section.

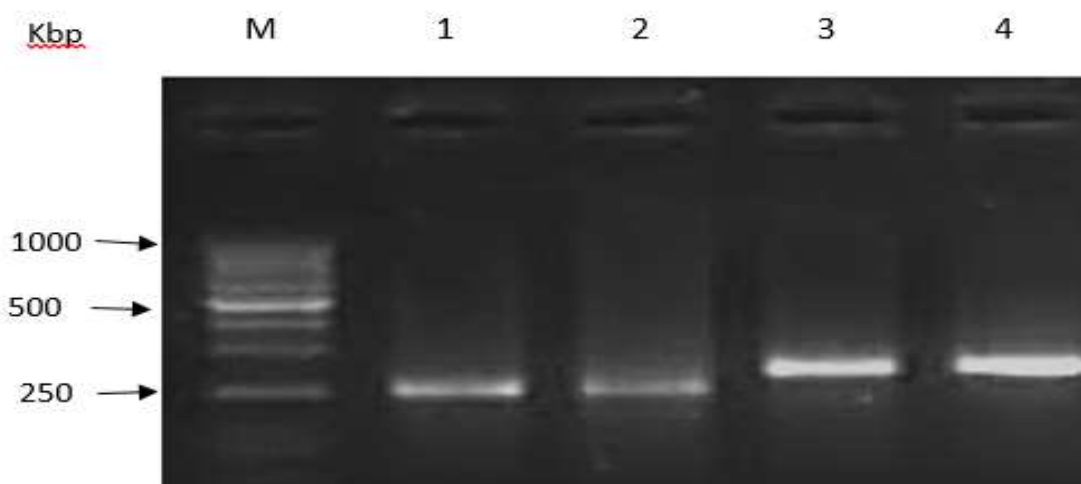


Figure 21: PCR products in the control section (Lane M represents a DNA marker, Lane 1-4 DNA in the samples collected after 0, 48, 96 and 144 hours respectively).

The PCR products obtained indicated that the sulfate-reducing bacteria: *Desulfovibro*, *Desulfobacter* and *Desulfococcus* were present in the planted section, while in the control section, *Desulfobacter* and *Desulfovibrio* were identified (Figure 21). Cypionka *et al.* (2005) reported that *Desulfococcus* is one of many strains of sulfate reducing bacteria that is sensitive to oxygen. This might have been the reason for the absence of *Desulfococcus* in the planted section. They indicated that sulfate-reducing bacteria uses membrane bound oxygen reductases and some other mechanisms such as antioxidative systems in order to scavenge oxygen effect. According to Neubauer *et al.* (2018), in the microbial competition of sulfate reducers and methanogens for electron donors, sulfate-reducing bacteria decrease with the decrease in sulfate and carbon source availability. The increase in hydraulic retention time results in the decrease in competitive pressure with the increase in the production of the carbon sources such as methane from the methylation of heavy metals such as mercury. The utilization of this carbon source by sulfate-reducing microorganisms led to the increase in microbial communities reported Neubauer *et al.* (2018).

The findings in Figure 21 are in relation to Neubauer's *et al.* (2018) findings because there were higher and rapid levels of sulfate removal in the planted section compared to the control section. This was due to the presence of macrophytes and oxygen which lead to the reduction in microbial population in the planted section with the decrease in sulfate due to high competition. This explains the abundance of bacterial DNA in the control section than in the planted section as there were no macrophytes in the control section and oxygen availability was decreasing in concentrations, which worked in favour for the survival of anaerobic sulfate-reducing bacteria. In addition to the above findings, Figure 22 shows the PCR products found in the planted section.

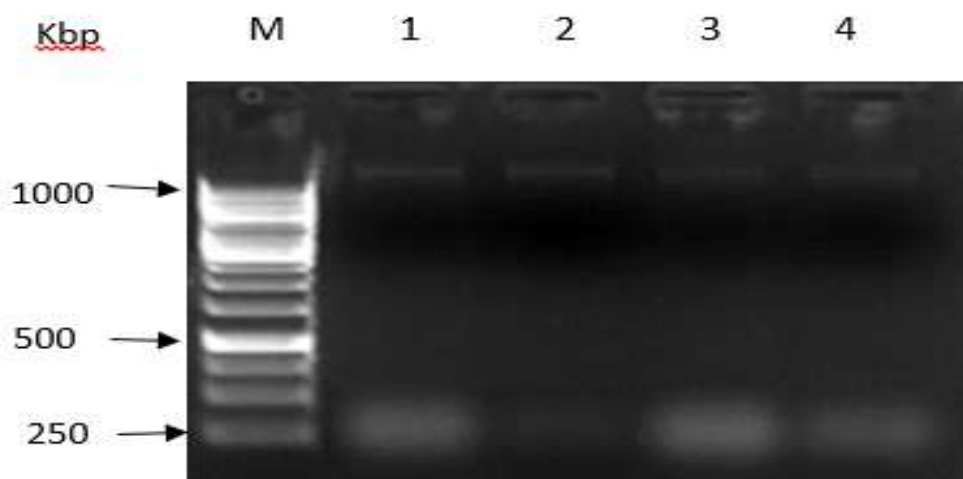


Figure 22: PCR products in the planted section. (Lane M represents a DNA marker, Lane 1-4 DNA in the samples collected after 0, 48, 96 and 144 hours respectively).

The PCR products obtained in the planted section indicated that the microbial communities were in high quantities in the Lane 1, which was the initial sample. The sulfate-reducing bacteria decreased with the decrease in sulfate availability and the increase in hydraulic retention time. This supported the fact that sulfate removal, sulfate competitors, macrophytes and oxygen negatively influenced sulfate-reducing population in the planted section, thus leading to their decrease. Figure 23 presents the different genera of sulfate-reducing bacteria in both sections of the hydroponic system.

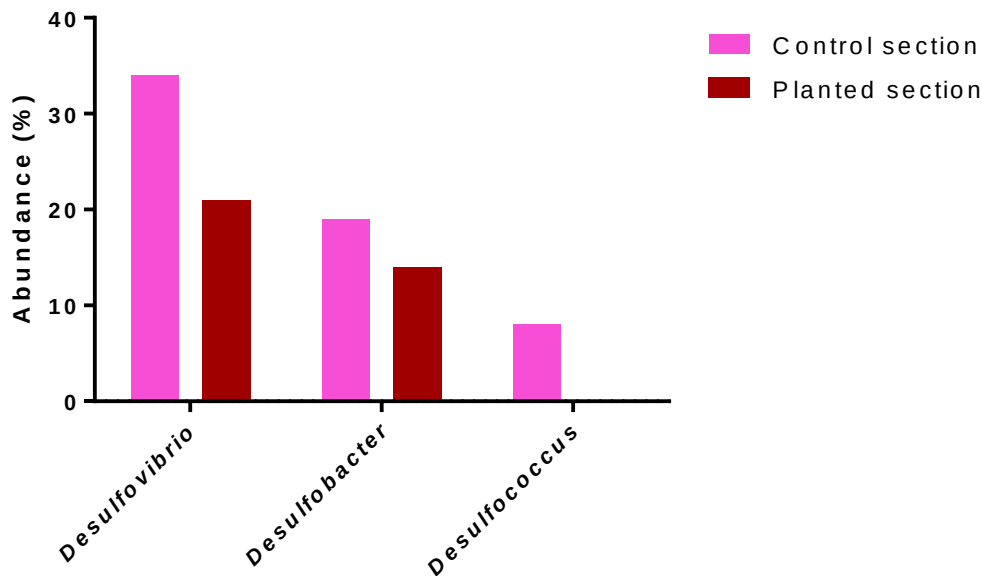


Figure 23: Microbial genera of sulfate-reducing bacteria in the hydroponic system.

Different genera of sulfate-reducing bacteria were present in both sections of the system. These genera included *Desulfovibrio*, *Desulfobacter*, and *Desulfococcus*. *Desulfococcus* was absent in the planted section due to its over-sensitiveness to oxygen while 8% of this strain bacteria were present in the control section. According to Cypionka *et al.* (2005), *Desulfococcus* is one of many oxygen sensitive strains of sulfate-reducing bacteria. Sulfate-reducing bacteria indicated abundant populations of the genus *Desulfovibrio* in both planted and control sections of the hydroponic system. It was 34% in the control section and 21% in the planted section. Figure 23 indicated that the percentages of *Desulfobacter* were 19% in the control section and 14% in the planted section. The findings in Figure 23 indicated that genera *Desulfovibrio* and *Desulfobacter* were able to survive under aerobic conditions. Dolla *et al.* (2006) reported that some strains of SRB have developed several defence strategies in order to survive exposure to oxygen such as enzymatic systems for reduction and elimination of oxygen. Oxygen reductases have an ability to oxidize sulfite with oxygen as an electron acceptor while forming ATP in the respiratory processes of sulfate-reducing bacteria. The

findings in Figure 23 indicated that the genera that were present in the hydroponic system have developed defence strategies against exposure to oxygen as reported by Dolla *et al.* (2006) who also reported that sulfate measured under aerobic conditions during daytime differed from sulfate removal measured at night and *Desulfovibrio* was present in the oxic environments due to its ability to withstand exposure to oxygen.

4.5 Conclusion

Based on the findings obtained, it can be concluded that the supply of different carbon source and temperature promoted the growth of sulfate-reducing bacteria and iron sulfide precipitation. The presence of different sulfate-reducing microorganisms, oxygen, and macrophytes led to a decrease in microbial population in the planted section and sulfate assimilation by plants might also have had an impact in the decline in sulfate-reducing bacteria in the planted section. The population dynamics of sulfate-reducing microorganisms in the control section did not decrease at the same rate as in the planted section. The presence of macrophytes reduced sulfate while decreasing microbial communities due to sulfate and carbon source starvation and oxygen availability since these microorganisms are anaerobic, while the absence of oxygen and macrophytes in the control section favoured the growth of sulfate-reducing bacteria. Sulfate-reducing bacteria were able to outcompete methanogens and utilized other carbon sources during sulfate starvation and increased in concentrations in the final samples collected in the control section due to the reduction in the competitive pressure and increase in carbon sources such as methane from sulfate oxidation. SRB population also increased in the planted section but at a lower rate compared to the control section due to macrophytes and oxygen presence.

4.6 Recommendations

It can be recommended that different carbon sources are to be added for nutrients provision to sulfate-reducing bacteria in the hydroponic system in order to facilitate their growth and activity. This will also lead to the increase in sulfate-reducing microorganisms, thus increasing sulfate removal. It can also be recommended that temperature be manipulated for the growth of sulfate reducing bacteria in order to obtain credible results.

4.7 References

- Achá, D., Iniguez, V., Roulet, M., Guimaraes, J.R.D., Luna, R., Alanoca, L. and Sanchez, S. (2005). Sulfate-reducing bacteria in floating macrophyte rhizospheres from an Amazonian floodplain lake in Bolivia and their association with Hg methylation. *Applied and Environmental Microbiology* **71**(11): 7531-7535.
- Ben-Dov E, Brenner A, Kushmaro A. (2007). Quantification of sulfate-reducing bacteria in industrial wastewater, by real-time polymerase chain reaction (PCR) using *dsrA* and *apsA* genes. *Microbial Ecology* **54**(3): 439-51.
- Cypionka, H., Widdel, F. and Pfennig, N. (2005). Survival of sulfate-reducing bacteria after oxygen stress, and growth in sulfate-free oxygen-sulfide gradients. *Federations of European Microbiological Societies for Microbiology Ecology* **1**(1): 45.
- Daly, K., Sharp, R.J. and McCarthy, A.J., 2000. Development of oligonucleotide probes and PCR primers for detecting phylogenetic subgroups of sulfate-reducing bacteria. *Microbiology* **146**(7): 1693-1705.
- Dar, S.A. (2007). Diversity and activity of sulfate-reducing bacteria in sulfidogenic wastewater treatment reactors (Doctoral dissertation, TU Delft, Delft University of Technology).
- Dolla, A., Fournier, M. and Dermoun, Z. (2006). Oxygen defense in sulfate-reducing bacteria. *Journal of Biotechnology* **126**(1): 87-100.
- D'Souza, G., Shitut, S., Preussger, D., Yousif, G., Waschina, S. and Kost, C. (2018). Ecology and evolution of metabolic cross-feeding interactions in bacteria. *Natural Product Reports* **35**(5): 455-488.
- Girguis, P.R., Cozen, A.E. and DeLong, E.F. (2005). Growth and population dynamics of anaerobic methane-oxidizing archaea and sulfate-reducing bacteria in a continuous-flow bioreactor. *Applied and Environmental Microbiology* **71**(7): 3725-3733.
- Gramp, J.P., Bigham, J.M., Jones, F.S. and Tuovinen, O.H. (2010). Formation of Fe-sulfides in cultures of sulfate-reducing bacteria. *Journal of Hazardous Materials* **175**(1-3): 1062-1067.

Icgen, B. and Harrison, S. (2006). Identification of population dynamics in sulfate-reducing consortia on exposure to sulfate. *Research in Microbiology* **157**(10): 922-927.

Karna, R., Hettiarachchi, G., Van Nostrand, J., Yuan, T., Rice, C., Assefa, Y. and Zhou, J. (2018). Microbial population dynamics and the role of sulfate-reducing bacteria genes in stabilizing Pb, Zn, and Cd in the terrestrial subsurface. *Soil Systems* **2**(4): 60.

Laue, H., Friedrich, M., Ruff, J. and Cook, A.M. (2001). Dissimilatory sulfite reductase (desulfoviridin) of the taurine-degrading, non-sulfate-reducing bacterium *Bilophila wadsworthia* RZATAU contains a fused DsrB-DsrD subunit. *Journal of Bacteriology* **183**(5): 1727-1733.

Liu, F., Zhang, G., Liu, S., Fu, Z., Chen, J. and Ma, C. (2018). Bioremoval of arsenic and antimony from wastewater by a mixed culture of sulfate-reducing bacteria using lactate and ethanol as carbon sources. *International Biodeterioration and Biodegradation* **126**: 152-159.

Meier, J., Piva, A. and Fortin, D. (2012). Enrichment of sulfate-reducing bacteria and resulting mineral formation in media mimicking pore water metal ion concentrations and pH conditions of acidic pit lakes. *Federation of European Microbiological Societies for Microbiology Ecology* **79**(1): 69-84.

Neubauer, S.C., Givler, K., Valentine, S. and Megonigal, J.P. (2018). Seasonal patterns and plant-mediated controls of subsurface wetland biogeochemistry. *Ecology* **86**(12): 3334-3344.

Niu, Z.S., Pan, H., Guo, X.P., Lu, D.P., Feng, J.N., Chen, Y.R., Tou, F.Y., Liu, M. and Yang, Y. (2018). Sulphate-reducing bacteria (SRB) in the Yangtze Estuary sediments: Abundance, distribution and implications for the bioavailability of metals. *Science of the Total Environment* **634**: 296-304.

Pereyra, L.P., Hiibel, S.R., Riquelme, M.P., Reardon, K.F. and Pruden, A. (2010). Detection and quantification of functional genes of cellulose-degrading, fermentative, and sulfate-reducing bacteria and methanogenic archaea. *Applied and Environmental Microbiology* **76**: 2192-2202.

Sigalevich, P., Baev, M.V., Teske, A. and Cohen, Y. (2000). Sulfate removal and possible aerobic metabolism of the sulfate-reducing bacterium *Desulfovibrio oxyclinae* in a chemostat

coculture with *Marinobacter* sp. strain MB under exposure to increasing oxygen concentrations. *Applied and Environmental Microbiology* **66**(11): 5013-5018.

CHAPTER 5: DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Introduction

Discarding of improperly treated industrial wastewater leads to high sulfate loads in natural water resources and groundwater (Geurts *et al.*, 2009). This negatively impacts the environment and human health, thus indirectly affecting the economy of the country. These challenges necessitate research in the development of wastewater treatment techniques that are environmentally friendly.

Constructed wetlands, therefore, can be used as a biological alternative for sulfate-rich water treatment due to their ability to use natural processes (that do not involve the use of chemicals) for sulfate degradation. The other advantages of constructed wetlands include less energy consumption, cost-effectiveness, and the simplicity of the systems that are easy to maintain and operate (Nelson *et al.*, 2003). Wetland technology also makes use of the systems that are designed to treat wastewater using biological processes associated with microbial communities, the substrate (soil and gravel) and macrophytes.

5.2 Sulfate removal and the mechanisms of removal from the hydroponic system

Sulfate was removed in both the planted and control sections and the final removal efficiency was 76% in the planted section and 56% in the control section. This indicated that the hydroponic system had the ability to remove sulfate from wastewater using the mechanisms of plant uptake and microbial degradation. The other mechanisms of sulfate removal in wetlands included chemical precipitation of metal sulfides and adsorption (Riggio *et al.*, 2018). Sulfate removal was also indicated by the reduced final concentrations of sulfate after treatment, which was 169 mg/l in planted section and 309 mg/l in the control section. These

findings suggested that the presence of macrophytes in the planted section improved sulfate removal as it was removed to levels below the acceptable amounts of sulfate in water (250 mg/l), while it was above the limit in the control section. Saidin *et al.* (2014) reported that the presence of macrophytes in wetlands intensifies sulfate removal through the supply of oxygen to the sulfate-reducing microbial communities within the macrophytes' rhizosphere in the roots. According to Thongnueakhaeng and Chaiprasert (2015), oxygen inhibited the growth of sulfate-reducing bacteria. This was due to the fact that sulfate-reducing bacteria are anaerobic microorganisms. The findings in this study showed that some strains of sulfate-reducing bacteria can survive under aerobic conditions. These findings are within the limits of constructed wetlands since Albalawneh *et al.* (2016) reported that wetlands are up 98% efficient in COD removal and 98.5% in sulfate removal. The mechanisms of sulfate removal were dependent on the physicochemical parameters.

5.3 Effects of physicochemical parameters on sulfate removal

The effect of pH, temperature, dissolved oxygen and COD on sulfate removal was evaluated in this study. The hydroponic system was located in Vulindlela Wastewater Treatment Plant and the environmental conditions were not controlled. The increasing dissolved oxygen and pH positively affected sulfate removal in the system, but DO was decreasing in the control section. *Bidens pilosa* L showed the potential to survive in the presence of sulfate contaminated water without the inhibition of growth. Geldenhuys (2003) reported that acidic pH below 5.5 is toxic to aquatic plants and corrosive to the water pipeline. The findings obtained in this study indicated that acidic pH did not negatively impact the hydroponic system and macrophytes. However, the relationship between sulfate removal and the temperature was inversely proportional. This indicated that the optimum temperatures of

this study were not favourable to the biological processes of sulfate removal. This study was conducted in winter. According to Chao *et al.* (2014) microbial-related and plant-mediated degradation processes of sulfate removal tend to be more effective in summer than in winter. Sawicka *et al.* (2012) also reported that the optimum temperature for mesophilic sulfate-reducing microorganisms is between 18°C and 40°C. While the relationship between COD and sulfate removal was also inversely proportional. This was indicated by the decreasing sulfate concentrations with the decrease in COD.

5.4 Population shift and dynamics of sulfate-reducing bacteria

Microbial population of sulfate-reducing bacteria indicated high abundance in the control section than in the planted section. *Desulfovibrio* is the strain of sulfate-reducing bacteria that was abundant in both sections. The strains of SRB that were present in both systems were *Desulfovibrio* and *Desulfobacter*. *Desulfococcus* was present in the control section but absent in planted section due to its extreme sensitiveness to oxygen. The microbial population in both sections was initially present at high levels but decreased with the depletion of carbon source and sulfate, increase in hydraulic retention time and competitive pressure and increased again towards the end of water treatment. The increase in the microbial population towards the end of water treatment was due to the reduction of competitive pressure, utilization of other available carbon sources and the fact that sulfate-reducing bacteria were able to outcompete other microorganisms such as methanogens while in the planted section they increased but not at the same rate as in the control section as they were reduced by the presence of oxygen when utilizing available carbon sources and increasing towards the end of water treatment. This negatively affected some sulfate reducing bacteria since they are obligate anaerobes.

5.5 Conclusion

The hydroponic system had a potential to remove sulfate from wastewater up to the acceptable levels except in the control section. The activity of sulfate-reducing microbial populations and macrophytes contributed to the high sulfate removal in the planted section. Meanwhile, the cold temperatures during the winter were not favourable for sulfate removal processes and may have interfered with activities of macrophytes and microorganisms. As established, the increase in dissolved oxygen and pH positively influenced sulfate removal while COD decreased with the decrease in sulfate concentrations. Population dynamics of sulfate-reducing bacteria were initially high in both sections of the hydroponic system but declined with the increase in hydraulic retention time and other factors like competitive pressure, carbon source and sulfate availability and oxygen presence, which was the main cause of the decline of the SRB population in the planted section.

5.6 Recommendations

In order to minimize sulfate implications on human health and the environment, it is recommended that people are educated about the detriments of high sulfate concentrations consumption, and how to remediate sulfate contaminated water. It can also be recommended that physicochemical parameters are optimized (especially temperature) with the prolonged hydraulic retention time. There should also be a provision for the carbon source for sulfate-reducing microorganisms (as a positive control) in the continual studies suggested to be carried out during summer because of favourable temperatures. The future studies should also include identification of other sulfate-reducing microorganisms. This will aid in understanding the interaction within the populations and communities of sulfate-reducing microorganisms.

5.7 References

- Albalawneh, A., Chang, T.K., Chou, C.S. and Naoum, S. (2016). Efficiency of a horizontal sub-surface flow constructed wetland treatment system in an arid area. *Water* **8**(2): 51.
- Chao, W., Zheng, S., Wang, P.F. and Jin Q.I. (2014). Effect of vegetation on the removal of contaminants: A review. *Journal of Hydrodynamics* **26**(4): 502.
- Geldenhuys, P. (2003). An integrated limestone/lime process for partial sulphate removal. *Journal of the Southern African Institute of Mining and Metallurgy* **103**(6): 345-353.
- Geurts, J.J., Sarneel, J.M., Willers, B.J., Roelofs, J.G., Verhoeven, J.T. and Lamers, L.P. (2009). Interacting effects of sulphate pollution, sulphide toxicity and eutrophication on vegetation development in fens: a mesocosm experiment. *Environmental Pollution* **157**(7): 2072-2081.
- Nelson, M., Alling, A., Dempster, W.F., Van Thillo, M. and Allen, J. (2003). Advantages of using subsurface flow constructed wetlands for wastewater treatment in space applications: Ground-based mars base prototype. *Advances in Space Research* **31**(7): 1799-1804.
- Riggio, V.A., Ruffino, B., Campo, G., Comino, E., Comoglio, C. and Zanetti, M. (2018). Constructed wetlands for the reuse of industrial wastewater: A case-study. *Journal of Cleaner Production* **171**: 723-732.
- Saidin, Z.H., Tajuddin, R.M., Kamarun, D. and Rahman, N.A. (2014). Performance of aquatic macrophytes on removal and accumulation of sulfate and potassium from domestic wastewater. *In Centre for Industry Education Collaboration*: 769-781.
- Sawicka, J.E., Jørgensen, B.B. and Brüchert, V. (2012). Temperature characteristics of bacterial sulfate removal in continental shelf and slope sediments. *Biogeosciences* **9**(8): 3425-3435.
- Thongnueakhaeng, W. and Chaiprasert, P. (2015). Effect of Dissolved Oxygen Concentrations on Specific Microbial Activities and Their Metabolic Products in Simultaneous Sulfur and Nitrogen Removal. *International Journal of Environmental Science and Development* **6**(4): 235.

APPENDICES

Appendix 1: Physicochemical parameters and sulfate removal in a hydroponic system.

Planted section

Sampling periods	pH	Temperature (°C)	Sulfate concentration (mg/l)
0	5.03	22.0	705
24	6.61	26	670
48	6.3	24.2	630
72	6.5	25	585
96	6.0	21	526
120	7.2	25	460
144	7.0	23.8	390
168	6.8	22.4	316
192	6.5	21.6	256
216	6.8	23	201
240	6.0	20.9	190
264	5.42	24.7	184
288	5.85	21.6	169

Control section

Sampling periods	pH	Temperature (°C)	Sulfate concentration (mg/l)
0	5.03	22.0	705
24	5.35	21.7	684
48	6.15	23.6	659
72	6.17	24.1	620
96	6.22	26.2	590
120	6.34	25.1	577
144	6.1	23.4	515
168	6.6	22.9	460
192	6.9	22	409
216	7.4	23.1	370
240	6.5	24.6	330
264	6.4	21	316
288	5.8	20.8	309

Appendix 2: Sulfate concentrations in macrophytes before and after treatment.

Sulfate concentration in mg/l (before treatment)	Sulfate concentration in mg/l (after treatment)
110	350
108	356
112	352

Appendix 3: Sulfate-reducing bacteria in the hydroponic system.

Keys: + Present

- Absent

Microorganisms and their percentages	Control section	Planted section
<i>Desulfovibrio</i>	+ (34%)	+ (21%)
<i>Desulfobacter</i>	+ (19%)	+ (14%)
<i>Desulfococcus</i>	+ (8 %)	- (0%)