

**The Use of Design Expert in Evaluating The Effect of pH,
Temperature and Hydraulic Retention Time on Biological Sulphate
Reduction in a Down-Flow Packed Bed Reactor**

by

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The Use of Design Expert in Evaluating The Effect of pH, Temperature and Hydraulic Retention Time on Biological Sulphate Reduction in a Down-Flow Packed Bed Reactor

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Abstract

Biological sulphate reduction (BSR) has been identified as a promising alternative technology for the treatment of acid mine drainage. BSR is a process that uses sulphate reducing bacteria to reduce sulphate to sulphide using substrates as nutrients under anaerobic conditions. The performance of BSR is dependent on several factors including substrate, pH, temperature and hydraulic retention time (HRT).

In a quest to find a cost effective technology, Mintek conducted bench-scale tests on BSR that led to the commissioning of a pilot plant at a coal mine in Mpumalanga province, South Africa. This current study forms part of the ongoing tests that are conducted to improve Mintek's process. The purpose of this study was to investigate the robustness of Mintek's process and to develop a tool that can be used to predict the process' performance with varying pH, temperature and HRT.

Design Expert version 11.1.2.0 was used to design the experiments using the Box-Behnken design. In the design, pH ranged from 4 to 6, temperature from 10 °C to 30 °C and HRT from 2 d to 7 d with sulphate reduction efficiency, sulphate reduction rate and sulphide production as response variables. Experiments were carried out in water jacketed packed bed reactors that were operated in a down-flow mode. The reactors were packed with woodchips, wood shaving, hay, lucerne straw and cow manure as support for sulphate reducing bacteria (SRB) biofilm. Cow manure and lucerne

pellets were used as the main substrates and they were replenished once a week. These reactors mimicked the pilot plant.

The data obtained were statistically analysed using response surface methodology. The results showed that pH did not have a significant impact on the responses ($p>0.05$). Temperature and HRT, on the other hand, greatly impacted the process ($p<0.05$) and the interaction between these two factors was found to be strong. Sulphate reduction efficiency and sulphate reduction rate decreased by over 60 % with a decrease in temperature 30 °C to 10 °C. Generally, a decrease in sulphide production was observed with a decrease in temperature. Overall, a decrease in HRT resulted in a decline of sulphate reduction efficiency and sulphide production but favoured sulphate reduction rate.

This study demonstrated that Mintek's process can be operated at pH as low as 4 without any significant impact on the performance. This decreases the lime requirements and sludge production during the pre-neutralisation stage by close to 50 %. There was, however, a strong interaction between temperature and HRT which can be used to improve the performance especially during the winter season.

Keywords: Acid mine drainage, Biological sulphate reduction, Sulphate reducing bacteria, Design Expert, Response Surface Methodology

DECLARATION

I, **Mukhethwa Judy Mukwevho**, hereby declare that this dissertation is my own original work (citation is provided where people's work has been used) and has never been submitted for another degree at this or other institutions.

Mukwevho MJ

Signature of candidate

10/09/2020

Date

DEDICATION

To my parents

Matodzi Milton Mukwevho and Thinamaano Mercy Mukwevho

and

To my siblings

Rendani Sydney Mukwevho and Mukovhe Clinton Mukwevho

For their love, support, prayers and constant encouragement throughout this journey.

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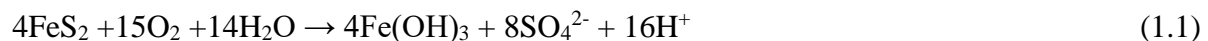
LIST OF ABBREVIATIONS

AMD	Acid Mine Drainage
BSR	Biological Sulphate Reduction
SRB	Sulphate Reducing Bacteria
MPB	Methane Producing Bacteria
HRT	Hydraulic Retention Time
ALD	Anoxic Limestone drains
OLC	Open Limestone Channel
ABC	Alkali-Barium-Calcium
PSS	Primary Sewage Sludge
RSBR	Recycling Sludge Bed Reactor
IMPI	Integrated Managed Passive
COD	Chemical Oxygen Demand
PSS	Primary Sewage Sludge
EPS	Extracellular Polymeric Substances
RSM	Response Surface Methodology
DoE	Design of Experiments
ANOVA	Analysis of Variance
OA	Orthogonal Array
SOB	Sulphide Oxidising Bacteria
LFCR	Linear Flow Channel Reactor
OTU	Operational Taxonomic Units

CHAPTER 1: INTRODUCTION

1.1. Background

High sulphate concentration, heavy metal-laden and acidic effluents are a result of many industrial processes. Of all the industrial processes, mining is the major contributor to these effluents. The phenomenon associated with the formation of such effluents is known as acid mine drainage (AMD). AMD may form in underground workings of deep mines that are in operation and those that are abandoned. Mines in operations mostly contribute little to this problem as the water is constantly pumped out in order to keep the water table at a lower level (Fernando et al., 2018, Johnson and Hallberg, 2005a). When mining operations stop, the pumping also stops allowing the exposed sulphide mineral to come into contact with water completing the oxidation process (Bijmans, 2008). AMD is formed by the oxidation of a sulphide bearing mineral such as pyrite in the presence of oxygen and water as shown in Equation (1.1) (Bwapwa et al., 2017). The oxidation process is mediated by acid-tolerant bacteria (Kuyucak, 2002). The oxidation process leads to the formation of acid which reacts with the country rock and leach out elements and also increases the solubility of heavy and toxic metals (Equeenuddin et al., 2010, McCarthy, 2011).



Sulphate concentration in AMD in South Africa is above 2000 mg/L and pH below 3. When AMD seeps into freshwater bodies, it has negative impacts on humans and aquatic organisms (Simate and Ndlovu, 2014). High sulphate concentration is known to have laxative effects in humans (Luptakova et al., 2013) and can also cause acute and chronic diseases (Kefeni et al., 2017). The recommended wastewater discharge sulphate concentration in South Africa is below 600 mg/L and the World Health Organization (WHO) recommends 250 mg/L for drinking water (Arnold et al., 2016b).

The treatment and management of AMD is one of the biggest environmental challenges facing the mining industry. In a quest to find new technologies to minimize the impacts caused by AMD,

research on the treatment of AMD has begun to gain momentum. Treatment methods can be divided into two processes namely active and passive processes. Active processes are those processes that require regular maintenance and are generally considered to be costly (Seervi et al., 2017). One such a process is the chemical treatment using neutralising agents such as hydrated lime, caustic soda and sodium hydroxide. Although this method can increase the pH and precipitate metals, it does not reduce sulphate concentration (Arnold et al., 2016b). Other examples of active processes include ion exchange, reverse osmosis and electro-dialysis. These methods are effective but require pre-treatment (Luptakova et al., 2013) and have limitations such as not being able to handle high metal content and generate waste brine that needs to be disposed of (Saha and Sinha, 2018).

Passive treatment processes are known to be cost-effective and sustainable as they require minimal to no maintenance and can operate for years (Skousen et al., 2018). A semi-passive process that has received attention over the years is the biological sulphate reduction (BSR) due to its sustainability and cost-effectiveness. BSR is a process that uses sulphate reducing bacteria (SRB), in anaerobic conditions, to reduce sulphate to sulphide while simultaneously removing metals as metal sulphides. The reduction process produces alkalinity which increases the pH. Like any other microbes, SRB require substrates to use as nutrients. Substrates used by SRB can be categorized as simple organic compounds and complex organic compounds. Simple organic compounds include ethanol, methanol, acetate, lactate and butyrate (Liamleam and Annachatre, 2007), and complex organic compounds include woodchips, manure and food waste (Sheoran et al., 2010). Although simple organic compounds are readily available for use by SRB, they are costly (Zagury et al., 2007) and therefore more BSR studies have been leaning towards using complex organic compounds as substrates (Salo et al., 2017, Zhang and Wang, 2014, Choudhary and Sheoran, 2011, Dev et al., 2015). The performance of BSR is dependent on the activity of SRB which is affected by several factors including pH, hydraulic retention time (HRT), substrate availability, temperature, redox potential and solid support for SRB growth (Sheoran et al., 2010). For this reason, it is important to consider these factors when designing a system to treat AMD biologically. Studies have been done to optimize and investigate the effect of different factors on sulphate reduction using design of experiments (DoE) (Dev et al., 2017, Dev et al., 2015, Najib et al., 2017). DoE is a systemic, rigorous approach to engineering problem-solving that applies principles and

techniques at the data collection stage so as to ensure the generation of valid, defensible and supportable engineering conclusions (NIST/SEMATECH, 2013). In this study, Design Expert software was used for designing the experiments using the Box-Behnken design. Response surface methodology (RSM) was used to statistically analyse the data. RSM is used for the estimation of interactions and quadratic effects on the response under investigation. RSM can also be used to make the process robust (NIST/SEMATECH, 2013).

This work forms part of an ongoing project by Mintek. Mintek piloted their BSR process at a coal mine in eMalahleni, Mpumalanga province, South Africa (Neale et al., 2017). This study was done to investigate what impact pH, temperature and HRT has on their process to test how robust it is. The experiments were carried out in lab scale reactors that mimicked the pilot plant. The results found will be used to improve Mintek's BSR process.

1.2. Aims

- To determine the effect of pH, temperature and hydraulic retention time on biological sulphate reduction.
- To develop mathematical models that can be used to understand the relationship and predict the impact of the above-mentioned factors
- Observe the amount of lime needed to adjust the AMD to different pH and the respective sludge produced.

1.3. Methodology

Design-Expert® Software (version 11.1.2.0, Stat Ease Inc., USA) was used to matrix pH (4 to 6), temperature (10 to 30 °C) and HRT (2 to 7 days) using the Box-Behnken design with a triplicate at the centre points. The Box-Behnken design was chosen because it is efficient and more economical (Bezerra et al., 2008). Once all the data was collected, response surface methodology was used to statistically analyse the data and to understand the individual effect and interaction between all the factors on sulphate reduction efficiency, sulphate reduction rate and sulphide production.

1.4. Main findings

In the present study, pH as low as 4 was found to have no significant impact on the process which was verified by analysis of variance. The effects of temperature and HRT on the process were significant. The interactions between HRT and temperature were stronger than those between temperature and pH and HRT and pH. Lower retention times required an increased frequency of substrates replenishment in order to keep sulphate reduction above 80 % at higher temperatures. The lime required to pre-neutralise AMD to pH 4 and the sludge formed was almost 50 % less than that observed at pH 5.

CHAPTER 2: LITERATURE REVIEW

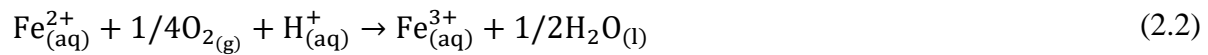
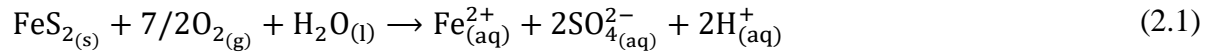
2.1. Acid mine drainage

Mining is one of the most important sectors in South Africa. It is without a doubt that most of the economic activities in South Africa are centred around mining activities in a nation with many minerals (Akcil and Koldas, 2006). The mining sector has been one of the main contributors to South Africa's economy and is the world's largest producer of chrome, gold, coal and platinum group metals (PGMs) (Langenhoven, 2017). The country is known to have one of the most sophisticated and developed mining industries in the world (Akcil and Koldas, 2006).

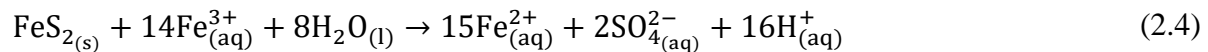
Acid mine drainage (AMD) is a widespread problem which is mainly caused by current or past mining activities (Akcil and Koldas, 2006, Equeenuddin et al., 2010). AMD is not only caused by mining activities, but mining activities are major contributors to this problem. Acidic, sulphur-rich and heavy metal laden wastewater are by-products of a variety of industrial operations such as galvanic processing, the scrubbing of flue gas at power stations. AMD is a major cause of environmental pollution and it has been a problem in South Africa and the world as a whole (Equeenuddin et al., 2010, Johnson and Hallberg, 2005a).

AMD is produced in a two stage process when a sulphide-bearing mineral such as pyrite (FeS_2 , also known as the fool's gold), chalcopyrite (CuFeS_2), sphalerite (ZnS), galena (PbS), to mention a few, are exposed to oxygen and water (Akcil and Koldas, 2006). From the above-mentioned sulphide minerals, pyrite is the most abundant mineral in the planet and it tends to be associated with the formation of AMD (Johnson and Hallberg, 2005a).

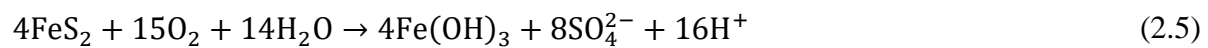
The first stage produces sulfuric acid and ferrous sulphate (McCarthy, 2011). The first oxidative reaction converts pyrite to dissolved ferrous ions (Fe^{2+}) and the two equivalent units of aqueous sulfuric acid (H^+ and SO_4^{2-}) increasing total dissolved solids and a decreasing pH, Equation (2.1). Depending on the oxygen concentration (dissolved or atmospheric), ferrous ion is further oxidized to produce ferric ions (Fe^{3+}) as shown in Equation (2.2) (Akcil and Koldas, 2006, McCarthy, 2011).



The second stage produces an orange-red ferric hydroxide and more sulfuric acid. At pH values between 2.3 and 3.5 ferric ion precipitates as ferric hydroxide ($\text{Fe}(\text{OH})_3$) while simultaneously lowering the pH as shown in Equation (2.3) (Akcil and Koldas, 2006). The remaining ferric ions that do not precipitate is used to further oxidize pyrite as shown in Equation (2.4).



The oxidation of pyrite is sometimes summarized as shown in Equation (2.5) which is a combination of Equation (2.1) to (2.3).



2.2. Impacts of AMD

The characteristics of AMD may differ from site to site depending on what is being mined (McCarthy, 2011). AMD is mostly characterized by low pH, high concentrations of heavy metals (such as iron, manganese, arsenic and aluminium), high concentration of sulphate and other toxic metals/substances. Due to the abovementioned characteristics, AMD can contaminate groundwater, surface water and soils severely (Akcil and Koldas, 2006). The severity of AMD impacts varies widely and is very much dependent on local conditions such as geomorphology, climate and the extent of AMD distribution (McCarthy, 2011).

Although mining and mineral processing industries create jobs in South Africa, this country is water-scarce and mine drainage affects its water resources negatively. Low dilution potential due

to low rainfall worsens salinity of water resources. AMD does not only affect water resources but also has an impact on sustainability parameters including ecological, social and economic concerns (Feris and Kotze, 2015).

The risk of exposure of AMD on human health is not adequately known, however, due to heavy metals concentration in AMD, they may accumulate in the biological food chain resulting in acute and chronic diseases (Simate and Ndlovu, 2014, Kefeni et al., 2017). Heavy metals are persistent in the environment and as a result, they contaminate the soil and accumulate in plant tissues which affect their growth (Simate and Ndlovu, 2014, Naidu et al., 2019). Aquatic organisms require pH between 6 and 9 so that they can perform their physiological functions, such as respiration, effectively (Simate and Ndlovu, 2014, Taylor et al., 2005). When AMD enters freshwater systems, it affects aquatic life as it significantly decreases the pH of freshwater bodies.

The sulphate concentration in AMD is a major problem due to its presence in high concentrations. In South Africa, the required sulphate discharge concentration is 200 – 600 mg/L and the World Health Organization (WHO) recommends 250 mg/L for drinking water (Arnold et al., 2016b). High sulphate concentration may have laxative effects on humans (Luptakova et al., 2013) and high sulphate concentration in industrial effluents may form complexes that destroy pipelines and crack concrete tanks (Christoe, 1976).

2.3. Treatment of AMD

Due to the impacts mentioned above, AMD remains a subject of interest. Although there are many technologies that have been implemented, each treatment technology may be unique to a specific type of AMD for different discharge standards (Arnold et al., 2016b). The treatment and management of different AMD effluents have been investigated at laboratory, pilot and even commercial scales (Gibert et al., 2002). However, some of these technologies were only tested in laboratory scale and therefore their effect not proven for full-scale implementation.

AMD can be treated in various ways. A more conventional way of treating AMD is the application of a chemical neutralising agent (such as lime, slaked lime, calcium carbonate, sodium hydroxide

and magnesium oxide and hydroxide) to adjust the pH and simultaneously precipitating some heavy metals (Johnson and Hallberg, 2005a, Raymund, 1947). In South Africa, lime neutralization is the most common treatment method applied. However, this method is not sustainable over a long term as it produces large quantities of sludge that may require further treatment and it is difficult to dispose of. Lime neutralization successfully removes the metals and increases pH but does not reduce the amount of sulphate significantly as the effluents contain high levels of sulphate between 1500 and 2000 mg/L (Arnold et al., 2016b). Moreover, neutralised AMD still contains a high concentration of dissolved salts which makes it unfit for human consumption, unusable for industrial applications and results in a harsh environment for aquatic life (Smit, 1999).

AMD remediation can be classified as abiotic and biotic which can be further be sub-divided into active and passive systems (Johnson and Hallberg, 2005a). Abiotic remediation entails the use of a chemical neutralising agent whereas biotic remediation uses microorganisms for the addition of alkalinity (Johnson and Hallberg, 2005a). Active systems refer to continual reagent, energy and labour inputs to neutralise acidic mine waters, precipitate metals, while passive treatment only requires occasional maintenance as they make use of natural and constructed wetlands (Johnson and Hallberg, 2005a, Taylor et al., 2005) and they require little human intervention. Passive systems are generally more economically attractive compared to active system but they have limitations such as not being able to handle acidity loads in excess of 100 – 150 kg CaCO₃ per day and they may not be able to achieve a specific metal reduction target (Taylor et al., 2005).

2.3.1. Active systems

2.3.1.1. Chemical precipitation

Treatment by chemical precipitation involved the addition of a chemical neutralising agent such as limestone, calcium carbonate, sodium hydroxide, caustic soda, magnesium hydroxide, ammonia and pebble quick lime which simultaneously precipitate some metal and increases the pH of AMD (Johnson and Hallberg, 2005a). However, this method produces large sludge quantities that are difficult to dispose of and does not significantly reduce sulphate (Arnold et al., 2016b).

2.3.1.2. Ion exchange

Ion exchange is a reversible process that involves the exchange of ions between an aqueous solution and cationic or anionic ion exchange resin. A regenerant such as NaCl and HCl can be used to regenerate spent resins for reuse. This process is mostly used to soften hard water and has advantages including 98 % water recovery (Saha and Sinha, 2018). However, this process may not be suitable for high sulphate concentrations, requires pre-treatment which makes it costly and large amounts of the regenerant that contain contaminants that are used have to be disposed of (Clifford et al., 2011, Saha and Sinha, 2018). Howard et al. (2009) developed a patented ion exchange process to treat AMD from a South Africa gold mine which was tested on pilot scale level.

2.3.1.3. Natural zeolites

Zeolites are naturally occurring hydrated aluminosilicate materials that, like ion exchange, are used to soften water and they have exceptional sorption and ion exchange properties (Clifford et al., 2011, Margeta et al., 2013). Natural zeolites are shown in literature to be used for the adsorption of heavy metals such as copper, iron, zinc, manganese and nickel in acid mine drainage (Ciosek and Luk, 2017, Markovic et al., 2015, Motsi et al., 2009). Zeolites are very effective in removing metals (efficiency > 80%) in wastewater and they are resistant to chemical, thermal, biological and mechanical changes (Margeta et al., 2013, Motsi et al., 2009).

2.3.1.4. Reverse osmosis

Reverse osmosis involves the use of membranes where salts and metal ions are removed by the membranes. Reverse osmosis has been investigated as an alternative treatment for acid mine drainage. Although this method is efficient, it is suitable for water with low metal concentration, membranes foul quickly and may require pre-treatment which makes the process costly (Saha and Sinha, 2018).

2.3.1.5. Electro-dialysis

Electro-dialysis is an electric potential driven membrane process that is used to remove ions using cation- and anion-selective membranes (Edzwald, 2010). A study conducted by Buzzi et al. (2013) showed that electro-dialysis with AMD containing iron resulted in precipitation on the cation membrane causing blockage and therefore reducing the efficiency. Although this process has high

water recovery and sulphate removal efficiencies (>97%), it requires pretreatment and cannot handle AMD with high iron content (Buzzi et al., 2013, Saha and Sinha, 2018).

2.3.2. Passive systems

2.3.2.1. Aerobic wetlands

Aerobic wetlands are shallow ponds that provide settling for suspended solids. These ponds have vegetation planted to improve wildlife habitat, aesthetics and to promote slow flow and attachment sites for flocs (Skousen et al., 2017, Taylor et al., 2005). Aerobic wetlands do not neutralise AMD, but they only provide aeration and residence time for metal precipitation (AMD). Due to their inability to neutralise AMD, aerobic wetlands must receive net-alkaline water that can neutralise the acidic AMD, however, limestone has to be added to generate net-alkaline conditions which improve long-term effectiveness (Skousen et al., 2017). Although aerobic wetlands are effective in removing Fe, they fail to sufficiently remove manganese (Taylor et al., 2005).

2.3.2.2. Anaerobic wetlands

Anaerobic wetlands are relatively deep water retention ponds that use organic matter such as spent mushroom, compost, hay and sawdust which strip oxygen from the water resulting in anaerobic conditions (Skousen et al., 2017, Taylor et al., 2005). These organic materials are mostly mixed with limestone to help with the generation of alkalinity. In anaerobic wetlands, sulphate reducing bacteria are present and they use the organic material as substrates and also assist in the generation of alkalinity which neutralises AMD. Although anaerobic wetlands have more enhanced treatment mechanisms, they are more suitable for treating small amount of AMD with low metal and acidic loads. They also require maintenance to replenish substrates as they get consumed and may be covered by metal precipitates (oxyhydroxides) which reduces efficiency (Skousen et al., 2017).

2.3.2.3. Vertical flow wetlands

Vertical flow wetlands are also referred to as successive alkalinity producing systems (SAPS) or reducing and alkalinity producing systems (RAPS) (Kepler and McCleary, 1997, Watzlaf et al., 2000). The treatment mechanism is similar to that of anaerobic wetlands, however, in vertical flow wetlands, AMD flows vertically and the hydraulic head forces AMD through the organic substrates

(Skousen et al., 2018). SAPS require high capital costs and they have to be continually monitored as they are more likely to clog due to substrate compaction (Taylor et al., 2005, Watzlaf et al., 2000).

2.3.2.4. Anoxic limestone drains (ALD)

ALDs are below the ground limestone trenches in which anoxic water is introduced (Seervi et al., 2017, Skousen and Ziemkiewicz, 1996). To prevent the oxidation of soluble ferrous iron to insoluble ferric species, ALDs are kept anoxic by means of capping the limestones with clay or plastic sheeting (Watzlaf et al., 2000). ALDs are effective for a specific kind of AMD, that is, AMD should not contain Fe^{3+} or Al as they precipitate as Fe and Al hydroxides respectively. This leads to the clogging of limestone and therefore decreasing the performance of ALDs, however, if working properly, should achieve pH of about 6 (Skousen and Ziemkiewicz, 1996).

2.3.2.5. Open limestone channel (OLC)

These are open channels that make use of coarse limestone. When AMD is introduced, limestone increases the alkalinity and raises the pH to between 6 and 8 while Fe, Al and other metals are being precipitated as hydroxides (Seervi et al., 2017). Due to limestone armouring with hydroxides, these channels' efficiency is reduced and as a result makes use of large amounts and more coarse limestone compared to ALD (Taylor et al., 2005).

2.3.2.6. Limestone diversion wells

Limestone diversion wells are cylindrical concrete tanks that were initially developed to treat acidic water from acid rain in Sweden (Arnold, 1991, Skousen et al., 2017). Water is fed to the well from an upstream dam that should have a hydraulic head of about 2.4 m (Arnold, 1991). Although diversion wells are effective in increasing pH and precipitating metals, they treat small flows and they require maintenance to replenish limestone and to remove debris (Schmidt and Sharpe, 2002).

2.3.2.7. Bioreactors

Over the years, biological sulphate reduction (BSR) using sulphate reducing bioreactors has been identified as a promising technology for the treatment of AMD. BSR uses sulphate reducing bacteria (SRB) to reduce sulphate to sulphide under anaerobic conditions. SRB use sulphate as a

terminal electron acceptor and substrates as electron donors (Moosa et al., 2002). Sulphate reducing bioreactors have been used in lab scale, pilot scale and full field-scale operations (Gibert et al., 2002). These systems depend on sulphate reduction by SRB for the treatment and they have an advantage of being able to handle acidic and heavy metal-rich wastewaters (Skousen et al., 2017). The start-up of these bioreactors tends to be slow because the SRB have to adapt AMD, substrates and form a biofilm on the solid support (Neculita et al., 2010, Skousen et al., 2017). The successfulness of bioreactors depend on the SRB activity which is controlled by the availability of substrates (Neculita et al., 2010), therefore, substrates have to be added periodically to provide nutrients for SRB (Tsukamoto et al., 2004, Zamzow et al., 2006).

Bioreactors are classified based on the flow mode (batch, semi-continuous, continuous), substrate state (gas, liquid, solid), biomass retention (attached, suspended) and whether it is a single-stage or multistage, with single-stage bioreactors being a more attractive option because they reduce construction costs (Gómez and Lens, 2017). Figure 2.1 shows some of the types of bioreactors used in biological sulphate reduction.

These bioreactors are able to produce alkalinity that increases pH, reduce sulphate to sulphide which in turn precipitates metals as metal sulphides. The mechanisms of metal removal in bioreactors may change over time in bioreactors, however, the most desired one is sulphide precipitation (Neculita et al., 2010). Other metal removal mechanisms include adsorption and precipitation of metals as carbonates, oxyhydroxides and hydroxides (Luptakova et al., 2013, Neculita et al., 2010).

The performance of bioreactors depends on many factors such as pH, temperature, substrate availability, redox potential, solid support for SRB, hydraulic retention time, sulphate concentration, sulphide and metal concentration (Gómez and Lens, 2017, Skousen et al., 2017). These factors have to be taken into account during the design stage.

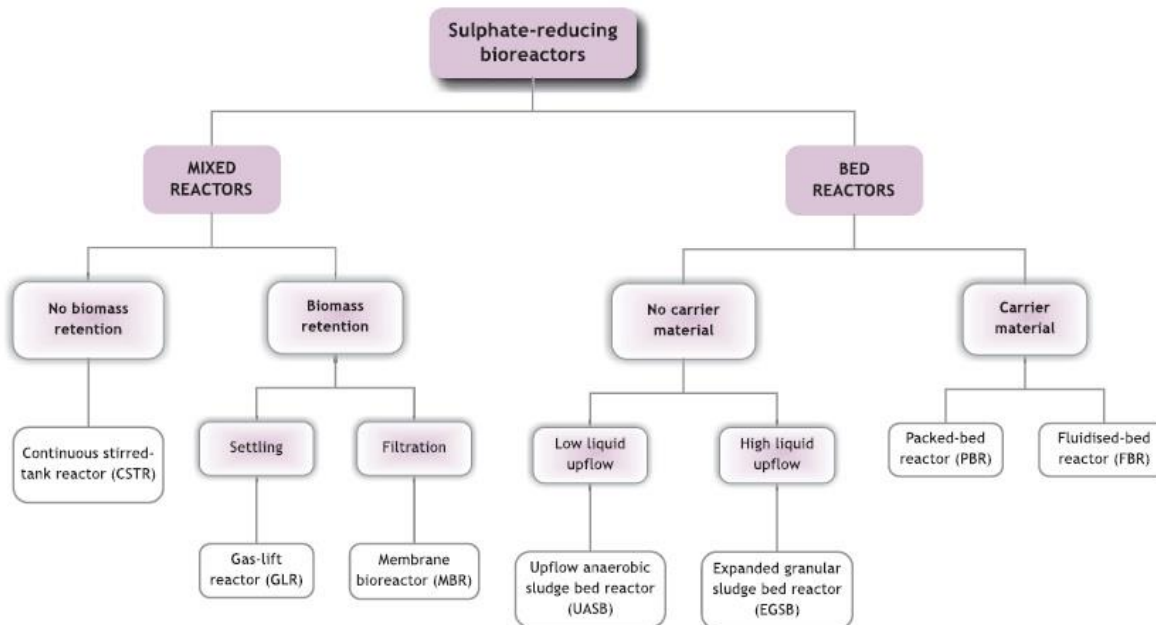


Figure 2.1: Bioreactors used in sulphate-reducing applications (Kousi et al., 2015)

2.3.3. AMD Treatment processes in South Africa

In a bid to combat the impacts of AMD in SA, various AMD treatment processes have been developed. SAVMIN, also known as the Ettringite process, is one of the processes that have been developed by Mintek in collaboration with Savannah Mining and The Wen group. The SAVMIN process involves the precipitation of metal hydroxides, calcium sulphate and ettringite ($3\text{CaO}\cdot 3\text{CaSO}_4\cdot \text{Al}_2\text{O}_3\cdot 31\text{H}_2\text{O}$) and it has been proposed by (Smit, 1999). The successfulness of this process relies on the regeneration and recycling of aluminium hydroxide which is used to precipitate ettringite. This process uses precipitation reactions to purify sulphate polluted water to produce water that is suitable for domestic, agricultural and industrial use. Gypsum ($\text{CaSO}_4\cdot 2\text{H}_2\text{O}$) and calcium carbonate (CaCO_3) are saleable by-products that are produced by this process. Although this process is able to produce portable water, it has a narrow operating pH band, complicated control and it requires huge amounts of lime which has cost implications. (DWAF, 2013).

Another process developed for the treatment of AMD is the GYP-CIX process which was developed by the Chemical Effluent Treatment Process Limited Company. This is a novel

desalination process that uses ion exchange resins to remove scaling ions such as sulphate, calcium and magnesium from pre-neutralised influent proposed by (Everett et al., 1993). This process has two stages with the first stage responsible for the removal of cations using cation exchange resins and the second stage removes anions using anion exchange resins. Both stages produce gypsum as a waste product. The exchange resins are regenerated in a batch reactor seeded with gypsum crystals to further improve the precipitation of gypsum which can be sold commercially (Bowell, 2004). A major disadvantage is the production of brine that may be difficult to dispose of and may require additional costs (INAP, 2003).

The Alkali-Barium-Calcium (ABC) process is another desalination process developed by the Council for Scientific and Industrial Research (CSIR). The feed water to this process is pre-treated with a neutralising agent such as lime, or calcium carbonate. The removal of sulphate is achieved by using barium carbonate (BaCO_3) which precipitates sulphate to levels below 200 mg/L as barium sulphate. This process consists of several process units which include AMD neutralisation using limestone, sulphate precipitation as gypsum using lime, sulphate precipitation as barite, filtration and disinfection, the recovery of BaSO_4 and Ca(OH)_2 from sludge and the production of elemental sulphur from the H_2S produced during the process at temperatures above 1000 °C. In addition to achieving low sulphate concentrations, some of the sludge produced during this process can be used as saleable by-products, therefore, reducing the amount of sludge that requires disposal. However, this process is incapable of removing monovalent ions from wastewater, it has not been demonstrated in large scale, it requires high thermal energy and it has high environmental risks due to parts of the process that involve operation at high temperatures (DWAF, 2013).

Table 2.1 shows biological technologies for the treatment of AMD that have been developed in South Africa.

Table 2.1: Biological technologies to treat AMD in South Africa (Harrison et al., 2014)

Process	Description
Rhodes BioSURE process	This process was developed at Rhodes University in the early 1900s with observations of enhanced degradation of complex organic wastes in sulphate reducing tannery ponds. Initially, this process relied on algal primary production to provide the electron donor for sulphate reduction. Primary sewage sludge (PSS) was later used as an electron donor and promising results were obtained which led to the development of the recycling sludge bed reactor (RSBR). Encouraging results from the RSBR led to a pilot plant design, then a demonstration scale plant followed by a full-scale plant at ERWAT Anchor sewage treatment works. This process was designed to remove sulphate to levels below 250 mg/L. However, the process was decommissioned due to utility changes.
Praques biological sulphate removal technology	This process was implemented in Witbank and made use of ethanol as the electron donor. A demonstration scale plant was commissioned and operated for several years. The plant's performance was encouraging with sulphate concentrations usually below 500 mg/L. Although this process' performance was relatively stable, there were challenges encountered including scaling of heat exchangers and high costs for the electron donor.
Integrated Managed Passive (IMPI) process	When IMPI was developed in 1995, it initially utilised lignocellulose material as an electron donor. The hydrolysis of lignocellulose was determined to be the rate-limiting. After more laboratory tests, a 4 stage process was established which made use of hay, woodchips and molasses as electron donors. The IMPI process consists of a degrading packed bed reactor, sulphide removal reactor, secondary sulphate removal reactor and a sulphide removal reactor. This process was designed to mainly increase the pH.

2.4. Sulphate reducing bacteria

SRB are a diverse group of prokaryotes that are considered the oldest and the most studied group of bacteria found in the environment (Wargin et al., 2007). This group of bacteria can be found in various environments including sediments, soil, geothermal vents (Kakooei et al., 2012, Sheoran et al., 2010) and humans and animals digestive tract (Kováč et al., 2018). The presence of SRB in the environment is detected by the production of H₂S which in turn precipitates iron as a black precipitate (Wargin et al., 2007).

In BSR, the performance is highly dependent of bacterial activity (Sheoran et al., 2010) and therefore, it is important to have an understanding of the role of the bacterial community in these systems for an improved design and performance (Johnson and Hallberg, 2005a, Sheoran et al., 2010). SRB use substrates as electron donors and sulphate ions as terminal electron acceptors under anaerobic conditions in BSR systems. Castro et al. (2000) classify SRB into 4 groups based on rRNA sequence analysis as follows:

- Gram-negative mesophilic SRB – This group of bacteria have various shapes including rod, oval, filamentous, spherical and coccoid and their growth temperature is between 20 °C and 40 °C. These bacteria belong to the genera *Desulfobulbus*, *Desulfomicrobium*, *Desulfomonas*, *Desulfovibrio*, *Desulfobacter*, *Desulfobacterium*, *Desulfococcus*, *Desulfomonile*, *Desulfonema*, *Desulfosarcina*.
- Gram-positive spore-forming SRB – Most of these bacteria have growth temperatures between 25 and 45 °C and some between 40 and 60 °C. They are known to form heat resistant endospores. This group of bacteria can be rod or straight to curve-shaped and they belong to the genus *Desulfotomaculum*.
- Bacterial thermophilic SRB – These are vibrioid to rod-shaped bacteria belonging to the genus *Thermodesulfobacterium* and have optimal growth temperatures between 65 and 70 °C, however, they can endure lower temperatures.
- Archaeal thermophilic SRB – These group of SRB are coccoid shaped have growth temperatures between 64 and 92 °C with the optimum temperature above 80 °C. This group of SRB belong to the genus *Archaeoglobus*.

2.5. Competition between SRB and MPB

In anaerobic systems, there is a competition between different groups of bacteria (Visser et al., 1993b) with the most common one being between methane producing bacteria (MPB) and sulphate reducers (Koschorreck, 2008). This competition between SRB and MPB is dependent on a number of factors including pH, temperature, substrate, undissociated sulphide concentration, reactor type, COD/SO₄²⁻ mole ratio, HRT and the physical structure of microbial cultures (Moon et al., 2015). The result of this competition determines the extent to which the end product (sulphide and methane) will be produced (Vallero, 2003). The recommended COD/SO₄²⁻ mole ratio for either sulphidogenesis and methanogenesis vary considerably in literature (Annachhatre and Suktrakoolvait, 2001b), however, it is generally known that low COD/SO₄²⁻ mole ratio (less than 5) favours sulphidogenesis and high COD/SO₄²⁻ mole ratio (greater than 6) favours methanogenesis (Choi and Rim, 1991, Mizuno et al., 1994). White and Gadd (1996) suggested that a COD/SO₄²⁻ mole ratio between 1 and 3 is favourable for SRB. Dar et al. (2008), on the other hand, reported that a COD/SO₄²⁻ mole ratio of 0.34 resulted in SRB outcompeting MPB. Due to the differences in COD/SO₄²⁻ mole ratios reported in literature, further investigations may be necessary to fully understand the competition between SRB and MPB (Moon et al., 2015).

Both SRB and MPB are capable of using acetate and hydrogen as substrate, however, when sulphate is available in excess, SRB utilises acetate and hydrogen and therefore outcompeting MPB (Oude Elferink et al., 1994).

As mentioned before, there is a pH dependent competition between SRB and methane producing bacteria (MPB) (Koschorreck, 2008, Visser et al., 1993a). At low pH (<6.5) and at higher pH (>8), SRB outcompetes MPB because MPB are more sensitive to pH (Lopes, 2007, Visser et al., 1993a). However, at near-neutral pH (6.5-8), they compete equally (Visser et al., 1993a). Contrarily, according to Gutierrez et al. (2009) and Omil et al. (1997), near-neutral pH favours SRB over MPB.

In a study done by Rintala and Lettinga (1992), it was found that MPB utilised acetate at 37 °C and SRB outcompeted MPB at 55 °C. Similarly, Visser et al. (1993b) showed that acetate was

mainly utilised by SRB at 55 °C. They also found sulphate reduction to be less sensitive to temperature increases than methanogenesis.

2.6. Factors affecting the performance of bioreactors

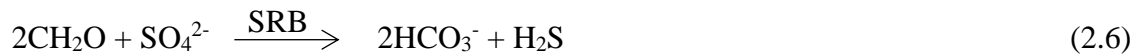
The efficiency of sulphate removal in bioreactors can be affected by many factors such as influent pH, temperature, metal concentration, carbon source/substrate concentration, hydraulic retention time, hydrogen sulphide concentration (Kaksonen and Puhakka, 2007), influent sulphate concentration and suitable nitrogen source (Dev et al., 2017). It is important to assess the effects of different operational parameters because in industry, reactors have high process stability but environmental changes such as temperature and pH changes can have severe impacts on reactor performance (Visser et al., 1993b) as these factors affect the activity of SRB (Tsukamoto et al., 2004).

Sulphate reduction in different types of bioreactors and using different kinds of carbon sources have been studied extensively (Choudhary and Sheoran, 2011, Jiménez-Rodríguez et al., 2010, Moloantoa, 2015, Salo et al., 2017, Zagury et al., 2007, Zhang and Wang, 2014). Some research have focussed on different carbon sources and how they affect sulphate reduction (Choudhary and Sheoran, 2011, Neale et al., 2017, Salo et al., 2017, Zhang and Wang, 2014), others have targeted their efforts toward the effect of hydraulic retention time (Jiménez-Rodríguez et al., 2010, Sipma et al., 2007, Zagury et al., 2007), some have been focussed on the effect of pH (Gutierrez et al., 2009, Moon et al., 2015, Omil et al., 1997, Sharma et al., 2014) and some have dedicated their focus to investigating the effect of temperature (Visser et al., 1993b, Baskaran, 2005, Rintala and Lettinga, 1992).

2.6.1. Substrate

Substrate, electron donor and carbon source are used almost interchangeably in literature to describe a source of energy required for growth by SRB. Choudhary and Sheoran (2011) define a carbon source as any type of carbon material which can be microbiologically degraded to simple organics that can be utilized by the SRB. The general reaction for this is shown in Equation (2.6) where CH_2O represents a simple source of organic carbon. The carbon source is oxidized by the

SRB to bicarbonate and hydrogen sulphide. The bicarbonate neutralises the acidity which in turn favours metal precipitation and the hydrogen sulphide reacts with metals to form insoluble metal sulphides, Equation (2.7) (Dvorak et al., 1992).



where M represents metals.

Substrates can be simple compounds and some are more complex organic waste materials used to provide carbon source and also to maintain the flow through the reactor (Sheoran et al., 2010). Some of the most widely used simple compounds are hydrogen, methanol, ethanol, acetate, lactate, sugar, butyrate, propionate, and molasses (Liamleam and Annachatre, 2007). Although some of these simple compounds such as ethanol, methanol and lactate are readily available to be used by SRB, they are expensive (Zagury et al., 2007). Complex organic sources can be wastes from agricultural and food processing industry and can further be classified as cellulosic and organic wastes (Kuyucak and St-Germain, 1994). Cellulosic wastes that have been used, include woodchips, hay and sawdust, and organic wastes employed include cow manure, goat manure, buffalo manure and chicken manure (Choudhary and Sheoran, 2011, Neale et al., 2017, Salo et al., 2017, Zhang and Wang, 2014).

Complex organic sources have been studied and rendered suitable to promote and sustain sulphate reduction. According to Zagury et al. (2007), a mixture of several wastes performs better than a single waste. They also mentioned that the efficiency is improved when a mixture of biodegradable sources such as animal manure or sludge are used with cellulosic sources such as sawdust, hay or woodchips. In a study done by Choudhary and Sheoran (2011), the use of single substrates (cellulosic and organic waste) was examined, which revealed that cellulosic substrates resulted in lower sulphate reduction (maximum of 25%) probably because they take too long to biodegrade. Organic wastes had higher sulphate reduction ranging between 43% and 54%. A study done by Salo et al. (2017) confirms that using a mixture of cellulosic and organic waste can improve

efficiency. In their study, sulphate reduction up to 95% was achieved by using woodchips, hay and manure as substrates. Most operational costs come from substrates. It is therefore important to choose a suitable substrate for better efficiency and at lower costs.

For complex organic material to provide nutrients to SRB, they have to be broken down into simple compounds that are usable by SRB as shown in Figure 2.2. When using complex organic compounds as substrates, it is advantageous to use a mixed SRB culture because the available bacteria in the mixed culture help with the breakdown of substrates. A mixed culture also adapts to changing operating conditions better than pure cultures (Colleran et al., 1995). Hydrolysis is the first step for organic matter degradation and it is a rate-limiting step which is facilitated by hydrolytic enzymes (Ersahin et al., 2011, Seyler et al., 2003). Hydrolysis is a process that breaks down big molecular compounds such as carbohydrates, protein and lipids to basic monomers which are further broken down into smaller molecular compounds such as organic acids and alcohols by fermenting bacteria (Ersahin et al., 2011, Sheoran et al., 2010). The disadvantage of using these substrates is that they are not readily available (Sheoran et al., 2010) as they have to undergo hydrolysis for the release of usable components such as lactate, acetate, butyrate and propionate.

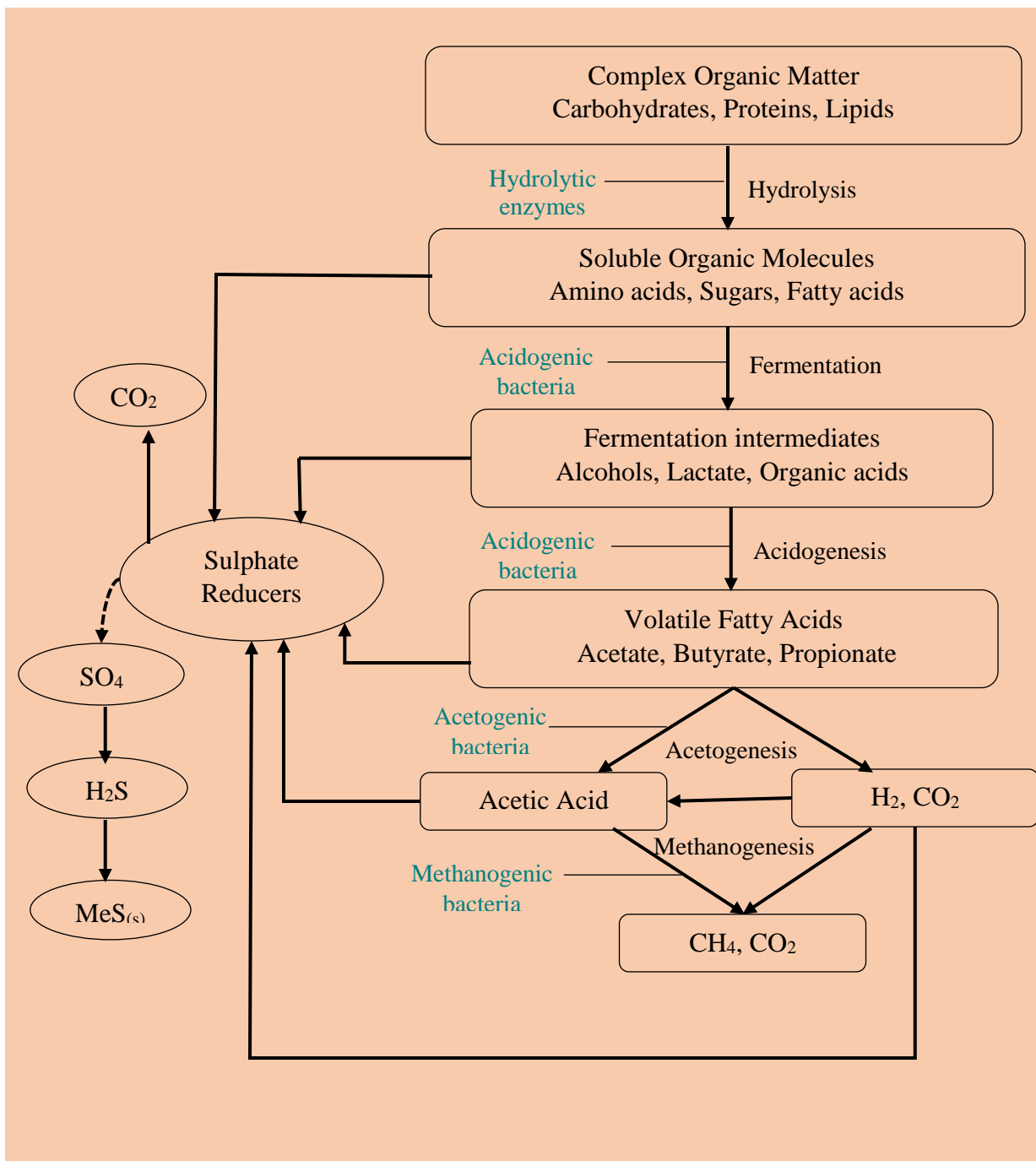


Figure 2.2: Anaerobic pathway for organic matter degradation (modified from Paulo et al. (2015))

2.6.2. Solid support for SRB

Solid support such as gravel is required by SRB so that they can establish microenvironments that are favourable for their survival in extreme conditions such as acidic conditions and the presence of oxygen (Lyew and Sheppard, 1997). Cellulosic organic compounds such as woodchips can also be used as support for SRB growth in bioreactors (Sheoran et al., 2010, Tsukamoto et al., 2004). An advantage of using cellulosic compounds is that they contribute as substrates although their contribution is small (Choudhary and Sheoran, 2011, Kuyucak and St-Germain, 1994). A porous surface is advantageous for efficient sulphate reduction by SRB compared to suspended bacteria (Glombitza, 2001). It is therefore generally preferred for the solid support to have large pore spaces, low surface area and a large void volume as it minimizes plugging in bioreactors (Sheoran et al., 2010). However, greater surface area is required for better treatment efficiency. Consequently, surface area and pore size need to be balanced in field reactors for better efficiency and longevity of bioreactors (Tsukamoto et al., 2004).

2.6.3. pH

SRB are known to be affected by pH and hence sulphate reduction is also impacted by pH. At low pH (<5.5), SRB are suppressed and the solubility of metal sulphides is increased (Dvorak et al., 1992) while at high pH (>9) SRB activity is inhibited. SRB perform better at near-neutral pH (Chaiprapat et al., 2011, Moon et al., 2015). Therefore sulphate reduction efficiency is at its optimum at pH close to neutral (Bijmans et al., 2011). Due to this, Lettinga et al. (1984) recommend adding a buffering agent to increase the influent pH above 6.5. In a study done by Moon et al. (2015), increasing pH from 6 to 7 led to an increase in sulphate reduction and Sharma et al. (2014) obtained highest sulphate reduction when pH was between 6.5 and 7.5.

Although sulphate reduction at low pH has been shown to be possible in different studies (Bijmans et al., 2011), a lot of studies suggests that bioreactors should be operated near-neutral pH as low pH inhibits sulphate reduction activity and increases the solubility of metal sulphides (Dvorak et al., 1991).

Most successful studies have been done at near-neutral pH, however, sulphate reduction has been shown to be possible at pH less than 5 albeit at low efficiencies. Christensen et al. (1996) showed

that sulphate reduction occurs as at pH between 3 and 4, however, at reduced efficiencies. Studies have shown that sulphate reduction efficiency was impacted at pH around 4, with sulphate reduction less than 45 % (Elliott et al., 1998, Sen and Johnson, 1999, Tsukamoto et al., 2004) whereas some studies have shown that sulphate reduction of above 70 % is possible (Jong and Parry, 2006, Lopes, 2007). The above-mentioned studies were conducted using readily available substrates such as lactate, lactic acid, glycerol, ethanol and sucrose. Low sulphate reduction efficiencies in some studies may be because the reactor pH is controlled (Sen and Johnson, 1999) whereas efficiencies may be higher in some because only the influent pH is controlled (Jong and Parry, 2006). As a result, there are uncertainties about the pH at which sulphate reduction occurs if only the influent pH is controlled as the effluent pH is always higher (Lopes, 2007).

A lot of effort has been made to isolate acid-tolerant SRB, however, they were found to be inactive below pH 5 (Küsel et al., 2001). It is suggested that SRB found in acidic environments are neutrophilic and that they probably inhabit near-neutral pH microsites in the acidic streams (Kimura et al., 2006, Küsel et al., 2001). Consequently, some authors believe that it is better to use a mixed SRB culture for the treatment than pure cultures because a mixed culture can withstand harsh conditions.

2.6.4. Temperature

Temperature plays an important role in the overall efficiency of biological sulphate reduction because it has a major effect on bacterial growth and activity (Lettinga et al., 1984). Changes in temperature in full-scale anaerobic reactors cannot be avoided as the wastewater will always be at a different temperature (Visser et al., 1993b), therefore it is important to consider this factor in the optimisation process. SRB can be classified into 3 groups based on their growth temperatures. The first group of SRB have an optimum growth temperatures between 25 and 40 °C (Bijmans et al., 2011, Sawicka et al., 2012) and they are known as mesophiles. The second group is known as moderate thermophiles and have an optimum temperature between 40 and 65 °C (Castro et al., 2000). The last group is the extremophiles and they operate at temperatures between 65 and 110 °C (Belkin et al., 1985).

Most known SRB are mesophilic (Salo et al., 2017) and as a result, most sulphate reducing bioreactors are operated between 25 and 45 °C (Bijmans et al., 2011). Lettinga et al. (1984) reported that the optimum temperature for mesophilic treatment is 38 °C, however, this is dependent on the type of reactor being used (Baskaran, 2005). Some species have their optimum at temperatures lower than 25 °C (Sawicka et al., 2012). Low temperatures are known for slowing down the SRB activity (Doshi, 2006). As a result, most studies in literature have been done at temperatures between 25 and 35 °C (Castro Neto et al., 2018, Chaiprapat et al., 2011, Ghigliazza et al., 2000, Jiménez-Rodríguez et al., 2010, Oyekol et al., 2007, Poinapen et al., 2009a). However, there are some studies that have been done on the effect of low temperature on SRB for sulphate and metal removal.

Some bacteria are cold-adapted and can grow well at temperature as low as 4 °C and if the bacteria population is increased it might counteract lower reaction rates at low temperatures (Hard et al., 2003). In a study done by Tsukamoto et al. (2004), temperature as low as 6 °C was found to be efficient while using ethanol and methanol as carbon sources. Drury (2000) reported that temperature affects sulphate reduction significantly and that the required HRT for 50 % sulphate reduction varies from 8 days at 17 °C to 41 days at 1 °C. Hard et al. (2003) suggested once SRB acclimates at high temperatures, they are not significantly affected by low temperature because the effect that low temperature has on SRB is their ability to acclimate.

2.6.5. Hydraulic retention time

Hydraulic retention time (HRT) affects the rate at which sulphate is removed from AMD. Short HRT can lead to flushing of biomass whereas very long HRT may lead to the depletion of available organic matter source or sulphate source for SRB (Sheoran et al., 2010, Jiménez-Rodríguez et al., 2010). According to Sheoran et al. (2010), it takes about 3 to 5 days of HRT for metals to precipitate. Long HRT favours SRB more than MPB (Mallelwar, 2013), this can be seen in a study done by Isa et al. (1986) who reported that there was an increase in sulphate reduction from $65 \pm 3.1\%$ to $98 \pm 2.6\%$ when they increased the HRT from 0.5 to 10 days. In another study done by Jiménez-Rodríguez et al. (2010) the maximum sulphate removal was obtained when HRT was 8 days however increasing HRT to 16 days led to a deceleration in sulphate removal and decreasing the HRT to 4 days led to a decrease in sulphate reduction. Neculita's 2007 study (cited in (Sheoran

et al., 2010)) shows that there was an increase in sulphate reduction from 18% to 27% when HRT was increased from 2.4 to 9 days.

Ghigliazza et al. (2000) achieved sulphate removal above 98% with HRT of 2 days when using propionate as a carbon source. In a study mentioned by Zagury et al. (2007) sulphate removal above 90% was achieved at HRT of 25 h using substrate consisting of yellow birch barks. In another study done by Içgen and Harrison (2006) using acetic acid as carbon source, sulphate reduction ranged between 90.7 % and 4 % when retention time was decreased from 240 h to 36 h. Oyekol et al. (2007) observed a decrease in volumetric sulphate reduction rate with a decrease in HRT and this was linked to washout of slower growing SRB species.

2.6.6. Redox potential

Redox potential is an important indicator of the overall reducing capacity in bioreactors and it can be used as an indicator of SRB growth and activity (Christensen et al., 1996, Garcia et al., 2001). A minimum of -100 mV is required for biological sulphate reduction to occur (Postgate, 1984). Lower redox potentials in the effluent confirm that sulphate reduction is taking place in passive systems with -300 mV being a good indicator of a strong reducing environment (Salo et al., 2017). Some authors have linked a decrease in redox to an increase in sulphate reduction (Gibert et al., 2004, Johnson and Hallberg, 2005b, Lyew and Sheppard, 1997, Salo et al., 2017).

2.6.7. Sulphide

Hydrogen sulphide is produced during the reduction of sulphate reduction as shown in Equation (2.6) and it is known for its toxicity. Hydrogen sulphide is known for causing problems such as odour, corrosion and sulphate reduction inhibition (Greben et al., 2005, Valdés et al., 2006). H_2S , HS^- and S^{2-} are three different forms of sulphide available in either liquid or gaseous form (Kaksonen and Puhakka, 2007). The three forms in which sulphide is present are pH dependent shown in Figure 2.3. H_2S is dominant at pH less than 6, HS^- is dominant between pH 6 and 11 and S^{2-} is dominant above pH 11. The un-dissociated form of sulphide, H_2S , is available in gaseous form at pH less than 6. At high concentrations, H_2S can be toxic to SRB by precipitating metal ions in active sites within bacterial cells which affects the metabolic coenzymes and denatures proteins (Kaksonen and Puhakka, 2007). Between pH 6 and 8, H_2S dissociates to HS^- which is

more suitable for metal precipitation. Therefore it is generally recommended that reactors operate at pH above 5 to achieve metal precipitation (Lopes, 2007).

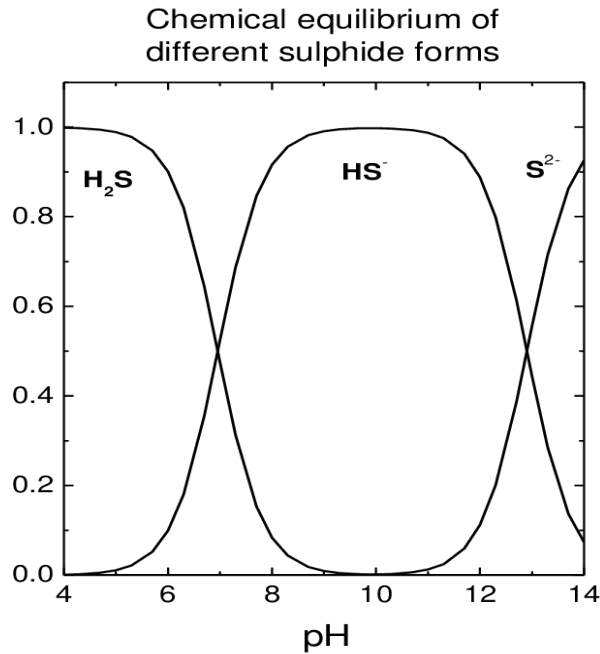


Figure 2.3: Chemical equilibrium of different forms of sulphide as a function of pH (Miloshova et al., 2003)

Hydrogen sulphide is toxic for both SRB and MPB and could possibly result in process failure (Visser et al., 1993a). Studies have shown that sulphide has inhibition effects on SRB growth (O'Flaherty et al., 1998, Visser et al., 1996), sulphate reduction activity (Kalyuzhnyi and Fedorovich, 1997, Yamaguchi et al., 1999) and substrate utilization activity (Kaksonen et al., 2004, Maillacheruvu and Parkin, 1996, Visser et al., 1996). Additionally, SRB sensitivity to sulphide is also dependent on the bacterial species (Hiligsmann et al., 1998, Maillacheruvu and Parkin, 1996, O'Flaherty et al., 1998).

2.6.8. Sulphate concentration

Sulphate concentration in AMD vary from site to site depending on what is being mined (McCarthy, 2011) and it influences sulphate reduction kinetics and SRB growth (Sheoran et al., 2010). Some authors have reported that high sulphate concentration has inhibitory effects on SRB growth (Al Zuhair et al., 2008, Oyekol et al., 2007). However, some reported that increasing the

initial sulphate concentration could improve the reaction rate and increase bacterial activity (Moosa et al., 2002). Al Zuhair et al. (2008) found that sulphate concentration above 2500 mg/L had an inhibitory effect on SRB growth in batch experiments. Similarly, in a study done by Oyekol et al. (2007) in a continuous reactor, sulphate inhibition was observed at feed concentration between 10000 mg/L and 15000 mg/L. Conversely, Moosa et al. (2002) observed an increase in sulphate reduction with an increase in sulphate concentration from 1000 mg/L to 10000 mg/L in experiments conducted in a continuous bioreactor.

2.6.9. Metal concentration

In BSR the production of hydrogen sulphide aids metal removal. Metals in the AMD precipitates out as metal sulphides. However, some studies suggest that in the BSR process, metals could be removed by binding into the bacteria cell wall (Jalali and Baldwin, 2000) and some suggest that bacteria produce extracellular polymeric substances (EPS) that take up soluble metals (Wang et al., 2014). Although there are other mechanisms, the primary mechanism of metal removal in BSR is metal sulphide precipitation (Brahmacharimayum et al., 2019, Dvorak et al., 1992).

Many heavy metals such as iron, copper, zinc and manganese are toxic to SRB because they are able to compete with essential ions, denature proteins and deactivate enzymes by reacting with their functional groups (Utgikar et al., 2002). Batch experiments done by Cabrera et al. (2006) showed high metal concentration could decrease sulphate reducing capacity, slow SRB growth and may cause death. Similarly, Jong and Parry (2003) conducted experiments in a continuous bioreactor and found that sulphate reduction decreased with an increase in initial metal concentration due to metal toxicity which decreased SRB metabolic activity. Dissolved heavy metals can reduce the total number and diversity of a mixed SRB culture and hence affecting the process (Utgikar et al., 2001). Metal sulphides may not be directly toxic to SRB, however, batch experiments showed that insoluble metal sulphides can inhibit SRB by forming precipitates which coats SRB and therefore blocking access to reactants such as sulphate and substrates (Utgikar et al., 2002).

2.7. Design of Experiments (DoE)

DoE, as defined by NIST/SEMATECH (2013), is a systemic, rigorous approach to engineering problem-solving that applies principles and techniques at the data collection stage so as to ensure the generation of valid, defensible and supportable engineering conclusions. DoE is a statistical method which is used for efficient planning of experiments so that the obtained data can be used to understand the relationship between experimental parameters and the response at minimal expenditure of engineering runs, time and money (Giles Jr et al., 2004, NIST/SEMATECH, 2013, Yuangyai and Nembhard, 2010). There are different experimental designs that can be used for DoE, however, the choice depends on the objectives of the experiment. For example, full factorial designs are primarily used for the screening process to understand factors that are important for a system whereas for response surface designs, the goal is generally optimization and to understand the interaction between independent factors using response surface methodology (RSM) (NIST/SEMATECH, 2013).

Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data with an objective to statistically predict and understand the system's behaviour (Bezerra et al., 2008). RSM was developed for the simplification of multivariable optimization process by reducing the number of experimental runs necessary for a thorough understanding of multiple variable interactions. An advantage of using RSM include less time required for experimentation due to reduced experimental runs and therefore a cost reduction of materials and reagents (Bezerra et al., 2008, Najib et al., 2017).

The most commonly used response surface designs are the central composite design (CCD) and Box-Behnken designs (Najib et al., 2017). CCD is based on a 2-level factorial design with centre and axial points to fit quadratic models. Box-Behnken designs, on the other hand, do not have runs at extreme conditions of all factors, however, they compensate by having better prediction precision in the centre of the factor space (StatEase, 2018). The mathematical model provided by both CCD and Box-Behnken is the same (Rakić et al., 2014). CCD is able to test extreme conditions above or below the specified range (Rakić et al., 2014), however, the Box-Behnken

designs contain fewer experiments and therefore more economical and efficient without sacrificing the accuracy of the model (Bezerra et al., 2008, StatEase, 2018).

The application of DoE in BSR is not new. Moon et al. (2015) investigated the effect of linoleic acid, pH and COD/SO₄²⁻ mole ratio on sulphate reduction using the Taguchi design. Taguchi designs, also known as orthogonal arrays (OA) are similar to factorial designs, however, they have minimum number of experiments and they are used in the design stage of processes and products to ensure good performance (NIST/SEMATECH, 2013). Dev et al. (2017) also used the Taguchi design to study the influence of pH, marine waste extract, total organic carbon, HRT and initial sulphate concentration on sulphate reduction efficiency. Najib et al. (2017) used CCD to understand the effect of initial sulphate concentration, pH, COD/SO₄²⁻ mole ratio and COD_{ethanol}/COD_{total} on sulphate reduction efficiency. The use of Box-Behnken design was demonstrated by (Dev et al., 2015). In their study, initial sulphate concentration, pH and marine waste extract were independent factors and sulphate reduction was the response variable.

CHAPTER 3: MATERIALS AND METHOD

3.1. Reactor setup

Three water-jacketed reactors were operated in parallel in a down-flow mode. Each reactor consisted of a base section that functioned as a stand and housed a conical section with an outlet at the bottom of the cone. Above the conical section was a perforated plate acting as a flow distributor with approximately 36 1 cm holes. Each reactor was 1 m tall and 0.15 m wide, resulting in a total internal volume of 17.7 L. The total working volume of each reactor was 8 L. A piece of silicone tubing, with an outer and inner diameter of 1 cm and 0.7 cm respectively, was connected at the bottom of the cone. The tube was routed up the column to the near top edge to enable gravity flow control. A T-piece was fitted at the top of the tube to assist in fluid level adjustment. The schematic diagram of the reactors used is shown in Figure 3.1.

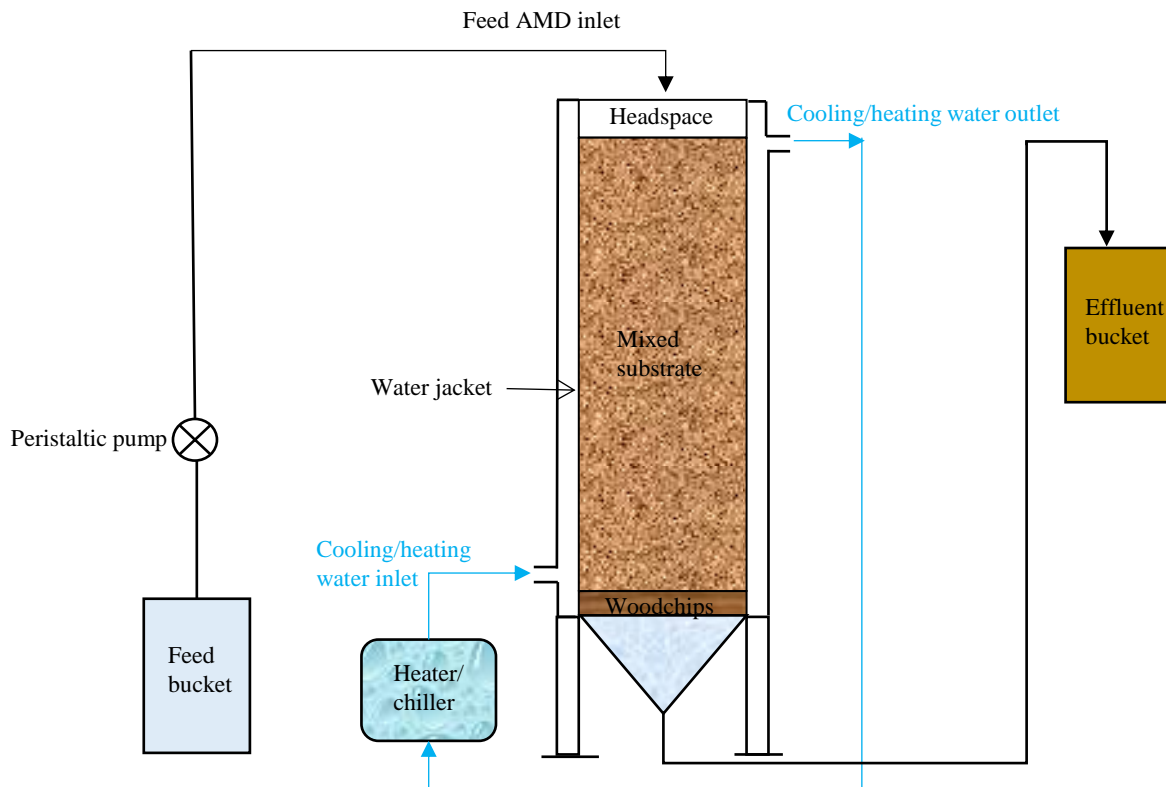


Figure 3.1: Schematic diagram of the reactors

To control the temperature, a PolyScience Whispercool® (PolyScience, Illinois, USA) heater/chiller was used. The water jacket of the reactor was connected in closed circuit with a heater/chiller unit with a built-in pump that recirculated water through the water jacket of the column. For operations lower than 20°C, ethylene glycol was used to allow temperatures inside the column to be controlled at a desired set-point. One litre of laboratory-grade ethylene glycol (>99 % (v/v)) was added to 11 L of distilled water which resulted in the chiller operating at 8.33 % (v/v) ethylene glycol.

The feed water for each reactor was stored in a 10 L or 25 L plastic buckets and pumped to the top of the column using a variable flowrate Watson Marlow 120 series peristaltic pump (Watson-Marlow Fluid Technology Group, Johannesburg, South Africa). The reactor overflow was collected in a 15 L bucket for each column as shown in Figure 3.2. A flexible tube was connected at the top and the other end was dipped inside the bioleach liquor which was used to trap excess H₂S produced during the biological sulphate reduction.

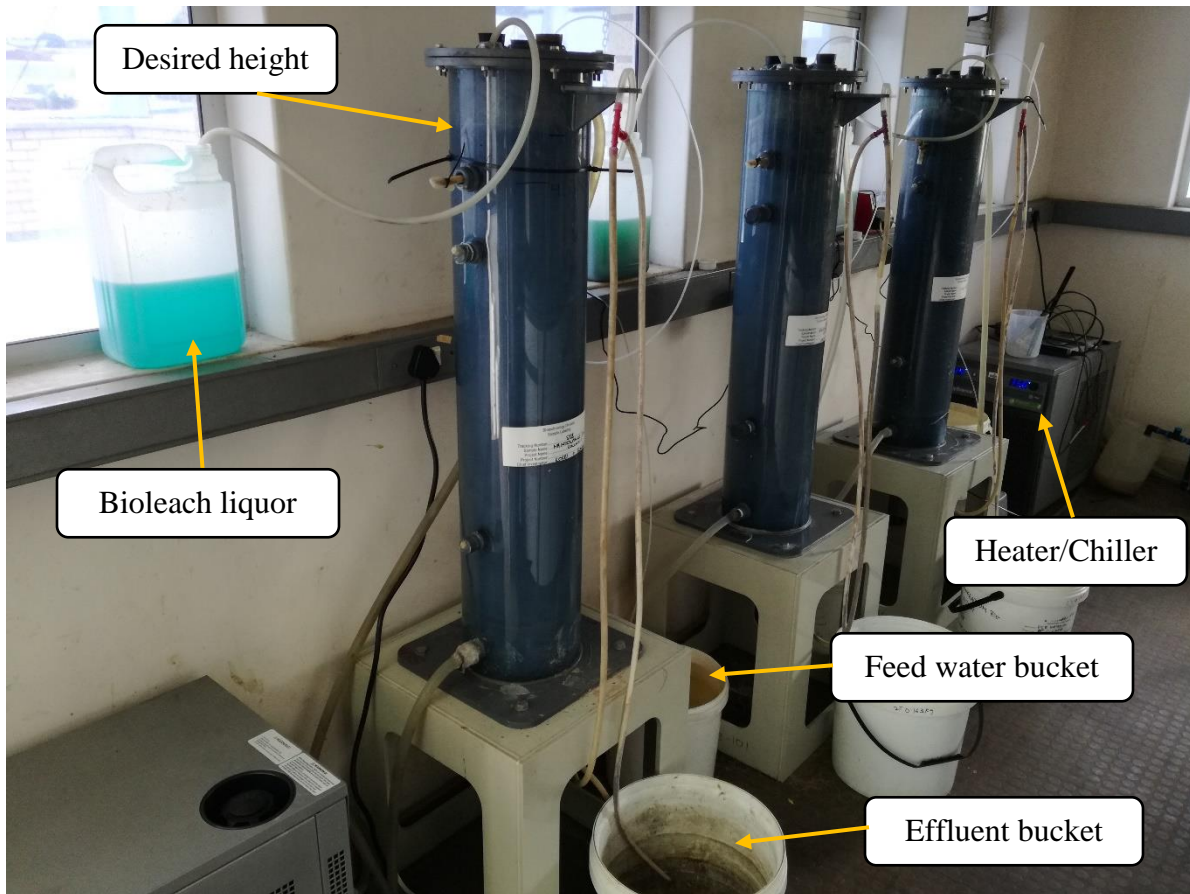


Figure 3.2: Experimental setup

3.2. Substrates

Initially, the three reactors were packed with 30 % (v/v) woodchips, 30 % (v/v) wood shavings, 20 % (v/v) hay, 10 % (v/v) lucerne straw and 10 % (v/v) cow manure. A 50 L bucket was used to measure the above-mentioned packing before they were blended and loaded into the reactors. The packing was used as a support for the biofilm. Although woodchips, wood shavings, hay and lucerne straw were used as just packing, it is hypothesized that they may have been helping with the sulphate reduction process because they have been mentioned as cellulosic substrates and investigated in some studies but it was found that they require more time to biodegrade and their contribution as substrates is small (Choudhary and Sheoran, 2011, Kuyucak and St-Germain, 1994).

Cow manure purchased from Lifestyle Home Garden, Johannesburg, South Africa and lucerne pellets purchased from Milmac Feeds, Fourways, South Africa were used as the main substrates. 186 ml (128 g) of cow manure and 186 ml (64 g) of lucerne pellets were added to the reactors and replenished once every week. Cow manure and lucerne pellets were not analysed, however, their physicochemical properties are shown in Table 3.1 and Table 3.2 as obtained from literature.

Table 3.1: Physicochemical properties of cow manure (Khater, 2015)

Bulk density (kg/m ³)	750
Moisture content (%)	58.3
Porosity	41.57
EC (dS/m)	4.2
Total organic carbon (%)	18.16
Total organic matter (%)	31.3
Total nitrogen (%)	0.93
Total phosphorus (%)	0.21
Total potassium (%)	0.17
C/N ratio	19.53:1

Table 3.2: Physicochemical properties of lucerne pellets (EquusFeeds, 2015).

Bulk density (g/cm ³)	0.66-0.69
Moisture content (%)	12
Ca:P ratio	1.5-2.3:1
Organic matter (%)	88.8
C/N ratio	15:1
Calcium (g/kg)	6-8
Phosphorus (g/kg)	3
Magnesium (g/kg)	2
Potassium (g/kg)	8

3.3. AMD and Inoculum

The AMD used for all experiments was collected from a coal mine in eMalahleni, Mpumalanga province, South Africa. The raw AMD sulphate concentration ranged between 2500 mg/L and 5200 mg/L with pH less than 3.

The anaerobic mixed sulphate reducing culture used was collected from a pilot plant that has been running in the same coal mine. The reactors were inoculated using 30 % (v/v) inoculum and 70 % (v/v) neutralised AMD. The inoculum was prepared by mixing 9 L of pilot plant sample with 21 L of mine water pre-adjusted to pH approximately 6.5.

3.4. Start-up and operating procedure for the reactors

The mixture of woodchips, wood shavings, hay, lucerne straw and cow manure were blended thoroughly (Figure 3.3) before loading into the reactors to the desired height of 0.985 m. Before loading the packing, a layer of woodchips, about 2 to 3 cm (Refer to Figure 3.1), was evenly spread over the distribution plate. This was done to prevent blocking of the holes on the distribution plate when the substrates migrated downwards. After filling the reactors with the packing material (Refer to Figure 3.2), they were each filled with the 30 % (v/v) inoculum adding 1 L at a time until the water reached the top of the bed (8 L was added). Initially, all reactors were operated in batch mode, that is without feeding AMD, for 10 days at a temperature of 30 °C to establish the biofilm.

To increase the growth of the biofilm, the inoculum was recirculated around the column for seven days commencing after day 10. Recirculation was obtained by pumping the inoculum from the bottom to the top of the column at a flow rate of 0.46 ml/min (approximately 12 day residence time). After a week of recirculation, the sulphate levels in the effluent was low (<100 mg/L) which showed that the SRB were active. To keep the SRB active, recirculation was stopped and AMD feed was commenced. The AMD feed contained sulphate at a concentration above 3000 mg/L at pH approximately 6. After two weeks of feeding, the flow rate was increased to 0.56 ml/min (10 days residence time) and the sulphate levels were continuously monitored. After a further two weeks, the flow rate was changed to 0.79 ml/min (7 days residence time) which was the starting point of the experiment. Since three factors were investigated, two of those factors had to be kept constant when one factor was changed. The pumps were operated at a flow rate of 0.79 ml/min of for 7 days residence time, 1.23 ml/min for 4.5 days residence time and 2.78 ml/min for 2 days residence time. The reactors were operated in continuous mode until steady state was reached. Each experiment was allowed to run for 4 volume changes before changing to the next experiment.



Figure 3.3: Blended woodchips, wood shavings, hay, lucerne straw and cow manure

3.5. Analysis

3.5.1. pH

The effluent pH was measured immediately after sampling. Samples were collected using a beaker and immediately the pH sensor was dipped in the sample and the reading was recorded once stabilised. A Metrohm pH sensor (Metrohm, Herisau, Switzerland) was used for pH measurements and it was calibrated for pH 4 and 7 buffer solutions before analysis. A Hamilton redox sensor (Hamilton, Bonaduz, Switzerland) was used for redox potential measurements and it was calibrated for s475 mV buffer solution before analysis.

3.5.2. Sulphate

Influent and effluent sulphate concentrations were measured using a Merck Spectroquant® Prove 300 (Merck, Darmstadt, Germany) which uses the turbidimetric method. In this method, sulphate ions react with barium to form slightly soluble barium sulphate which results in turbidity and is measured in the photometer. All samples were filtered using a 0.22 µm membrane syringe filters (Labcon, Randburg, South Africa) before analysis to prevent suspended solids from interfering with the results. Samples were analysed immediately after collection.

3.5.3. Sulphide

Potentiometric determination of hydrogen sulphide using 0.1 M AgNO₃ was used to determine the total sulphide concentration in the effluent. A Metrohm Titrando (Metrohm, Herisau, Switzerland) was used for sulphide titrations using AgNO₃. At pH below 6 sulphide is mainly in the form of hydrogen sulphide, between 6 and 11 it is mainly in the form of bisulphide and above pH 11 it is mainly as in the form S²⁻ as shown in Figure 2.3. The Metrohm Titrando measure sulphide in the form of S²⁻, due to this the reactor effluent pH (which was between 5 and 7.6) had to be adjusted to above 11. This was achieved by adding 0.15 – 0.2 mL of 10 M NaOH in 15 ml centrifuge cells before sample collection.

3.5.4. Microbial analysis

3.5.4.1. Inoculum

As mentioned above, the inoculum used was collected from the effluent of one of the reactors operating at the coal mine. The reactors at the coal mine were inoculated with cow manure. The microbial communities of the collected inoculum were identified as described by Salo et al. (2017); The microbial communities were characterised with high throughput amplicon sequencing which targeted the prokaryotic 16S rRNA gene. Bact_0341F/Bact_805R (Herlemann et al., 2011, Klindwort et al., 2013) primers were used, targeting the variable region V3-V4 of the 16S rRNA gene. The amplicons were paired-end sequenced on the Illumina MiSeq platform. The Ion torrent sequences were trimmed and the quality checked as reported by Rajala et al. (2016). The MiSeq sequences were paired using the default quality score values assigned in QIIME version 1.9 (Caporaso et al., 2010). The sequence data were subsequently analysed with the QIIME software, chimeric sequence reads were removed from the dataset with the USEARCH-algorithm (Edgar, 2010) by de novo detection and through similarity searches against the Greengenes reference dataset (Version gg_13_8) (DeSantis et al., 2006). Sequence reads were grouped into Operational Taxonomic Units (OTUs) at minimum 97% sequence homology using the open OTU picking method in QIIME. Taxonomic assignments for the OTUs were based on the Greengenes (gg_13_8) reference database.

3.5.4.2. Reactor effluent

Samples for microbial analysis were taken from the effluent. About 50 ml sample was taken and filtered using a vacuum filter through 0.45 µm nitrocellulose membrane filters. The membrane filters were dried at 50 °C for 30 minutes. The identification of the effluent microbial communities was conducted by Inqaba Biotec. The samples were analysed using a full length 16S rRNA gene amplicons metagenomic analysis. The samples were sequenced on the Sequel system by PacBio. Raw subreads were then processed through the SMRTlink (version 6.0) Circular Consensus Sequences (CCS) algorithm to produce accurate highly reads (>QV40). The results were then processed through USEARCH (Edgar, 2010) and taxonomic information was determined based on the Ribosomal Database Project's 16s database v16 (Cole et al., 2013).

3.6. Design of experiments

Design Expert® (version 11.1.2.0, Stat Ease Inc., USA), a statistical tool that helps with the design of experiments, was used to design the experiments and for data analysis. The effect and interactions between three independent factors were studied. The independent variables were pH, temperature and hydraulic retention time with three corresponding response variables namely sulphate reduction, sulphate reduction rate and sulphide production. This was achieved by using the Box-Behnken design and response surface methodology (Bezerra et al., 2008). A three-factor Box-Behnken design with a triplicate at the centre point was used. Table 3.3 shows the coded and the actual values and for the three chosen independent variables. A total of 15 experiments were designed, however, two additional experiments that were conducted were added manually, run 16 and 17 in Table 3.4.

Table 3.3: Box-Behnken design for 3 factors in experimental design

Code	Factors	Factor range and levels (coded)		
		-1	0	1
A	pH	4	5	6
B	Temperature (°C)	10	20	30
C	HRT (d)	2	4.5	7

Table 3.4: Designed experiments and results

Run	Independent variables			Response variables		
	A: pH	B: Temperature (°C)	C: HRT (d)	Sulphate reduction efficiency (%)	Sulphate reduction rate (mol/m ³ /d)	Sulphide production (mg/L)
1	5	20	4.5	76.94	4.57	693.19
2	5	30	7	98.73	7.66	411.55
3	4	30	4.5	90.34	5.70	549.73
4	5	30	2	65.61	9.75	533.77
5	6	20	2	50.28	8.97	369.36
6	5	10	2	25.48	4.56	144.54
7	5	10	7	41.74	1.66	403.18
8	5	20	4.5	84.46	4.99	696.08
9	4	10	4.5	22.76	1.67	236.74
10	6	30	4.5	96.87	6.17	510.24
11	6	20	7	97.56	5.63	410.08
12	4	20	2	55.17	9.89	358.28
13	4	20	7	93.43	5.35	626.11
14	5	20	4.5	84.38	4.98	797.30
15	6	10	4.5	30.38	1.88	332.22
16	6	30	7	98.40	7.54	396.43
17	5	30	4.5	96.57	6.46	631.65

Table 3.5 shows the order in which they were conducted.

Table 3.5: The order in which experiments were conducted

Reactor 1	Reactor 2	Reactor 3
pH 6, 30 °C, 7 d	pH 5, 30 °C, 7 d	pH 5, 20 °C, 4.5 d
pH 6, 30 °C, 4.5 d	pH 5, 30 °C, 4.5 d	pH 5, 20 °C, 4.5 d
pH 6, 20 °C, 7 d	pH 4, 20 °C, 7 d	pH 6, 10 °C, 4.5 d
pH 4, 30 °C, 4.5 d	pH 5, 20 °C, 4.5 d	pH 5, 10 °C, 2 d
pH 5, 30 °C, 2 d	pH 5, 10 °C, 7 d	
pH 6, 20 °C, 2 d	pH 4, 10 °C, 4.5 d	
pH 4, 20 °C, 2 d		

3.7. Statistical analysis

RSM was used to understand the interactions between the factors. This was achieved by fitting the experimental data into a polynomial quadratic equation to obtain regression coefficients as shown in Equation (3.1).

$$Y = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij} x_i x_j \quad (3.1)$$

where Y is the response variable, b_0 is the constant term, b_i is the linear coefficient, b_{ii} is the quadratic coefficient, b_{ij} is the interaction coefficient, and x_i and x_j are the values of the coded variables. In this study, sulphate reduction efficiency (%), sulphate reduction rate (mol/m³/d) and sulphide production (mg/L) were chosen as response variables and therefore they were fitted into Equation (3.1). Analysis of variance (ANOVA) was used to evaluate the validity and significance of the fitted model. The coefficient of determination R², adjusted R², lack of fit, adequate precision, F-value and p-value were used to further evaluate the quality and accuracy of the model. The coefficient of determination, R², and adjusted R² are used to measure how well the data fits the regression line. The lack of fit determines how well the model fits the data. Adequate precision is a measure of the signal to noise ratio. The model F-value measures how the data fluctuates around

the mean (Najib et al., 2017) while the p-value measures the level of significance. In the present study, the significance level was set at 0.05.

3.8. Pre-neutralisation experiment

The aim of this experiment was to determine the amount of lime required to adjust the mine water pH to a certain pH and to determine the amount of sludge formed. This was done in 10 L buckets and 25 L buckets. An arbitrary amount of hydrated lime was weighed before the neutralisation process then slowly added to the mine water while stirring at 300 rpm and 400 rpm for 10 L and 25 L buckets respectively. When the pH was at the desired point, the remaining lime was weighed and the difference between the lime before and after the neutralisation process was calculated to get the amount of lime consumed. The sludge was allowed to settle in the buckets for 2 days before the water was drained. The remaining sludge and water mixture was transferred into 1 L and 2 L measuring cylinder for 10 and 25 L buckets respectively. The sludge was then allowed to settle for 3 days and the volume was recorded. Each experiment was done twice. Figure 3.4 shows the sludge before and after settling in measuring cylinders.

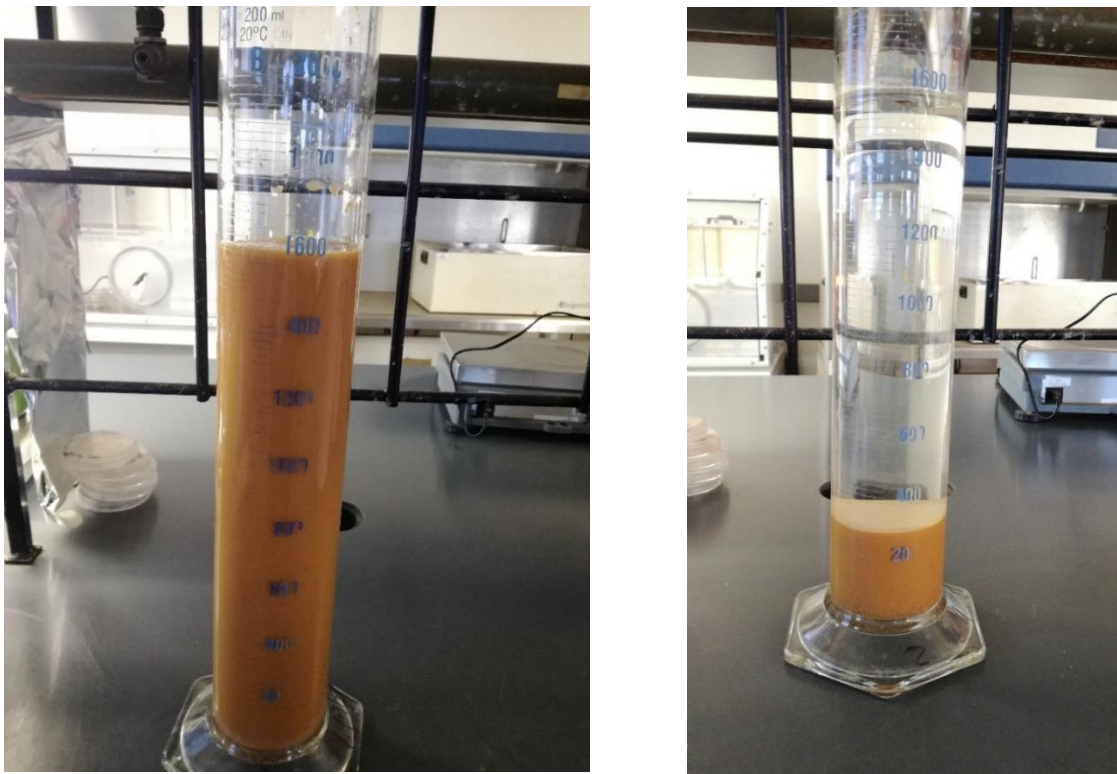


Figure 3.4: Sludge settling initially (left) and after 3 days (right)

CHAPTER 4: MICROBIAL ANALYSIS AND MODEL VALIDATION

4.1. Microbial analysis

High throughput sequence analysis showed that majority of the microbial community in the inoculum belonged to Bacteroidetes (38.7 %), Lentisphaera (32 %), Firmicutes (9 %), Proteobacteria (10.5 %) and OD1 (9.5 %) phyla as shown in Figure 4.1. These group of bacteria are heterotrophic fermenters (Bauer et al., 2006, Choi et al., 2013, Ramakrishna, 2013, Wrighton et al., 2012). Additionally, Bacteroidetes have a wide variety of hydrolytic enzymes which makes them capable of degrading high molecular weight organic matter such as polysaccharides and protein (Bauer et al., 2006) whereas OD1 bacteria reduces sulfur (Wrighton et al., 2012).

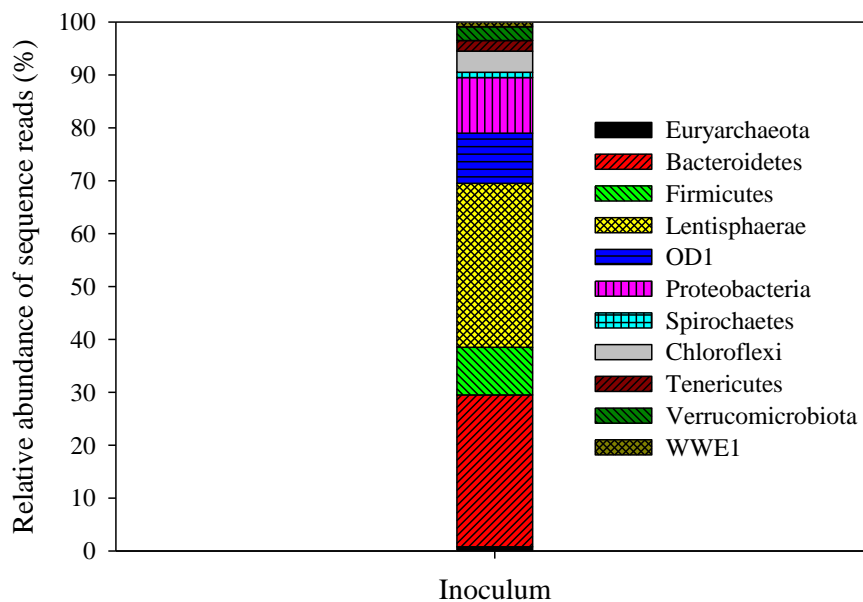


Figure 4.1: Relative abundances of bacterial phyla observed in the inoculum (Salo et al., 2017)

The relative abundance of SRB genera in the inoculum is shown in Figure 4.2. The graph shows that the relative abundance of SRB was 1.1 %. There were only 2 SRB genera detected namely *Desulfococcus* and *Desulfovibrio*. In bioreactors that use complex organic compounds as substrates, SRB has shown to have lower abundance compared to other bacteria. For example, in a study done by Hiibel et al. (2011), ethanol-fed reactors had SRB accounting for approximately 70 % of the bacterial community whereas there was only about 2 – 10 % of SRB in reactors that used lignocellulose substrates such as woodchips, hay and horse manure.

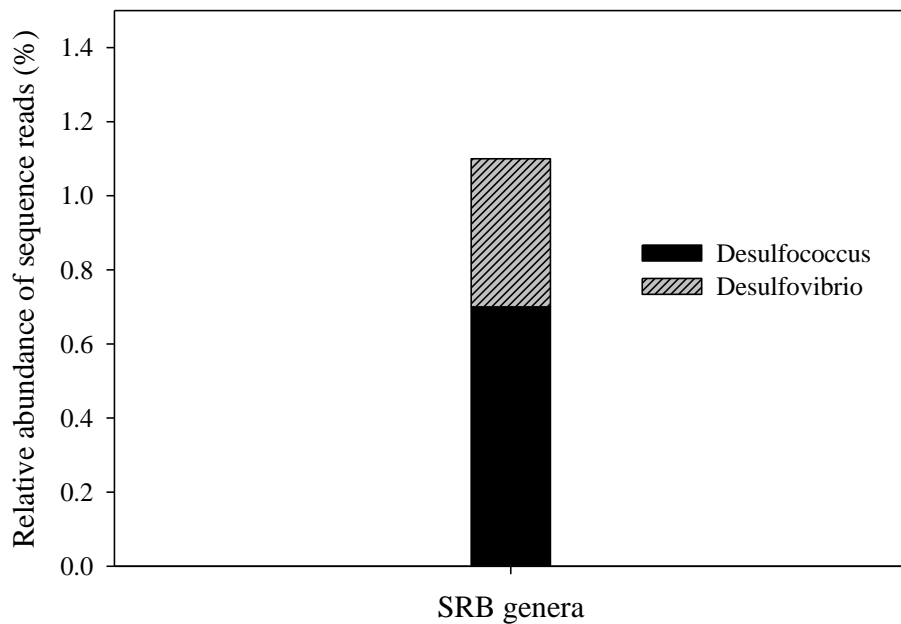


Figure 4.2: Relative abundance of SRB genera in the inoculum (Salo et al., 2017)

After concluding the experiments, the effluents from all the reactors were collected and analysed using metagenomic analysis. This was done to compare the microbial community in the inoculum and that at the end of the experiments to see if there is a major difference between the two. The most abundant bacterial phyla in the reactor effluent were Firmicutes (75.15 %) and Bacteroidetes (11.8 %) for reactor 1 (R1), Firmicutes (38.15 %), Proteobacteria (27.89 %) and Microgenomates (12.38) for reactor 2 (R2) and Firmicutes (67.98 %), Proteobacteria (16.32 %) and Bacteroidetes (14.27 %) for reactor 3 (R3) as depicted in Figure 4.3. Majority of the identified phyla were heterotrophic fermenters as observed in the inoculum as well. Although Microgenomates was not

present in the inoculum, it was detected in R2 effluent and it is also has a fermentative lifestyle according to Hu et al. (2016).

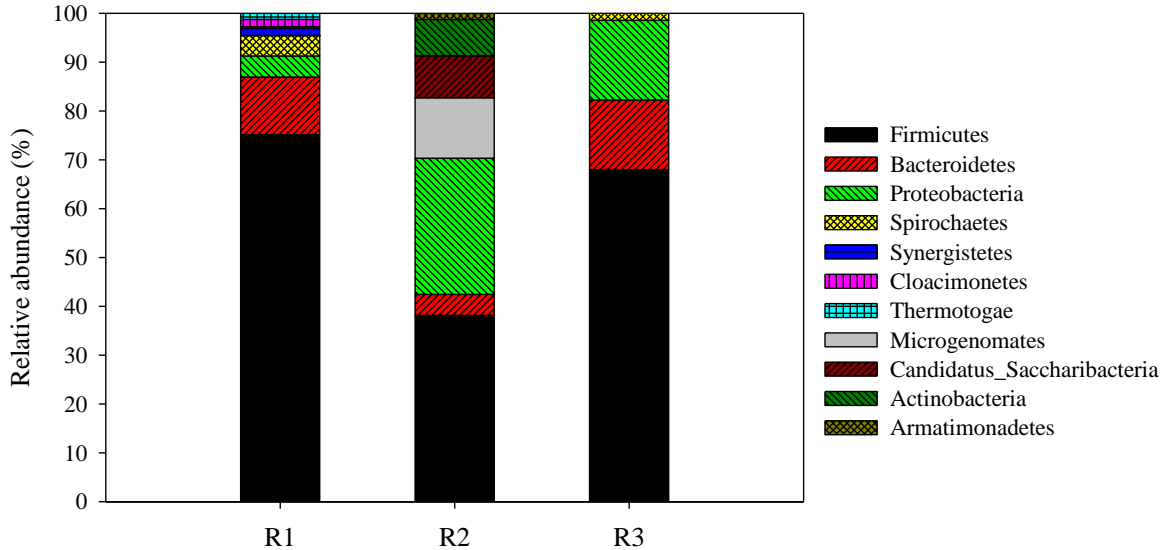


Figure 4.3: Relative abundance of bacterial phyla observed in the reactor effluent

Figure 4.4 shows the relative abundance of different bacterial genera observed in R1, R2 and R3 effluents. The most abundant genus in R1 was *Trichococcus* (75.49 %) which belongs to the Firmicute phylum. *Trichococcus* is a facultative anaerobe which is capable of degrading organic matter in cold environments (Holzapfel and Wood, 2014, van Gelder et al., 2012). These bacteria have been used to ferment substrates that were used by SRB (Stams et al., 2009). Its presence is not surprising as some experiments were conducted at 10 °C which might suggest that it was responsible for degrading the substrate at such low temperatures. *Clostridium_sensu_stricto* which belongs to the Firmicute phylum contributed 28.21 % of the bacterial community in R2. This group of bacteria are obligate anaerobe that can be isolated from manure and sewage sludge (Vos et al., 2011). R2 effluent also included sulphur oxidisers such as *Thiofaba* and *Sulfurimonas*. These group of bacteria oxidizes sulphur to sulphate or sulphuric acid and may be attributed to decreased reactor performance (Arnold et al., 2016a). The presence of these sulphur oxidisers may be due to the changing conditions inside the reactors such as low temperatures and short HRT (Salo et al., 2017). The only SRB genus observed was *Desulfotomaculum* in R2 and it contributed 1.47 % of the

bacterial community. In R3 effluent, the most abundant bacteria was *Lactobacillus* (37.95 %) which is a facultative anaerobe fermenter found in cow dung (Han et al., 2014) and belongs to the Firmicutes phylum.

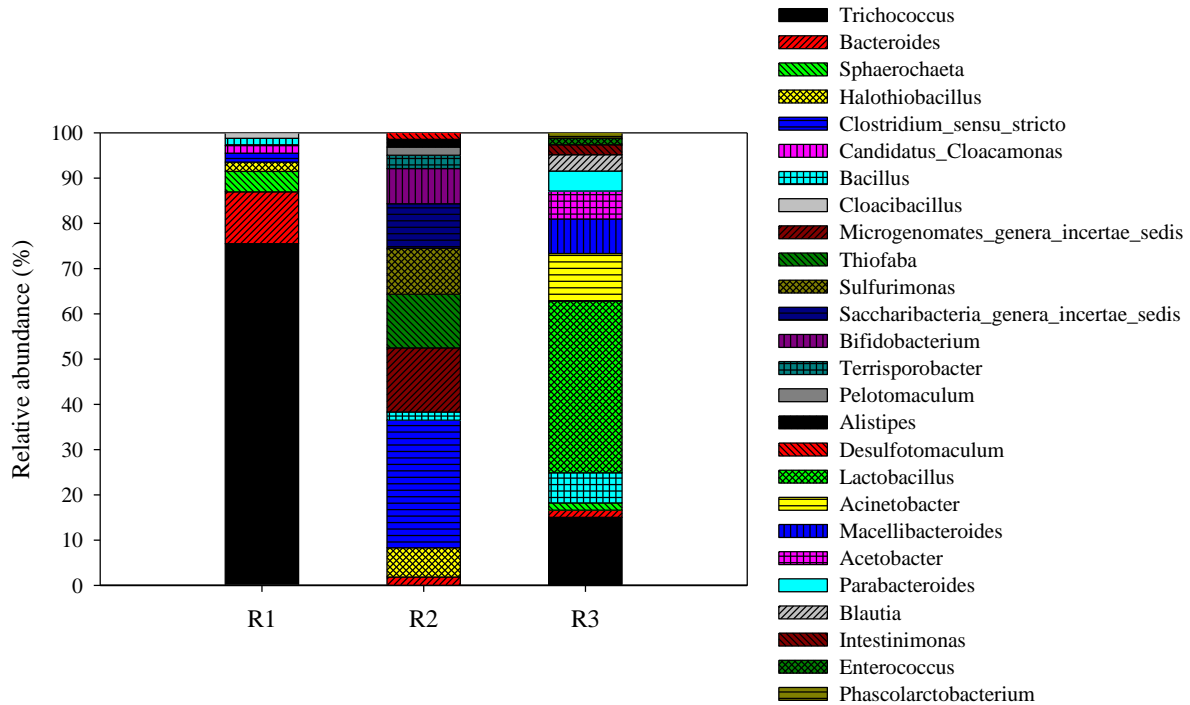


Figure 4.4: Relative abundance of bacterial genera observed in the reactor effluent

The microbial community observed in both the inoculum and reactor effluents were dominated by non-SRB. However, the dominating bacteria were those found in the gut and in livestock manure which was expected as cow manure was used as a substrate for these experiments. There was a difference in bacterial groups detected in the inoculum and those detected in the reactor effluents. It is possible that the bacterial community might have evolved due to changing reactor conditions (Oyekol et al., 2010) and that the reactor conditions might have favoured the growth of non SRB. Furthermore, it should be noted that only the reactor effluents were analysed and therefore the lack of SRB in the effluent could mean that SRB were firmly attached to the reactor bed. The microbial community in both the inoculum and reactor effluents was diverse. This is usually the case in reactors that use complex organic compounds as substrates because other bacterial groups are

needed to break down complex organic compounds into simple compounds usable by SRB (Hiibel et al., 2011).

4.2. Model validation and statistical analysis


The experiments were conducted according to the design of experiment that was done using Design Expert as shown in Table 3.4. The average of each run was calculated then input in design expert. Analysis of variance (ANOVA) was done for all the responses namely, sulphate reduction efficiency, sulphate reduction rate and sulphide production. The ANOVA results were used to validate the models and further validations were obtained from the diagnostic section in the Design Expert software.

4.2.1. Elimination of an outlier experiment

In statistics, Cook's distance is used to evaluate points that are influential in multilinear regression analysis (Jayakumar and Sulthan, 2015). Cook's distance is a way of finding points, considered as outliers, that affect the regression model negatively and any value greater than 1 is considered an outlier (Deviant, 2011, Hair et al., 1998). Initial analysis in Design Expert had indicated that run 6 (10 °C, pH 5 and HRT 2 d) in Table 3.4 is an outlier. This was evident from the sulphate reduction efficiency and sulphate reduction rate Cook's distance plots shown in Figure 4.5 and Figure 4.6 respectively. As a result of run 6 being an outlier, the rest of Design Expert analyses were done without run 6. Cook's distance plots for the analyses without run 6 are shown in APPENDIX B.

Design-Expert® Software

Sulphate reduction efficiency

Color points by value of Sulphate reduction efficiency:
22.7574  98.7296

Std # 9 **Run # 6**
X: 6
Y: 1.71347

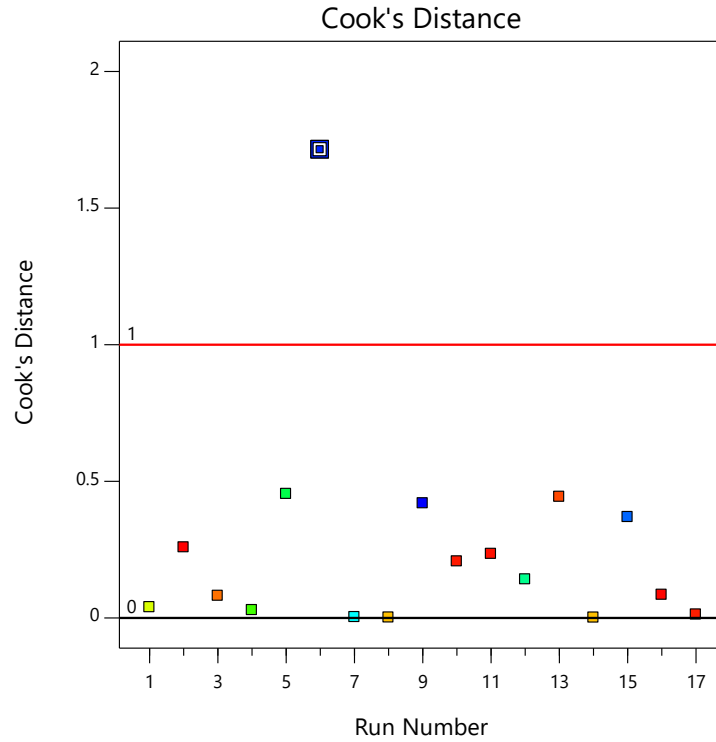



Figure 4.5: Cook's distance plot for sulphate reduction efficiency

Design-Expert® Software

Suphate reduction rate

Color points by value of Suphate reduction rate:
1.66499  9.89196

Std # 9 **Run # 6**
X: 6
Y: 1.76606

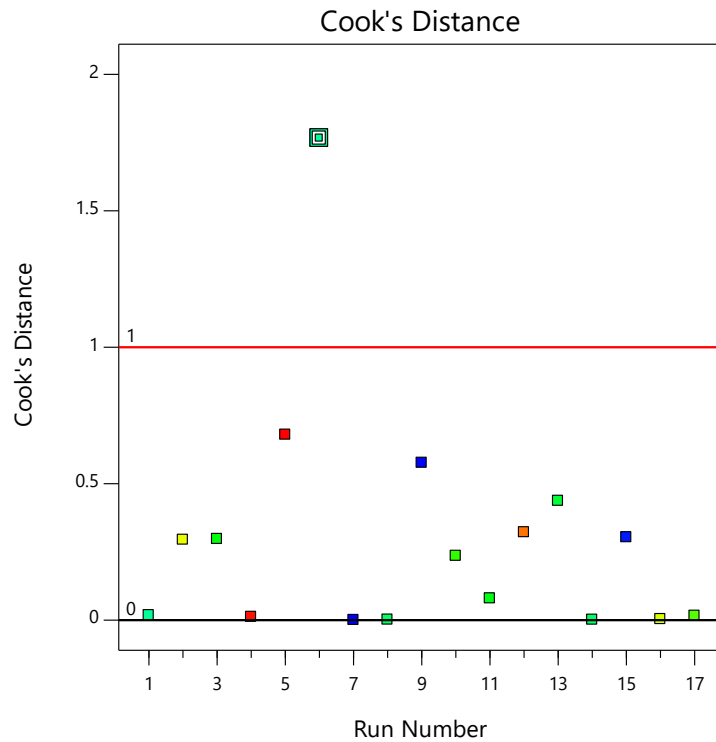


Figure 4.6: Cook's distance plot for sulphate reduction rate

4.2.2. Analysis of Variance

ANOVA was used to evaluate the reliability and the quality of the fitted model as shown in Table 4.1. In order to confirm the significance of the models, the F-value has to be relatively high and the probability (p-value) has to be low (<0.05) (Sun et al., 2016). The F-values and p-values were 101.70, 221.37, 6.78 and <0.0001 , <0.0001 , 0.0151 for sulphate reduction efficiency, sulphate reduction rate and sulphide production respectively. All the F-values rendered the models to be significant. There is a 0.01 %, 0.01 % and 1.51 % chance that the large F-values for sulphate reduction efficiency, sulphate reduction rate and sulphide production, respectively, could be due to noise. P-values less than 0.05 indicates that the model terms are significant to a 95% confidence level. In all the models, the terms A, AB, AC and A^2 were insignificant including BC for sulphide production. The lack of fit was found to be non-significant for all the models, implying that the models fitted the data well.

The coefficient of determination (R^2) and the adjusted R^2 measures how well the data fits the line and according to Henseler et al. (2009), an R^2 value greater than 0.7 is substantial. The R^2 and the adjusted R^2 values were 0.9935 and 0.9837, 0.9970 and 0.9925, 0.9104 and 0.7761 for sulphate reduction efficiency, sulphate reduction rate and sulphide production respectively. The desirable value of the adequate precision, which is a measure of signal to noise ratio, is 4. When adequate precision is greater than 4, it implies that the signal is adequate and the model can be used to navigate the design space. Adequate precision of 46.208, 29.362 and 7.338 was obtained for sulphate reduction efficiency, sulphate reduction rate and sulphide production respectively.

Table 4.1: ANOVA results for response surface quadratic model

Response	Source of variation	Sum of squares	df	Mean square	F-value	p-value
Sulphate reduction efficiency (%)	Model	10155.47	9	1128.39	101.70	< 0.0001 ^a
	A-pH	59.11	1	59.11	5.33	0.0604 ^b
	B-Temperature	6519.62	1	6519.62	587.61	< 0.0001 ^a
	C-HRT	2442.99	1	2442.99	220.19	< 0.0001 ^a
	AB	2.65	1	2.65	0.2389	0.6424 ^b
	AC	2.11	1	2.11	0.1897	0.6784 ^b
	BC	72.74	1	72.74	6.56	0.0429 ^a
	A ²	3.68	1	3.68	0.3316	0.5856 ^b
	B ²	1808.36	1	1808.36	162.99	< 0.0001 ^a
	C ²	370.19	1	370.19	33.37	0.0012 ^a
	Residual	66.57	6	11.10		
	Lack of Fit	29.32	4	7.33	0.3937	0.8060 ^b
	Pure Error	37.25	2	18.62		
	Correction Total	10222.04	15			
	R²	0.9935				
Adjusted R²	0.9837					

df – degree of freedom

^aSignificant

^bNot significant

Response	Source of variation	Sum of squares	df	Mean square	F-value	p-value
Sulphate reduction rate (mol/m ³ /d)	Model	101.69	9	11.30	221.37	< 0.0001 ^a
	A-pH	0.5037	1	0.5037	1.49	0.2617 ^b
	B-Temperature	25.50	1	25.50	499.63	< 0.0001 ^a
	C-HRT	20.96	1	20.96	410.67	< 0.0001 ^a
	AB	0.0093	1	0.0093	0.1822	0.6844 ^b
	AC	0.1504	1	0.1504	2.95	0.1369 ^b
	BC	1.78	1	1.78	34.89	0.0010 ^a
	A ²	0.0577	1	0.0577	1.13	0.3287 ^b
	B ²	2.58	1	2.58	50.60	0.0004 ^a
	C ²	23.09	1	23.09	452.42	< 0.0001 ^a
	Residual	0.3062	6	0.0510		
	Lack of Fit	0.1926	4	0.0481	0.8472	0.6045 ^b
	Pure Error	0.1137	2	0.0568		
	Correction Total	102.00	15			
	R²	0.9970				
	Adjusted R²	0.9925				

df – degree of freedom

^aSignificant

^bNot significant

Response	Source of variation	Sum of squares	df	Mean square	F-value	p-value
Sulphide production (mg/L)	Model	3.383E+05	9	37591.42	6.78	0.0151 ^a
	A-pH	558.93	1	558.93	0.1008	0.7617 ^b
	B-Temperature	97841.75	1	97841.75	17.64	0.0057 ^a
	C-HRT	36223.31	1	36223.31	7.16	0.0317 ^a
	AB	728.38	1	728.38	0.1313	0.7295 ^b
	AC	5715.99	1	5715.99	1.03	0.3492 ^b
	BC	21245.79	1	21245.79	3.83	0.0981 ^b
	A ²	32467.47	1	32467.47	5.85	0.0519 ^b
	B ²	1.236E+05	1	1.236E+05	22.28	0.0033 ^a
	C ²	83355.06	1	83355.06	15.03	0.0082 ^a
	Residual	33278.32	6	5546.39		
	Lack of Fit	26247.37	4	6561.84	1.87	0.3779 ^b
	Pure Error	7030.95	2	3515.48		
	Cor Total	3.716E+05	15			
	R²	0.9104				
Adjusted R²	0.7761					

df – degree of freedom

^aSignificant

^bNot significant

The experimental data was fitted into a quadratic model and the regression coefficients were calculated. The full quadratic models in terms of coded factors are shown in Equation (4.1) to (4.3). Supplementary ANOVA results are shown in APPENDIX A.

$$\begin{aligned}
 \text{Sulphate reduction efficiency} = & + 82.86 + 2.65 * A + 33.79 * B + 21.31 * C \\
 & - 0.77 * AB - 0.69 * AC - 5.67 * BC \\
 & + 1.08 * A^2 - 23.64 * B^2 + 10.51 * C^2 \quad (4.1)
 \end{aligned}$$

$$\begin{aligned}
\text{Sulphate reduction rate} = & + 4.92 + 0.23 * A + 2.11 * B + 1.97 * C \\
& + 0.05 * AB - 0.18 * AC + 0.89 * BC \\
& - 0.13 * A^2 - 0.89 * B^2 + 2.63 * C^2
\end{aligned} \tag{4.2}$$

$$\begin{aligned}
\text{Sulphide production} = & + 712.22 - 8.14 * A + 130.91 * B + 77.46 * C \\
& - 12.79 * AB - 35.82 * AC - 96.97 * BC \\
& - 113.15 * A^2 - 195.46 * B^2 - 157.74 * C^2
\end{aligned} \tag{4.3}$$

4.2.3. Diagnostics

The diagnostic section in design expert provides plots that can be used to further validate the accuracy and quality of the model. These include the normal plot of residuals, also known as normal probability plot, residual vs predicted plot and the plot of the predicted vs actual response values. The normal plot of residuals, shown in Figure 4.7, shows whether the residuals follow a normal distribution. If the points are closer to the straight line, then the residuals are normally distributed (Najib et al., 2017). The residual vs predicted, shown in Figure 4.8, can be used to check for outliers and to check if a transform is required. The model's quality is proved by random scatters that are evenly distributed above and below the horizontal axis (Najib et al., 2017). The predicted and the actual response values, Figure 4.9, are fairly clustered along the diagonal line which confirms that the model is accurate and robust (Najib et al., 2017, Talib et al., 2017).

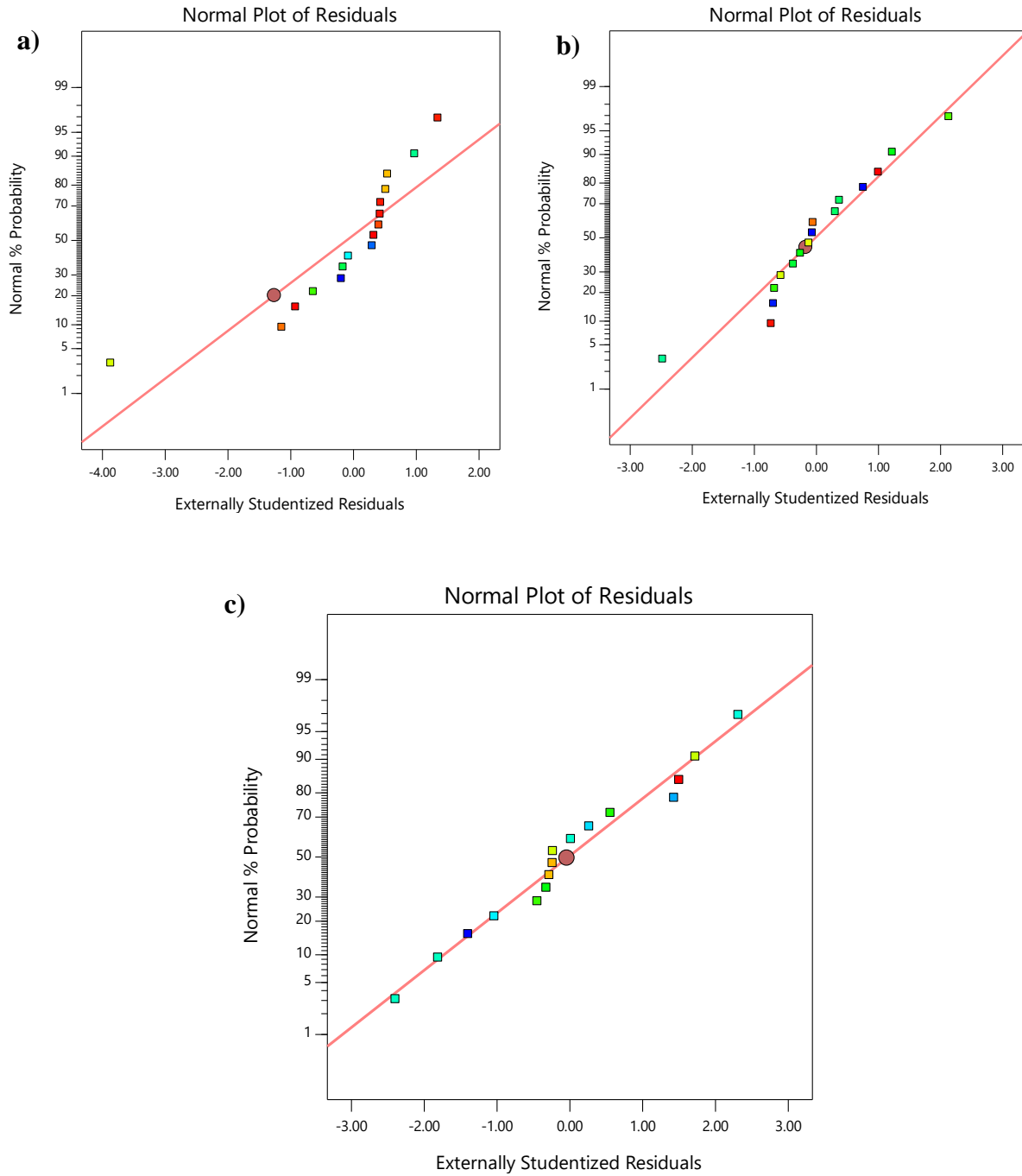


Figure 4.7: Normal probability plot for a) sulphate reduction efficiency, b) sulphate reduction rate and c) sulphide production

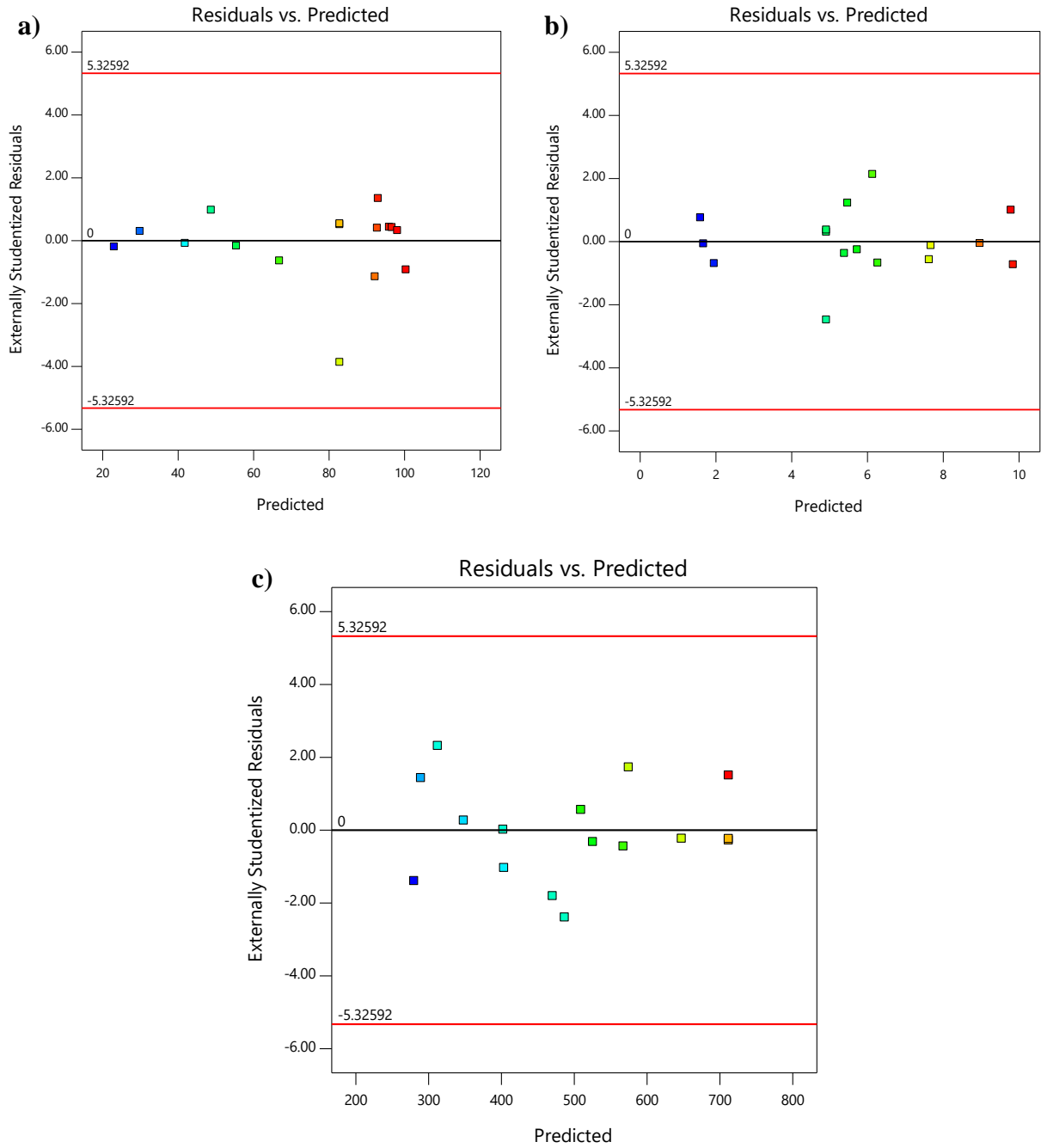


Figure 4.8: Residual vs predicted plot for a) sulphate reduction efficiency, b) sulphate reduction rate and c) sulphide production

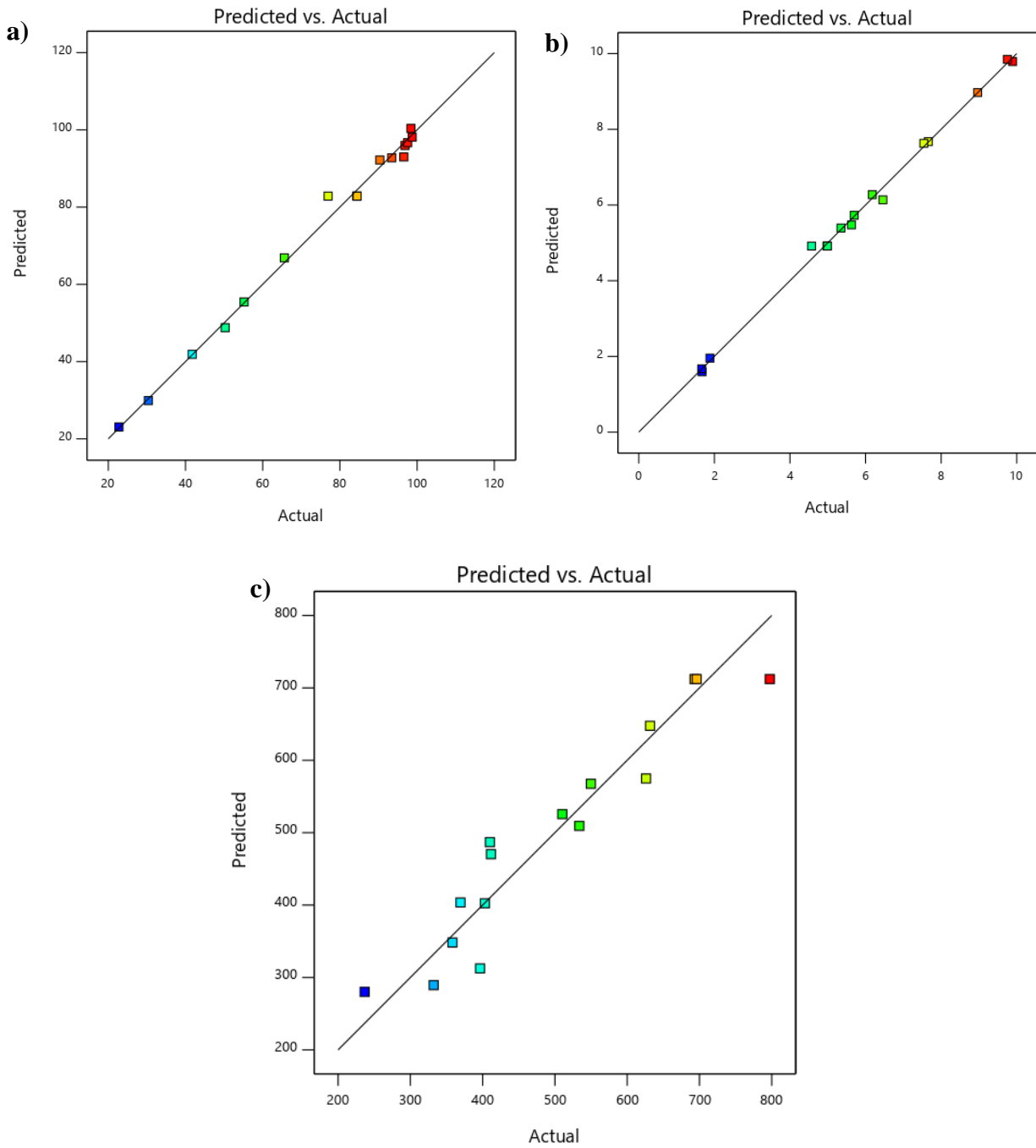


Figure 4.9: Predicted vs actual response values plot for a) sulphate reduction efficiency, b) sulphate reduction rate and c) sulphide production

CHAPTER 5: EFFECTS OF INDIVIDUAL PARAMETERS

In this section, individual effects of independent variables on the responses are shown where one factor is changed at a time while others are kept constant at the centre point. The sensitivity of a factor is shown by a steep slope.

5.1. The effect of pH

From Figure 5.1a and Figure 5.1b, a pH increase from 4 to 6 is shown to have almost no impact on sulphate reduction efficiency and sulphate reduction rate. There was a slight increase in sulphide production from about 600 mg/L to approximately 700 mg/L (Figure 5.1c) when pH increased from 4 to 5 followed by a slight decrease to 600 mg/L with an increase in pH from 5 to 6, however, the change was not significant. This is in agreement with ANOVA analysis shown in Table 4.1 that shows that the model term A (pH) is not significant for all responses.

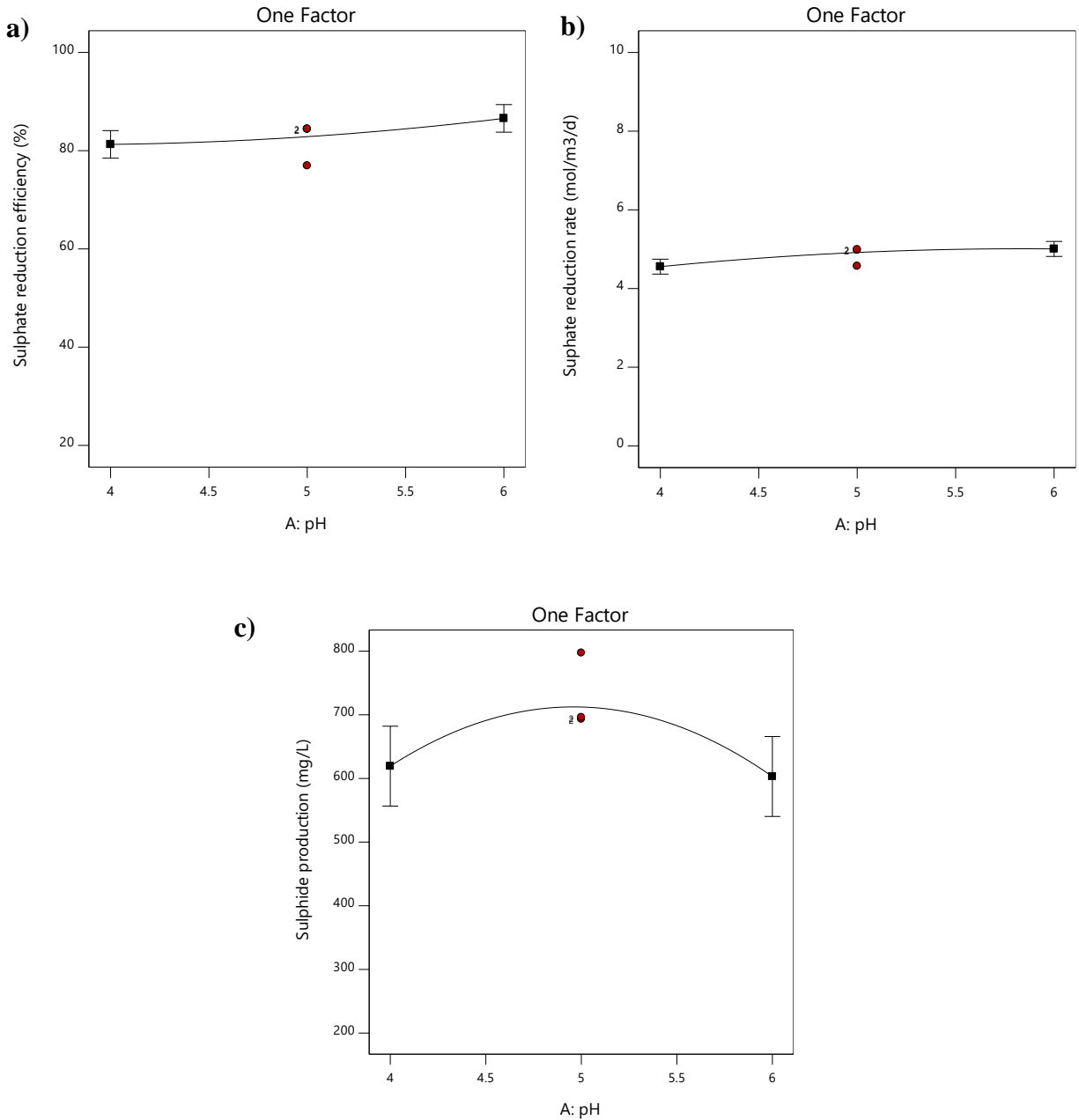


Figure 5.1: Effect of pH on a) sulphate reduction efficiency, b) sulphate reduction rate and c) sulphide production at 20 °C and HRT of 4.5 d

Experiments at 30 °C, HRT 4.5 d and pH 4, 5 and 6 were chosen to show the effect of decreasing pH on sulphate reduction efficiency over time as shown in Figure 5.2. From the graph, it is clear that a decrease in pH from 6 to 5 had no significant impact on sulphate reduction efficiency ($p = 0.69$). A further decrease in pH from 5 to 4 had a slight impact on sulphate reduction efficiency ($p = 0.005$). Sulphate reduction, however, was still maintained above 87 % even at pH as low as 4.

Low pH (<5.5) is known to suppress sulphate reduction (Dvorak et al., 1992, Lettinga et al., 1984). Other studies have shown that sulphate reduction was possible at pH 4 and less albeit at reduced efficiencies (< 45 %) (Christensen et al., 1996, Elliott et al., 1998, Sen and Johnson, 1999, Tsukamoto et al., 2004). For example, Elliott et al. (1998) found that at pH 3 only 14.4 % sulphate reduction was possible with no sulphide production detected. In the current study, Design expert has predicted that at pH 3 sulphate reduction efficiency of over 65 % is possible with sulphide production of over 260 mg/L. Some studies, on the other hand, have shown that sulphate reduction of above 70 % is possible at pH 4 (Jong and Parry, 2006, Lopes, 2007). Using a mixed culture of SRB could have been favourable for this study. Some authors suggest that a mixed SRB culture should be used because it can withstand harsh conditions compared to pure cultures (Kimura et al., 2006, Küsel et al., 2001). The effluent pH was maintained above 6.9 for all pHs, Figure 5.3. The increase in pH shows that SRB metabolic activity was not inhibited by low initial pH hence the system was able to consume the acidity (Castro Neto et al., 2018). In the current study, it was demonstrated that sulphate reduction is not impacted by a decrease in pH from 6 to 4. This could imply that lower costs need to be expended to get AMD to pH levels above 5.5 and this could have a positive impact on the process' operating expenses

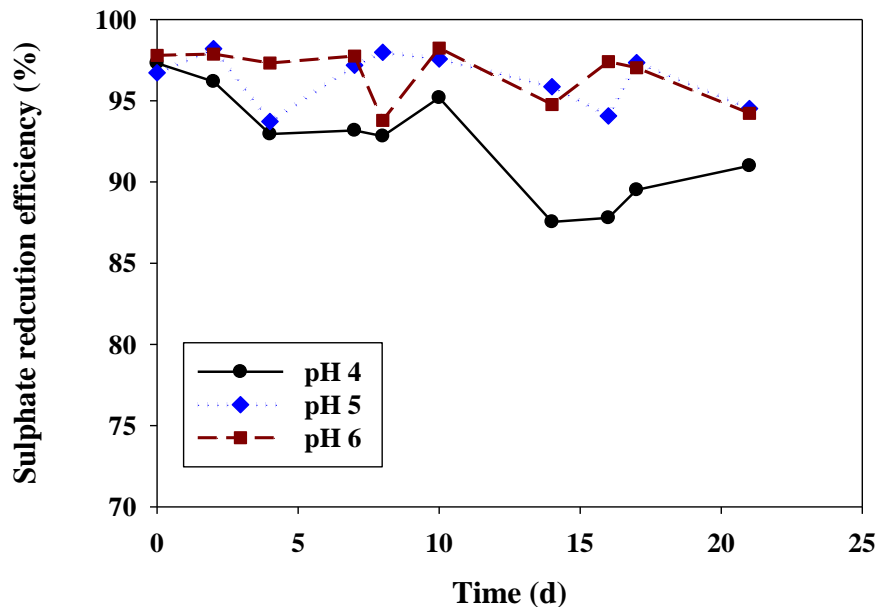


Figure 5.2: Sulphate reduction efficiency at 30 °C and HRT of 4.5 d

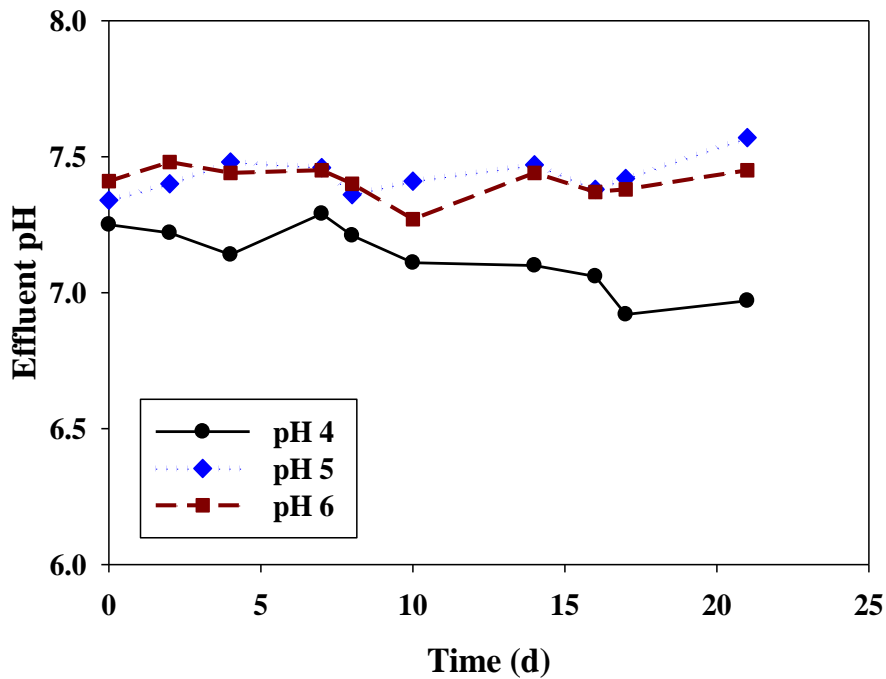


Figure 5.3: Effluent pH at 30 °C and HRT of 4.5 d

5.1.1.1. Pre-neutralisation lime requirements and sludge formation

Table 5.1 shows the results obtained for the pre-neutralisation experiments. From the table, it is clear that pH adjustment to pH 4 requires less lime than that required for pH adjustment to pH 5 and 6. Lime required to adjust the pH to 4 was approximately 50 % less than that required to adjust the pH to 5 in both 10 L and 25 L buckets. Lime added to adjust the pH to 4 was 2.50 ± 0.065 g and 5.38 ± 0.075 g in 10 L and 25 L buckets respectively whereas it was 5.62 ± 0.585 g and 10.9 ± 1.1 g in 10 L and 25 L buckets respectively for pH 5. Lime requirement for pH 6 was similar to that for pH 5 for 10 L buckets. However, for 25 L buckets, the amount of lime required was 18.23 ± 2.345 g for pH 6 compared to 10.9 ± 1.1 g. This shows that the difference in the amount of lime required to adjust pH to 6 increases drastically with an increase in the AMD volume.

The disadvantage of using lime neutralisation is the production of large sludge quantities that may be difficult to dispose of (Arnold et al., 2016b). The sludge produced is lower at pH 4 than pH 5

and 6. The results shown below shows that there is a significant reduction in sludge production if the pH is adjusted to 4 as opposed to 5 or 6. The sludge produced at pH 5 was 97.5 ± 7.5 ml and 310 ± 10 ml for 10 L and 25 L buckets respectively which was almost double that produced at pH 4 (47.5 ± 2.5 ml and 152.5 ± 12.5 ml for 10 L and 25 L buckets respectively).

The results show that operating at pH 4 (or lower) does not only reduce sludge formation (due to low lime dosages which result in decreased metal precipitation) but reduce lime consumption which in turn will have a positive impact on the operating expenditure of Mintek's process.

Table 5.1: Lime added and sludge formed during the pre-neutralisation

Target pH	Bucket Volume (L)	Raw AMD pH	Lime added (g)	Final pH	Sludge (ml)
pH 4	10	2.73 ± 0.035	2.50 ± 0.065	4.09 ± 0.02	47.5 ± 2.5
	25	2.50 ± 0.065	5.38 ± 0.075	4.07 ± 0.01	152.5 ± 12.5
pH 5	10	2.67 ± 0.035	5.62 ± 0.585	5.07 ± 0.015	97.5 ± 7.5
	25	2.8 ± 0.09	10.9 ± 1.1	5.11 ± 0.005	310 ± 10
pH 6	10	2.75 ± 0.01	5.8 ± 0.44	6.04 ± 0.015	137.5 ± 7.5
	25	2.71 ± 0.055	18.23 ± 2.345	6.1 ± 0.02	405 ± 25

5.2. The effect of temperature

The model term B (temperature) was significant for all the responses (Table 4.1). According to Figure 5.4a and Figure 5.4b, increasing temperature from 10 °C to 30 °C resulted in an increase in sulphate reduction efficiency and sulphate reduction rate. This was due to the impact that low temperature has on the metabolic activity of SRB (Doshi, 2006, Lettinga et al., 1984). A decrease in temperature from 30 °C to 10 °C led to a decrease in sulphate reduction efficiency of more than 60 %.

Initially, dissolved sulphide concentration correlated well with sulphate reduction until about 23 °C, shown in Figure 5.4c. Sulphate reduction increased until 30 °C, however, sulphide production did not increase as expected after approximately 23 °C. A study done by Zinatizadeh et al. (2011)

showed that the fraction of gaseous H_2S emitted increases with an increase in temperature. Therefore, it is presumed that the increase in temperature, in this case, could possibly have increased the H_2S gas emission and therefore a decrease in sulphide in solution with a further increase in temperature from 23 °C to 30 °C. This implies that there could be an increase in sulphide gas production at temperatures above 23 °C.

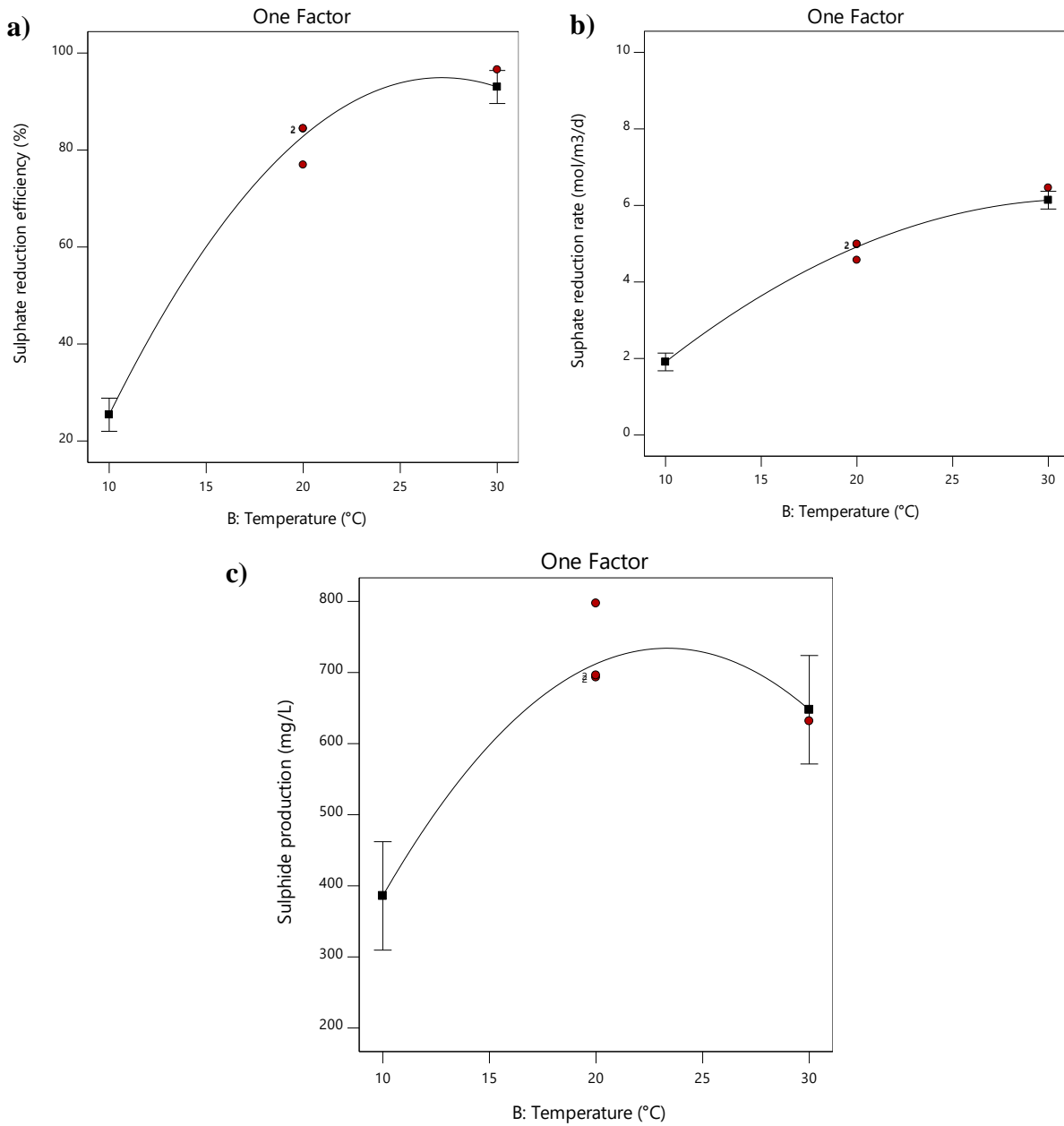


Figure 5.4: Effect of temperature on a) sulphate reduction efficiency, b) sulphate reduction rate and c) sulphide production at pH 5 and HRT of 4.5 d

Sulphate reduction efficiency was impacted by a decrease in temperature from 30 °C to 20 °C at HRT of 4.5 d (Figure 5.5a) as compared to HRT of 7 d (Figure 5.5d). At HRT of 4.5 d, sulphate reduction efficiency was an average of 96 % at 30 °C. At 20 °C, sulphate reduction efficiency decreased to about 75 % at day 8 followed by an increase to about 80 % at day 13 and was maintained at that efficiency for the duration of the experiment. A further decrease to 10 °C resulted in a decrease in sulphate reduction efficiency to about 14 % at day 8 followed by an increase from day 13 to approximately 30 % until day 21. A similar trend was observed for sulphate reduction rate, Figure 5.5b. Sulphate reduction rate was maintained between 5.8 and 6.8 mol/m³/d at 30 °C. A decrease from 5.2 mol/m³/d to approximately 4.2 mol/m³/d was observed at day 8 at 20 °C. However, the reduction rate increased to between 4.5 and 5 mol/m³/d from day 13 till day 21. At 10 °C, the reduction rate was below 2.3 mol/m³/d. The sulphide concentration was steady at around 640 mg/L at 30 °C and around 230 mg/L at 10 °C whereas it was between 600 and 820 mg/L at 20 °C.

At HRT 7 d, there was no significant impact ($p = 0.06$) on sulphate reduction efficiency when temperature was decreased from 30 °C to 20 °C (Figure 5.5d). Sulphate reduction efficiency was maintained above 96 % for both temperatures. A further decrease in temperature to 10 °C resulted in a decrease in sulphate efficiency to between 35 % and 46 % for the duration of the experiment. In Figure 5.5e, sulphate reduction rate was between 7 and 8.1 mol/m³/d at 30 °C, between 5 and 6.2 mol/m³/d at 20 °C and just below 2 mol/m³/d at 10 °C. Sulphide concentration followed a similar trend for all temperatures as shown in Figure 5.5f. This was presumably due to low emission rate at longer HRT (Zinatizadeh et al., 2011), hence the similar sulphide concentration for the duration of the experiment at 30 °C, 20 °C and 10 °C.

The results presented in this section shows that a decrease in temperature from 30 °C to 20 °C did not impact sulphate reduction efficiency drastically as compared to a decrease to 10 °C. The drastic decrease in sulphate reduction can be attributed to the decreased SRB metabolic activity at low temperatures (Doshi, 2006). Sheoran et al. (2010) mentioned that sulphate reduction efficiency is likely to drop by 50 % or more when temperature is decreased from 20 °C to 10 °C or lower. Greben et al. (2002) found that SRBs were not too sensitive to temperatures between 15 °C and 20 °C, however, the SRB activity was impacted at temperatures lower than 15 °C. In their study,

sulphate reduction rate was reduced by 46 % at temperatures lower than 15 °C. Similarly, Ferrentino et al. (2017) reported that sulphate reduction rate was stable between 15 and 20 °C, however, it decreased by more than 55 % at temperatures lower than 15 °C. Virpiranta et al. (2019), on the other hand, observed a 42 % decrease in sulphate reduction rate with a decrease in temperature from 22 °C to 16 °C. In a study done by Marais et al. (2018), sulphate reduction efficiency decreased by approximately 38 % as temperature was decreased from 30 °C to 10 °C.

The results also show that sulphate reduction efficiency was higher at 20 °C and HRT of 7 d (Figure 5.5d) compared to 20 °C and HRT of 4.5 d (Figure 5.5a). Poinapen et al. (2009b) found that longer HRT was required at 20 °C in order to achieve sulphate reduction efficiency of greater than 90 %. A similar observation was made in this study where sulphate reduction efficiency was approximately 80 % at HRT of 4.5 d and above 96 % at 7 d. This implies that in colder months the model will suggest running the system at longer HRTs.

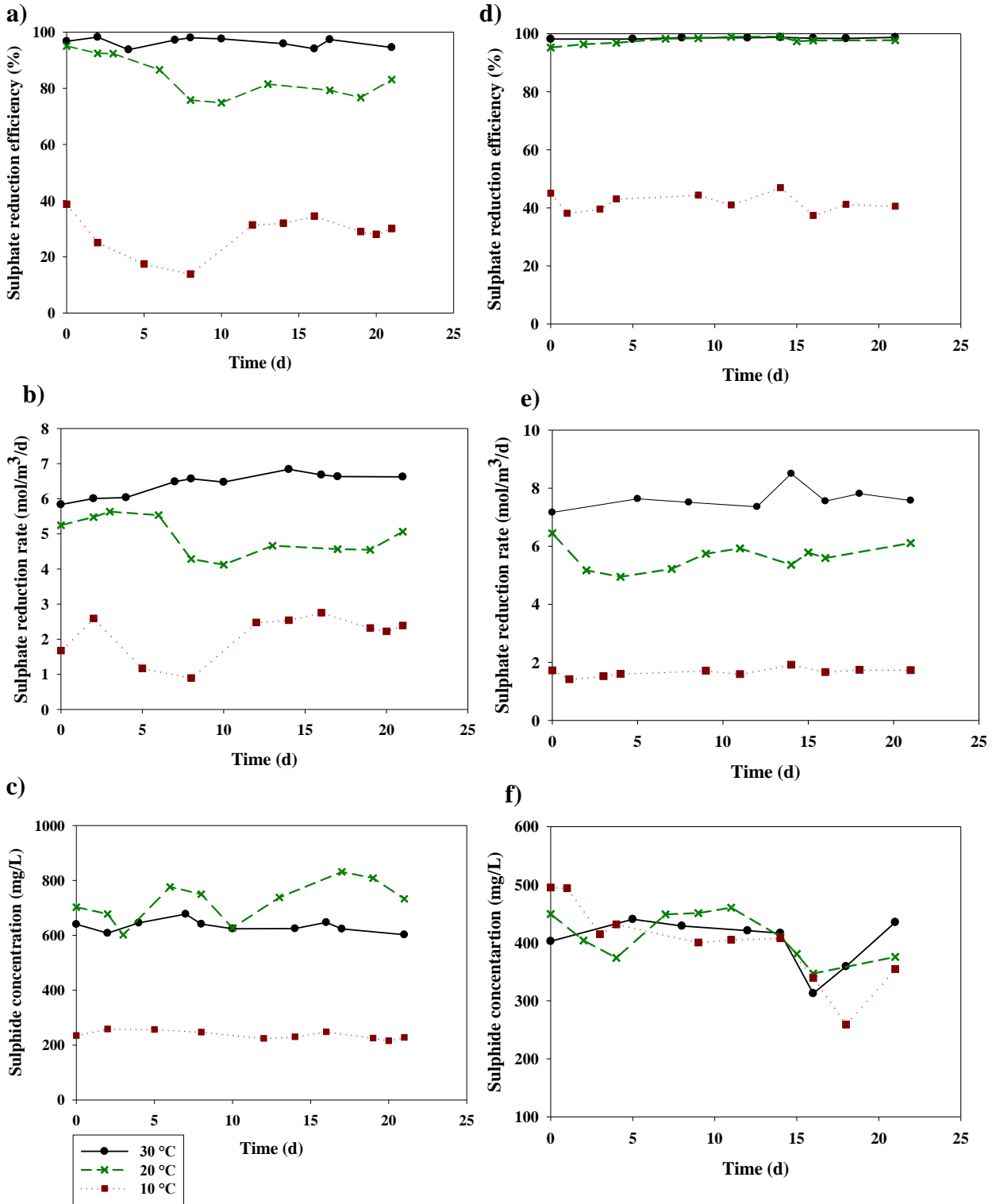


Figure 5.5: Sulphate reduction efficiency, sulphate reduction rate and sulphide concentration at HRT of 4.5 d (a, b, c) and HRT of 7 d (d, e, f)

5.3. The effect of HRT

The effect of HRT on sulphate reduction efficiency, sulphate reduction rate and sulphide production is shown in Figure 5.6. Sulphate reduction efficiency increased with an increase in HRT (Figure 5.6a). Sulphate reduction rate decreased with an increase in HRT from HRT of 2 d to HRT of 5 d followed by a slight increase with an increase in HRT to 7 d. Although sulphate reduction efficiency decreased with decreasing HRT, sulphate reduction rate increased with a decrease in HRT due to higher feed rates. Longer HRT resulted in higher sulphate reduction efficiency but lower sulphate reduction rates. Similar observations were made in earlier studies (Gibert et al., 2004, Glombitza, 2001, Greben and Maree, 2000, Mallelwar, 2013).

Sulphide increased with an increase in HRT reaching maximum at HRT of approximately 5 d followed by a slight decrease with an increase in HRT. Low sulphide concentration is observed at HRT of 2 d due to low sulphate reduction efficiency which was a result of reduced contact time between SRB and substrates (Mallelwar, 2013) due to high feeding rates. The slight decrease in sulphide observed may possibly be due to metal precipitation as a result of sufficient contact time between metals and sulphide at HRT greater than 5 d (Sheoran et al., 2010).

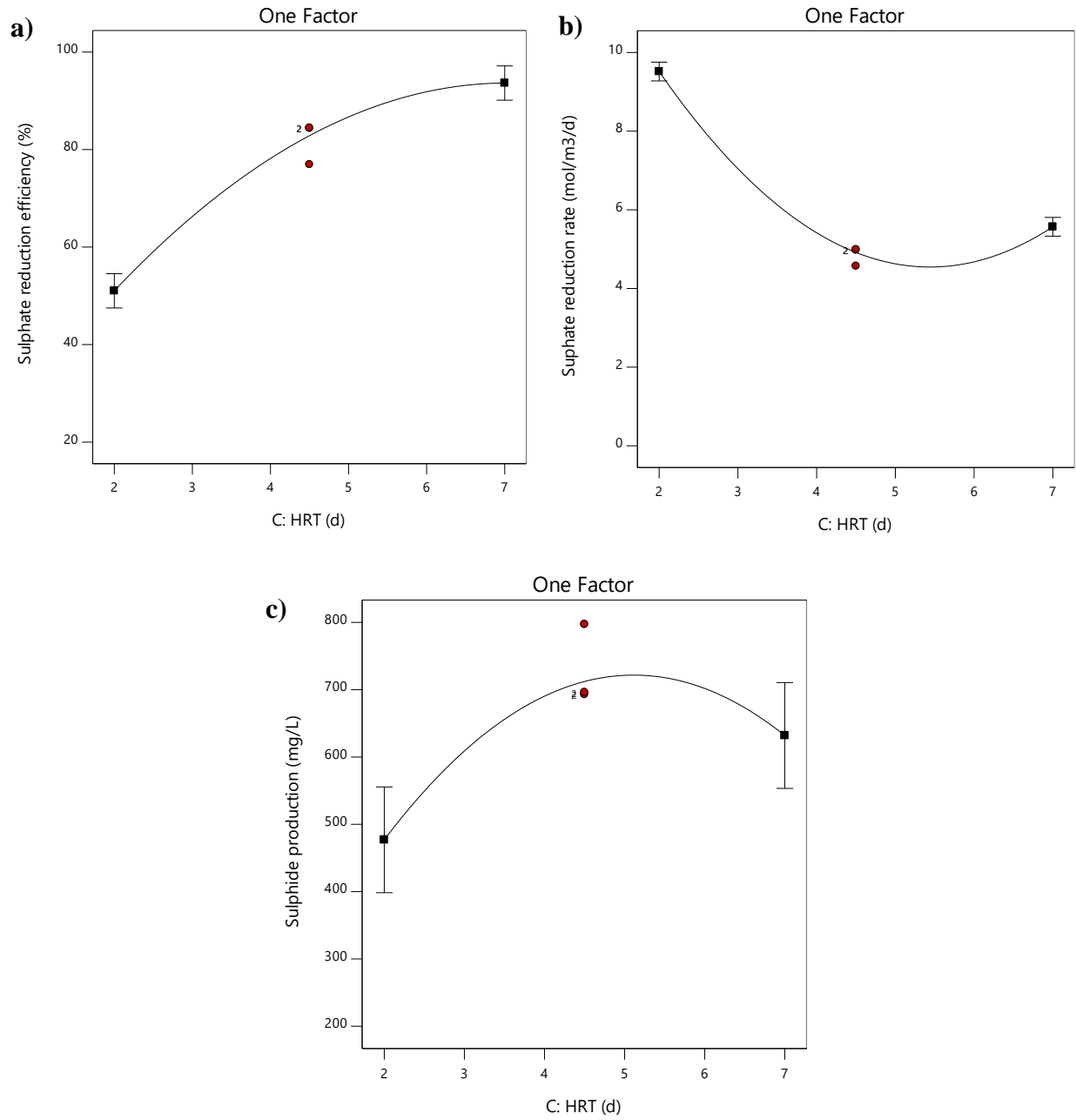


Figure 5.6: Effect of HRT on a) sulphate reduction efficiency, b) sulphate reduction rate and c) sulphide production at 20 °C and pH 5

Figure 5.7 shows how the system behaved over time with a decrease in HRT from 7 d to 2 d. Experiments at 30 °C, pH 5 and HRT 2 d, 4.5 d and 7 d were chosen to show the effect of decreasing HRT on sulphate reduction efficiency, sulphate reduction rate and sulphide production over time. From Figure 5.7a, sulphate reduction efficiency was slightly impacted by a decrease in HRT from 7 d to 4.5 d. Sulphate reduction efficiency was maintained above 98 % at HRT of 7 d. Decreasing HRT to 4.5 d led to a slight decrease in sulphate reduction efficiency to about 93 % after 4 days, however, it increased and was stable above 95 % for the duration of the experiment. Sulphate reduction rate was maintained between 6.5 and 8.9 mol/m³/d at HRT 7 and between 6 and 6.5 mol/m³/d with a decrease in HRT to 4.5 d (Figure 5.7b). Sulphide concentration decreased from about 530 mg/L (day 5) to above 350 mg/L (day 14) and was maintained between 350 and 380 mg/L for the duration of the experiment at HRT of 7 d as shown in Figure 5.7c. Sulphide concentration was stable between 600 and 640 mg/L at HRT of 4.5 d.

Decreasing HRT further to 2 d had a significant impact on sulphate reduction efficiency, sulphate reduction rate and sulphide production. A decrease in sulphate reduction to 50 % was observed when HRT was decreased to 2 d. Interestingly, after replenishing, sulphate reduction increased to 87.2 % at day 3. After 5 days of not replenishing the substrates, sulphate reduction decreased to 37.5 % (day 8). At day 8, the substrates were replenished and this led to an increase in sulphate reduction to 82.3 % (day 11). The same trend was observed until the end of the experiment (day 21). A similar trend at HRT of 2 d was observed for sulphate reduction rate and sulphide concentration. Sulphate reduction rate reached a minimum of approximately 5.5 mol/m³/d before replenishing and a maximum of approximately 12.9 mol/m³/d after replenishing. Sulphide production, on the other hand, had a minimum of about 380 mg/L before replenishing and a maximum of approximately 680 mg/L after replenishing.

Low HRT has a major impact on the performance of BSR. Some studies have shown that sulphate reduction efficiency decreases with a decrease in HRT due to various reasons including incomplete oxidation of acetate (Kaksonen et al., 2004) and SRB washout (Icgen and Harrison, 2006, Oyekol et al., 2007, Sipma et al., 2007). However, in this study, it is presumed that low HRT resulted in the washout of substrates. This became evident when sulphate reduction improved upon replenishing. These results show that at 30 °C, sulphate reduction is still possible at HRT as low

as 2 d, however, to maintain it above 80 % the substrates have to be replenished more often. A study done by Poinapen et al. (2009a) using primary sewage sludge (PSS) as substrate demonstrated that a decrease in HRT did not have an impact on sulphate reduction. This was because the PSS was fed into the reactor together with the synthetic AMD, therefore a decrease in HRT implied that the PSS loading was increasing. In other words, the substrate loading was increased with a decrease in HRT which was not the case in this study. Hence it is presumed that the substrates were washed out quicker.

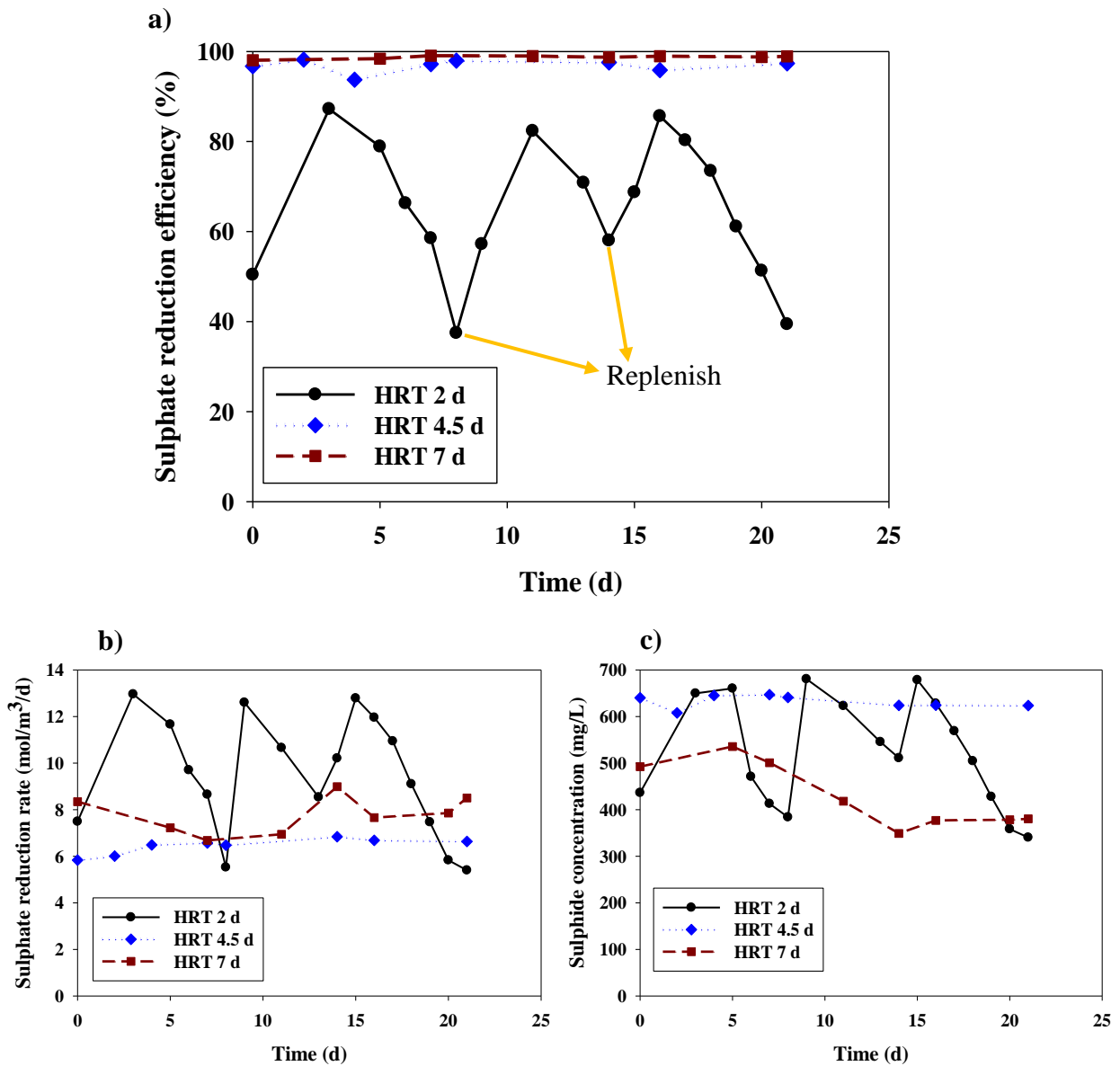


Figure 5.7: Effect of HRT over time at 30 °C and pH 5 for a) sulphate reduction efficiency, b) sulphate reduction rate and c) sulphide production

The experiment at 30 °C, pH 5 and HRT of 2 d was conducted over a period of 21 days due to the observed decrease in sulphate reduction before replenishing the substrates and an increase after replenishing. After it was established that the trend was possibly due to the washout of substrates, the remaining experiments at HRT of 2 d were conducted over a period of 12 days. Figure 5.8 depicts sulphate reduction efficiency at 10 °C, 20 °C and 30 °C. From this graph, it is observable that sulphate reduction efficiency increased after the replenishing of substrates (day 5) with decreasing temperature.

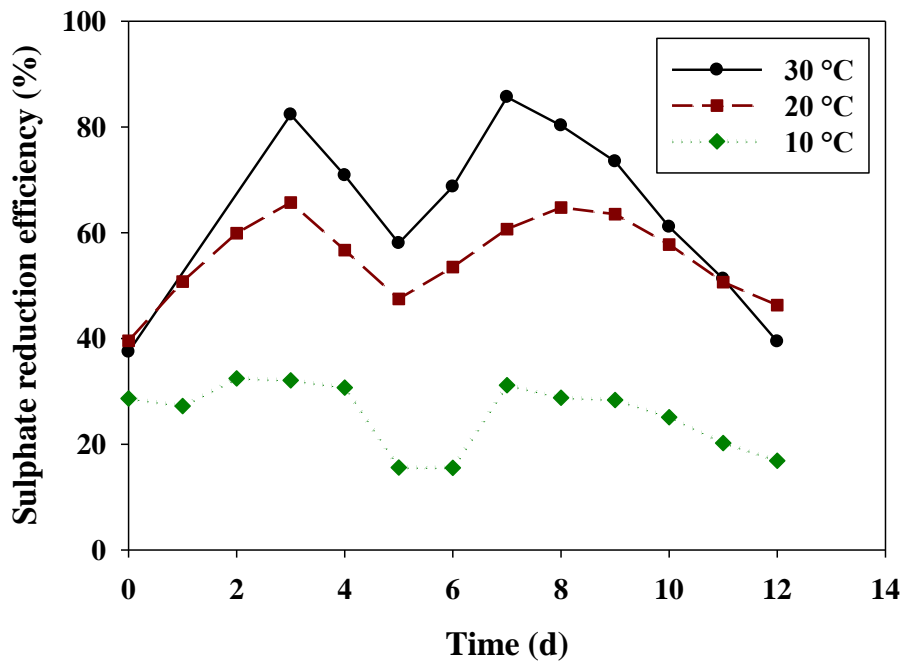


Figure 5.8: Sulphate reduction efficiency at different temperatures at HRT of 2 d

The presented results show that sulphate reduction is possible at HRT as low as 2 d, however, this requires frequent replenishing of substrates which might not be economical for large scale as it would require more labour and it could have cost implications on the purchasing of substrates.

CHAPTER 6: RESPONSE SURFACE DIAGRAMS, OPTIMIZATION AND SULPHIDE MANAGEMENT

Interactive effects between factors are discussed in this chapter. The Design Expert software was also used to obtain the optimized solution. The applicability of the developed sulphate reduction efficiency model is also explained. The last section of this chapter has details on how the sulphide produced will be further processed to reduce its toxic effects.

6.1. Temperature and pH

Figure 6.1a, b and c show the response surface diagrams for sulphate reduction efficiency, sulphate reduction rate and sulphide production. The interaction between pH and temperature (AB) is illustrated. For sulphate reduction efficiency and sulphate reduction rate, increasing the pH at all temperatures had no impact as depicted in Figure 6.1a and b. Temperature, on the other hand, had a significant impact on both sulphate reduction efficiency and rate. Sulphate reduction increased with an increase in temperature at constant pHs. Increasing both pH and temperature simultaneously had an impact on sulphide production. Sulphide production increased until a maximum was reached as shown in Figure 6.1c, this was followed by a decrease with a further increase in temperature and pH. According to the response surface diagram, the impact temperature had at constant pHs was greater than the impact that pH had at constant temperatures. The simultaneous influence of temperature and pH on all responses, however, was found to be insignificant as shown in Table 4.1.

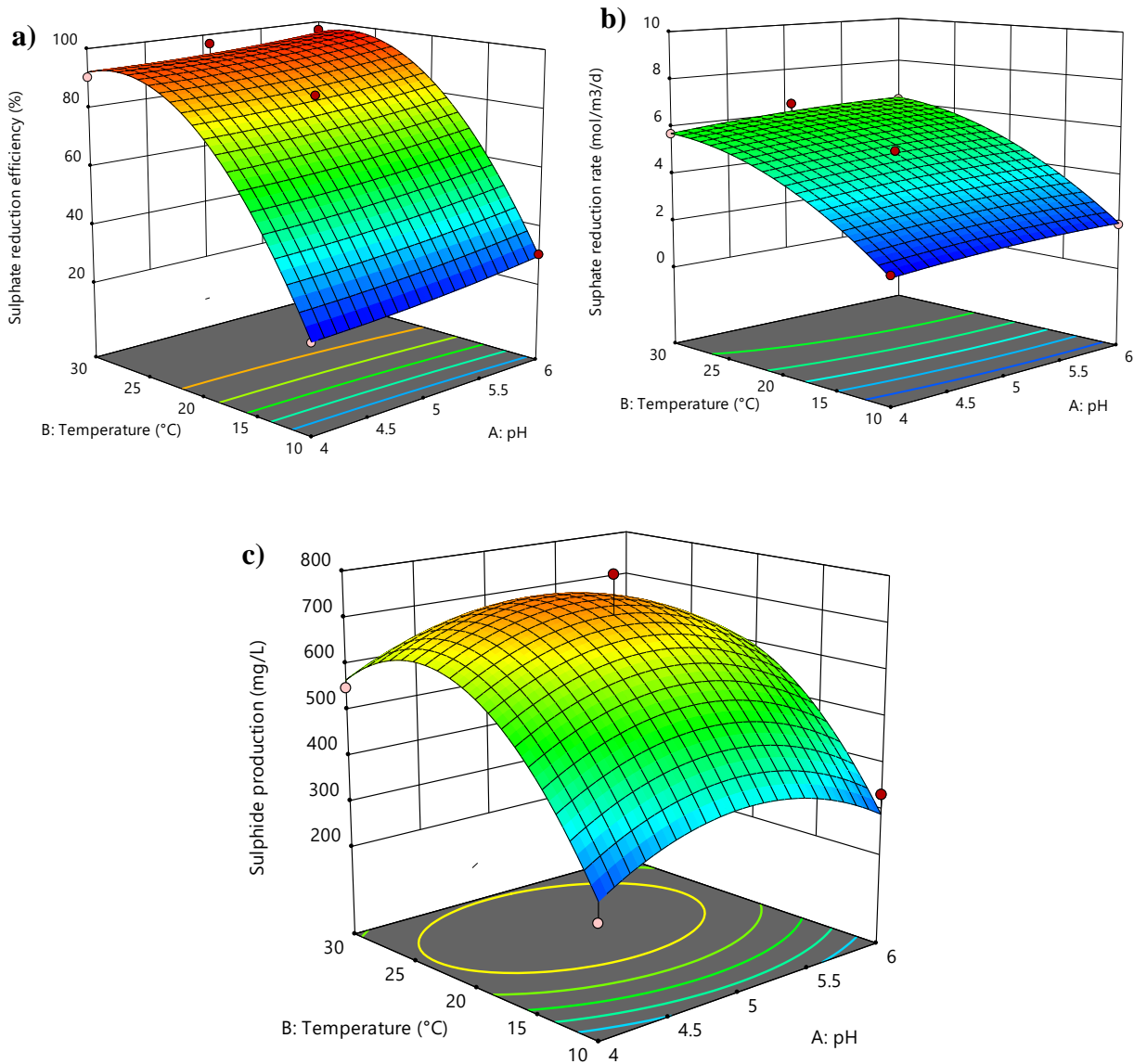


Figure 6.1: Surface response for a) sulphate reduction efficiency, b) sulphate reduction rate and c) sulphide production with respect to temperature and pH at HRT of 4.5 d

6.2. HRT and pH

The interactive effect between pH and HRT (AC) on sulphate reduction efficiency, sulphate reduction rate and sulphide production when temperature was constant at the centre point is shown in Figure 6.2a, b and c. From all the graphs, pH is shown to not have any significant impact on all the responses at all HRTs. Sulphate reduction efficiency increased with an increase in HRT at all pHs as shown in Figure 6.2a. The impact of HRT was greater for sulphate reduction rate which

increased considerably with a decrease in HRT as illustrated in Figure 6.2b. Sulphate reduction rate reached a maximum of approximately 9.8 mol/m³/d at all pHs at HRT of 2 d. Sulphide production, on the other hand, increased with an increase in HRT until a maximum was reached at about HRT 5 d, Figure 6.2c. This was followed by a slight decrease with a further increase in HRT. These graphs prove that the interaction between pH and HRT was not significant as shown in Table 4.1.

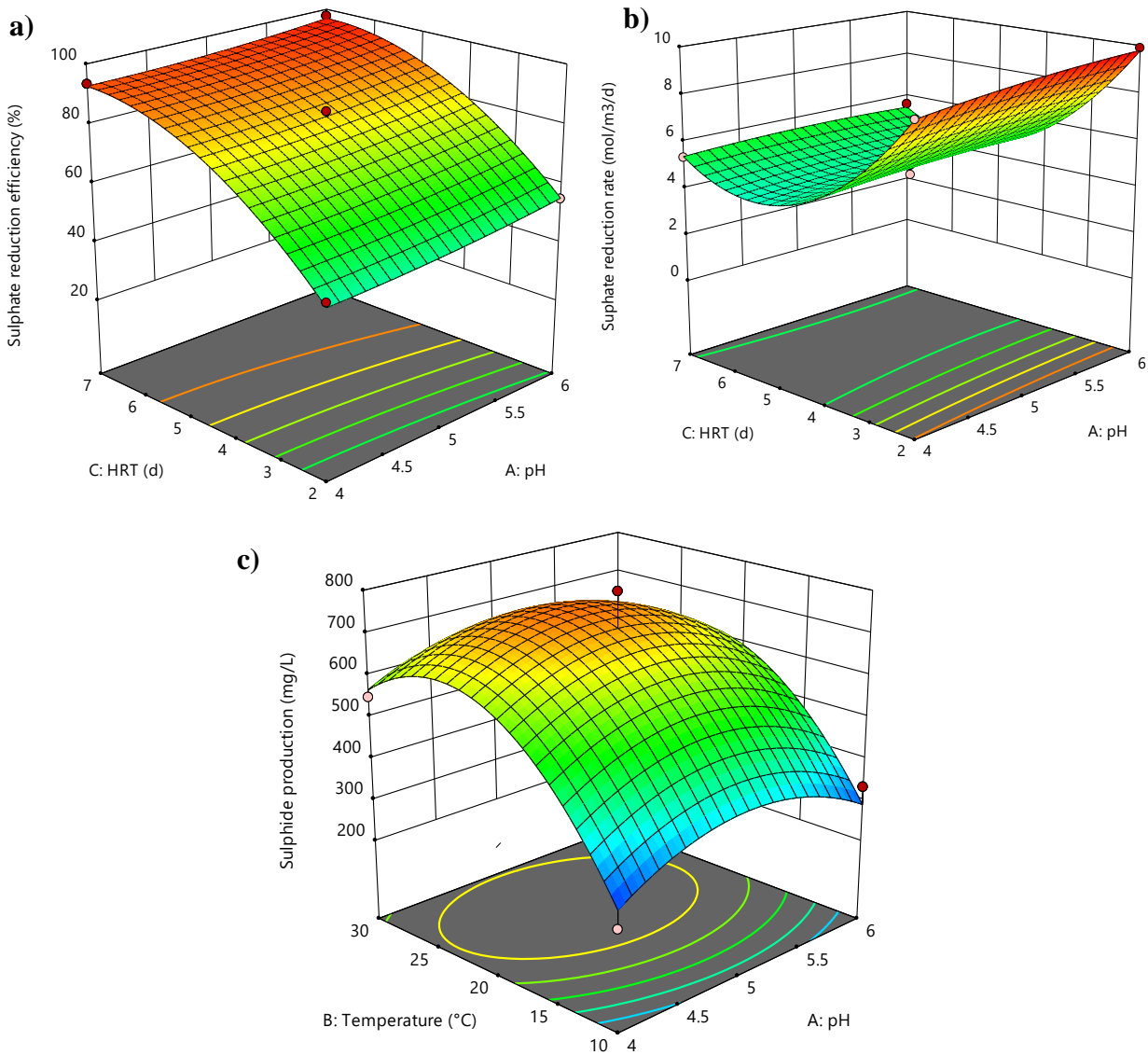


Figure 6.2: Surface response for a) sulphate reduction efficiency, b) sulphate reduction rate and c) sulphide production with respect to HRT and pH at temperature of 20 °C

6.3. Temperature and HRT

Figure 6.3a, b and c depict the surface response diagram for the interactive effect of temperature and HRT (BC) on sulphate reduction efficiency, sulphate reduction rate and sulphide production while pH remained constant at the centre point. The interaction between model term BC was found to be significant for sulphate reduction efficiency and sulphate reduction rate but insignificant for sulphide production as reported in Table 4.1. In Figure 6.3a, sulphate reduction efficiency increased with a simultaneous increase in temperature and HRT. From the graph, temperature had a more impact on sulphate reduction efficiency compared to HRT. For example, at 30 °C, sulphate reduction efficiency decreased from almost 100 % at HRT of 7 d to just above 60 % at HRT of 2 d. On the other hand, at HRT of 7 d, it decreased to approximately 40 % with a decrease in temperature from 30 °C to 10 °C. This could mean that for large scale operations, HRT should be increased to higher than 7 d to compensate for lower temperatures during winter seasons in order to obtain higher sulphate reduction efficiencies.

Conversely, the increase in sulphate reduction rate caused by a decrease in HRT was greater at all temperatures than that caused by an increase in temperature at all HRTs as shown in Figure 6.3b. The higher sulphate reduction rate was approximately 10 mol/m³/d which was observed at 30 °C and HRT of 2 d. This means that there should be a careful consideration between sulphate reduction rate and efficiency and which one is more important in order to have treated AMD that complies with the discharge standards. In Figure 6.3c, sulphide production increased with a simultaneous increase in HRT and temperature until a maximum was reached. This was followed by a decrease with a further increase in both HRT and temperature. Just like with sulphate reduction efficiency, the impact of temperature on sulphide production was greater than that caused by HRT.

The presented results showed that temperature and HRT have a strong interaction. For optimal operations, HRT and temperature should be changed seasonally. In summer seasons, the reactors could operate at low enough HRT that will not require frequent replenishing. However, in winter seasons, HRT should be high enough to meet the discharge standards.

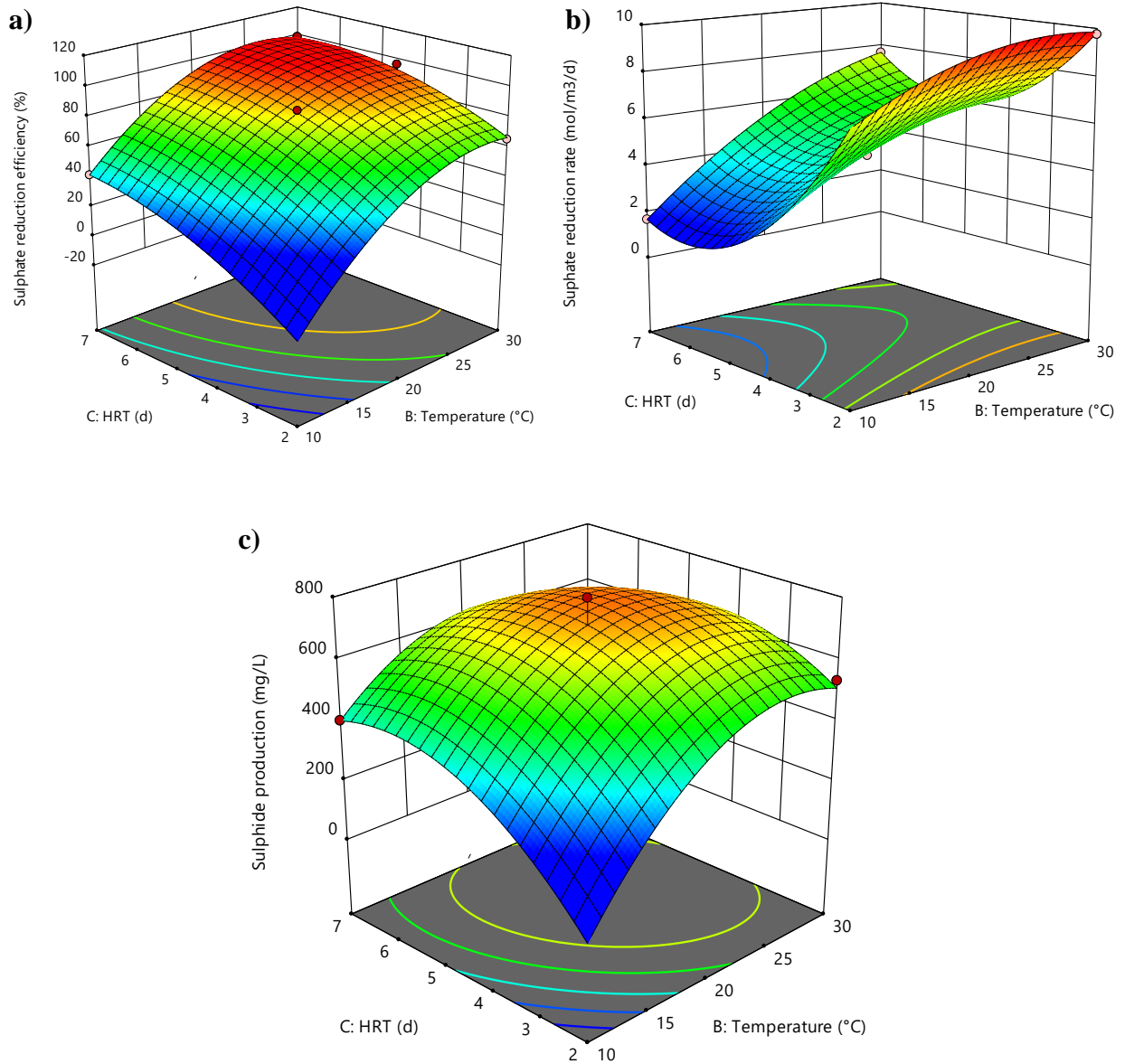


Figure 6.3: Surface response for a) sulphate reduction efficiency, b) sulphate reduction rate and c) sulphide production with respect to temperature and HRT at pH 5

6.4. Optimization

The numerical optimization section in design expert allows one to maximize the desirability function. The desirability level varies from 0 to 1, with level 0 indicating that one of the responses is outside the specified limit and a level closer to 1 indicating that the corresponding factor combination is closer to optimal (Kasim et al., 2019). The optimization process was carried out by setting the factor and response goals as shown in Table 6.1. The goal was to maximize sulphate reduction efficiency simultaneously with the rate at which sulphate is reduced while minimizing sulphide production. The pH was chosen to be minimized because it was found to not have any impact on the process and therefore it should be kept as low as possible. Although sulphide production is dependent on the efficiency and the rate at which sulphate is reduced, the goal was set to minimize because it is toxic. The optimized solution is shown in Table 6.2.

Table 6.1: Goals and limits applied for optimization

Factor/Response	Goal	Lower limit	Upper limit
pH	Minimize	4	6
Temperature (°C)	Is in range	10	30
HRT (d)	Is in range	2	7
Sulphate reduction efficiency (%)	Maximize	22.76	98.7298
Sulphate reduction rate (mol/m ³ /d)	Maximize	1.66	9.89196
Sulphide production (mg/L)	Minimize	144.54	797.3

Table 6.2: Optimized solution

Factor			Response			Desirability
pH	Temperature (°C)	HRT (d)	Sulphate reduction efficiency (%)	Sulphate reduction rate (mol/m ³ /d)	Sulphide production (mg/L)	
4	30	4.481	92.081	5.737	567.42	0.716

The optimized solution found in design expert was similar to an experiment that formed part of the 17 experiments that were conducted. The predicted and the actual response values with their respective percent difference are shown in Table 6.3. This provides confidence that the models developed from this study are robust and accurate.

Table 6.3: Comparison between optimized response values and experimental values

Factor/Response	Predicted	Experimental	Percent difference
pH	4	4	-
Temperature (°C)	30	30	-
HRT (d)	4.481	4.5	0.42
Sulphate reduction efficiency (%)	92.081	90.35	1.90
Sulphate reduction rate (mol/m ³ /d)	5.737	5.7	0.66
Sulphide production (mg/L)	567.42	549.73	3.17

6.5. Application of the developed models

DoE does not only help with understanding the behaviour of systems but it is also used as a decision-making tool by evaluating the consequences of different scenarios (Kasim et al., 2007). This section serves as an example of how the developed tool can be used for decision-making and evaluating different scenarios focusing on sulphate reduction efficiency.

In an open system, temperature is one factor that cannot be controlled. The average temperatures at eMalahleni are shown in Table 6.4. From the table, it can be seen that the maximum average temperature is 20.1 °C in summer and the minimum average temperature is 8.7 °C in winter.

Table 6.4: Average temperatures in eMalahleni throughout the year (Climate-Data.org, 2020)

Month	Average temperatures (°)
January	20.1
February	19.6
March	18.3
April	15.3
May	11.9
June	8.7
July	8.7
August	11.4
September	14.9
October	17.6
November	16.6
December	19.6

The average influent sulphate concentration in the raw AMD that was used throughout the experiment was 3368.2 mg/L. The maximum allowable discharge concentration for sulphate in South Africa is 600 mg/L (Arnold et al., 2016b). Assuming that the influent concentration is 3368.2 mg/L, a sulphate reduction efficiency of 82.2 % will be required to achieve 600 mg/L sulphate in the effluent. The goals were set as shown in Table 6.5 and Table 6.6 and for summer and winter seasons respectively. From Table 6.5, the minimum HRT required to achieve 82.2 % sulphate reduction efficiency in summer is 4.54 d with a desirability of 0.997.

Table 6.5: Goals and limits to solve for minimum HRT required to achieve minimum sulphate reduction efficiency to achieve discharge standards in summer

Factor/Response	Goal	Lower limit	Upper limit	Predicted	Desirability
pH	Minimize	4	6	4.07	
Temperature (°C)	Target: 20.1	10	30	20.1	
HRT (d)	Is in range	2	7	4.57	
Sulphate reduction efficiency (%)	Target: 82.2	22.76	98.73	82.2	0.997
Sulphate reduction rate (mol/m ³ /d)	Is in range	1.66	9.89	4.45	
Sulphide production (mg/L)	Is in range	144.54	797.3	628.35	

Although extrapolation is not recommended in Design Expert software, it was used to estimate the highest sulphate reduction efficiency possible at the goals set for winter seasons as shown in Table 6.6. The maximum possible sulphate reduction efficiency that could be achieved is 31.15 % at HRT of 7.66 d if the reactor temperature is 8.7 °C with a desirability of 0.294. This shows that the discharge standard will not be met if the reactor temperature is that low. It should be noted that in an open system, the air temperature may not necessarily be the reactor temperature. The reactor temperature may not decrease to temperatures as low as 8.7 °C in winter. Some pilot plant studies have shown that the performance of the bioreactors did not decrease despite freezing temperatures (Gusek, 2002, Hedin, 2013, Nordwick et al., 2006). Some authors suggest that SRB increase in numbers to compensate for decreased substrate degradation caused by low temperatures and therefore maintaining the same sulphate reduction rates even in winter (Fortin et al., 2000). It is possible that the performance of Mintek's process might not drastically change during winter seasons provided that HRT is high enough to allow sufficient time for sulphate reduction to occur.

Table 6.6: Goals and limits to solve for minimum HRT required to achieve minimum sulphate reduction efficiency to achieve discharge standards in winter

Factor/Response	Goal	Lower limit	Upper limit	Predicted	Desirability
pH	Minimize	4	6	4	
Temperature (°C)	Equal to: 8.7	-	-	8.7	
HRT (d)	Is in range	2	16	7.66	
Sulphate reduction efficiency (%)	Target: 82.2	22.76	98.73	31.15	0.294
Sulphate reduction rate (mol/m ³ /d)	Is in range	1.66	9.89	1.75	
Sulphide production (mg/L)	Is in range	144.54	797.3	236.74	

6.6. Sulphide management

Sulphide gas produced during BSR is considerably more hazardous than sulphate (Marais et al., 2020, Rose, 2013). The common methods of sulphide removal are physicochemical processes such as direct air stripping and oxidation. However, these methods result in secondary toxic wastes and have high energy demand (Annachhatre and Suktrakoolvait, 2001a). Biological sulphide oxidation using sulphide oxidising bacteria (SOB) has been identified as a clean alternative for the removal of sulphide. Biological sulphide oxidation is a process that entails the use of sulphide oxidising bacteria to oxidise sulphide to elemental sulphur (S^0) as shown in Equation (6.1). The oxidation of sulphide to elemental sulphur is a result of incomplete oxidation. The complete oxidation of sulphide results in the formation of sulphate as shown in Equation (6.2). It is therefore recommended that the ratio of sulphide to oxygen be kept at 2:1 to prevent complete oxidation to sulphate (Marais et al., 2020). A floating sulphur biofilm is used to facilitate sulphide oxidation. The biofilm acts as a barrier that regulates oxygen mass transfer in order to promote the incomplete oxidation of sulphide to elemental sulphur (Van Hille and Mooruth, 2011).



In a study done by Van Hille and Mooruth (2011), degrading packed bed reactors were used for the reduction of sulphate and linear flow channel reactor (LFCR) for sulphide oxidation. In their study, they obtained 82.2 % sulphide oxidation of which 93 % was elemental sulphur. Marais et al. (2020) conducted a study based on previous work done by Van Hille and Mooruth (2011). They designed an LFCR hybrid that integrates both sulphate reduction and sulphide production in the same reactor. The designed hybrid reactor achieved 97 % sulphate reduction and sulphide oxidation between 95 and 100 %. Likewise, Celis-García et al. (2008) conducted a study in a down-flow fluidized reactor where sulphate reduction and sulphide oxidation were coupled in one reactor. In their study, sulphate reduction efficiency was between 75 % and 77 % with 50 % sulphur recovery.

The management of sulphide gas produced during the BSR process was beyond the scope of this project. However, Mintek is currently working on an integrated system where there is a stage after the BSR process for the oxidation of sulphide to elemental sulphur using SOBs. The goal for Mintek's integrated process is to produce treated AMD that is suitable for irrigation. The schematic diagram for the integrated process is shown in Figure 6.4. Initial studies conducted showed that 78 % of sulphide can be converted to elemental sulphur. Additionally, the treated AMD have the potential to be used for irrigating crops like maize sorghum and potatoes. However, more tests have to be conducted in order to optimise the process.

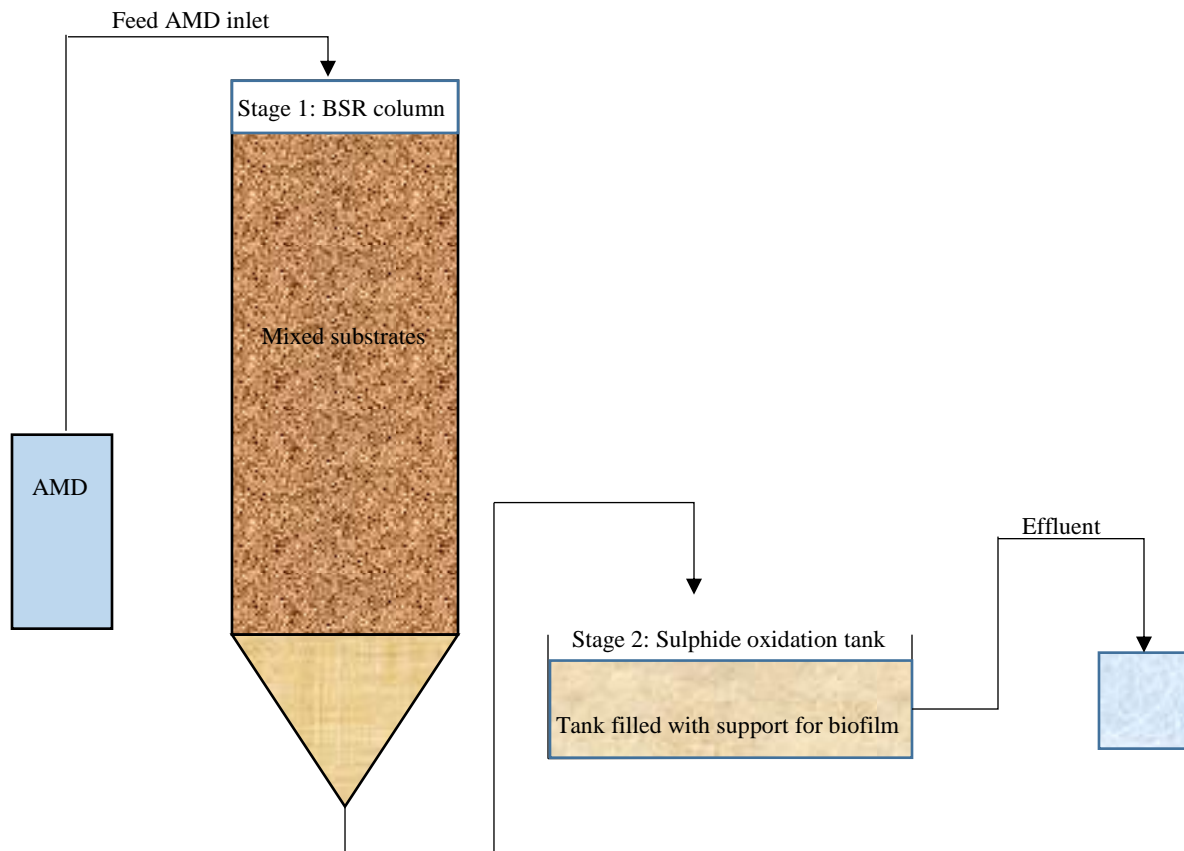


Figure 6.4: Integrated BSR and biological sulphide oxidation process

CHAPTER 7: CONCLUSION AND RECOMMENDATIONS

7.1. Conclusion

Mining activities are a major cause of effluents that are acidic, contain high concentrations of sulphate and are heavy metal laden. In South Africa, most of these effluents contain sulphate concentration above 2000 mg/L and the discharge limit is less than 600 mg/L. It is therefore important to reduce sulphate to dischargeable limits.

This study investigated the effect of pH, temperature and hydraulic retention time (HRT) on biological sulphate reduction in down-flow packed bed reactors using lucerne pellets and cow manure as main substrates that were replenished once every week. The experiments were designed using the Box-Behnken design in the Design Expert software. The pH was matrixed from 4 to 6, temperature from 10 °C to 30 °C and HRT from 2 d to 7 d and the chosen response variables were sulphate reduction efficiency, sulphate reduction rate and sulphide production. The obtained data was then analysed statistically using response surface methodology.

Some studies in literature suggest that operating at pH lower than 5 can lead to the suppression of sulphate reducing bacteria (SRB) and therefore reduce the efficiency. This study, however, has shown that operating at pH 4 does not have a significant impact on sulphate reduction. The analysis of variance (ANOVA) results also found the pH factor to be insignificant for all the response variables ($p < 0.05$). The ability to operate at pH 4 as opposed to pH 5 showed a positive impact on the lime requirement and sludge production. The lime required for pre-neutralisation to pH 4 and the sludge produced at pH 4 was approximately 50 % less than at pH 5.

Overall, sulphate reduction efficiency and sulphate reduction declined with a decrease in temperature which was attributed to the decrease in metabolic activity of SRB that is a result of low temperatures. At HRT of 7 d, sulphate reduction efficiency at 30 °C and 20 °C was maintained above 96 % for the duration of the experiment. At HRT of 4.5 d, a decrease in temperature from 30 °C to 20 °C led to a decrease in sulphate reduction efficiency from an average of 96 % to an

average of 80 %. A further decrease resulted in a drastic decrease in both sulphate reduction efficiency and rate. Sulphide production, on the other hand, was mostly impacted by temperatures lower than 20 °C at HRT of 4.5 d. At HRT of 7 d, sulphide production was similar for all temperatures which was presumably due to lower emission rates at longer HRTs. ANOVA analysis showed that temperature had a significant effect on all responses with a p-value of <0.0001 for sulphate reduction efficiency and sulphate reduction rate and 0.0057 for sulphide production.

A decrease in HRT from 7 d to 4.5 d at 30 °C did not have any significant impact on sulphate reduction efficiency and rate. Conversely, sulphide concentration was higher at HRT of 4.5 d compared to 7 d. This was likely due to increased metal sulphide precipitation at longer HRT. At HRT of 2 d, all the responses were found to increase upon the replenishment of substrates and drastically decrease after that. This was attributed to the washout of substrates due to higher feed rates.

Response surface diagrams showed that the interactive effects between temperature and HRT were stronger than those between pH and HRT and pH and temperature. This was expected as pH was proven to not have a significant impact on the process. This means that the process' performance is greatly impacted by temperature and HRT. This study developed a tool that can be used by Mintek to evaluate how the process will react to particular conditions within the range investigated.

7.2. Recommendations

It is recommended that a more in-depth microbiology study be conducted to find out how different conditions affect the bacterial community inside the reactor and in the reactor effluent and how the abundance of SRB culture may affect the reactor performance.

This study was conducted over a narrow range of pH and it was found that the lowest pH investigated did not have a significant influence on the process. It is therefore recommended that further studies are done to test the effect of pH lower than 4.

The present study also showed that the substrates were broken down and washed out quicker at HRT of 2 d. It is also recommended that the substrates be digested to get the necessary electron

donors so that they can be fed as a liquid, this way they are readily available for SRB and the effect at lower residence time can be fully understood.

It is presumed that at temperatures higher than 23 °C, sulphide gas production increases and as a result of sulphide emission rate increases and it is therefore not detected in solution. A confirmation study should be conducted in order to understand the production and behaviour of sulphide beyond 23 °C.

It is also recommended that this study be tested at a real-time pilot facility on an active mining site that discharges effluent daily. This will enable the model to be tested and its operational benefits quantified.

APPENDIX A: ANOVA ANALYSIS

Coefficients

The coefficient estimate represents the expected change in response per unit change in factor value when all remaining factors are held constant. The intercept in an orthogonal design is the overall average response of all the runs. The coefficients are adjustments around that average based on the factor settings. When the factors are orthogonal the VIFs are 1; VIFs greater than 1 indicate multicollinearity, the higher the VIF the more severe the correlation of factors. As a rough rule, VIFs less than 10 are tolerable.

Sulphate reduction efficiency

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	82.86	1	1.83	78.39	87.32	
A-pH	2.65	1	1.15	-0.1593	5.46	1.06
B-Temperature	33.79	1	1.39	30.38	37.20	1.48
C-HRT	21.31	1	1.44	17.80	24.83	1.44
AB	-0.7714	1	1.58	-4.63	3.09	1.11
AC	-0.6874	1	1.58	-4.55	3.17	1.11
BC	-5.67	1	2.22	-11.10	-0.2515	1.77
A ²	1.08	1	1.87	-3.49	5.65	1.24
B ²	-23.64	1	1.85	-28.18	-19.11	1.22
C ²	-10.51	1	1.82	-14.96	-6.06	1.19

Sulphate reduction rate

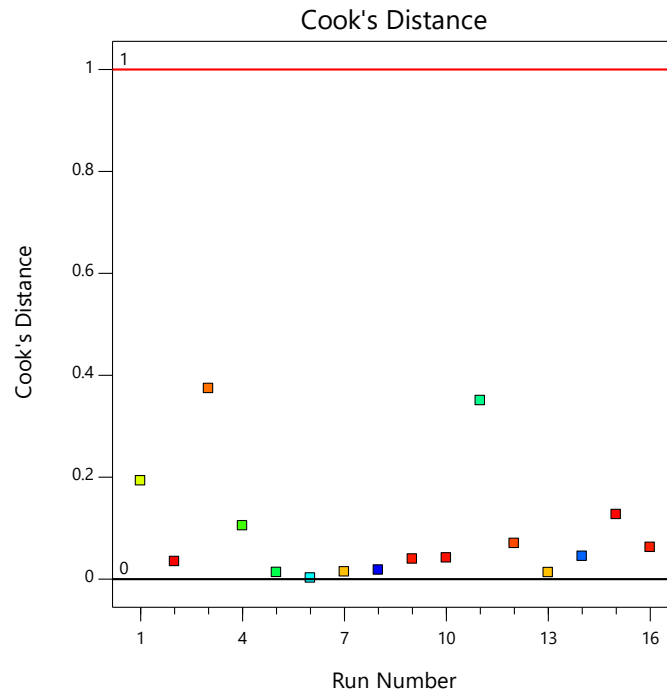
Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	4.92	1	0.1238	4.61	5.22	
A-pH	0.2257	1	0.0778	0.0353	0.4161	1.06
B-Temperature	2.11	1	0.0946	1.88	2.34	1.48
C-HRT	-1.97	1	0.0974	-2.21	-1.74	1.44
AB	0.0457	1	0.1070	-0.2162	0.3076	1.11
AC	-0.1838	1	0.1070	-0.4457	0.0782	1.11
BC	0.8878	1	0.1503	0.5201	1.26	1.77
A ²	-0.1347	1	0.1267	-0.4447	0.1753	1.24
B ²	-0.8936	1	0.1256	-1.20	-0.5862	1.22
C ²	2.63	1	0.1234	2.32	2.93	1.19

Sulphide production

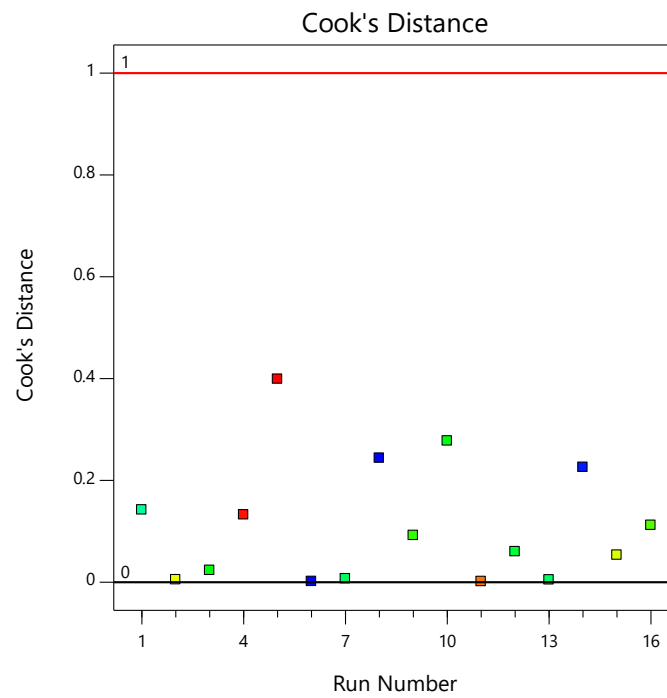
Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	712.22	1	40.81	612.35	812.08	
A-pH	-8.14	1	25.65	-70.91	54.62	1.06
B-Temperature	130.91	1	31.17	54.64	207.18	1.48
C-HRT	77.46	1	32.11	-1.12	156.03	1.44
AB	-12.79	1	35.29	-99.13	73.56	1.11
AC	-35.82	1	35.29	-122.17	50.52	1.11
BC	-96.97	1	49.55	-218.21	24.26	1.77
A ²	-101.05	1	41.76	-203.24	1.15	1.24
B ²	-195.46	1	41.41	-296.78	-94.14	1.22
C ²	-157.74	1	40.69	-257.30	-58.17	1.19

APPENDIX B: COOK'S DISTANCE PLOTS

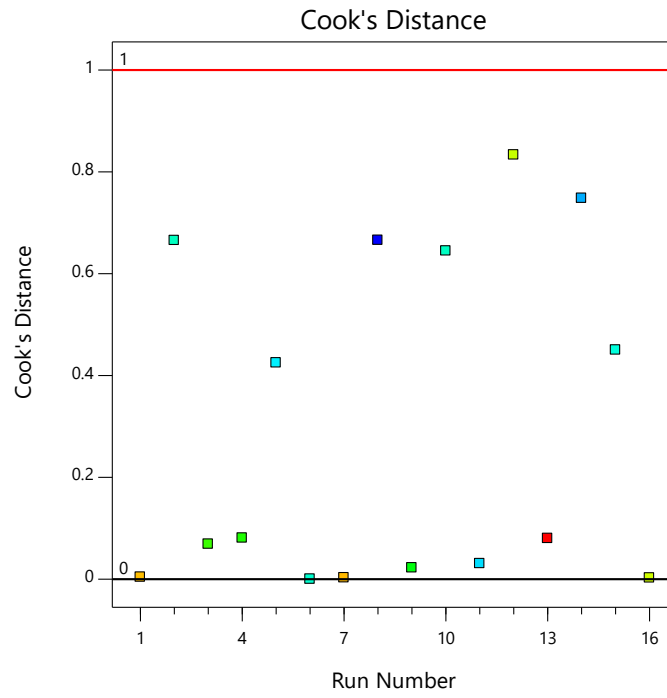
Sulphate reduction efficiency



Sulphate reduction rate



Sulphide production



REFERENCES

- Akcil, A. & Koldas, S., 2006, Acid Mine Drainage (AMD): causes, treatment and case studies, *J. Clean. Prod.*, 14(12-13), 1139-1145.
- Al Zuhair, S., El-Naas, M. H. & Al Hassani, H., 2008, Sulfate inhibition effect on sulfate reducing bacteria, *Journal of Biochemical Technology*, 1(2), 39-44.
- Annachhatre, A. & Suktrakoolvait, S., 2001a, Biological sulfide oxidation in a fluidized bed reactor, *Environmental technology*, 22(6), 661-672.
- Annachhatre, A. P. & Suktrakoolvait, S., 2001b, Biological sulfate reduction using molasses as a carbon source, *Water Environment Research*, 73(1), 118-126.
- Arnold, D. E. Diversion wells—a low-cost approach to treatment of acid mine drainage. Twelfth West Virginia Surface Mine Drainage Task Force Symposium, 3-4 April 1991 Morgantown, West Virginia, USA. 3-4.
- Arnold, M., Mäkinen, M., Salo, M. & Bomberg, M. 2016a. Microbiology and Chemistry Interactions in a Biological Sulphate Reducing Process. *International Mine Water Association*.
- Arnold, M., Mariekie, G. & Ritva, M., 2016b, Technologies for sulphate removal with valorisation options *IMWA*, 1343 – 1345.
- Baskaran, V. K., 2005, *Kinetics of anaerobic sulphate reduction in immobilised cell bioreactors*, MSc Thesis, University of Saskatchewan, Saskatoon, Canada.
- Bauer, M., Kube, M., Teeling, H., Richter, M., Lombardot, T., Allers, E., Würdemann, C. A., Quast, C., Kuhl, H., Knaust, F. & Woebken, D., 2006, Whole genome analysis of the marine Bacteroidetes ‘Gramella forsetii’ reveals adaptations to degradation of polymeric organic matter, *Environmental Microbiology* 8(12), 2201-2213.
- Belkin, S., Wirsn, C. O. & Jonnasch, H. W., 1985, Biological and abiological sulfur reduction at high temperatures, *Applied and Environmental Microbiology*, 49(5), 1057-1061.
- Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S. & Escalera, L. A., 2008, Response surface methodology (RSM) as a tool for optimization in analytical chemistry, *Talanta*, 76(5), 965-977.

- Bijmans, M., Buisman, C., Meulepas, R. & Lens, P., 2011, Sulfate reduction for inorganic waste and process water treatment, *Comprehensive Biotechnology*, 6(2), 435-446.
- Bijmans, M. F. 2008. *Sulfate reduction under acidic conditions for selective metals recovery*, Ph.D Thesis, Wageningen University, Wageningen, The Netherlands.
- Bowell, R. J., 2004, A review of sulphate removal options for mine waters, *International Mine Water Association*, 2, 75 - 91
- Brahmacharimayum, B., Mohanty, M. P. & Ghosh, P. K., 2019, Theoretical and Practical Aspects of Biological Sulfate Reduction: A Review, *Global NEST Journal*, 21(2), 222-244.
- Buzzi, D. C., Viegas, L. S., Rodrigues, M. a. S., Bernardes, A. M. & Tenório, J. a. S., 2013, Water recovery from acid mine drainage by electrodialysis, *Minerals Engineering*, 40, 82-89.
- Bwapwa, J., Jaiyeola, A. & Chetty, R., 2017, Bioremediation of acid mine drainage using algae strains: A review, *South African Journal of Chemical Engineering*, 24, 62-70.
- Cabrera, G., Pérez, R., Gomez, J., Abalos, A. & Cantero, D., 2006, Toxic effects of dissolved heavy metals on *Desulfovibrio vulgaris* and *Desulfovibrio* sp. strains, *Journal of hazardous materials*, 135(1-3), 40-46.
- Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I. & Huttley, G. A., 2010, QIIME allows analysis of high-throughput community sequencing data, *Nature methods*, 7(5), 335.
- Castro, H. F., Williams, N. H. & Ogram, A., 2000, Phylogeny of sulfate-reducing bacteria, *FEMS Microbiol Ecology*, 21(1), 1-9.
- Castro Neto, E., Aguiar, A., Rodriguez, R. & Sancinetti, G., 2018, Acid mine drainage treatment and metal removal based on a biological sulfate-reducing process *Brazilian Journal of Chemical Engineering*, 35(2), 543-552.
- Celis-García, L. B., González-Blanco, G. & Meraz, M., 2008, Removal of sulfur inorganic compounds by a biofilm of sulfate reducing and sulfide oxidizing bacteria in a down-flow fluidized bed reactor, *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology*, 83(3), 260-268.
- Chaiprapat, S., Preechalertmit, P., Boonsawang, P. & Karnchanawong, S., 2011, Sulfidogenesis in Pretreatment of High-Sulfate Acidic Wastewater Using Anaerobic Sequencing Batch Reactor and Upflow Anaerobic Sludge Blanket Reactor, *Environmental Engineering Science*, 28(8), 597-604.

- Choi, A., Yang, S. J., Rhee, K. H. & Cho, J. C., 2013, *Lentisphaera marina* sp. nov., and emended description of the genus *Lentisphaera*, *International journal of systematic and evolutionary microbiology*, 63(4).
- Choi, E. & Rim, J. M., 1991, Competition and inhibition of sulfate reducers and methane producers in anaerobic treatment, *Water Science and Technology*, 23(7-9), 1259-1264.
- Choudhary, R. P. & Sheoran, A. S., 2011, Comparative study of cellulose waste versus organic waste as substrate in a sulfate reducing bioreactor, *Bioresour Technol*, 102(6), 4319-24.
- Christensen, B., Laake, M. & Lien, T., 1996, Treatment of acid mine water by sulfate-reducing bacteria; results from a bench scale experiment, *Water Research*, 30(7), 1617-1624.
- Christoe, J., 1976, Removal of sulfate from industrial wastewaters, *Journal (Water Pollution Control Federation)*, 2804-2808.
- Ciosek, A. & Luk, G., 2017, Kinetic modelling of the removal of multiple heavy metallic ions from mine waste by natural zeolite sorption, *Water*, 9(7), 482.
- Clifford, D., Sorg, T. & Ghurye, G. 2011. Ion exchange and adsorption of inorganic contaminants. *Water Quality & Treatment: A Handbook on Drinking Water*. McGrawHill.
- Climate-Data.Org. 2020. *Emalahleni Climate* [Online]. Available: <https://en.climate-data.org/africa/south-africa/mpumalanga/emalahleni-641/> [Accessed 06 March 2020].
- Cole, J. R., Wang, Q., A, F. J., Chai, B., MCGarrel, D. M., Sun, Y., Brown, C. T., Porrás-Alfaro, A., Kuske, C. R. & Tiedje, J. M., 2013, Ribosomal Database Project: data and tools for high throughput rRNA analysis, *Nucleic acids research*, 42(D1), D633-D642.
- Colleran, E., Finnegan, S. & Lens, P., 1995, Anaerobic treatment of sulphate-containing waste streams, *Antonie van Leeuwenhoek*, 67(1), 29-46.
- Dar, S. A., Kleerebezem, R., Stams, A. J., Kuenen, J. G. & Muyzer, G., 2008, Competition and coexistence of sulfate-reducing bacteria, acetogens and methanogens in a lab-scale anaerobic bioreactor as affected by changing substrate to sulfate ratio, *Applied microbiology and biotechnology*, 78(6), 1045-1055.
- Desantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P. & G.L, A., 2006, Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB, *Applied and Environmental Microbiology*, 72(7), 5069-5072.

- Dev, S., Roy, S. & Bhattacharya, J., 2017, Optimization of the operation of packed bed bioreactor to improve the sulfate and metal removal from acid mine drainage, *J Environ Manage*, 200, 135-144.
- Dev, S., Roy, S., Das, D. & Bhattacharya, J., 2015, Improvement of biological sulfate reduction by supplementation of nitrogen rich extract prepared from organic marine wastes, *International Biodeterioration & Biodegradation*, 104, 264-273.
- Deviant, S. 2011. *The Practically Cheating Statistics Handbook*, Lulu. com.
- Doshi, S. M., 2006, Bioremediation of acid mine drainage using sulfate-reducing bacteria, *US Environmental Protection Agency, Office of Solid Waste and Emergency Response and Office of Superfund Remediation and Technology Innovation*, 65.
- Drury, W. J., 2000, Modeling of sulfate reduction in anaerobic solid substrate bioreactors for mine drainage treatment, *Mine Water and the Environment*, 19(1), 19-29.
- Dvorak, D. H., Hedin, R. S., Edenborn, H. M. & McIntire, P. E., 1991, Treatment of Metal-Contaminated Water using Bacterial Sulfate Reduction: Results from Pilot-Scale Reactors, *American Society for Surface Mining and Reclamation*, 109 - 122.
- Dvorak, D. H., Hedin, R. S., Edenborn, H. M. & McIntire, P. E., 1992, Treatment of metal-contaminated water using bacterial sulfate reduction: Results from pilot-scale reactors, *Biotechnology and bioengineering*, 40(5), 609-616.
- Dwaf 2013. Feasibility study for a long-term solution to address the acid mine drainage associated with the East, Central and West Rand underground mining basins.
- Edgar, R. C., 2010, Search and clustering orders of magnitude faster than BLAST, *Bioinformatics*, 26(19), 2460-2461.
- Edzwald, J. K. 2010. *Water Quality and Treatment A Handbook on Drinking Water*, McGrawHill.
- Elliott, P., Ragusa, S. & Catcheside, D., 1998, Growth of sulfate-reducing bacteria under acidic conditions in an upflow anaerobic bioreactor as a treatment system for acid mine drainage, *Water Research*, 32(12), 3724-3730.
- Equeenuddin, S. M., Tripathy, S., Sahoo, P. K. & Panigrahi, M. K., 2010, Hydrogeochemical characteristics of acid mine drainage and water pollution at Makum Coalfield, India, *J. Geochem. Explor.*, 105(3), 75-82.
- Equusfeeds. 2015. *Lucerne cubes* [Online]. Available: <http://www.equusfeeds.co.za/lucerne-cubes/> [Accessed 18 August 2020].

- Ersahin, M. E., Ozgun, H., Dereli, R. K. & Ozturk, I., 2011, Anaerobic treatment of industrial effluents: an overview of applications, *Waste water-treatment and reutilization*, 9-13.
- Everett, D. J., Du Plessis, J. & Gussman, H. W., 1993, The treatment of underground mine waters for the removal of calcium and sulphates by a GYP-CIX process, *International Mine Water Association & Zambia Consolidated Copper Mines Limited: The first African Symposium on Mine Dainage and Environment Protection from Mine Waste Water Disposal*, 463-491.
- Feris, L. & Kotze, L. J., 2015, The regulation of acid mine drainage in South Africa: law and governance perspectives, *Potchefstroom Electronic Law Journal/Potchefstroomse Elektroniese Regsblad*, 17(5), 2104 - 2163.
- Fernando, W. a. M., Ilankoon, I. M. S. K., Syed, T. H. & Yellishetty, M., 2018, Challenges and opportunities in the removal of sulphate ions in contaminated mine water: A review, *Minerals Engineering*, 117, 74-90.
- Ferrentino, R., Langone, M. & Andreottola, G., 2017, Temperature effects on the activity of denitrifying phosphate accumulating microorganisms and sulphate reducing bacteria in anaerobic side-stream reactor, *J. Environ. Bio. Res.*, 1(1), 1.
- Fortin, D., Goulet, R. & Roy, M., 2000, Seasonal cycling of Fe and S in a constructed wetland: the role of sulfate-reducing bacteria, *Geomicrobiology Journal*, 17(3), 221-235.
- Garcia, C., Moreno, D. A., Ballester, A., Blazquez, M. L. & Gonzalez, F., 2001, Bioremediation of an industrial acid mine water by metal-tolerant sulphate-reducing bacteria, *Minerals Engineering*, 14(9), 997-1008.
- Ghigliazza, R., Lodi, A. & Rovatti, M., 2000, Kinetic and process considerations on biological reduction of soluble and scarcely soluble sulfates, *Resources, Conservation and Recycling*, 29(3), 181-194.
- Gibert, O., De Pablo, J., Cortina, J. & Ayora, C., 2002, Treatment of acid mine drainage by sulphate-reducing bacteria using permeable reactive barriers: a review from laboratory to full-scale experiments, *Reviews in Environmental Science and Biotechnology*, 1(4), 327-333.
- Gibert, O., De Pablo, J., Cortina, J. L. & Ayora, C., 2004, Chemical characterisation of natural organic substrates for biological mitigation of acid mine drainage, *Water Research*, 38(19), 4186-4196.

- Giles Jr, H. F., Mount Iii, E. M. & Wagner Jr, J. R. 2004. *Extrusion: the definitive processing guide and handbook*, William Andrew.
- Glombitza, F., 2001, Treatment of acid lignite mine flooding water by means of microbial sulfate reduction, *Waste Management*, 21(2), 197-203.
- Gómez, D. K. V. & Lens, P. N. 2017. Metal Recovery from Industrial and Mining Wastewaters. *Sustainable Heavy Metal Remediation*. Springer.
- Greben, H., Bologo, H. & Maree, J., 2002, The effect of different parameters on the biological volumetric and specific sulphate removal rates, *Water S.A.*, 33-37.
- Greben, H. & Maree, J. The effect of reactor type and residence time on biological sulphate and sulphide removal rates. WISA Biennial Conference 2000, 28 May - 1 June 2000 Sun City, South Africa. Citeseer.
- Greben, H., Maree, J., Eloff, E. & Murray, K., 2005, Improved sulphate removal rates at increased sulphide concentration in the sulphidogenic bioreactor, *Water SA*, 31(3), 351-358.
- Gusek, J. J., 2002, Sulfate-reducing bioreactor design and operating issues: is this the passive treatment technology for your mine drainage, *National Association of Abandoned Mine Land Programs, Park City, Utah*.
- Gutierrez, O., Park, D., Sharma, K. R. & Yuan, Z., 2009, Effects of long-term pH elevation on the sulfate-reducing and methanogenic activities of anaerobic sewer biofilms, *Water Res*, 43(9), 2549-57.
- Hair, J. F., Black, W. C., Babin, B. J., Anderson, R. E. & Tatham, R. L. 1998. *Multivariate data analysis*, Prentice hall Upper Saddle River, NJ.
- Han, H., Ogata, Y., Yamamoto, Y., Nagao, S. & Nishino, N., 2014, Identification of lactic acid bacteria in the rumen and feces of dairy cows fed total mixed ration silage to assess the survival of silage bacteria in the gut, *Journal of dairy science*, 97(9), 5754-5762.
- Hard, B. C., Higgins, J. P. & Mattes, A., 2003, Bioremediation of acid rock drainage using sulphate-reducing bacteria, *Proceedings of Sudbury: Mining and Environment, Sudbury, Ontario, May, 25-28*.
- Harrison, S. T. L., Van Hille, R. P., Mokone, T., Motleleng, L., Smart, M., Legrand, C. & Marais, T. 2014. Addressing the Challenges Facing Biological Sulphate Reduction as a Strategy for AMD Treatment: Analysis of the reactor stage: raw materials products and process kinetics. University of Cape Town.

- Hedin, R. S. Temperature Independent Removal of Iron in a Passive Mine Water System. *In: WOLKERDORFER, B. F., ed. International Mine Water Association Annual Conference: Reliable Mine Water Technology*, 2013 Golden CO, USA.
- Henseler, J., Ringle, C. M. & Sinkovics, R. R. 2009. The use of partial least squares path modeling in international marketing. *New challenges to international marketing*. Emerald Group Publishing Limited.
- Herlemann, D. P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J. J. & Anderson, A. F., 2011, Transitions in bacterial communities along 2000 km salinity gradient of the Baltic sea, *The ISME journal*, 5(11), 1571-1579.
- Hübel, S. R., Pereyra, L. P., Breazea, M. V. R., Reisman, D. J., Reardon, K. F. & Pruden, A., 2011, Effect of Organic Substrate on the Microbial Community Structure in Pilot-Scale Sulfate-Reducing Biochemical Reactors Treating Mine Drainage, *Environmental Engineering Science*, 28(8), 563-572.
- Hilgsmann, S., Jacques, P. & Thonart, P., 1998, Isolation of highly performant sulfate reducers from sulfate-rich environments, *Biodegradation*, 9(3-4), 285-292.
- Holzappel, W. H. & Wood, B. J. 2014. *Lactic acid bacteria: biodiversity and taxonomy*, Oxford, John Wiley & Sons.
- Howard, D., Grobler, C., Robinson, R. & Cole, P. Sustainable purification of mine water using ion exchange technology. International Mine Water Conference, 2009. 447-453.
- Hu, P., Tom, L., Singh, A., Thomas, B. C., Baker, B. J., Piceno, Y. M., Anderson, G. L. & Banfield, J. F., 2016, Genome-Resolved Metagenomic Analysis Reveals Roles for Candidate Phyla and Other Microbial Community Members in Biogeochemical Transformations in Oil Reservoirs, *MBio*, 7(1), e01669-15.
- Icgen, B. & Harrison, S., 2006, Exposure to sulfide causes populations shifts in sulfate-reducing consortia, *Research in microbiology*, 157(8), 784-791.
- Inap 2003. Treatment of sulphate in mine Effluents.
- Isa, Z., Grusenmeyer, S. & Verstraete, W., 1986, Sulfate reduction relative to methane production in high-rate anaerobic digestion: microbiological aspects, *Applied and Environmental Microbiology*, 51(3), 580-587.

- Jalali, K. & Baldwin, S. A., 2000, The role of sulphate reducing bacteria in copper removal from aqueous sulphate solutions, *Water Research*, 34(3), 797-806.
- Jayakumar, G. D. S. & Sulthan, A., 2015, Exact distribution of Cook's distance and identification of influential observations, *Hacettepe Journal of Mathematics and Statistics*, 44(1), 165-178.
- Jiménez-Rodríguez, A. M., Durán-Barrantes, M. D. L. M., Borja, R., Sánchez, E., Colmenarejo, M. & Raposo, F., 2010, Biological sulphate removal in acid mine drainage using anaerobic fixed bed reactors with cheese whey as a carbon source, *Lat. Am. Appl. Res.*, 40(4), 329-335.
- Johnson, D. B. & Hallberg, K. B., 2005a, Acid mine drainage remediation options: a review, *Sci. Total Environ.*, 338(1-2), 3-14.
- Johnson, D. B. & Hallberg, K. B., 2005b, Biogeochemistry of the compost bioreactor components of a composite acid mine drainage passive remediation system, *Science of the Total Environment*, 388(1-2), 81-93.
- Jong, T. & Parry, D. L., 2003, Removal of sulfate and heavy metals by sulfate reducing bacteria in short-term bench scale upflow anaerobic packed bed reactor runs, *Water research*, 37(14), 3379-3389.
- Jong, T. & Parry, D. L., 2006, Microbial sulfate reduction under sequentially acidic conditions in an upflow anaerobic packed bed bioreactor, *Water Res.*, 40(13), 2561-71.
- Kakooei, S., Ismail, M. C. & Ariwahjoedi, B., 2012, Mechanisms of microbiologically influenced corrosion: a review, *World Appl. Sci. J*, 17(4), 524.
- Kaksonen, A. H., Franzmann, P. D. & Puhakka, J. A., 2004, Effects of hydraulic retention time and sulfide toxicity on ethanol and acetate oxidation in sulfate-reducing metal-precipitating fluidized-bed reactor, *Biotechnology and Bioengineering*, 86(3), 332-343.
- Kaksonen, A. H. & Puhakka, J. A., 2007, Sulfate Reduction Based Bioprocesses for the Treatment of Acid Mine Drainage and the Recovery of Metals, *Engineering in Life Sciences*, 7(6), 541-564.
- Kalyuzhnyi, S. & Fedorovich, V., 1997, Integrated mathematical model of UASB reactor for competition between sulphate reduction and methanogenesis, *Water Science and Technology*, 36(6-7), 201-208.

- Kasim, M. S., Harun, N. H., Hafiz, M. S. A., Mohamed, S. B. & Mohamad, W. N. F. W. 2007. Multi-Response Optimization of Process Parameter in Fused Deposition Modelling by Response Surface Methodology. *International Conference on Engineering Design*. Paris, France.
- Kasim, M. S., Harun, N. H., Hafiz, M. S. A., Mohamed, S. B. & Mohamad, W. N. F. W., 2019, Multi-Response Optimization of Process Parameter in Fused Deposition Modelling by Response Surface Methodology, *Int. J. Recent Technol.*, 8(3), 327-338.
- Kefeni, K. K., Msagati, T. A. & Mamba, B. B., 2017, Acid mine drainage: prevention, treatment options, and resource recovery: a review, *Journal of Cleaner Production*, 151, 475-493.
- Kepler, D. A. & McCleary, E. C. Passive aluminum treatment successes. Proceedings of the 18th West Virginia Surface Mine Drainage Task Force Symposium, April 15–16, 1997.
- Khater, E.-S. G., 2015, Some physical and chemical properties of compost, *International Journal of Waste Resources*, 5(1), 1-5.
- Kimura, S., Hallberg, K. B. & Johnson, D. B., 2006, Sulfidogenesis in low pH (3.8–4.2) media by a mixed population of acidophilic bacteria, *Biodegradation*, 17(2), 57-65.
- Klindwort, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. & Glöckner, F. O., 2013, Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies, *Nucleic acids research*, 41(1), e1-e1.
- Koschorreck, M., 2008, Microbial sulphate reduction at a low pH, *FEMS Microbiol Ecol*, 64(3), 329-42.
- Kousi, P., Remoundaki, E., Hatzikioseyan, A. & Tsezos, M., 2015, Sulphate-reducing bioreactors: current practices and perspectives, *International water Association (IWA)*.
- Kováč, J., Vítězová, M. & Kushkevych, I., 2018, Metabolic activity of sulfate-reducing bacteria from rodents with colitis, *Open Medicine*, 13(1), 344-329.
- Küsel, K., Roth, U., Trinkwalter, T. & Peiffer, S., 2001, Effect of pH on the anaerobic microbial cycling of sulfur in mining-impacted freshwater lake sediments, *Environmental and Experimental Botany*, 46(3), 213-223.
- Kuyucak, N., 2002, Role of microorganisms in mining: generation of acid rock drainage and its mitigation and treatment, *ejmp & ep (European Journal of Mineral Processing and Environmental Protection)*, 2(3), 179-196.

- Kuyucak, N. & St-Germain, P. In situ treatment of acid mine drainage by sulfate reducing bacteria in open pits: scale-up experiences. Proceedings of the International Land Reclamations and Mine Drainage Conference and the Third International Conference on the Abatement of Acidic Drainage, 1994. 303-310.
- Langenhoven, H. 2017. *Chamber of Mines South Africa Facts and Figures 2016* [Online]. Available: <http://www.chamberofmines.org.za/industry-news/publications/facts-and-figures> [Accessed 12 April 2018].
- Lettinga, G., Pol, L. H., Koster, I., Wiegant, W., De Zeeuw, W., Rinzema, A., Grin, P., Roersma, R. & Hobma, S., 1984, High-rate anaerobic waste-water treatment using the UASB reactor under a wide range of temperature conditions, *Biotechnology and genetic engineering reviews*, 2(1), 253-284.
- Liamleam, W. & Annachatre, A. P., 2007, Electron donors for biological sulfate reduction, *Biotechnol Adv*, 25(5), 452-63.
- Lopes, S. I. C., 2007, *Sulfate reduction at low pH in organic wastewaters*, PhD Thesis, Wageningen University.
- Luptakova, A., Kotulicova, I., Macingova, E. & Jencarova, J., 2013, Bacterial elimination of sulphates from mine waters, *Chemical engineering transactions*, 35, 853-858.
- Lyew, D. & Sheppard, J. D., 1997, Effects of physical parameters of a gravel bed on the activity of sulphate-reducing bacteria in the presence of acid mine drainage, *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology*, 70(3), 223-230.
- Maillacheruvu, K. Y. & Parkin, G. F., 1996, Kinetics of growth, substrate utilization and sulfide toxicity for propionate, acetate, and hydrogen utilizers in anaerobic systems, *Water Environment Research*, 68(7), 1099-1106.
- Mallewar, P. C., 2013, *Effect of Linoleic Acid and Hydraulic Retention Time on Anaerobic Sulfate Reduction in High Rate Reactors*, Masters Thesis, University of Windsor.
- Marais, T., Huddy, R., Van Hille, R. & Harrison, S. The Effect of Temperature on the Kinetics of Sulphate Reduction and Sulphide Oxidation in an Integrated Semi-Passive Bioprocess for Remediating Acid Rock Drainage. In: WOLKERDORFER, C., SARTZ, L., WEBER, A., BURGESS, J. & TREMBLAY, G., eds. 11th ICARD|IMWA|MWD Conference - Risk to Opportunity, 10-4 September 2018 Pretoria, South Africa.

- Marais, T. S., Huddy, R., Harrison, S. T. L. & Van Hille, R., 2020, Demonstration of simultaneous biological sulphate reduction and partial sulphide oxidation in a hybrid linear flow channel reactor, *Journal of Water Process Engineering*, 34, 101143.
- Margeta, K., Logar, N. Z., Šiljeg, M. & Farkaš, A., 2013, Natural zeolites in water treatment—how effective is their use, *Water treatment*, 5, 81-112.
- Markovic, R., Gardic, V., Obradovic, L., Djordjievski, S., Stevanovic, Z., Stevanovic, J. & Gvozdenovic, M., 2015, The application of a natural zeolite for acid mine drainage purification, *Materials Transactions*, 56(12), 2053-2057.
- Mccarthy, T. S., 2011, The impact of acid mine drainage in South Africa, *South African Journal of Science*, 107(5/6), 1 -7.
- Miloshova, M., Baltés, D. & Bychkov, E., 2003, New chalcogenide glass chemical sensors for S₂- and dissolved H₂S monitoring, *Water Science and Technology*, 47(2), 135-140.
- Mizuno, O., Li, Y. & Noike, T., 1994, Effects of sulfate concentration and sludge retention time on the interaction between methane production and sulfate reduction for butyrate, *Water Science and Technology*, 30(8), 45.
- Moloantoa, K. M., 2015, *Biogeochemical and kinetics characterization of sulfate reducing microbial communities enriched from mine drainages*, Magister Scientiae, University of the Free State.
- Moon, C., Singh, R., Veeravalli, S., Shanmugam, S., Chaganti, S., Lalman, J. & Heath, D., 2015, Effect of COD:SO₄²⁻ Ratio, HRT and Linoleic Acid Concentration on Mesophilic Sulfate Reduction: Reactor Performance and Microbial Population Dynamics, *Water*, 7(12), 2275-2292.
- Moosa, S., Nemati, M. & Harrison, S., 2002, A kinetic study on anaerobic reduction of sulphate, Part I: Effect of sulphate concentration, *Chemical Engineering Science*, 57(14), 2773-2780.
- Motsi, T., Rowson, N. & Simmons, M., 2009, Adsorption of heavy metals from acid mine drainage by natural zeolite, *International Journal of Mineral Processing*, 92(1-2), 42-48.
- Naidu, G., Ryu, S., Thiruvengkatahari, R., Choi, Y., Jeong, S. & Vigneswaran, S., 2019, A critical review on remediation, reuse, and resource recovery from acid mine drainage, *Environmental pollution*.

- Najib, T., Solgi, M., Farazmand, A., Heydarian, S. M. & Nasernejad, B., 2017, Optimization of sulfate removal by sulfate reducing bacteria using response surface methodology and heavy metal removal in a sulfidogenic UASB reactor, *J. Environ. Chem. Eng.*, 5(4), 3256-3265.
- Neale, J. W., Muller, H. H., Gericke, M. & Muhlbauer, R., 2017, Low-Cost Biological Treatment of Metal- and Sulphate-Contaminated Mine Waters, *IMWA*, 1, 453 - 460.
- Neculita, C. M., Zagury, G. J. & Kulnieks, V. Short-term and long-term bioreactors for acid mine drainage treatment. Proceedings of the Annual International Conference on Soils, Sediments, Water and Energy, 2010. 2.
- Nist/Sematech. 2013. *e-Handbook of Statistical Methods* [Online]. Available: <http://www.itl.nist.gov/div898/handbook/> [Accessed 27 September 2018].
- Nordwick, S., Zaluski, M., Park, B. & Bless, D. Advances in development of bioreactors applicable to the treatment of ARD. Proceedings from the 7th International Conference on Acid Rock Drainage (ICARD), St. Louis (MO), 2006. 1410-1420.
- O'flaherty, V., Mahony, T., O'kenedy, R. & Colleran, E., 1998, Effect of pH on growth kinetics and sulphide toxicity thresholds of a range of methanogenic, syntrophic and sulphate-reducing bacteria, *Process Biochemistry*, 33(5), 555-569.
- Omil, F., Bakker, C., Pol, L. H. & Lettinga, G., 1997, Effect of pH and low temperature shocks on the competition between sulphate reducing bacteria and methane producing bacteria in UASB reactors, *Environmental technology*, 18(3), 255-264.
- Oude Elferink, S. J. W. H., Visser, A., Pol, L. W. H. & Stams, A. J. M., 1994, Sulfate reduction in methanogenic bioreactors, *FEMS Microbiology Reviews*, 15(2-3), 119-136.
- Oyekol, O. O., Van Hille, R. P. & Harrison, S. T., 2010, Kinetic analysis of biological sulphate reduction using lactate as carbon source and electron donor: Effect of sulphate concentration, *Chemical Engineering Science*, 65(16), 4771-4781.
- Oyekol, O. O., Van Hille, R. P. & Harrison, S. T. L., 2007, Effect of sulphate concentration on the community structure and activity of sulphate reducing bacteria, *Advanced Materials Research*, 20, 513-515.
- Paulo, L. M., Stams, A. J. & Sousa, D. Z., 2015, Methanogens, sulphate and heavy metals: a complex system, *Reviews in Environmental Science and Bio/Technology*, 14(4), 537-553.

- Poinapen, J., Wentzel, M. W. & Ekama, G., 2009a, Biological sulphate reduction with primary sewage sludge in an upflow anaerobic sludge bed (UASB) reactor. Part 1: Feasibility study, *Water S.A.*, 35(5), 525-534.
- Poinapen, J. P., Ekama, G. E. & Wentzel, M. W., 2009b, Biological sulphate reduction with primary sewage sludge in an upflow anaerobic sludge bed (UASB) reactor—Part 3: Performance at 20 C and 35 C, *Water SA*, 35(5).
- Postgate, J. R. 1984. *The sulphate reducing bacteria*, second ed. University Press, Cambridge, UK.
- Rajala, P., Bomberg, M., Huttunen-Saarivirta, E., Priha, O., Tausa, M. & Carpén, L., 2016, Influence of chlorination and choice of materials on fouling in cooling water system under brakish seawater, *Materials*, 9(6), 475.
- Rakić, T., Kasagić-Vujanović, I., Jovanović, M., Jančić-Stojanović, B. & Ivanović, D., 2014, Comparison of full factorial design, central composite design, and box-behnken design in chromatographic method development for the determination of fluconazole and its impurities, *Analytical letters*, 47(8), 1334-1347.
- Ramakrishna, B. S., 2013, Role of the gut microbiota in human nutrition and metabolism, *Journal of Gastroenterology and Hepatology*, 28, 9-17.
- Raymund, H. J., 1947, Acid Mine Drainage, *Industrial and Engineering Chemistry*, 39(5), 642 - 646.
- Rintala, J. & Lettinga, G., 1992, Effects of temperature elevation from 37 to 55 C on anaerobic treatment of sulphate rich acidified wastewaters, *Environmental technology*, 13(9), 801-812.
- Rose, P., 2013, Long-term sustainability in the management of acid mine drainage wastewaters—development of the Rhodes BioSURE Process, *Water SA*, 39(5), 582-592.
- Saha, S. & Sinha, A., 2018, A review on treatment of acid mine drainage with waste materials: A novel approach, *Global Nest Journal*, 20(3), 512-528.
- Salo, M., Bomberg, M., Grewar, T., Seepei, L., Mariekie, G. & Arnold, M., 2017, Compositions of the Microbial Consortia Present in Biological Sulphate Reduction Processes During Mine Effluent Treatment, *IMWA*, 1, 14 - 21.
- Sawicka, J. E., Jørgensen, B. B. & Brøcher, 2012, Temperature characteristics of bacterial sulfate reduction in continental shelf and slope sediments, *Biogeosciences*, 9(8), 3425-3435.

- Schmidt, K. L. & Sharpe, W. E., 2002, Passive treatment methods for acid water in Pennsylvania, *Penn State College of Agricultural Sciences, Pennsylvania*.
- Seervi, V., Yadav, H., Srivastav, S. & Jamal, A., 2017, Overview of active and passive systems for treating acid mine drainage, *IARJSET*, 4(5), 131-137.
- Sen, A. M. & Johnson, B., 1999, Acidophilic sulphate-reducing bacteria: candidates for bioremediation of acid mine drainage, *Process Metallurgy*, 9, 709-718.
- Seyler, J., Figueroa, L., Ahmann, D., Wildeman, T. & Robustelli, M. Effect of solid phase organic substrate characteristics on sulfate reducer activity and metal removal in passive mine drainage treatment systems. Proceedings of National Meeting of the American Society of Mining and Reclamation and the 9th Billings Land Reclamation Symposium, 3-6 June 2003 Montana, USA.
- Sharma, K., Derlon, N., Hu, S. & Yuan, Z., 2014, Modeling the pH effect on sulfidogenesis in anaerobic sewer biofilm, *Water Res*, 49, 175-85.
- Sheoran, A. S., Sheoran, V. & Choudhary, R. P., 2010, Bioremediation of acid-rock drainage by sulphate-reducing prokaryotes: A review, *Minerals Engineering*, 23(14), 1073-1100.
- Simate, G. S. & Ndlovu, S., 2014, Acid mine drainage: Challenges and opportunities, *Journal of Environmental Chemical Engineering*, 2(3), 1785-1803.
- Sipma, J., Osuna, M. B., Lettinga, G., Stams, A. J. & Lens, P. N., 2007, Effect of hydraulic retention time on sulfate reduction in a carbon monoxide fed thermophilic gas lift reactor, *Water Res*, 41(9), 1995-2003.
- Skousen, J., Zipper, C. E., Rose, A., Ziemkiewicz, P. F., Nairn, R., McDonald, L. M. & Kleinmann, R. L., 2017, Review of passive systems for acid mine drainage treatment, *Mine Water and the Environment*, 36(1), 133-153.
- Skousen, J. G. & Ziemkiewicz, P. F. 1996. *Acid mine drainage control and treatment*, Citeseer.
- Skousen, J. G., Ziemkiewicz, P. F. & McDonald, L. M., 2018, Acid mine drainage formation, control and treatment: Approaches and strategies, *The Extractive Industries and Society*.
- Smit, J. P., 1999, The treatment of polluted mine water, *International Mine Water Association*, II, 467 - 471.
- Stams, A. J., Huisman, J., Encina, P. a. G. & Muyzer, G., 2009, Citric acid wastewater as electron donor for biological sulfate reduction, *Applied microbiology and biotechnology*, 83(5), 957-963.

- Statease. 2018. *Response Surface Designs* [Online]. Available: <https://www.statease.com/docs/v11/designs/rsm/#rsm> [Accessed 27 September 2018].
- Sun, Y., Wei, J., Zhang, J. P. & Yang, G., 2016, Optimization using response surface methodology and kinetic study of Fischer–Tropsch synthesis using SiO₂ supported bimetallic Co–Ni catalyst, *J. Nat. Gas Sci. Eng.*, 28, 173-183.
- Talib, N. a. A., Salam, F., Yusof, N. A., Ahmad, S. a. A. & Sulaiman, Y., 2017, Optimization of peak current of poly (3, 4-ethylenedioxythiophene)/multi-walled carbon nanotube using response surface methodology/central composite design, *RSC Adv.*, 7(18), 11101-11110.
- Taylor, J., Pape, S. & Murphy, N. A summary of passive and active treatment technologies for acid and metalliferous drainage (AMD). Proceedings of the in Fifth Australian workshop on Acid Mine Drainage, 2005.
- Tsukamoto, T., Killion, H. & Miller, G., 2004, Column experiments for microbiological treatment of acid mine drainage: low-temperature, low-pH and matrix investigations, *Water Research*, 38(6), 1405-1418.
- Utgikar, V. P., Chen, B. Y., Chaudhary, N., Tabak, H. H., Haines, J. R. & Govind, R., 2001, Acute toxicity of heavy metals to acetate-utilizing mixed cultures of sulfate-reducing bacteria: EC100 and EC50, *Environmental Toxicology and Chemistry: An International Journal*, 20(12), 2662-2669s.
- Utgikar, V. P., Harmon, S. M., Chaudhary, N., Tabak, H. H., Govind, R. & Haines, J. R., 2002, Inhibition of sulfate-reducing bacteria by metal sulfide formation in bioremediation of acid mine drainage, *Environ. Toxicol.*, 17(1), 40-48.
- Valdés, F., Muñoz, E., Chamy, R., Ruiz, G., Vergara, C. & Jeison, D., 2006, Effect of sulphate concentration and sulphide desorption on the combined removal of organic matter and sulphate from wastewaters using expanded granular sludge bed (EGSB) reactors, *Electronic Journal of Biotechnology*, 9(4), 371-378.
- Vallero, M. V. G., 2003, *Sulfate reducing processes at extreme salinity and temperature: extending its application window*, Thesis, Wageningen University, Wageningen, Netherlands.
- Van Gelder, A. H., Aydin, R., Alves, M. M. & Stams, A. J., 2012, 1, 3-Propanediol production from glycerol by a newly isolated *Trichococcus* strain, *Microbial biotechnology*, 5(4), 573-578.

- Van Hille, R. & Mooruth, N., 2011, Investigation of sulphide oxidation kinetics and impact of reactor design during passive treatment of mine water, *Water Research Council, WRC Report No. KV, 268(10)*.
- Virpiranta, H., Taskila, S., Leiviskä, T., Rämö, J. & Tanskanen, J., 2019, Development of a process for microbial sulfate reduction in cold mining waters—Cold acclimation of bacterial consortia from an Arctic mining district, *Environmental Pollution*, 252, 281-288.
- Visser, A., Gao, Y. & Lettinga, G., 1993a, Effects of pH on methanogenesis and sulphate reduction in thermophilic (55°C) UASB reactors, *Bioresource Technology*, 44(2), 113-121.
- Visser, A., Gao, Y. & Lettinga, G., 1993b, Effects of short-term temperature increases on the mesophilic anaerobic breakdown of sulfate containing synthetic wastewater, *Water Research*, 27(4), 541-550.
- Visser, A., Pol, L. H. & Lettinga, G., 1996, Competition of methanogenic and sulfidogenic bacteria, *Water Science and Technology*, 33(3), 99-110.
- Vos, P., Garrity, G., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F. A., Schleifer, K.-H. & Whitman, W. B. 2011. *Bergey's manual of systematic bacteriology: Volume 3: The Firmicutes*, Springer Science & Business Media.
- Wang, J., Li, Q., Li, M. M., Chen, T. H., Zhou, Y. F. & Yue, Z. B., 2014, Competitive adsorption of heavy metal by extracellular polymeric substances (EPS) extracted from sulfate reducing bacteria, *Bioresource Technology*, 163, 374-376.
- Wargin, A., Olańczuk-Neyman, K. & Skucha, M., 2007, Sulphate-reducing bacteria, their properties and methods of elimination from groundwater, *Polish Journal of Environmental Studies*, 16(4), 639-644.
- Watzlaf, G. R., Schroeder, K. & Kairies, C. Long-term performance of alkalinity-producing passive systems for the treatment of mine drainage. Proceedings of the 2000 National Meeting of the American Society for Surface Mining and Reclamation, 2000.
- White, C. & Gadd, G. M., 1996, Mixed sulphate-reducing bacterial cultures for bioprecipitation of toxic metals: factorial and response-surface analysis of the effects of dilution rate, sulphate and substrate concentration, *Microbiology*, 142(8), 2197-2205.
- Wrighton, K. C., Thomas, B. C., Sharon, I., Miller, C. S., Castelle, C. J., Verberkmoes, N. C., Wilkins, M. J., Hettich, R. L., Lipton, M. S., Williams, N. H. & Long, P. E., 2012,

- Fermentation, hydrogen, and sulfur metabolism in multiple uncultivated bacterial phyla, *Science*, 337(6102), 1661--1665.
- Yamaguchi, T., Harada, H., Hisano, T., Yamazaki, S. & Tseng, I. C., 1999, Process behavior of UASB reactor treating a wastewater containing high strength sulfate, *Water Research*, 33(14), 3182-3190.
- Yuanyuai, C. & Nembhard, H. 2010. Design of experiments: a key to innovation in nanotechnology. *Emerging nanotechnologies for manufacturing*. Elsevier.
- Zagury, G., Neculita, C. & Bussiere, B. Passive treatment of acid mine drainage in bioreactors: short review, applications, and research needs. 60th Canadian Geotechnical Conference & 8th Joint CGS/IAH-CNC Groundwater Conference, 21-24 October 2007 Ottawa, Ontario, Canada. 14439-14446.
- Zamzow, K., Tsukamoto, T. & Miller, G., 2006, Waste from biodiesel manufacturing as an inexpensive carbon source for bioreactors treating acid mine drainage, *Mine Water and the Environment*, 25(3), 163-170.
- Zhang, M. & 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.
- Zinatizadeh, A. A., Bonakdari, H., Pirsahab, M. & Gharacheh, E., 2011, Response surface analysis and statistical modeling of sulfide generation from municipal wastewater, *CLEAN–Soil, Air, Water*, 39(5), 444-459.