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Evaluating the Effect of pH, Temperature, and Hydraulic Retention Time on Biological Sulphate Reduction Using Response Surface Methodology
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Abstract: Biological sulphate reduction (BSR) has been identified as a promising alternative for treating acid mine drainage. In this study, the effect of pH, temperature, and hydraulic retention time (HRT) on BSR was investigated. The Box–Behnken design was used to matrix independent variables, namely pH (4–6), temperature (10–30 °C), and HRT (2–7 days) with the sulphate reduction efficiency and sulphate reduction rate as response variables. Experiments were conducted in packed bed reactors operating in a downflow mode. Response surface methodology was used to statistically analyse the data and to develop statistical models that can be used to fully understand the individual effects and the interactions between the independent variables. The analysis of variance results showed that the data fitted the quadratic models well as confirmed by a non-significant lack of fit. The temperature and HRT effect were significant (p < 0.0001), and these two variables had a strong interaction. However, the influence of pH was insignificant (p > 0.05).

Keywords: acid mine drainage; sulphate reduction; sulphate reducing bacteria; response surface methodology

1. Introduction

Acid mine drainage (AMD) is a widespread problem that is considered the most important pollution problem caused by mining industries worldwide. AMD is formed when a sulphide-bearing mineral comes into contact with oxygen and water during or after the closure of mining operations. This oxidation process leads to the formation of sulphuric acid, which further reacts with the sulphide mineral and other exposed minerals and leaches out toxic heavy metals such as lead, cadmium, and arsenic [1–3]. Pyrite is the most common pathway for AMD formation, and its oxidation is shown in Equation (1) [4].

\[ 4\text{FeS}_2 + 15\text{O}_2 + 14\text{H}_2\text{O} \rightarrow 4\text{Fe(OH)}_3 + 8\text{SO}_4^{2-} + 16\text{H}^+ \]  

AMD is characterised by low pH, a high concentration of sulphate, and high concentrations of heavy metals such as iron, manganese, arsenic, zinc, copper, and aluminum. In South Africa, sulphate is considered one of the major contributors to water quality issues for mining operations as it is typically above 2000 mg/L in AMD. As a result, the maximum sulphate discharge levels should be less than 600 mg/L [5].
Conventionally, AMD is treated by neutralisation using lime or calcium carbonate, which precipitate metals and increase the pH but do not effectively reduce sulphate concentration in the mining effluent to levels suitable for discharge. This method is costly and produces large sludge volumes that are difficult to dispose of. Due to this, more research on AMD treatment has been done over the years, and biological sulphate reduction (BSR) has been identified as a promising alternative treatment for AMD. BSR is a process where sulphate is metabolically converted to sulphide by sulphate-reducing bacteria (SRB), and it simultaneously increases pH and precipitate metals under anaerobic conditions \[6,7\]. This process requires an electron donor and uses sulphate as a terminal electron acceptor. Electron donors, also known as substrates, that have been used include simple organic compounds such as ethanol, methanol, and butyrate \[8–10\], and complex organic compounds such as manure, food waste, woodchips, sludge, and hay \[11–15\]. Simple organic compounds are preferred as they are readily available; however, they are expensive \[16\]. Most studies have been leaning towards using complex compounds for BSR as they are considered to be cost-effective. SRB oxidises the organic matter, denoted by CH\(_2\)O in Equation (2), to produce alkalinity and hydrogen sulphide, which binds to the metals and precipitates them as metal sulphides, as shown in Equation (3).

\[
\begin{align*}
2\text{CH}_2\text{O} + \text{SO}_4^{2-} \xrightarrow{\text{SRB}} 2\text{HCO}_3^- + \text{H}_2\text{S} \\
\text{H}_2\text{S} + \text{M}^{2+} &\rightarrow \text{MS} + 2\text{H}^+ 
\end{align*}
\]

where M represents metals.

The performance of BSR is highly dependent on the availability of organic substrate, pH, temperature, and hydraulic retention time (HRT). Most known SRBs are mesophilic, and they perform optimally at neutral pH. Low pH and high pH suppress and inhibit SRBs, respectively \[17–19\], whereas low temperatures slow down the metabolic activity of SRBs \[20\]. Due to the sensitivity of SRB to temperature and pH, most research was done at neutral pH and temperatures greater than 20 °C \[17,21–23\]. HRT affects the rate at which sulphate is removed from AMD. Short retention times are known to washout biomass, whereas long retention times may lead to the depletion of organic matter if complex organic compounds are used \[10,11\].

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 the objective of statistically predicting and understanding the system’s behaviour \[24\]. RSM was developed for the simplification of multivariable experimental design enabling the reduction of the number of experiments that are required to identify ideal variables for a process. An advantage of using RSM includes less time required for experimentation due to reduced experimental runs and therefore a cost reduction of materials and reagents \[24,25\].

In the literature, studies have been focused on increasing the pH of mining influent while simultaneously precipitating dissolved heavy metals \[15,26–30\]. However, the main focus of this study was primarily on the reduction of sulphate in AMD. Although previous studies have used response surface methodology to investigate how different factors affect biological sulphate reduction \[25,31\], as far as the authors are aware, there is no published work on how pH, HRT, and temperature affects biological sulphate reduction using response surface methodology. This study forms part of the ongoing BSR work at Mintek, which has a pilot plant running at a coal mine in Mpumalanga, South Africa. This study aimed to investigate the effects of operational factors—namely pH, temperature and HRT—on sulphate reduction efficiency and sulphate reduction rate using response surface methodology. Additionally, the purpose of this study was to develop mathematical models that can be used to predict how the pilot plant would behave when factors are changed within the investigated range. This will help operate the pilot plant such that the sulphate discharge standard is met. The lab-scale reactors were set up to mimic the pilot plant.
2. Materials and Methods

2.1. Reactor Set-Up

In the current study, three laboratory-scale water-jacketed reactors were operated in parallel in a downflow mode. The schematic diagram of the lab-scale reactors is shown in Figure 1. The lab-scale packed bed reactors contained the following components: feed and effluent buckets, peristaltic pumps (Watson-Marlow Fluid Technology Group, Johannesburg, South Africa), water-jacketed columns, and a PolyScience Whispercool® (PolyScience, Niles, IL, USA) heater/chiller for temperature control. 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 with 1 cm holes. The reactors were 1 m in height and 0.15 m in internal diameter, and they had a total working volume of 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 near the top edge. A T-piece was fitted at the top of the tube to assist in fluid level adjustment. 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. The feed water for each reactor was stored in 10 L or 25 L plastic buckets, where it was pumped to the top of the column using a variable flowrate Watson Marlow 120 series peristaltic pump. The reactor overflow was collected in a 15 L bucket for each column.

Figure 1. Schematic diagram of the reactors.

2.2. Substrates

Initially, the three lab-scale reactors were packed with 30% woodchips, 30% wood shavings, 20% hay, 10% lucerne straw, and 10% cow manure measured by volume. A mixture of the above-mentioned substrates was blended and loaded into the reactors. Woodchips, wood shavings, hay, and lucerne straw are cellulosic compounds that can contribute as substrates, although their contribution is small [32,33]; hence, they were used as a support for the biofilm. Above the perforated plate, a 2–3 cm layer of woodchips was evenly spread to prevent the holes on the perforated plate
from blocking when the substrates migrated downwards. Cow manure purchased from Lifestyle, Johannesburg, South Africa and lucerne pellets purchased from Milmac Feeds, Fourways, South Africa were used as the main substrates. Then, 186 mL (128.07 g) of cow manure and 186 mL (63.69 g) of lucerne pellets were added on top of the reactor packing and replenished once every week.

2.3. AMD and Inoculum

The AMD used for all experiments was collected from a coal mine in eMalahleni, Mpumalanga province, South Africa. The raw AMD was characterised by pH less than 3 and a sulphate concentration ranging between 2500 mg/L and 5200 mg/L. The anaerobic mixed culture used was collected from one of the reactors that were operating at the Mintek’s pilot plant at the coal mine. The pilot plant had been operating for 10 months at HRT varying between 5 days and 7 days, influent pH > 5 with sulphate reduction efficiency above 90%, sulphide concentration varying between 200 mg/L and 700 mg/L, and it was packed with the same mixture as that used in lab-scale reactors. The lab-scale reactors were inoculated with a mixture of mine water adjusted using hydrated lime to pH approximately 6.5 (70% v/v) and the inoculum (30% v/v). For the duration of the study, the flow rate varied between 1.14 L/day and 4 L/day with a sulphate loading rate between 0.36 g/L/day and 2.6 g/L/day. Hydrated lime was used for all pH adjustments.

2.4. Sampling and Analysis

The effluent pH was measured immediately after the samples were taken. Samples were collected using a beaker; then, the pH meter was immersed into 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 pH 7 buffer solutions before analysis.

For sulphate analysis, a turbidimetric method was used to measure the influent and effluent sulphate concentration. This was achieved by using a Merck Spectroquant® Prove 300 (Merck, Darmstadt, Germany). All the samples were filtered using 0.22 µm membrane syringe filters before analysis to prevent interferences from suspended solids. Samples were analyzed immediately after collection.

The potentiometric determination of hydrogen sulphide using 0.1 M AgNO₃ was used to determine the total sulphide concentration in the effluent. A Metrohm Titrand (Metrohm, Herisau, Switzerland) was used for sulphide titrations using AgNO₃.

2.5. Experimental Design

Design-Expert® (version 11.1.2.0, Stat Ease Inc., Minneapolis, MN, USA), a statistical tool that helps with the design of experiments (DoE), was used to design the experiments. The Box–Behnken model with 3 centre points was used for the design. A total of 16 experiments were conducted. The effect of three factors—namely, pH ranging from 4 to 6 in 1 unit steps, temperature ranging from 10 °C to 30 °C in steps of 10 °C, and HRT ranging from 2 days to 7 days in steps of 2.5 days—was studied. The pH range was selected after it was found in the literature that most SRB perform better near neutral pH, and that at pH less than 4, SRB are suppressed and therefore affecting their performance [17,34]. The temperature range was selected after considering the average temperatures at eMalahleni throughout the year, and HRT was selected based on previous studies done at Mintek. The levels of the chosen variables in the design of experiments are shown in Table 1. The sulphate reduction efficiency and sulphate reduction rate were the corresponding response variables, as shown in Table 2.
Table 1. Box–Behnken design for three factors in experimental design.

| Code | Factors   | Factor Range and Levels (Coded) |
|------|-----------|---------------------------------|
| A    | pH        | −1 0 1                           |
| B    | Temperature (°C) | 4 5 6          |
| C    | HRT (days) | 10 20 30                         |

Table 2. Experimental runs and obtained responses.

| Run | A: pH | B: Temperature (°C) | C: HRT (days) | Sulphate Reduction Efficiency (%) | Sulphate Reduction Rate (mol/m³/day) |
|-----|-------|----------------------|---------------|-----------------------------------|--------------------------------------|
| 1   | 5     | 20                   | 4.5           | 76.94                             | 4.57                                 |
| 2   | 5     | 30                   | 7             | 98.73                             | 7.66                                 |
| 3   | 4     | 30                   | 4.5           | 90.34                             | 5.70                                 |
| 4   | 5     | 30                   | 2             | 65.61                             | 9.75                                 |
| 5   | 6     | 20                   | 2             | 50.28                             | 8.97                                 |
| 6   | 5     | 10                   | 7             | 41.74                             | 1.66                                 |
| 7   | 5     | 20                   | 4.5           | 84.46                             | 4.99                                 |
| 8   | 4     | 10                   | 4.5           | 22.76                             | 1.67                                 |
| 9   | 6     | 30                   | 4.5           | 96.87                             | 6.17                                 |
| 10  | 6     | 20                   | 7             | 97.56                             | 5.63                                 |
| 11  | 4     | 20                   | 2             | 55.17                             | 9.89                                 |
| 12  | 4     | 20                   | 7             | 93.43                             | 5.35                                 |
| 13  | 5     | 20                   | 4.5           | 84.38                             | 4.98                                 |
| 14  | 6     | 10                   | 4.5           | 30.38                             | 1.88                                 |
| 15  | 6     | 30                   | 7             | 98.40                             | 7.54                                 |
| 16  | 5     | 30                   | 4.5           | 96.57                             | 6.46                                 |

2.6. Statistical Analysis

Response surface methodology [24] was used to understand the interactions between the independent variables. This was achieved by fitting the experimental data into a polynomial quadratic equation to obtain regression coefficients, as shown in Equation (4).

\[
Y = b_0 + \sum b_i x_i + \sum b_{ij} x_i^2 + \sum b_{ij} x_i x_j
\]  

(4)

where \(Y\) is the response variable, \(b_0\) is the constant term, \(b_i\) is the linear coefficient, \(b_{ij}\) 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, the sulphate reduction efficiency (%) and sulphate reduction rate (mol/m³/day) were chosen as response variables and therefore were fitted into Equation (4). Analysis of variance (ANOVA) was used to evaluate the validity and significance of the fitted model. The coefficient of determination \(R^2\), adjusted \(R^2\), predicted \(R^2\), lack of fit, adequate precision, F-value, and p-value were used to further evaluate the quality and accuracy of the model. In the present study, the significance level was set at 0.05.

3. Results and Discussion

3.1. Statistical Analysis

The data obtained from the 16 experiments that were conducted were fitted into polynomial quadratic equations as shown in Equations (5) and (6) in terms of coded factors.

Sulphate reduction efficiency

\[
= + 82.86 + 2.65 \times A + 33.79 \times B + 21.31 \times C - 0.7714 \times AB - 0.6874 \times AC - 5.67 \times BC + 1.08 \times A^2 - 23.64 \times B^2 + 10.51 \times C^2
\]  

(5)
The reliability, quality, and accuracy of the fitted quadratic models were evaluated using analysis of variance (ANOVA), as shown in Table 3. The significance of the models is confirmed by high F-values and low p-values [35]. The models were significant as confirmed by low probability values of less than 0.0001 and high F-values of 101.70 for sulphate reduction efficiency and 221.37 for sulphate reduction rate. The reported F-values imply that there is only a 0.01% chance that the differences could be due to noise. For this study, the lack of fit for both models was insignificant, which shows that the data fitted the models well.

Table 3. ANOVA results for the fitted quadratic model.

| Response                          | Source of Variation | Sum of Squares | df | Mean Square | F-Value | p-Value |
|-----------------------------------|---------------------|----------------|----|-------------|---------|---------|
| Sulphate reduction efficiency (%) | Model               | 10,155.47      | 9  | 1128.39     | 101.70  | <0.0001 |
|                                   | A-pH                | 59.11          | 1  | 59.11       | 5.33    | 0.0604  |
|                                   | B-Temperature       | 6519.62        | 1  | 6519.62     | 587.61  | <0.0001 |
|                                   | C-HRT               | 2442.99        | 1  | 2442.99     | 220.19  | <0.0001 |
|                                   | AB                  | 2.65           | 1  | 2.65        | 0.2389  | 0.6424  |
|                                   | AC                  | 2.11           | 1  | 2.11        | 0.1897  | 0.6784  |
|                                   | BC                  | 72.74          | 1  | 72.74       | 6.56    | 0.0429  |
|                                   | A^2                 | 3.68           | 1  | 3.68        | 0.3316  | 0.5856  |
|                                   | B^2                 | 1808.36        | 1  | 1808.36     | 162.99  | <0.0001 |
|                                   | C^2                 | 370.19         | 1  | 370.19      | 33.37   | 0.0012  |
|                                   | Residual            | 66.57          | 6  | 11.10       |         |         |
|                                   | Lack of Fit         | 29.32          | 4  | 7.33        | 0.3937  | 0.8060  |
|                                   | Pure Error          | 37.25          | 2  | 18.62       |         |         |
|                                   | Correction Total    | 10,222.04      | 15 |             |         |         |

| Sulphate reduction rate (mol/m^3/day) | Source of Variation | Sum of Squares | df | Mean Square | F-Value | p-Value |
|--------------------------------------|---------------------|----------------|----|-------------|---------|---------|
| Model                                | 101.69              | 9              | 11.30 | 221.37 | <0.0001 |
| A-pH                                 | 0.5037              | 1              | 0.5037 | 1.49   | 0.2617  |
| B-Temperature                        | 25.50               | 1              | 25.50 | 499.63 | <0.0001 |
| C-HRT                                | 20.96               | 1              | 20.96 | 410.67 | <0.0001 |
| AB                                   | 0.0093              | 1              | 0.0093 | 0.1822 | 0.6844  |
| AC                                   | 0.1504              | 1              | 0.1504 | 2.95   | 0.1369  |
| BC                                   | 1.78                | 1              | 1.78  | 34.89  | 0.0010  |
| A^2                                  | 0.0577              | 1              | 0.0577 | 1.13   | 0.3267  |
| B^2                                  | 2.58                | 1              | 2.58  | 56.60  | 0.0004  |
| C^2                                  | 23.09               | 1              | 23.09 | 452.42 | <0.0001 |
| Residual                             | 0.3062              | 6              | 0.0510|         |         |
| Lack of Fit                          | 0.1926              | 4              | 0.0481| 0.8472 | 0.6045  |
| Pure Error                           | 0.1137              | 2              | 0.0568|         |         |
| Correction Total                     | 102.00              | 15             |      |         |         |

df—degree of freedom; ^1 Significant; ^2 Not significant.

Fit statistics are shown in Table 4. The coefficient of determination $R^2$ is a statistical parameter that measures how well the data fits the line. Adjusted $R^2$ is a version of $R^2$ that is always smaller than $R^2$, and predicted $R^2$ measures the predictive accuracy of the model [25]. A model is considered well fitted when the $R^2$ value is greater than 0.8 [25]. $R^2$, adjusted $R^2$, and predicted $R^2$ were found to be 0.9935, 0.9837, and 0.9716 for sulphate reduction efficiency and 0.9970, 0.9925, and 0.9853 for sulphate reduction rate. The difference between the predicted and adjusted $R^2$ should be less than 0.2 for the model to be considered well fitted and able to make satisfactory predictions. For this study, predicted and adjusted $R^2$ were in agreement with this. Adequate precision measures the signal-to-noise ratio, and a ratio greater than 4 is desirable. The values of adequate precision were 29.36 and 46.21 for sulphate reduction efficiency and sulphate reduction rate, respectively, which indicates an adequate signal.
The diagnostic section provides plots that can be used to further validate the accuracy of the model. The normal probability plots illustrated in Figure 2 show that the residuals are normally distributed as the points are closer to the line. Residuals vs. predicted, as shown in Figure 3, proved the models’ quality by having random scatters that are evenly distributed above and below the horizontal axis [25]. The correlation between predicted and actual values is shown in Figure 4. The clustering of the values along the diagonal line confirms that the model is accurate and robust [25,36].

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### Table 4. Fit statistics.

|                          | $R^2$   | Adjusted $R^2$ | Predicted $R^2$ | Adequate Precision |
|--------------------------|---------|----------------|-----------------|--------------------|
| Sulphate reduction efficiency (%) | 0.9935  | 0.9837         | 0.9716          | 29.36              |
| Sulphate reduction rate (mol/m³/day) | 0.9970  | 0.9925         | 0.9853          | 46.21              |

Figure 2. Normal plot of residuals for (a) sulphate reduction efficiency and (b) sulphate reduction rate.

Figure 3. Plot of residuals vs. predicted for (a) sulphate reduction efficiency and (b) sulphate reduction rate.
3.2. Effect of Individual Factors

The effect of individual factors on the responses is shown in this section. One factor is changed at a time while keeping other factors at the centre point. The steeper the slope, the more sensitive a response is to the factor.

3.2.1. pH

Figure 5a,b shows the effect of pH on sulphate reduction efficiency and sulphate reduction rate, respectively. From the graphs, both responses slightly increase with an increase in pH from pH 4 to pH 6; however, the increase is not significant. This is evident in Table 3, where pH was insignificant for both responses. This shows that SRBs were not suppressed at an initial pH of 4, which is considered too low for SRB to grow [37]. This could imply that lower costs need to be expended for pH adjustment, and this could have a positive impact on the process’s operating expenses. Sulphate reduction at pH approximately 4 was highly impacted in some studies [38–40], which may be because the reactor pH was controlled [39]. However, Jong and Parry [34] found a sulphate reduction efficiency of above 80% when the reactor’s influent pH was 4.07. For this study, only the influent pH was controlled, and the average effluent pH, for experiments that had an influent pH of 4, was above 6, as shown in Figure 6. Jong and Parry [34] also observed an effluent pH of above 6. 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 [41].
Figure 5. pH effect on (a) sulphate reduction efficiency and (b) sulphate reduction rate at 20 °C and hydraulic retention time (HRT) of 4.5 days.

3.2.2. Temperature

The effect of temperature is depicted in Figure 7a,b. Decreasing temperature from 30 to 20 °C with HRT and pH at the centre point had a minimal impact on SRB activity, which was expected, as 20 °C
is in a range that supports the growth and activity of SRB, as also observed in other studies [42–44].
A further decrease in temperature from 20 to 10 °C slowed down the metabolic activity significantly. Sheoran et al. [10] suggested that sulphate reduction is likely to decrease by 50% at temperatures lower than 10 °C compared to sulphate reduction at 20 °C. Similarly, the same effect can be observed from the graphs. The sulphate reduction efficiency and sulphate reduction rate decrease by more than 50% with a decrease in temperature from 20 to 10 °C. According to the graphs, the sulphate reduction efficiency decreases from approximately 80% to about 25%, and the sulphate reduction rate decreases from approximately 5 to 2 mol/m³/day when the temperature is decreased from 20 to 10 °C with HRT and pH kept constant at the centre point.

**Figure 7.** Temperature effect on (a) sulphate reduction efficiency and (b) sulphate reduction rate at pH 5 and HRT of 4.5 days.

3.2.3. HRT

Figure 8a shows that the sulphate reduction efficiency response increases as the HRT increases from 2 to 7 days. Conversely, the sulphate reduction rate decreases with increasing HRT from 2 to approximately 7 days, followed by a slight increase with a further increase in HRT to 7 days, as shown in Figure 8b. Although sulphate reduction efficiency was observed to decrease with decreasing HRT, the sulphate reduction rate increased with a decrease in HRT due to higher feed rates. A longer HRT resulted in higher sulphate reduction efficiency but lower sulphate reduction rates. Similar observations were made in earlier studies [45–48].

Figure 9a,b shows the effect of HRT over time at 30 °C and pH 5. When HRT was decreased from 7 to 4.5 days, there was only a slight decrease in both the sulphate reduction efficiency and sulphate reduction rate. An interesting observation was made when the HRT was decreased from 4.5 days to 2 days. At a HRT of 2 days, both the sulphate reduction efficiency and sulphate reduction rate increased rapidly upon replenishing the substrates. This was followed by a decrease after a maximum was reached. Although some studies show that HRT leads to a decrease in sulphate reduction due to SRB washout [49,50], it is presumed in this study that the decrease in sulphate reduction efficiency and sulphate reduction rate was a result of substrates washout [51]. This was evident when both responses improved upon the replenishing of substrates. A study done by Poinapen et al. [52] using primary sewage sludge (PSS) as a 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 8.** HRT effect on (a) sulphate reduction efficiency and (b) sulphate reduction rate at 20 °C and pH 5.

**Figure 9.** Effect of HRT over time at 30 °C and pH 5 for (a) sulphate reduction efficiency and (b) sulphate reduction rate.

### 3.3. Interactions between Factors

The interactive effects between pH, temperature, and HRT on sulphate reduction efficiency and sulphate reduction rate are shown in this section. The interaction between temperature and pH illustrated in Figure 10a,b shows that both the sulphate reduction efficiency and sulphate reduction rate decrease with a decrease in temperature. Furthermore, it is clear from the graphs that pH does not affect both responses, especially at low temperatures. At maximum temperature, the responses slightly increase with an increase in pH.
increase in sulphate reduction rate caused by a decrease in the HRT was greater at all temperatures than decreased to approximately 40% with a decrease in temperature from 30 to 10 °C and HRT. However, temperature had more impact on the sulphate reduction efficiency compared to HRT. The interaction between temperature and pH (AB) and between pH and HRT (AC) were not strong, which is proven by ANOVA analysis in Table 3.

However, HRT is shown to have different effects on the responses. The sulphate reduction efficiency increases with an increase in HRT, whereas the overall trend for the sulphate reduction rate is that it increases with decreasing HRT. The graphs in Figure 10a,b show that the interactions between pH and temperature (AB) and between pH and HRT (AC) were not strong, which is proven by ANOVA analysis in Table 3.

Strong interactions were observed between HRT and temperature, as depicted in Figure 12a,b. In Figure 12a, the sulphate reduction efficiency increases with a simultaneous increase in temperature and HRT. However, temperature had more impact on the sulphate reduction efficiency compared to HRT. For example, at 30 °C, the sulphate reduction efficiency decreased from almost 100% at an HRT of 7 days to just above 60% at an HRT of 2 days. On the other hand, at an HRT of 7 days, it decreased to approximately 40% with a decrease in temperature from 30 to 10 °C. Conversely, the increase in sulphate reduction rate caused by a decrease in the HRT was greater at all temperatures than

**Figure 10.** Interactive effects between temperature and pH on (a) sulphate reduction efficiency and (b) sulphate reduction rate.

**Figure 11.** Interactive effects between HRT and pH on (a) sulphate reduction efficiency and (b) sulphate reduction rate.
that caused by an increase in temperature at all HRTs, as shown in Figure 12b. The highest sulphate reduction rate was approximately 10 mol/m³/day, which was observed at 30 °C and an HRT of 2 days.

![Figure 12. Interactive effects between HRT and temperature on (a) sulphate reduction efficiency and (b) sulphate reduction rate.](image)

In this section, it was shown that pH had an insignificant interaction with both temperature and HRT. However, HRT and temperature were shown to have strong interactions. The information obtained and the mathematical models developed will be used to evaluate the performance of the pilot plant. For example, due to the difficulty in controlling temperature in an open system, the models developed will be used to determine at what HRT and pH the pilot plant should operate during winter and summer seasons to compensate for drops in sulphate reduction associated with temperature changes. Considering that pH had no effect within the range investigated, it presumed that the only parameter that will be controlled is the HRT. However, further tests will be done on the pilot plant to confirm this.

3.4. Optimisation

RSM 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 [53]. The numerical optimisation section in design expert allows one to maximise 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 [53]. The optimisation process was carried out with the goal to maximise the sulphate reduction efficiency simultaneously with the sulphate reduction rate by minimising the pH and setting the temperature at 10 °C, 20 °C, and 30 °C. The HRT was set to be in range between 2 days and 7 days. At 10 °C, a 41.89% sulphate reduction efficiency and 1.67 mol/m³/day sulphate reduction rate can be achieved at pH 5 and HRT of 7 days. However, the desirability was low at 0.068. This could mean that more retention time, beyond that which was investigated in this study, will be required to achieve a higher sulphate reduction efficiency and sulphate reduction rate. At 20 °C, a sulphate reduction efficiency of 92.56% and sulphate reduction rate of 5.06 mol/m³/day can be achieved at pH 4 and HRT 6.7 days, and the desirability was 0.756. A sulphate reduction efficiency of 94.46% and sulphate reduction rate of 5.65 mol/m³/day can be achieved at 30 °C, pH 4, and HRT 4.8 days, and the desirability was 0.8. This shows that at higher temperatures, the pilot plant can operate at lower HRTs.
3.5. Sulphide

Hydrogen sulphide is produced during the reduction of sulphate, as shown in Equation (2), and it is known for its toxicity. Hydrogen sulphide causes problems such as odour, corrosion, and sulphate reduction inhibition [54,55]. The sulphide concentration for all experiments in this study ranged between 114 and 798 mg/L. Due to the high sulphide concentrations observed, there are further tests that are currently being done at Mintek for a downstream process that will use sulphide oxidising bacteria to oxidise sulphide to elemental sulphur (S⁰), as shown in Equation (7). 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 (8). Therefore, it is recommended that the ratio of sulphide to oxygen be kept at 2:1 to prevent complete oxidation to sulphate [56].

\[
\text{HS}^- + \frac{1}{2} \text{O}_2 \rightarrow \text{S}^0 + \text{OH}^-
\] (7)

\[
\text{HS}^- + 2\text{O}_2 \rightarrow \text{SO}_4^{2-} + \text{H}^+
\] (8)

4. Conclusions

In the current study, RSM was used to statistically analyse the data. ANOVA results showed that the sulphate reduction efficiency and sulphate reduction rate models were significant and adequate, as proven by statistical indexes including lack of fit, coefficient of variation, and adequate precision. Individually, the pH effect was insignificant for both responses, and therefore, its interaction with other independent variables was also not significant. However, there was a strong interaction between HRT and temperature. Additionally, a decrease in HRT impacted the sulphate reduction rate positively due to increased flow rates. Conversely, it had a negative impact on the sulphate reduction efficiency, which was likely due to substrates washout. This study developed mathematical models that were found to be statistically significant. These models can be used as decision-making tools by using them to predict how the process will react to different conditions within the investigated range. This will help adjust controllable factors such as pH and HRT when the temperature fluctuates.

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