Effect of iron and manganese concentration on the sulfate reducing process in acid mine drainage

S Sudarno, N Hardyanti, K Serafina, and A Oktaviana

1 Departemen Environmental Engineering, Engineering Faculty, Diponegoro University
Jl. Prof. H. Soedharto, S.H Tembalang, Semarang, Indonesia, 50275

Abstract. Mining activities produce acid mine drainage that can be treated with microorganisms, and in this case, sulfate reducing bacteria. Iron and manganese effect on the sulfate reduction process can be investigated using anaerobic batch and continuous reactors with various iron and manganese concentrations. For batch reactors, the highest efficiency of sulfate reduction and sulfate reduction rate was obtained by 0 mg Mn/l reactor. Both efficiency and rate continued to decline with the increasing of iron and manganese concentration. The temperature for all reactors remained stable. The sediment color in 0 mg Fe/l and 0 mg Mn/l reactors were remain black, while the sediment color in the rest of the reactors changed from black to brown. The COD concentration for all the reactors decreased and the highest efficiency was 35,50% which was obtained by 0 mg Fe/l reactors. The highest efficiency of iron reduction was 99,23% and was achieved by 60 mg Fe Mn/l reactor. The effect for continuous and batch reactors are quite identical.

Keywords: sulfate reducing bacteria, batch reactor, continuous reactor, sulfate, iron, manganese

1. Introduction
There are plenty contaminants in mining activities, and sulfate is one of major contaminants in mining water effluent, which is better known as acid mine drainage or AMD [1]. Acid mine drainage formed from the oxidation of mineral sulfides, producing sulfuric acid [2]. Beside sulfate, acid mine drainage also contains a numerous amount of heavy metals, preferably iron and manganese. The presence of both sulfate and heavy metals cause a serious threat to the environment, for sulfate can (1) promote secondary water quality impacts related to sulfur (S) redox processes and biological hydrogen sulfide (H2S) production; (2) stimulate methylation of mercury to methylmercury, the most toxic and bioaccumulative form of mercury; (3) enhance biodegradation of organic matter in soils; and (4) promote release of nutrients and potentially toxic compounds from sediments during biologically-mediated sulfate reduction processes [3], while heavy metals can inhibit the growth of sulfate reducing bacteria. Due to that reasons, all mining coorporation will have to provide a certain treatment to treat acid mine drainage.

The treatment for acid mine drainage can be done actively or passively. In the focus of excluding sulfate in acid mine water, various processing methods have been done either physically, chemically, and biologically. The chemical treatments can be done using lime injection and open limestone channel. Physical treatments using permeable reactive and lastly biological treatment using sulfate
reducing bacteria. Biological treatment provides a more efficient and cheaper treatment than chemical or physical treatment [4].

Sulfate treatment is biologically done by reducing sulfate using microorganisms called sulfur reducing bacteria which are the microorganisms that live in environment containing high sulfate concentration. Sulfate reducing bacteria reduce sulfate to H₂S anaerobically and oxidize organic material to H₂ using sulfate ions as electron acceptor and produce hydrogen sulfate and bicarbonate [5].

Biological treatment can be performed on anaerobic batch and continuous reactors with suspended growth for the growth method. The growth and activity of microorganisms play an important role in biological sulphate reduction process. Thus, it is necessary to create an suitable condition for microorganisms inside the reactor, in this case the sulphate reducing bacteria group. In addition, the heavy metal content is also found in acid mine drainage. The heavy metals found are mostly Fe (iron) and Mn (manganese) [6] which can affect the sulfate reduction process. Even so, both metals are important for anaerobic bacterial metabolism [7]. Other than that, Fe and Mn can act as binders of sulfate reduction products, ie sulfides and will be precipitated into metal sulphides thus making it easier to be removed [8]. However, in too high concentrations it can also be toxic to bacteria [9].

To inspect the effect of variations in concentration of iron and manganese on the sulfate reduction process can be indicated by the concentration of sulphate, pH, temperature, color, COD (Chemical Oxygen Demand) and heavy metal concentrations contained in water. This research will examine the removal of sulfate contained in wastewater using artificial method in the presence of processing using sulfate reducing bacteria from various sources with variation of type and concentration of heavy metals in artificially made acid mine drainage.

2. Method
This study was conducted with an experimental method and aims to find the effect of a particular treatment on another in controlled conditions [10]. The observations were conducted in an artificial condition regulated by the researchers. The study was conducted using a laboratory-scale reactor. At the beginning of the study, tests were conducted on bacterial sources to determine the most efficient source of bacteria to remove sulfate by contacting sediments and inoculants with artificial waste in a batch reactor for 7 days. The parameters tested were pH, sulfate concentration, temperature, and DO.

The sources of the bacteria tested were from Ayoma inoculants, Biosystem inoculants, laundry septic tanks and Rawa Pening Lake. Both inoculants are artificial inoculants that are about to be tested for their potential. The laundry septic tank sediment was chosen because it is an anaerobic sediment that pollutes the environment [11] so it is used so as not to pollute. Swamp sediments were chosen because they were the best stagnant sediments in reducing sulfate based on a research conducted by Fahruddin (2009) [12]. The most efficient source of bacteria is then grown and tested further to determine the effect of heavy metals on sulfate reduction process.

In testing the effect of heavy metals, the reactor used consisted of two types of reactor, an anaerobic batch and anaerobic continuous. Anaerobic batch reactor required 5 reactors. They are K1 (Control 1), K2 (Control 2), IC 50 (50% Inhibitory Concentration), MTC (Maximum Tolerated Concentration), 100 (Fe 100 mg / L and Mn 100 mg / L). The study was conducted for 14 days. Every single day, pH, sulfate concentration, and temperature was measured. Then, every three days, COD concentration was measured. Lastly, every five days iron and manganese was measured. After that, the bacteria would also be tested in an anaerobic continuous reactor for 23 days. It had 4 different concentration each stages: Normal, IC 50, MTC, and 100. Every single day, pH, sulfate concentration, and temperature was measured. Then, every three days, COD concentration was measured.

3. Results and discussions
3.1. Potential of sulfate reduction of inoculant and sediment samples
Potential test is an analysis performed to determine which inoculants or sediments are most effective in reducing sulfate in water. The most effective inoculants or sediments in sulfate reduction will further be used for analyzing the effects of heavy metals on sulfate reduction.
The sulfate potential test was carried out twice, at concentrations of 56.4 mg/l SO$_4^{2-}$ based on artificial coal acid water making methods by Faisol Asip (2015) [13], and the second with the real mining concentrations of 1780,1 mg/l [14] with the similar method.

Table 1. Sulfate reduction potential on 56.4 mg/l concentration for ayoma inoculants, biosystem inoculants, laundry septic tank sediment, dan Rawa Pening Lake sediment

| Inoculants/Sediment      | Parameter | Day - | 0     | 1     | 2     | 3     | 4     | 5     | 6     | 7     |
|--------------------------|-----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Biosystem                | SO$_4^{2-}$ (mg/L) |       | 23.58 | 46.08 | 42.89 | 38.17 | 34.14 | 29.14 | 31.92 | 42.33 |
|                          | pH        |       | 5.87  | 6.42  | 5.75  | 4.99  | 5.45  | 5.38  | 5.52  | 5.85  |
| Ayoma                    | SO$_4^{2-}$ (mg/L) |       | 44.28 | 65.39 | 59.42 | 55.39 | 46.78 | 56.78 | 62.75 | 55.11 |
|                          | pH        |       | 3.45  | 3.25  | 3.36  | 3.41  | 3.49  | 3.48  | 3.49  | 3.52  |
| Laundry septic tank      | SO$_4^{2-}$ (mg/L) |       | 32.89 | 44.00 | 42.19 | 40.67 | 38.58 | 35.39 | 45.53 | 37.19 |
|                          | pH        |       | 5.32  | 6.28  | 6.05  | 5.96  | 6.05  | 6.19  | 6.22  | 6.39  |
| Rawa Pening Lake         | SO$_4^{2-}$ (mg/L) |       | 41.36 | 66.78 | 56.50 | 51.78 | 50.53 | 47.89 | 72.06 | 51.50 |
|                          | pH        |       | 5.2   | 5.59  | 5.25  | 4.73  | 4.82  | 4.87  | 4.8   | 5.44  |

Table 2. Sulfate reduction potential on 1780,1 mg/l concentration for ayoma inoculant, biosystem inoculant, laundry septic tank sediment, dan Rawa Pening Lake sediment

| Inoculants/Sediment      | Parameter | Day - | 0     | 1     | 2     | 3     | 4     | 5     | 6     | 7     |
|--------------------------|-----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Biosystem                | SO$_4^{2-}$ (mg/L) |       | 1074.56 | 813.44 | 780.11 | 716.22 | 3102.33 | 2347.22 | 1028.22 | 827.33 |
|                          | pH        |       | 3.37  | 3.23  | 3.03  | 3.42  | 3.2    | 3.22  | 3.29  | 3.28  |
| Ayoma                    | SO$_4^{2-}$ (mg/L) |       | 869.00 | 702.33 | 724.56 | 827.33 | 2960.67 | 2287.56 | 1875.33 | 927.33 |
|                          | pH        |       | 3.43  | 3.34  | 3.34  | 3.33  | 3.2    | 3.27  | 3.32  | 3.36  |
| Laundry septic tank      | SO$_4^{2-}$ (mg/L) |       | 746.78 | 721.78 | 610.67 | 796.78 | 963.44 | 724.56 | 712.33 | 627.33 |
|                          | pH        |       | 3.85  | 3.75  | 3.78  | 3.79  | 3.7    | 3.69  | 3.65  | 3.66  |
| Rawa Pening Lake         | SO$_4^{2-}$ (mg/L) |       | 1030.11 | 702.33 | 707.89 | 852.33 | 1332.89 | 812.11 | 787.44 | 630.11 |
|                          | pH        |       | 3.66  | 3.57  | 3.38  | 3.42  | 3.3    | 3.32  | 3.32  | 3.36  |

Based on these data, the sample which has the best sulfate reduction capability is the sediment tank sample. The laundry septic tank sediment has the largest average sulfate reduction rate with a value of 164.68 mg SO$_4^{2-}$/L.day.

3.2. Effect of heavy metal and manganese metal on sulfate reduction process

The heavy metal content can affect the sulfate reduction process performed by the sulphate reducing bacteria by deactivating the enzymes and destroying the proteins in bacteria [15]. To understand more about the influence of heavy metals, the experiment was done by using laundry septic tank sediments as the best source of bacteria in the analysis of sulfate reduction process.

The main stages of the research consist of seeding and acclimation stage for 28 days on an intermittent completed stirred tank reactor, then proceed with the running stage on the anaerobic batch reactor for 14 days.

3.2.1. Seeding and acclimatization Stage

Seeding and acclimatization are needed so that bacteria have a chance to adapt to the new environment before the bacteria being used for further research. At this stage, artificial waste with a sulfate concentration of 4–2000 mg SO$_4^{2-}$/L was put into a reactor containing 850 ml of laundry septic tank for 28 days. In addition, the pH was controlled between 6-7.5 since that is the optimum pH range for sulphate reducing bacteria to grow [16]. This stage is done by two methods, namely gradual method and shock method.
In the gradual method, the sulfate concentration of +2000 mg SO\(_4^{2-}\) / L is done gradually with each +500 mg SO\(_4^{2-}\) / L per week. Giving the same amount of concentrations each week is done to prove that sulphate reducing bacteria have been able to adapt and grow. This can be evidenced by the increasing rate of sulfate reduction each week. Viewed from the visual change and reduction rate, gradual acclimatization shows optimum growth. In the first week, the sediment still showed a brown color and a reduction rate of only 22.7 mg / l per day. In the second week, the color change in the sediment partly turns black and the reduction rate rises to 37, 6375 mg / l per day. By the third week, most of the sediment had changed color to black and the reduction rate increased to 50,986 mg / l per day. Finally, by the fourth week, all the sediments changed to black and the reduction rate increased to 61.514 mg / l per day. The visual change can be seen in Figure 1.

![Figure 1. Change of sediment color every week on gradual acclimatization](image)

In the shock method, the concentration of sulfate concentration of +2000 mg SO\(_4^{2-}\) / L was done directly to prove that sulphate reducing bacteria require adaptation and can not be directly forced to full activity and grow at high sulfate concentration conditions. The sulfate concentration had decreased on the first day and continued to decrease significantly until the third day. This indicates that the sulphate reducing bacteria already use sulfate to the maximum so as to achieve equilibrium condition. On the 4th day until the 16th day, the sulphate concentration did not change / stagnate. This indicates that sulphate reducing bacteria have been unable to grow or die.

In contrast to the gradual acclimatization, in shock acclimatization occurs a high reduction rate in the first week and stagnant in the following weeks. In addition, significant visual changes occurred on day 10 from chocolate to black completely. The rate of reduction in the first week reached 118 mg / l per day. In the following weeks, the rate of reduction is constant between 0.4 mg / l per day to 2.5 mg / l per day. This proves bacteria were not able to reduce sulfate again in the second and third weeks. The visual change can be seen in Figure 2.

![Figure 2. Change of sediment color every week on shock acclimatization](image)

From both methods, it can be determined that the gradual method is better for growing sulphate reducing bacteria.

### 3.2.2. Running anaerobic batch reactor

The running stage is done after the seeding and acclamation stage. At this stage, the reactor is operated batch for 14 days for each concentration of heavy metal of iron and manganese, namely: K1 (Control 1): the value of iron (Fe) 0 mg / l; K2 (Control 2): the value of manganese metal (Mn) is 0 mg / l; IC 50 (50% Inhibitory Concentration): Based on the journal Samia Azabou (2006) [9], 50% inhibitory concentration is a condition in which concentrations of ferrous and manganese metals become inhibitors for bacteria causing bacterial growth ability to decline by 50%. The concentration is in iron (Fe) of 41 mg / L and in manganese (Mn) of 31 mg / L; MTC (Maximum Tolerated Concentration): maximum
tolerated concentration is a condition in which metal concentrations are inhibitors for bacteria that cause bacteria to not grow at all. The concentration is on iron (Fe) of 60 mg/L and in manganese (Mn) of 60 mg/L; and finally 100 (Fe 100 mg/L and Mn 100 mg/L): In reactor 100. A maximum concentration of concentration above maximum tolerated concentration of iron (Fe) of 100 mg/L and manganese (Mn) of 100 mg/L was performed.

The results of the five reactors are divided into seven parameters, namely:

• Sulfate Concentrations

Sulfate concentration is the most important parameter in determining the success of the sulfate reduction process. The results of the sulfate concentration test for 14 days on each reactor are described in Table 4 and illustrated in Figure 3 below.

| Day | K1     | K2     | IC 50  | MTC   | 100   |
|-----|--------|--------|--------|-------|-------|
| 0   | 817.61 | 794.69 | 722.47 | 808.58| 734.28|
| 1   | 771.78 | 766.22 | 648.86 | 817.61| 535.67|
| 2   | 578.72 | 516.92 | 612.75 | 618.31| 453.03|
| 3   | 566.92 | 583.58 | 519.00 | 600.94| 565.53|
| 4   | 475.25 | 491.92 | 514.14 | 541.92| 505.11|
| 5   | 519.69 | 482.19 | 476.64 | 530.81| 423.86|
| 6   | 546.78 | 537.75 | 453.03 | 422.47| 501.64|
| 7   | 411.36 | 494.00 | 555.81 | 554.42| 423.86|
| 8   | 528.72 | 548.17 | 535.67 | 453.03| 490.53|
| 9   | 500.94 | 439.83 | 506.50 | 516.22| 470.39|
| 10  | 464.14 | 429.42 | 525.25 | 490.53| 490.53|
| 11  | 489.14 | 437.06 | 457.89 | 448.17| 509.97|
| 12  | 509.97 | 455.11 | 586.36 | 581.50| 523.17|
| 13  | 505.11 | 464.83 | 536.36 | 571.08| 519.69|
| 14  | 495.39 | 467.61 | 544.00 | 582.89| 515.53|

Figure 3. Sulfate concentration graph for anaerobic batch reactors
From Figure 3, the trend of sulfate reduction from each reactor consists of three stages:
Stage 1: Sulfate concentration decreases as the active bacteria reduces sulfate and also assisted by the adsorption process. Stage 2: There is fluctuation in the sulfate reduction process. Stage 3: Stability in the sulfate reduction process occurs.

These three stages occurred at different times of each reactor. The difference in duration at each stage indicates the effect of heavy metal concentration. Phase one lasted for four days at K1 and K2 reactors. However, at reactor IC 50 and MTC, stage 1 lasted for six days. This is suitable according to a journal by Samia Azabou (2006) [9] which stated that the higher the metal concentration, the longer the lag phase (the phase where the cell number is relatively constant). The longer lag phase slowed down the bacteria's ability to reduce sulfate [17]. However, the reactor 100 shows an anomaly, ie stage 1 which lasted for two days which can be caused by the inaccuracy of the testing process. It was proved by the lowest concentration of sulfate at reactor 100 occurred on the fifth day. Just like IC 50 and MTC reactors, the lowest sulfate concentration is obtained at the end of stage 1. Stage 2 of each reactor got the same duration. The lowest sulfate concentration at K1 and K2 reactors occurs at stage 2. Finally, stage 3 took place after the bacteria loses its reduction / inability to grow.

Furthermore, based on the data in Table 3, we can calculate the efficiency and sulfate reduction rate of each reactor. The results of the calculation of efficiency and sulfate reduction rate of each reactor can be seen in Table 4 below

| Reactor | Reduction Efficiency (%) | Reduction rate (mg/L.hari) |
|---------|--------------------------|---------------------------|
| K1      | 40,62                    | 24,207                    |
| K2      | 46,58                    | 29,128                    |
| IC 50   | 37,99                    | 23,807                    |
| MTC     | 33,54                    | 21,028                    |
| 100     | 40,1                     | 25,128                    |

Based on Table 4, the best efficiency and sulfate reduction rate is found in K2 reactor, followed by K2 reactor. This is suitable with a journal written by Samia Azabou (2006) [9] which states that the manganese (Mn) metal has higher toxicity than the ferrous metal (Fe), hence the K2 reactor which has no manganese metal has the efficiency and the rate best sulphate reduction.

Furthermore, the efficiency and sulfate reduction rates decrease with increasing concentrations of metals which can be seen from the efficiency and sulfate reduction rates of 50 IC reactors and MTC reactors. This is suitable with a journal written by Monica Martins (2009) [17] which states that the higher the metal concentration, the smaller the reduction efficiency and the slower the rate of sulfate reduction.

Finally, there was an anomaly in reactor 100 where the efficiency and sulfate reduction rate are above IC 50 reactor and MTC reactor. This may be due to the high metal content causing the HS-produced by the sulphate reducing bacteria through a sulfate reduction process directly bounded. Thus, causing the reduction process in stage 1 was rapidly happened.

• pH
The pH is a controlled parameter of the sulfate reduction process because the optimum pH conditions are 6-7.5 [5]. The pH test results for 14 days on each reactor are described in Table 5 below

| Day | Fe 0       | Mn 0       | IC 50       | MTC       | 100       |
|-----|------------|------------|-------------|-----------|-----------|
| 0   | 4.10*-6.8  | 4.12*-6.75 | 4.09*-6.75  | 2.82*-6.76| 1.88*-6.75|
| 1   | 6.8        | 6.7        | 6.73        | 6.75      | 6.8       |
| 2   | 6.6        | 6.65       | 6.67        | 6.88      | 6.83      |
Based on Table 5 it can be seen that the initial pH (day 0) on the five reactors in an acidic atmosphere. This is a non-optimal condition for sulphate reducing bacteria, although sulphate reducing bacteria can survive up to pH 2.5. However, to obtain optimal results, it is necessary to do the conditioning to achieve a pH of 6-7.5. Therefore, it takes conditioning to keep the pH in the reactor optimal for bacteria. The pH conditioning in the reactor is carried out using NaOH.

Then, we can see the difference of the pH conditions from each reactors. The greatest reduction was shown by the K2 reactor, then followed by the K1 reactor. Then, at reactor IC 50 and MTC showed a similar decrease in pH. Finally, the reactor 100 showed a slower pH decline.

This decrease in pH indicates the activity of sulfate reduction into H$_2$S and HS$^-$ which then bind to the metal anion and produces H$^+$ and 2H$^+$ and causes acidity in the water so that the pH drops [8]. Thus, although pH increases should indicate the activation of sulphate reducing bacteria, in this case, the decrease in pH signifies the activation of sulphate reducing bacteria because H$_2$S or HS which is the result of sulfate reduction bonded with metals and yields H$^+$ that decreases pH.

Based on the above explanation, it can be seen that the most active sulphate reducing bacteria is in K2 reactor. This is suitable with a journal written by Samia Azabou (2006) [9] which states that manganese (Mn) metal has higher toxicity than ferrous metals (Fe), therefore K2 reactors which have no manganese metal are altogether the highest bacterial activity. The most inactive sulphate reducing bacteria is in the reactor 100 which is suitable with a journal written by Samia Azabou (2006) [9], that states that the higher the metal concentration the bacterial growth will be slower.

| Day | Fe 0    | Mn 0    | pH IC 50 | MTC | 100 |
|-----|---------|---------|----------|-----|-----|
| 3   | 6.62    | 6.35*-6.99 | 6.56     | 6.94 | 6.79 |
| 4   | 6.56    | 6.74     | 6.93     | 6.82 | 6.74 |
| 5   | 6.6     | 6.69     | 6.65     | 6.83 | 6.89 |
| 6   | 6.49    | 6.66     | 6.67     | 6.95 | 6.54 |
| 7   | 6.28    | 6.55     | 6.57     | 6.72 | 6.62 |
| 8   | 6.18    | 6.49     | 6.66     | 6.68 | 6.61 |
| 9   | 5.99*-6.84 | 6.32   | 6.73     | 6.62 | 6.57 |
| 10  | 6.79    | 6.21     | 6.53     | 6.33 | 6.56 |
| 11  | 6.52    | 6.12     | 6.49     | 6.3  | 6.62 |
| 12  | 6.37    | 6.08     | 6.45     | 6.36 | 6.68 |
| 13  | 6.28    | 5.94*-6.46 | 6.32     | 6.3  | 6.65 |
| 14  | 6.24    | 6.42     | 6.25     | 6.28 | 6.63 |
| Σ   | 1.41    | 1.49     | 0.5      | 0.48 | 0.12 |

*) = pH is not optimal (need conditioning)

- **Temperature**
  Temperature is used to observe bacterial activity. The optimal temperature for the sulfate reduction process is 35°C [16]. In addition, low temperature can slow down bacterial activity. Sulfate reducing bacteria can live up to 6°C [18]. The results of temperature measurements inside the reactors are relatively stable with a range of 26-28°C.

- **Color**
  The color of the sediment (visual changes) may indicate the presence of sulphate reducing bacteria in the reactor [19]. In the first week, there was no change in sediment color on the five reactors. The sediments on the five reactors showed black color indicating the presence of sulphate reducing bacteria in the reactor [19]. This is reinforced by the smell of rotten eggs in the reactor indicating a sulfate reduction process to H$_2$S.

  Furthermore, in the second week we can see the color difference at reactor IC 50, MTC, 100. The color changed from black into brown which indicates the death of sulphate reducing bacteria [19]. The color change in each reactor for 14 days can be observed in Figures 2 and 3 below.
In there, it can also be seen the differences in color density. Reactor IC 50 has the lowest concentration and the concentration keeps increasing as the metal concentration increases. The occurrence of sulfate reducing bacteria in IC 50, MTC, and 100 reactors in accordance with the journal Samia Azabou (2006) [9] which states that at concentrations of 50% metal inhibitory concentration, the inability of bacteria to grow and the higher the concentration of the metal will be increasingly toxic for sulphate reducing bacteria.

• COD (Chemical Oxygen Demand)

Chemical Oxygen Demand (COD) is a parameter used to view bacterial activity through the number of oxygen required by bacteria to degrade organic compounds. The organic compound used as a carbon source is glucose (C\textsubscript{12}H\textsubscript{6}O\textsubscript{12}). The concentration of COD on the five reactors is measured every 3 days with the aim of monitoring the growth of sulphate reducing bacteria through monitoring the declining concentration of COD. The initial concentration of COD for each reactor is 2400 mg / l as it resulted an optimum COD / SO\textsubscript{4}\textsuperscript{2-} value (± 2.72) [5]. The COD concentration for 14 days on each reactor are described in Table 6. Furthermore, based on the data in Table 6, we can calculate the COD / SO\textsubscript{4}\textsuperscript{2-} ratio of each reactor. The calculation of the efficiency of the reduction and comparison of COD / SO\textsubscript{4}\textsuperscript{2-} for each reactor can be seen in Table 7.

**Table 6** COD Concentration on batch reactors

| Day | K1   | K2   | IC 50 | MTC  | 100  |
|-----|------|------|-------|------|------|
| 0   | 2400 | 2400 | 2400  | 2400 | 2400 |
| 5   | 1556 | 2361 | 1588  | 1603 | 1853 |
| 8   | 1564 | 2213 | 1689  | 1548 | 2025 |
| 11  | 1603 | 2033 | 1658  | 1439 | 1705 |
| 14  | 1548 | 1869 | 1681  | 1556 | 1689 |

**Table 7** COD/SO\textsubscript{4}\textsuperscript{2-} on batch reactor

| Day | K1  | K2  | IC 50 | MTC | 100  |
|-----|-----|-----|-------|-----|------|
| 0   | 2,877 | 2,743 | 2,737 | 2,737 | 2,737 |
| 5   | 2,995 | 4,897 | 3,331 | 3,020 | 3,669 |
| 8   | 2,958 | 4,036 | 3,153 | 3,418 | 4,128 |
| 11  | 3,277 | 4,651 | 3,621 | 3,221 | 3,343 |
| 14  | 3,126 | 3,996 | 3,091 | 2,670 | 3,276 |
In Table 6, the concentration of COD on the five reactors has decreased. This indicates the presence of active bacterial activity. Although in Color has been discussed about sulfate reducing bacteria at reactor IC 50, MTC, and 100 cannot grow, the drop of COD concentration on all three reactors was still high enough. This may indicate the presence of other bacteria growing on the reactor and using glucose present in the reactor. Based on Table 7, it was found that the best COD / SO\(_{4}^{2-}\) ratio was in the MTC reactor, with the value closest to 2.72, followed by IC 50, K1, 100 and K2 reactor reactors. Despite the variation of values, the COD / SO\(_{4}^{2-}\) ratio is still within a safe range of 1.5-4 [5].

• Metals (Iron and Manganese)
Metal is a parameter tested to see the precipitation of the metal due to the bonding of metal on the sulfate reduction product, ie sulfide, into an insoluble metal sulfide [8]. In addition, varying the metal concentrations were also tested to see the metal toxicity of bacteria [9]. In this study, ferrous and manganese metals were tested every five days. However, there is an anomaly in the manganese concentration test results which can be seen from the concentration of manganese metal at 50, MTC and 100 IC reactors on day 0 which should be the same as the concentration of ferrous metals. This may be caused by an inaccuracy in the test. Therefore, the result of iron metal is used as a reference. The results of testing of ferrous and manganese metals can be seen in Tables 8 and 9.

Table 8. Iron concentration on anaerobic batch reactors

| Hari | K1 | K2 | IC 50 | MTC | 100 |
|------|----|----|-------|-----|-----|
| 0    | 1.06 | 10.31 | 50.78 | 98.40 | 101.60 |
| 5    | 0.657 | 0.711 | 0.656 | 0.722 | 1.352 |
| 10   | 0.783 | 0.783 | 0.661 | 0.842 | 0.933 |
| 14   | 0.734 | 2 | 0.694 | 0.755 | 5.493 |

Table 9. Manganese concentration on anaerobic batch reactors

| Hari | K1 | K2 | IC 50 | MTC | 100 |
|------|----|----|-------|-----|-----|
| 0    | 14.53 | 0.737 | 14.31 | 14.94 | 14.82 |
| 5    | 11.86 | 14.27 | 14.59 | 14.74 | 8.01 |
| 10   | 14.7 | 14.25 | 14.43 | 14.83 | 14.6 |
| 14   | 14.48 | 14.45 | 14.54 | 14.86 | 14.76 |

The concentration of ferrous metals decreased significantly on the 5th day, indicating that there was physical adsorption by sediment and metal bonding by sulfide [8]. In addition, since the pH of the reactor is approximately neutral, there is also precipitation of ferrous metal as (oxy) hydroxides [17]. On the 10th and 14th days, no significant changes from the previous test result (5th day) indicating that there is no metal binding. From Table 8, it can be calculated the efficiency and rate of iron metal declination and can be seen in Table 10.

Table 10. Iron reduction efficiency and rate on anaerobic batch reactors

| Reaktor | Reduction Efficiency (%) | Reduction Rate (mg/L.day) |
|---------|--------------------------|--------------------------|
| K1      | 30.56                    | 0.02                     |
| K2      | 80.61                    | 0.59                     |
| IC 50   | 98.63                    | 3.58                     |
| MTC     | 99.23                    | 6.97                     |
| 100     | 94.59                    | 6.86                     |

From Table 10, it can be seen that the highest result is in MTC reactor, followed by IC 50, 100, K2, and finally K1. This indicates that the iron metal can be precisely precipitated even though the concentration of metal at reactor is high.
3.2.3. Running anaerobic continuous reactor

The next running stage is done after the batch reactor stage. At this stage, the reactor is operated continuously for 23 days for each concentration of heavy metal of iron and manganese changed every few days, namely: K1 (Control 1): the value of iron (Fe) 10 mg / l and the value of manganese metal (Mn) is 10 mg / l; IC 50 (50% Inhibitory Concentration): Based on the journal Samia Azabou (2006) [9], 50% inhibitory concentration is a condition in which concentrations of ferrous and manganese metals become inhibitors for bacteria causing bacterial growth ability to decline by 50%. The concentration is in iron (Fe) of 41 mg / L and in manganese (Mn) of 31 mg / L; MTC (Maximum Tolerated Concentration): maximum tolerated concentration is a condition in which metal concentrations are inhibitors for bacteria that cause bacteria to not grow at all. The concentration is on iron (Fe) of 60 mg / L and in manganese (Mn) of 60 mg / L; and finally 100 (Fe 100 mg / L and Mn 100 mg / L): In reactor 100. A maximum concentration of concentration above maximum tolerated concentration of iron (Fe) of 100 mg / L and manganese (Mn) of 100 mg / L was performed. The results can be seen in Table 11 below.

| Day | pH  | Temperature (°C) | Sulfate (mg/L) | COD (mg/L) | COD/SO4 |
|-----|-----|------------------|----------------|------------|---------|
| 0   | 6,7 | 25,8             | 1959,3         | 4642,2     | 2,369336|
| 1   | 6,46| 26               | 1630,1         |            |         |
| 2   | 6,4 | 25,9             | 1731,5         |            |         |
| 3   | 6,96| 25,6             | 1742,6         |            |         |
| 4   | 6,93| 26,2             | 1656,5         |            |         |
| 5   | 6,89| 25               | 1659,3         |            |         |
| 6   | 6,32| 27,4             | 1627,3         | 3142,2     | 1,930881|
| 7   | 6,49| 27,5             | 1556,5         |            |         |
| 8   | 6,44| 27,6             | 1399,6         |            |         |
| 9   | 6,39| 25,5             | 1344,0         |            |         |
| 10  | 6,36| 26,9             | 1425,9         |            |         |
| 11  | 6,38| 27,7             | 1524,6         |            |         |
| 12  | 6,23| 27,7             | 1307,9         | 3665,6     | 2,802704|
| 13  | 6,17| 27,5             | 1281,5         |            |         |
| 14  | 5,91| 27,6             | 1403,7         |            |         |
| 15  | 5,72| 26,8             | 1430,1         |            |         |
| 16  | 5,66| 26,9             | 1395,4         |            |         |
| 17  | 5,83| 25,9             | 1303,7         | 3439,1     | 2,63788 |
| 18  | 6,13| 25,6             | 1378,7         |            |         |
| 19  | 6,02| 27               | 1380,1         |            |         |
| 20  | 6,05| 27               | 1234,3         |            |         |
| 21  | 5,68| 27,3             | 1226,6         | 3314,1     | 2,701743|
| 22  | 5,72| 27,1             | 1277,3         |            |         |
| 23  | 5,8 | 27,2             | 1281,5         |            |         |
The results of the five reactors are divided into five parameters, namely:

- **Sulfate Concentrations**
  Sulfate concentration is the most important parameter in determining the success of the sulfate reduction process. The results of the sulfate concentration test for 23 days are illustrated in Figure 6 below.

\[
\text{Figure 6. Sulfate concentration graph on continuous anaerobic reactor}
\]

Based on the Figure 6, the best efficiency and sulfate reduction rate is found on the first week, which contained 10 mg/l Fe and Mn. Then, followed by the second week and so on.

- **pH**
  The pH is a controlled parameter of the sulfate reduction process because the optimum pH conditions are 6-7.5 \[5\]. The pH test results for 28 days on each reactor are illustrated in Figure 7 below.

\[
\text{Figure 7. pH test results’ graph on continuous anaerobic reactor}
\]

Based on Figure 7 it can be seen that the pH value was slowly decreasing each week and the reduction rate was slowly decreasing as well. That is because the bacteria were less active each week caused by the metal toxicity.

- **Temperature**
  Temperature is used to observe bacterial activity. The optimal temperature for the sulfate reduction process is 35°C \[19\]. In addition, low temperature can slow down bacterial activity. Sulfate reducing bacteria can live up to 6°C \[18\]. The results of temperature measurements inside the reactors are relatively stable with a range of 26-28°C.

- **Color**
The color of the sediment (visual changes) may indicate the presence of sulphate reducing bacteria in the reactor [19]. In the first week, there was no change in sediment color on the reactor. The sediments showed black color indicating the presence of sulphate reducing bacteria in the reactor [19]. This is reinforced by the smell of rotten eggs in the reactor indicating a sulfate reduction process to H₂S.

Furthermore, in the second week we can see the color difference at reactor. The cream color on the top part of the sediment had gone, indicating the glucose was already used by the bacteria. After that, on the third week it can be seen that there was an orange bubbles float indicating that there is a iron hydroxides forming. Then the last week, the color changed from black into brown which indicates the death of sulphate reducing bacteria [19]. The color change in each reactor for 23 days can be observed in Figure 8 below

![Figure 8. Visual changes on continuous anaerobic reactor](image)

**• COD (Chemical Oxygen Demand)**

Chemical Oxygen Demand (COD) is a parameter used to view bacterial activity through the number of oxygen required by bacteria to degrade organic compounds. The organic compound used as a carbon source is glucose (C₁₂H₂₆O₁₂). The concentration of COD on the five reactors is measured every week with the aim of monitoring the growth of sulphate reducing bacteria through monitoring the declining concentration of COD. The initial concentration of COD for each reactor is 4600 mg / l as it resulted a close to optimum COD / SO₄²⁻ value (± 2.72) [5]. The COD concentration for 23 days on each reactor are described in Table 6. Furthermore, based on the data in Table 11, we can calculate the COD / SO₄²⁻ ratio of each reactor. The calculation of the efficiency of the reduction and comparison of COD / SO₄²⁻ for each reactor can be seen in Table 11. The COD concentration can be seen in Figure 9 below
3.3. Multiple Linear Regression Analysis

From the analysis of several parameters above, can be done linear regression analysis with SPSS application to know the influence level of heavy metal concentration to sulfate reduction. The following results show that the concentrations of ferrous and manganese metals have a significant influence on sulfate concentration.

4. Conclusions and recommendations

4.1. Conclusion

Based on the results and discussion above can be concluded as follows:
1. Variations in concentrations of ferrous and manganese metals have an influence on sulfate reduction processes on the efficiency parameters of sulfate reduction, pH, and color. Variations in concentrations of ferrous and manganese metals have a significant influence on sulfate reduction efficiency parameters.

4.2. Suggestions

Based on the above results and discussion can be taken several suggestions, as follows:
1. It is necessary to research the reduction process until the stability occurs for 5-7 days with testing parameters more often to see the optimum reduction process
2. Increased accuracy when making artificial waste to fit the desired waste characteristics.
3. Further research on the variation of heavy metal composition with different heavy metals is required.
5. References

[1] R. Indonesia. KepMen LH No. 113 Tahun 2003 tentang Baku Mutu Air Limbah bagi usaha dan Atau kegiatan Pertambangan Batubara. 2003.
[2] Rudy Sayoga Gautama. Pengelolaan Air Asam Tambang. Bimbingan Teknis, Reklamasi dan Pascatambang Pada Kegiatan Pertambangan mineral dan Batubara – DITJEN MINERAL DAN BATUBARA, KESDM. 2012.
[3] Abfertiawan, M. S. dan R. S. Gautama, 2011, Development of Catchment Area Approach in Management of Acid Mine Drainage, “Mine Water – Managing the Challenges”, Prosiding IMWA 2011, Aachen, Germany.
[4] Taylor, Jeff., Page, Murphy. 2005. A Summary of Passive and Active Treatment Technologies for Acid and Metalliferous Drainage (AMD). Prepared for the Australian Centre for Minerals Extension and Research (ACMER). Australia: Earth Systems.
[5] Najib, Tahereh. 2017. Optimization of sulfate removal by sulfate reducing bacteria using response surface methodology and heavy metal removal in sulfidogenic UASB reactor. Journal of Environmental Chemical Engineering 5 (2017) 3256–3265.
[6] Buzzi, D. C., L. S. Viegas, D. C. R. Espinosa, M. A. S. Rodrigues, Bernardes, J. A. S. Tenorio, 2011, The Use of Microfiltration and Electrodialysis for Treatment of Acid Mine Drainage, “Mine Water – Managing the Challenges”, Prosiding IMWA 2011, Aachen, Germany.
[7] Eric D. van Hullebusch, Sudarno Utomo, Marcel H. Zandvoort, Piet N. L. Lens. Comparison of three sequential extraction procedures to describe metal fractionation in anaerobic granular sludges. 2004.
[8] Irene Sánchez-Andrea, Jose Luis Sanz, Martijn FM Bijmans, Alfon JM Stams. Sulfate reduction at low pH to remediate acid mine drainage. Journal of hazardous materials Vol. 269. 2014.
[9] S. Azabou, T. Mechichi, B.K.C. Patel, S. Sayadi, Isolation and characterization of a mesophilic bacteria heavy-metals-tolerant sulphate-reducing bacterium Desulfovibrio sp. from an enrichment culture using phosphogypsum as a sulphate source, J. Hazard. Mater. 140 (2007) 264–270.
[10] Sugiyono, 2009, Metode Penelitian Kuantitatif, Kualitatif dan R&D, Bandung: Alfabeta.
[11] Nurandani Hardyanti, Suparni Setyowati Rahayu. Fitoremediasi Phospat Dengan Pemanfaatan Enceng Gondok (Eichhornia Crassipes)(Studi Kasus Pada Limbah Cair Industri Kecil Laundry). Jurnal Presipitasi 2 (1), 28-332007.
[12] Fahruddin, 2009, Pengaruh Jenis Sedimen Wetland Dalam Reduksi Sulfat pada Limbah Air Asam Tambang (AAT), Jurnal Teknik Lingkungan, Vol. 10, No. 1, Hal. 26-30, Jakarta.
[13] Faisol Asip. Pengaruh Adsorben Diatomaceous Earth terhadap Penurunan Kadar Besi dan Ion Sulfat dari Air Asam Tambang. 2015.
[14] Simarmata, B.P., 2010, Geologi serta Survei Awal Air Asam Tambang dan Kaitannya dengan Karakteristik Geologi Daerah Simpangempat, Kecamatan Bungur dan Sekitaranya, Kabupaten Tapin, Kalimantan Geological Engineering Dept., College of Technology “STTNAS Yogyakarta”, unpublished.
[15] Aguilera M., Cabrera A., Incerti C., Fuentes S., Russel N.J., Cormenzana A.R., dan Mercedes M-S. 2007. Chromohalobactersalarius sp. nov., a moderately halophilic bacterium isolated from a solar saltern in Cabo de Gata, Almeria, southern Spain. International Journal of Systematic and Evolutionary Microbiology 57: 1238-1242.
[16] S. Al Zuhair, S. Al Zuhair, M.H. El-Naas, H. Al Hassani, Sulfate inhibition effect on sulfate reducing bacteria, J. Biochem. Technol. 1 (2008) 39–44.
[17] Martins, Monica. 2009. Characterization and activity studies of highly heavy metal resistant sulphate-reducing bacteria to be used in acid mine drainage decontamination. Journal of Hazardous Materials 166 (2009) 706–713
[18] Tsukamoto I, et al. 2004. Inactivation effect of sonication and chlorination on Saccharomyces cerevisiae. Calorimetric analysis. Ultrasound in Medicine & Biology 29(3-4):167-72

[19] Sumich, James dan Morrisey, John. 2004. Introduction to the Biology of Marine Life. Massachusetts: Jones and Bartlett Publishers.

Acknowledgments
Authors wish to express sincere gratitude for assistance or encouragement and support from lecturers and colleagues, also laboratory staff and special work by technical staff, then for financial support from Diponegoro University, Faculty of Engineering and material support from PT Pakar Ipal Indonesia.