Performance of the Sulfate Reduction Process in Acid Mine Drainage (AMD): Comparison of Reactor Operation Modes and Fe/Mn Tolerance with Different Microbial Sources

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ABSTRACT

Acid mine drainage (AMD) generates acidic wastewater containing high concentrations of sulfate and dissolved metals, which can cause severe environmental degradation. Sulfate-reducing bacteria (SRB) can be applied in biological sulfate reduction for wastewater treatment because they neutralize acidity and transform dissolved metals into sulfide precipitates. This study evaluated SRB performance under two acclimatization strategies and varying Fe and Mn concentrations in different reactor operation modes. Gradual acclimatization was conducted by stepwise sulfate elevation to approximately 2,000 mg/L, whereas shock acclimatization involved direct exposure to 2,000 mg/L sulfate. Batch reactors were operated for 14 days with Fe and Mn concentrations ranging from 10 to 100 mg/L, followed by continuous reactor operation for 22 days to assess medium-term performance. Gradual acclimatization resulted in higher sulfate reduction, greater pH increase, and better sediment stability than shock acclimatization. In batch operation, the highest sulfate reduction efficiency was 46.58% with a reduction rate of 29.13 mg/L·day under Fe-dominant conditions, namely 10 mg/L Fe without Mn. By contrast, Mn dominance reduced the efficiency to 40.62%. Higher Fe and Mn

concentrations, including the inhibitory concentration (IC_{50}) and maximum tolerable concentration (MTC), further suppressed performance, whereas extreme concentrations of 100 mg/L resulted in sulfate removal mainly through chemical precipitation. Continuous reactors demonstrated greater resilience to metal stress. Overall, gradual acclimatization and continuous operation improved AMD treatment performance, with Mn identified as the more toxic inhibitor of SRB activity.

Keywords: Anaerobic batch reactor; continuously stirred tank reactor; Fe; Mn; reduction; sulfate; sulfate-reducing bacteria

1. INTRODUCTION

Water pollution at both global and national scales is strongly influenced by the mining industry, which is a major source of heavy metals and acidity in aquatic environments. Open-pit mining can negatively affect the environment, particularly water resources, by removing protective soil layers and exposing sulfide-rich bedrock such as pyrite (FeS_2). This exposure promotes sulfide mineral oxidation and leads to the formation of acid mine drainage (AMD) (Johnson et al., 2005). AMD formation decreases pH to highly acidic levels and increases sulfate concentrations and the dissolution of heavy metals such as iron (Fe), manganese (Mn), aluminum (Al), copper (Cu), and zinc (Zn) (Johnson et al., 2005; Nordstrom et al., 2015). High sulfate content in AMD contributes to acidity accumulation through sulfate ion hydrolysis into sulfuric acid and increases metal mobility and toxicity in aquatic environments (Sheoran et al., 2006). This process is triggered by excessive sulfate in AMD. Sulfate promotes chemical and biochemical reactions when sulfide minerals interact with oxygen and water, producing acid, sulfate ions, and dissolved heavy metals that enter the environment (Johnson et al., 2005; Nordstrom et al., 2015). The combination of low pH and dissolved heavy metals creates severe ecotoxic conditions for aquatic and soil organisms, potentially causing metabolic disorders, tissue damage, and reduced life expectancy across various species (Akcil et al., 2006, Bigham et al., 2000; Kefeni et al., 2017). Degradation of drinking water and irrigation sources occurs because of AMD-induced water quality deterioration, while the accumulation of heavy metals in soil reduces the fertility and productivity of agricultural land (Johnson et al., 2005; Kefeni et al., 2017). Therefore, effective AMD management is essential, particularly through sulfate reduction techniques that neutralize acidity and precipitate heavy metals. In biological processes, sulfate reduction relies on sulfate-reducing bacteria (SRB), which convert sulfate into sulfide. The sulfide then reacts with metal ions to form stable, low-solubility metal sulfide precipitates (Kaksonen et al., 2007).

SRB-based biological methods have been widely investigated because of their efficiency and sustainability in AMD treatment. These bacteria can grow and function under low-pH conditions, enhancing sulfate reduction, improving reactor efficiency, and shortening residence time compared with chemical or physical treatment methods (Perala et al., 2022; Sánchez-Andrea et al., 2013). SRB utilize organic electron donors, represented by chemical oxygen demand (COD), in anaerobic metabolism to reduce sulfate to sulfide. The generated sulfide reacts with dissolved metals such as Fe and Mn to form insoluble metal sulfide precipitates, thereby reducing metal and sulfate concentrations in AMD (Perala et al., 2022; Prianto, 2016). Various reactor configurations have been tested, including batch systems, intermittent systems such as sequencing batch reactors (SBRs), and continuous systems, using different inoculum sources. Because operating environments are subject to continuous change, research has examined gradual and shock acclimatization strategies to maintain sulfate reduction performance (Sandrawati et al., 2019).

Recently, various SRB-based sulfate reduction bioreactors have been developed (Habe et al., 2020; Xue et al., 2023). Prakash et al. (2023) reduced sulfate concentrations in wastewater through anaerobic treatment with voltage application, whereas Lu et al. (2016) reported stable and effective removal of COD and SO_4^{2-}. Sulfate concentration affects treatment efficiency, with an optimum around 300 mg/L, while higher concentrations may inhibit SRB activity (Li et al., 2010). Variations in the COD/SO_4^{2-} ratio alter microbial structure and treatment efficiency (Xue et al., 2023). Heavy metals such as Cu, Ni, and Mn can inhibit SRB activity at high concentrations (Miran et al., 2017). Conventional AMD treatment methods are less capable of maintaining long-term stability and often fail to accommodate microbial adaptation dynamics (Hajee et al., 2024). Therefore, a more systematic acclimatization strategy is needed to enhance SRB tolerance to dynamic environmental conditions. Various SRB acclimatization approaches, including batch and continuous-flow systems (Miranda et al., 2022), sodium sulfate increment strategies (Shuquan et al., 2020), and acclimatization to pH variation (Yu et al., 2020), have shown that SRB can effectively improve sulfate reduction efficiency under relatively stable operating conditions. However, real applications require strategies that address fluctuating operational environments. Accordingly, this study evaluated gradual and shock acclimatization to maintain sulfate reduction performance under disturbance conditions.

Gradual acclimatization enables SRB communities to develop resistance to increasing sulfate and heavy metal concentrations. Progressive exposure to harsher conditions can improve system performance and stability while reducing the probability of operational failure (Wang et al., 2022). In contrast, shock acclimatization exposes microbial communities abruptly to extreme conditions to test and strengthen their adaptation to unpredictable AMD characteristics. SRB may develop endurance under shock acclimatization, which can improve bioremediation potential by enabling survival during rapid sulfate and metal fluctuations without severe cell mortality (Xiao et al., 2025). Hardyanti et al. (2018) reported that SRB require time to adapt to increasing sulfate concentrations and to develop sustainable metabolic activity and growth for long-term operation. Shock acclimatization exposes SRB to elevated sulfate levels to assess their ability to withstand sudden changes, allowing rapid selection of tolerant microorganisms but potentially causing system instability. Although previous studies have examined sulfate reduction using SRB-based systems and evaluated acclimatization or heavy metals separately, limited attention has been given to the combined evaluation of acclimatization strategy and Fe–Mn loading in relation to sulfate reduction performance and sediment stability across different reactor operation modes. Therefore, this study evaluates the response of an SRB-based system to gradual and shock acclimatization, followed by assessment of Fe and Mn effects under batch and continuous operation. The novelty of this study lies in integrating acclimatization strategy, metal stress, and reactor operation mode into a sequential experimental assessment of sulfate reduction performance and system stability. Comparing these methods provides insight into trade-offs among adaptation rate, sulfate reduction efficiency, and system stability (Nurandani et al., 2018). This study assesses both strategies to determine the most suitable approach for sustainable AMD treatment in laboratory and field settings by evaluating SRB performance under baseline and severe conditions that simulate real AMD fluctuations.

2. METHODS

2.1. Materials

This study used two main components: natural sediment and an artificial inoculant as SRB sources. Natural sediment was collected from mining sites and waters exposed to AMD, whereas the artificial inoculant was cultivated in nutrient medium. The SRB inoculum was derived from sediment obtained from a laundry septic tank. The SRB inoculum was selected through a preliminary batch potential test, in which 100 mL of washed and dried sediment was incubated with synthetic wastewater containing 1,400 mg/L SO_4^{2-} for 21 days until the highest sulfate removal was achieved. The water source had a pH of 6.95, salinity of 221 ppm, and dissolved oxygen concentration of 0.81 mg/L. The selected inoculum was inoculated and acclimatized in an anaerobic reactor fed with synthetic AMD, in which sulfate was gradually increased to approximately 2,000 mg/L over 28 days.

Two reactor configurations were used. Batch experiments were conducted in five identical anaerobic batch reactors made of plastic. Each anaerobic batch reactor had a volume of 0.6 L and was equipped with a gas vent hose to remove air and reduce biogas pressure while maintaining anaerobic conditions. Each reactor was filled with 0.6 L of acclimated culture. Continuous experiments were conducted in a single cylindrical completely stirred tank reactor (CSTR) with a total volume of 8.5 L and a working volume of 8.2 L. The reactor was continuously mixed using a motorized paddle and fed with synthetic AMD in top-feed mode through a feed pump. The flow rate was maintained constant using a control valve to provide a hydraulic retention time (HRT) of 3 days during the running phase. A longer retention time of approximately 28 days was used during seeding and acclimatization. Both configurations were operated under anaerobic conditions at room temperature, ranging from 25 to 28 °C. Temperature was monitored daily but not actively controlled, with care taken to maintain it below approximately 35 °C. The batch reactor was operated for 14 days, and the continuous reactor was operated for 22 days, with samples collected daily. Each parameter, including sulfate, COD, pH, and temperature, was measured in duplicate, and the reported values represent the average of duplicate measurements. Key performance metrics, including sulfate and COD removal efficiencies and reduction rates, are expressed as the mean ± standard deviation of duplicates. Because a single reactor was operated for each metal condition, this study used analytical replication at the measurement level rather than independent reactor-level replication. Therefore, formal inferential statistical comparisons between conditions were not performed. The reaction medium was adjusted to support anaerobic sulfate reduction using electron donors, including lactate, ethanol, and glucose, as carbon and energy sources (Rasool et al., 2015). Sulfate served as the electron acceptor for SRB during anaerobic respiration (Li et al., 2018).

2.2. SRB Acclimatization Strategy

The gradual SRB acclimatization strategy involved increasing sulfate loading by 500 mg/L weekly until the target concentration of 2,000 mg/L was reached. This method was applied to help SRB maintain sulfate reduction activity and efficiency while preventing inhibition caused by sudden exposure to high sulfate concentrations (Sreekutty et al., 2024). Acclimatization required physical monitoring, particularly sediment color change from brown to dark black, which indicates sulfate reduction activity (Posumah et al., 2018). Monitoring included sulfate, COD, pH, and reduction rate to assess operational stability and effectiveness. Successful sulfate reduction was indicated by decreasing sulfate concentrations, with SRB electron donor requirements supported by an appropriate COD/SO_4^{2-} ratio. The system achieved optimal performance under neutral to slightly alkaline pH conditions, while the reduction rate reflected the operational performance of the system (Dong et al., 2023).

The shock method is an SRB acclimatization approach that exposes microorganisms directly to high sulfate concentrations at the initial stage of acclimatization (Sato et al., 2022). This method began with an initial sulfate concentration of approximately 2,000 mg/L. Direct application of high sulfate concentrations aimed to evaluate the ability of SRB to adapt and survive under extreme conditions associated with sudden sulfate concentration changes (Bernardez et al., 2012). The acclimatization process required physical observations to monitor changes in sediment properties, including rapid color darkening and development of a brittle texture (Posumah et al., 2018; Yu et al., 2020).

The two acclimatization methods were evaluated to determine their effects on SRB colony stability and sulfate reduction efficiency according to the organic compound transformation pathway shown in Figure 1. The process begins with the breakdown of organic compounds through hydrolysis and fermentation, producing simpler substances, including organic acids and acetate, which SRB use to produce sulfide through sulfate reduction. Methanogenic microorganisms provide a competing metabolic route, indicating that operational parameters and acclimatization methods determine whether the system favors sulfide production by SRB or methane generation by methanogens. The comparison between gradual and shock loading illustrates how microbial metabolic processes can be controlled under high-sulfate AMD conditions.

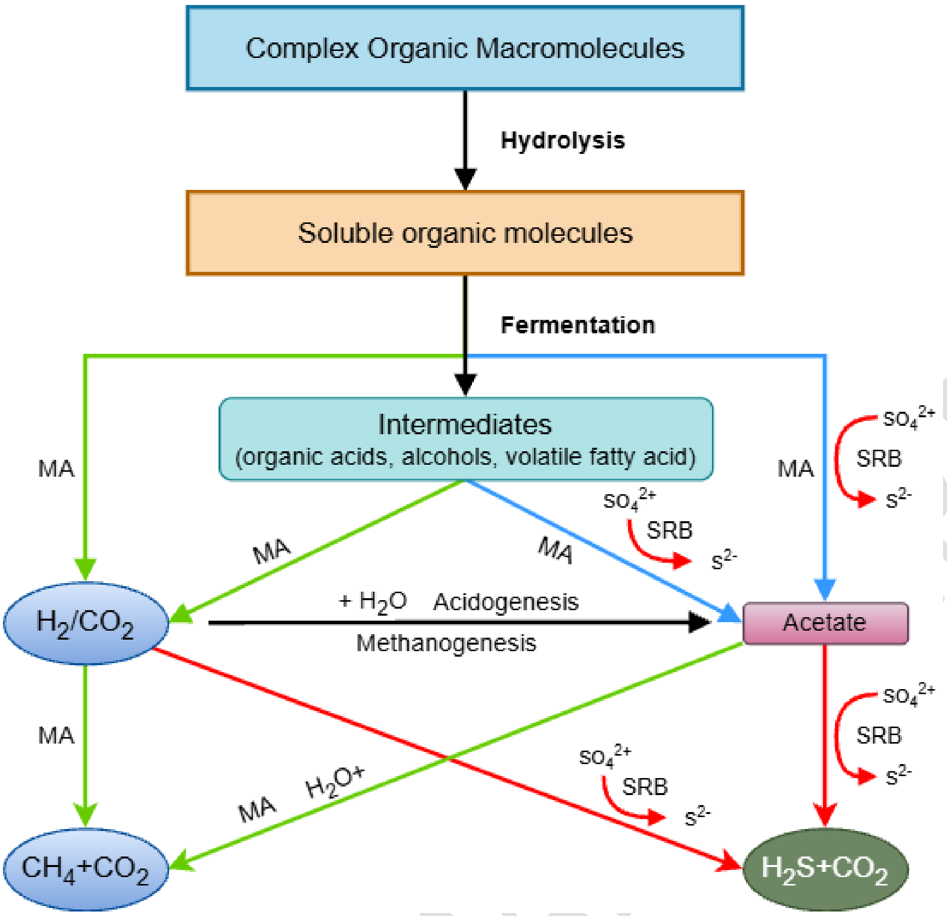

Figure 1. Schematic Diagram of Anaerobic Digestion Pathways with Sulfate Reduction

2.3. Effect of Fe and Mn

This study examined the effect of varying iron (Fe) and manganese (Mn) concentrations on sulfate reduction efficiency by SRB. Batch metal toxicity testing is a validated and widely used method for isolating the specific effects of inhibitory compounds and studying inhibition kinetics under controlled conditions (Utgikar et al., 2003). The experiment was conducted for 14 days using anaerobic batch reactors. The observed parameters included sulfate concentration, temperature, pH, COD, and Fe and Mn concentrations. Monitoring these parameters is essential for interpreting overall system performance (Lens et al., 1998).

One of the main objectives of this study was to analyze the performance of SRB derived from laundry septic tank sediment, which had previously been selected as the most effective bacterial source and contained heavy metals at certain concentrations. Septic tank sediment was selected as the SRB inoculum based on its performance during preliminary screening and its suitability as an anaerobic microbial source. Septic systems provide reducing conditions and contain diverse anaerobic microbial consortia capable of utilizing organic substrates, including sulfate-reducing microorganisms. Because microbial community profiling was not conducted in the present study, the inoculum is considered a mixed anaerobic consortium rather than a taxonomically characterized SRB culture. This stage began with a 28-day seeding and acclimatization process to adapt the bacteria to synthetic wastewater conditions with a high sulfate concentration of approximately 2,000 mg/L. The long acclimatization phase allowed cell encapsulation in biofilms, expression of resistance genes, and selection of tolerant populations, which is important before acute toxicity testing (Jong et al., 2003; Kaksonen et al., 2007). The main test was then conducted for 14 days using five different Fe and Mn concentration conditions. The tested concentration variations were K1, K2, IC_{50}, MTC, and 100. Condition K1 contained 10 mg/L Mn and 0 mg/L Fe, whereas K2 contained 10 mg/L Fe and 0 mg/L Mn. The IC_{50} condition represented the 50% inhibitory concentration, with 41 mg/L Fe and 31 mg/L Mn. The MTC condition represented the maximum tolerable concentration, with 60 mg/L Fe and 60 mg/L Mn. The 100 condition used 100 mg/L Fe and 100 mg/L Mn, exceeding the MTC limit. These conditions were used to test bacterial resistance, evaluate the potential for metal precipitation resulting from sulfate reduction, and observe the dominance of abiotic removal mechanisms, such as sulfide precipitation, over biological mechanisms (Xu et al., 2020). Each concentration variation was tested in a separate reactor under identical operational conditions so that the effects of Fe and Mn could be observed. Identical parallel reactor design minimizes confounding variables and is a basic principle in toxicological studies, ensuring that differences in results can be directly attributed to differences in metal concentration treatments (Persoone et al., 1993).

2.4. Effect of Fe and Mn in the Continuous Reactor

The running stage was conducted after seeding and acclimatization to evaluate reactor performance under continuous and stable operating conditions (Nurandani et al., 2018). The reactor operated continuously for 22 days, during which Fe and Mn concentrations were adjusted according to four concentration schedules. The experiment began with Fe and Mn concentrations of 10 mg/L, which were maintained for seven days. During the second week, Fe and Mn concentrations were adjusted to their IC_{50} values and maintained for seven days. The MTC values were then applied and maintained for five days. In the final segment, Fe and Mn concentrations were maintained at 100 mg/L for four days. This sequence was designed to observe SRB responses to gradually increasing metal concentrations under continuous operation (Nurandani et al., 2018). The duration of each phase was determined based on the need for the biological system to achieve a quasi-steady-state condition after each change in metal loading, particularly because SRB-based reactors are sensitive to environmental changes (Anekwe et al., 2023). With an HRT of 3 days across all reactors, each test phase encompassed more than one HRT cycle to allow representative sulfide removal before the next concentration increase (Anekwe et al., 2023). During the running phase, sulfate concentration was not externally adjusted and depended solely on the remaining sulfate in the reactor. This approach enabled a more realistic evaluation of the ability of SRB to utilize sulfate naturally under metal stress (Nurandani et al., 2018). The process evaluation system measured sulfate, COD, pH, Fe, and Mn concentrations to determine biological process effectiveness and the stability of the SRB-based AMD treatment system.

3. RESULTS AND DISCUSSION

3.1. Comparison of Gradual and Shock Acclimatization Strategies on Sulfate Reduction Performance and System Stability

The acclimatization phase determines the survival of SRB during their initial exposure to AMD treatment systems because it establishes their ability to function under harsh and variable environmental conditions. The inoculated SRB population requires time to develop physiological activity before it can perform sulfate reduction metabolism in an AMD environment characterized by acidity, high sulfate concentrations, and dissolved heavy metals. The main role of acclimatization is to form a more stable microbial community that is resistant to the extreme conditions typical of AMD. Without sufficient acclimatization, SRB performance may decline, potentially leading to biological process failure in the reactor.

Two primary acclimatization methods were examined: gradual acclimatization and shock acclimatization. In gradual acclimatization, the SRB system is exposed to stepwise increases in sulfate concentration, allowing environmental adaptation while reducing toxic effects (Nurandani et al., 2018). This method improves system stability and maintains sulfate reduction performance, although it requires a longer adaptation period. Shock acclimatization evaluates microbial community resistance through direct exposure to high sulfate concentrations, simulating sudden field fluctuations in AMD levels. This method provides a rapid assessment of microbial response to high sulfate loading. However, under the conditions tested in this study, the shock-acclimatized reactor showed less stable performance during subsequent operation. The comparison of these two methods was used to assess how adaptation strategies influence AMD treatment performance through sulfate reduction efficiency, pH stability, COD consumption patterns, and sediment characteristics associated with metal precipitation.

3.2. Gradual Acclimatization Method

The AMD treatment system underwent gradual acclimatization by incrementally increasing sulfate concentration by approximately 500 mg/L every seven days until it reached approximately 2,000 mg/L. Figure 2 shows the concentrations of sulfate, COD, and pH during gradual acclimatization. The same dose increment was applied weekly to support the development of SRB resistance to increasing environmental stress (Johnson et al., 2005). This approach is relevant for AMD treatment systems because SRB can be sensitive to sudden changes in sulfate concentration and severe chemical fluctuations (Kaksonen et al., 2007).

Visual assessments of reactor sediment were conducted during acclimatization to monitor SRB development. The sediment color changed from blackish brown at the beginning of the process to deep black after four weeks of operation. This change indicates that SRB colonies had become active and that sulfate reduction produced sulfide compounds (Nurandani et al., 2018). The black color is associated with the formation of metal sulfide minerals, including FeS, which serves as an indicator of successful sulfate bioreduction (Neculita et al., 2007).

Sulfate concentrations decreased consistently throughout the acclimatization period, indicating that SRB used sulfate as the primary electron acceptor during acclimation (Muyzer et al., 2008). COD also decreased as SRB metabolic activity increased because organic compounds were consumed as electron donors in the sulfate reduction process (Kaksonen et al., 2007). The system pH shifted toward neutral conditions because bioreduction consumes protons and creates alkaline conditions, supporting the application of SRB in AMD treatment (Johnson et al., 2005). Therefore, progressive sulfate loading provided a more controlled transition toward the target sulfate concentration, allowing the reactor to maintain sulfate reduction activity while sediment structure developed progressively. This pattern suggests that gradual exposure may support greater process stability during reactor acclimatization.

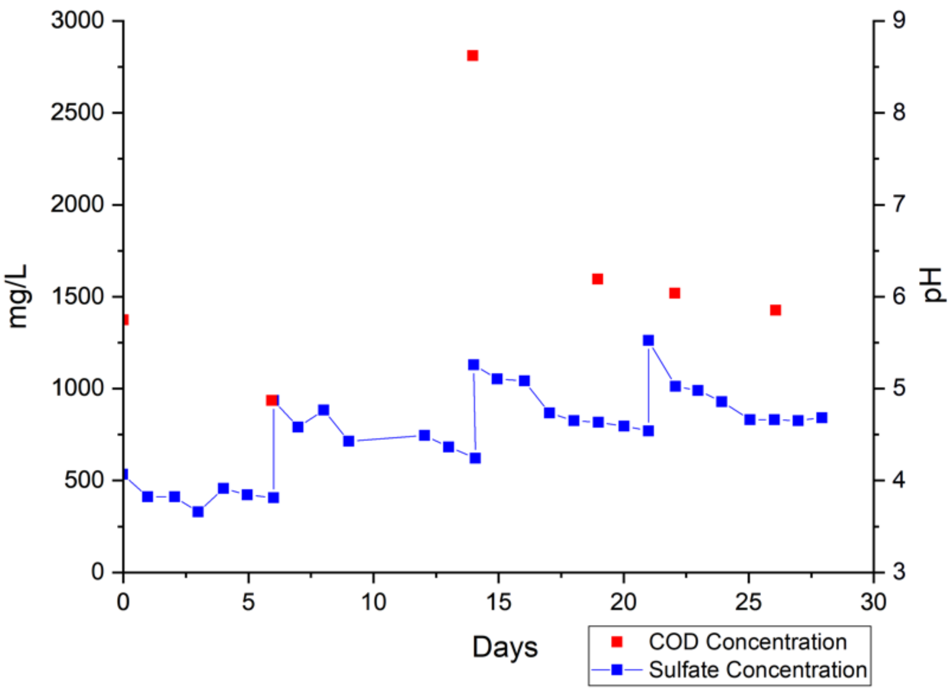

Figure 2. Sulfate Concentration, COD, and pH During Gradual Acclimatization

3.3. Shock Acclimatization Method

In the shock acclimatization approach, SRB were directly inoculated into a reactor containing a high sulfate concentration of approximately 2,000 mg/L. Glucose was added directly to the system as a carbon source and electron donor together with the sulfate concentration. This approach was designed to simulate extreme field conditions in AMD systems, where sulfate concentrations and organic loads may fluctuate and increase suddenly (Kaksonen et al., 2007). This concentration was applied to demonstrate that SRB require adaptation and cannot immediately become fully active or grow optimally under high-sulfate conditions. The concentrations of sulfate, COD, and pH during shock acclimatization are shown in Figure 3.

Visually, the sediment in the reactor changed markedly from brown to completely black by day 10. The color and consistency changes indicated the presence of metal sulfides and initial SRB activity in the reactor. The brown color was associated with the presence of glucose, whereas the black color in the shock reactor indicated the formation of metal sulfides. However, the formed sediment had a fragile and loose texture, indicating that the microbial colonies were unstable and that the biomass structure was weak (Neculita et al., 2007). This finding suggests that although sulfate reduction occurred, the formation of a strong biological sediment matrix did not develop optimally.

From a chemical perspective, sulfate concentration decreased rapidly in the early phase of operation. This decline was largely associated with non-biological mechanisms, such as physical adsorption of sulfate onto reactor media and initial chemical reactions with dissolved metal ions before the microbial community reached a stable condition (Johnson et al., 2005). COD concentration also decreased sharply at the beginning of the process because of rapid consumption by active SRB and supporting chemical reactions. However, COD then tended to stagnate, indicating a decline or cessation in microbial metabolic activity (Muyzer et al., 2008). The rapid initial increase in pH occurred because of proton consumption during sulfate reduction, followed by fluctuations that reflected biological system instability and disruption of reaction equilibrium in the reactor (Kaksonen et al., 2003).

Interpretively, the initial increase in sulfate reduction, COD removal, and pH was mainly caused by a combination of non-biological reactions and early SRB activity before the microbial community experienced full toxic stress. However, after the initial phase, system performance tended to stagnate under shock loading. This pattern suggests that sudden exposure to the target sulfate concentration imposed greater environmental stress on the SRB system and limited the maintenance of sulfate reduction activity. This may have reduced SRB activity and contributed to performance stagnation under shock loading (Muyzer et al., 2008). Under the tested conditions, the loose sediment structure and stagnation in treatment performance indicate lower system stability during shock acclimatization.

The shock acclimatization condition represents the shock-loading phenomenon that commonly occurs under real field conditions. This method can be used to evaluate how SRB respond to sudden environmental shifts and may serve as a reactor start-up strategy for selecting microorganisms capable of surviving extreme conditions before introduction into the main reactor (Kaksonen et al., 2007). Although this technique can induce initial SRB activation, the severe early environmental stress may lead to metabolic stress, cell death, and unstable microbial populations, thereby limiting system development and performance (Muyzer et al., 2008).

Under the experimental conditions of this study, gradual acclimatization showed more stable treatment performance than shock acclimatization, as indicated by progressive sulfate reduction and sediment development. Gradual acclimatization resulted in a more stable and linear increase in sulfate reduction performance, accompanied by the formation of stronger and denser bacterial colonies in the sediment, which enhanced reactor system stability (Neculita et al., 2007).

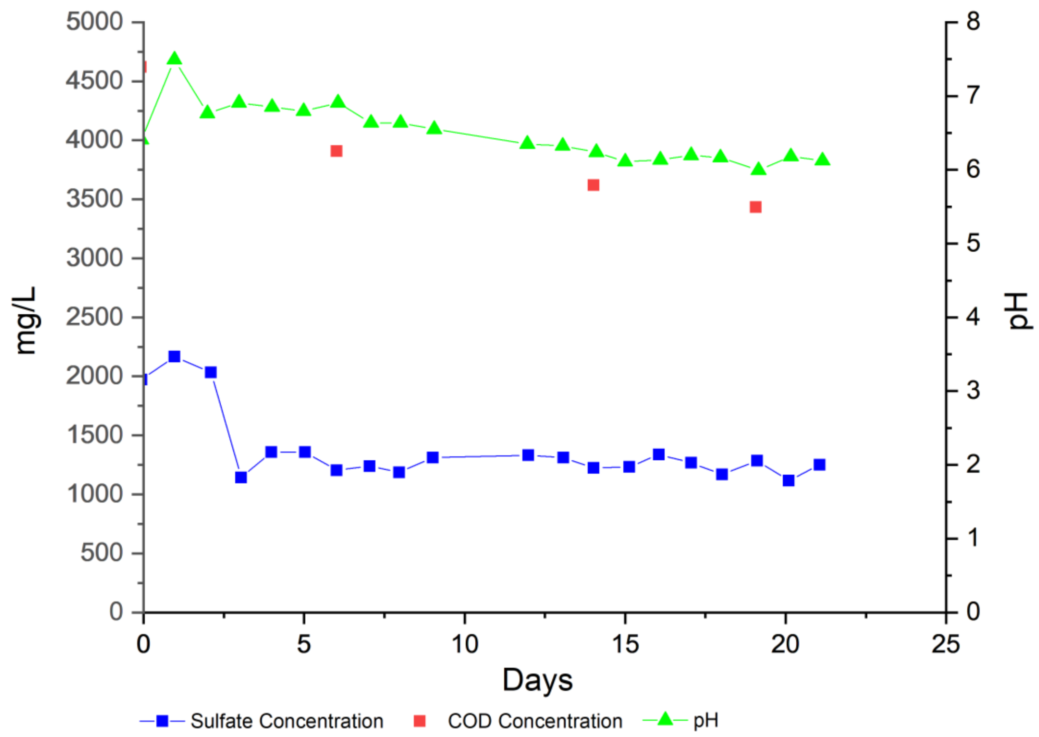

Figure 2. Sulfate, COD, and pH Concentrations During Shock Acclimatization

3.4. Effect of Fe and Mn on Sulfate Reduction Performance and Sediment Stability in Anaerobic Batch Reactors

AMD treatment systems mediated by SRB convert sulfate into sulfide, which then reacts with metals to form metal sulfides and reduce dissolved metal concentrations. However, excessive heavy metal concentrations can affect sulfate reduction by inactivating enzymes and damaging bacterial proteins (Johnson et al., 2005). In this study, Fe and Mn were tested as key metals influencing AMD treatment performance. The presence of Fe and Mn plays complex and sometimes contradictory roles. Fe and Mn are dominant components in many AMD streams, and at certain concentrations, these metals can be toxic to microorganisms, including SRB consortia, by disrupting enzyme function, damaging cell membrane integrity, and inducing oxidative stress (Utgikar et al., 2002). However, Fe and Mn also play important roles in sulfide precipitation. Sulfide ions (S^{2-}) produced by SRB-mediated sulfate reduction can react with Fe^{2+} and Mn^{2+} cations to form insoluble sulfide precipitates, such as FeS in the form of mackinawite or pyrite and MnS. This process simultaneously removes metals from solution (Kaksonen et al., 2007; Lens et al., 1998). Precipitation not only reduces excessive heavy metal concentrations but also supports the formation and stabilization of an anaerobic sediment matrix. Therefore, a comprehensive understanding of the interaction between metal toxicity and deposition mechanisms is important for optimizing the design and performance of SRB-based bioreactors for AMD treatment (Johnson et al., 2005). This process may also increase system pH, thereby altering metal solubility and reaction kinetics (Ayangbenro et al., 2018). The experimental objective of this study was specifically designed to evaluate the effect of varying Fe and Mn concentrations on sulfate reduction efficiency and sediment stability in anaerobic batch systems. The effects of Fe and Mn concentration variations were evaluated using two main performance parameters: sulfate reduction efficiency as a proxy for SRB biological activity and the stability of the formed sediment as an indicator of long-term metal immobilization in batch anaerobic reactor systems (Bijmans et al., 2010; Kaksonen et al., 2007). The interpretation of these results is important for distinguishing biological effects, namely SRB metabolic activity, from chemical effects, namely abiotic or biologically induced metal sulfide precipitation.

In this study, sulfate reduction and sediment stability were evaluated in anaerobic batch reactors for 14 days. This incubation period is commonly used to observe microbial adaptation kinetics and reaction rates in batch systems (Sahinkaya et al., 2010; Kieu et al., 2011). The test used five Fe and Mn concentration variations: K1, K2, IC_{50}, MTC, and 100. K1 contained 10 mg/L Mn and 0 mg/L Fe and served as the Mn-dominant control. K2 contained 10 mg/L Fe and 0 mg/L Mn and served as the Fe-dominant control. The IC_{50} condition contained 41 mg/L Fe and 31 mg/L Mn, representing the 50% inhibitory concentration, or the concentration that reduces bacterial growth by 50%. The MTC condition contained 60 mg/L Fe and 60 mg/L Mn, representing the maximum tolerable concentration. The 100 condition contained 100 mg/L Fe and 100 mg/L Mn, representing an extreme condition exceeding the tolerance threshold. This concentration design aimed to identify the toxicity thresholds of each metal and investigate the synergistic or antagonistic effects of Fe and Mn on SRB activity and sulfide sediment formation. This methodology was guided by Azabou et al.

(2007), who reported that Mn is more toxic than Fe. Based on this finding, differences in bacterial performance between conditions without Mn and without Fe were anticipated.

Monitoring sulfate concentration for 14 days showed markedly different patterns in each reactor, illustrating the influence of metal loading variation on sulfate reduction efficiency, as presented in Figure 4. These results indicate that complex and variable reduction dynamics were influenced by the applied metal treatment. The sulfate concentration–time curves for all reactors can generally be divided into three phases: a rapid initial decline phase supported by physical adsorption and initial bacterial activity, a fluctuation or slow-decline phase, and a stabilization phase in which sulfate concentration tends to remain constant, indicating equilibrium conditions or depletion of readily degradable substrates (Kaksonen et al., 2007; Sahinkaya et al., 2010). The duration and slope (Khan et al.) of each phase were strongly influenced by metal concentration, with higher concentrations tending to prolong the lag phase and slow the reduction rate (Utgikar et al., 2003). This three-phase sulfate reduction pattern is consistent with biological dynamics in nutrient-limited and stress-affected systems.

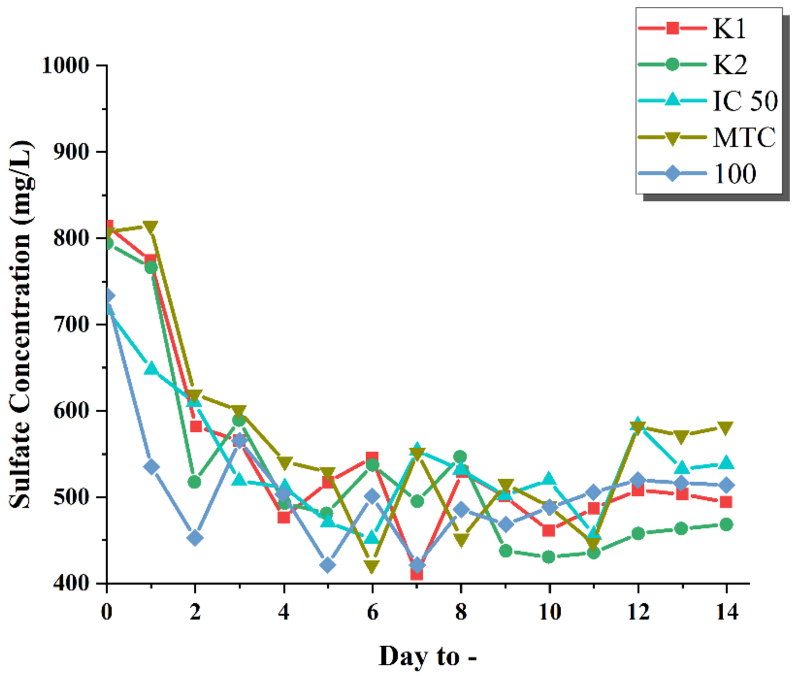

Figure 3. Sulfate Concentration Profile During Reactor Operation

Table 1. Efficiency and Sulfate Reduction Rate of Each Reactor

Variation	Reduction Efficiency (%)	Reduction Rate (mg/L.day)
K1 (Mn)	40.62	24.21
K2 (Fe)	46.58	29.13
IC_{50}	37.99	23.81
MTC	33.54	21.03
100	40.10	25.13

Based on Table 1, SRB performance was strongly influenced by Fe and Mn concentrations. The K2 reactor, containing 10 mg/L Fe and 0 mg/L Mn, achieved the highest efficiency of 46.58% with a reduction rate of 29.13 mg/L·day. This indicates that bacteria functioned optimally under low-Fe and Mn-free conditions, as Fe can act as a nutrient while the absence of Mn reduces toxicity. The K1 reactor, containing 10 mg/L Mn and 0 mg/L Fe, showed lower efficiency of 40.62% and a reduction rate of 24.21 mg/L·day, confirming that Mn toxicity was greater than Fe toxicity. Mn^{2+} can inhibit key enzymes and cause oxidative stress (Azabou et al., 2007). Under the IC_{50} and MTC conditions, efficiencies decreased to 37.99% and 33.54%, respectively, because of stronger inhibitory effects, extended lag phases, and enzymatic inhibition (Jong et al., 2003; Utgikar et al., 2003). At 100 mg/L of each metal, efficiency increased to 40.10%, likely because of chemical precipitation, in which sulfide ions (HS^-/H_2S) bound Fe^{2+} to form FeS. This process lowered measured sulfate without necessarily reflecting high biological activity (Rickard, 1995; Xu et al., 2020). These results indicate that sulfate removal in the batch reactor was not governed exclusively by biological SRB activity. The performance decrease under IC_{50} and MTC conditions suggests increasing metal stress, whereas the partial recovery observed at 100 mg/L may reflect a greater contribution of chemical sulfide precipitation. Therefore, sulfate removal efficiency should be interpreted together with metal concentration data and the potential contribution of abiotic precipitation. The profiles of iron and manganese concentrations during reactor operation are shown in Figures 5 and 6.

The decrease in sulfate concentration was caused by two interrelated mechanisms. The first mechanism was biological reduction by SRB, in which sulfate was used as the terminal electron acceptor and organic compounds, represented by COD, were used to produce sulfide ions (HS^-/H_2S). This mechanism depends on SRB viability and activity, which are inhibited at high heavy metal concentrations, especially Mn. This inhibition reduced sulfate reduction efficiency in the IC_{50} and MTC reactors because heavy metals interfere with electron transport, denature proteins, and promote the generation of reactive oxygen species (ROS) (Jong et al., 2003). The second mechanism was chemical precipitation, in which biologically produced sulfide reacted with metal cations, especially Fe^{2+}, to form insoluble precipitates such as FeS. This abiotic process occurs rapidly in Fe-rich environments and can reduce measured sulfate without intense biological activity, as observed in reactors with high Fe concentrations of 100 mg/L. Fe^{2+} and H_2S react rapidly at neutral to slightly acidic pH (Rickard, 1995). Interpretation of sulfate removal therefore requires separation of biological and chemical contributions because chemical precipitation can overestimate SRB performance and resilience (Sani et al., 2001; Bijmans et al., 2010). Metal toxicity studies in SRB systems should monitor sulfide production, cellular ATP, or viable cell counts to confirm biological activity alongside dissolved sulfate measurements (Kaksonen et al., 2007).

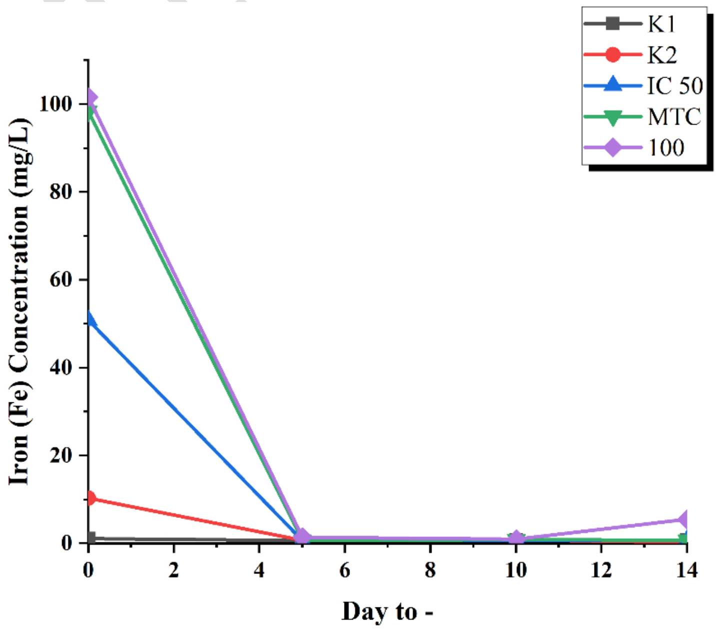

Figure 4. Iron Concentration Profile During Reactor Operation

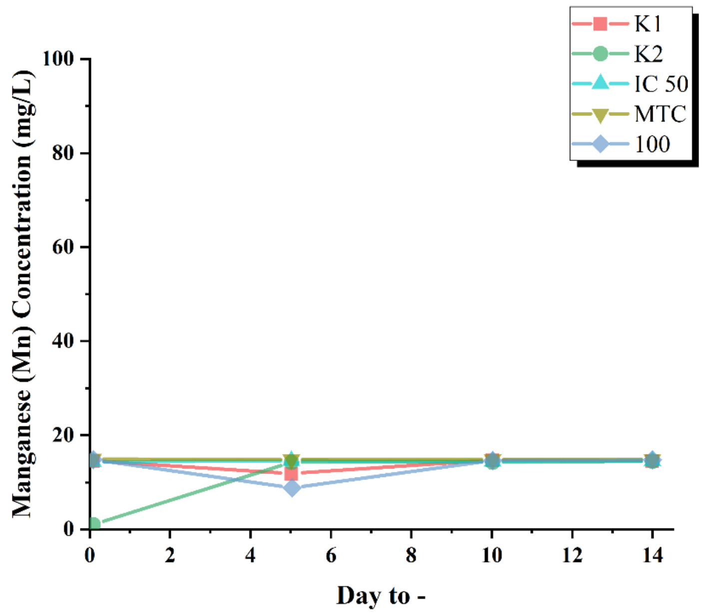

Figure 5. Manganese Concentration Profile During Reactor Operation

Monitoring results showed contrasting behavior between Fe and Mn. Fe concentrations decreased significantly, especially by day 5, because of adsorption onto sediment particles and chemical precipitation between Fe^{2+} and sulfide ions (HS^{-}), forming FeS (Kaksonen et al., 2007; Rickard et al., 2005). The highest Fe reduction efficiency was achieved in the MTC reactor at 99.23%, followed by the IC_{50} and 100 conditions. In the K2 reactor containing 10 mg/L Fe, the efficiency reached 80.61%. These data demonstrate the important role of Fe in sulfide precipitation, where Fe acts as the main sink for sulfide and removes Fe and sulfate/sulfide species from the liquid phase (Vallero et al., 2004). In contrast, Mn showed high stability and low removal. For example, the K1 reactor containing 10 mg/L Mn achieved only 30.56% removal. Mn is more resistant to adsorption and precipitation because MnS has higher solubility than FeS (Azabou et al., 2007). Consequently, Mn tends to remain in the dissolved phase and maintain toxic pressure on microorganisms.

The fundamental differences in the chemical behavior of Fe and Mn have important implications for the physical and biological stability of sediments in the reactors. In general, very high heavy metal concentrations can disrupt sediment structure and inhibit microbial activity through toxicity mechanisms. However, Fe has a dual effect. Although Fe can be toxic at high concentrations, it can also form solid FeS precipitates that contribute to sediment compaction and stability. In contrast, Mn did not significantly contribute to sediment stabilization because of its high solubility and limited sulfide precipitate formation. Based on these observations, Fe exhibited more tolerant and functional behavior than Mn in SRB-containing systems. This is attributable to the dual role of Fe as a sulfide-precipitating agent and contributor to sediment stability, resulting in lower inhibitory effects and potential synergy with the sulfate reduction process (Kaksonen et al., 2007). However, at very high concentrations, such as those applied in the MTC and 100 conditions, both Fe and Mn exerted considerable toxic stress and directly reduced the efficiency of purely biological sulfate reduction.

Table 2. Efficiency and Rate of Iron Concentration Decrease

Variation	Decrease Efficiency (%)	Decrease Rate (mg/L.day)
K1 (Mn)	30.56	0.02
K2 (Fe)	80.61	0.59
IC_{50}	98.63	3.58
MTC	99.23	6.97
100	94.59	6.86

3.5. Effect of Fe and Mn on Sulfate Reduction Performance and Sediment Stability in Continuous Reactors

Sustainable AMD treatment systems must maintain operational stability and consistent performance during field-scale operation. Continuous reactors operate at constant flow rates, enabling continuous AMD treatment and stable reductions in heavy metal concentrations (Cui et al., 2012). Previous studies have shown that continuous reactor systems can achieve high removal rates for dissolved metals in AMD, including 99.8% Fe removal and neutralization of wastewater pH (Strosnider et al., 2011). Continuous reactors also support the maintenance of anaerobic conditions, which is essential for improving SRB performance and achieving sulfate reduction and metal removal through biological processes (Afsar et al., 2022).

A major challenge in continuous reactor operation for AMD treatment is heavy metal accumulation, particularly Fe and Mn. The sediment–water interface becomes a site for metal accumulation because environmental chemical factors, including pH and redox potential, influence metal distribution. Dissolved metal concentrations increase under low-pH conditions because Fe and Mn become more soluble and mobile. Conversely, precipitation and adsorption become more effective at immobilizing Fe and Mn when pH increases. Accumulation of Fe and Mn at harmful levels can damage microbial ecosystems, including SRB communities responsible for sulfate reduction. Fe and Mn deposits in sediment can also affect physical sediment stability by changing structure and porosity. Therefore, long-term continuous reactor performance is vulnerable to physical and biological changes in the treatment environment. This experiment was conducted to evaluate how different Fe and Mn concentrations affected sulfate reduction performance and sediment properties in a continuous reactor from day 1 to day 22. Understanding interactions between heavy metals and microorganisms in sustainable systems is essential for maintaining process stability and ensuring the sustainability of AMD treatment. Five metal concentration levels were evaluated.

Table 3. Fe and Mn Concentration Variations Used in the Experiment

Variation	**Fe Concentration (mg/L)**	**Mn Concentration (mg/L)**	**Description**
K1 (Mn)	0	10	Mn-dominated control
K2 (Fe)	10	0	Fe-dominated control
IC_{50}	41	31	50% inhibitory concentration
MTC	60	60	Maximum tolerable concentration
100	100	100	Extreme concentration

The metal concentration variations used in the continuous reactor system were designed to be identical to those used in the batch system, allowing direct comparison between the two reactor configurations. This approach ensured that sulfate reduction efficiency and sediment stability results were attributable to differences in reactor system operation rather than initial conditions or influent composition. The evaluation was therefore more objective because both systems received the same treatment variations, enabling assessment of how reactor design influenced system responses. The following data show the sulfate concentration results obtained from the reactors during the 22-day testing period.

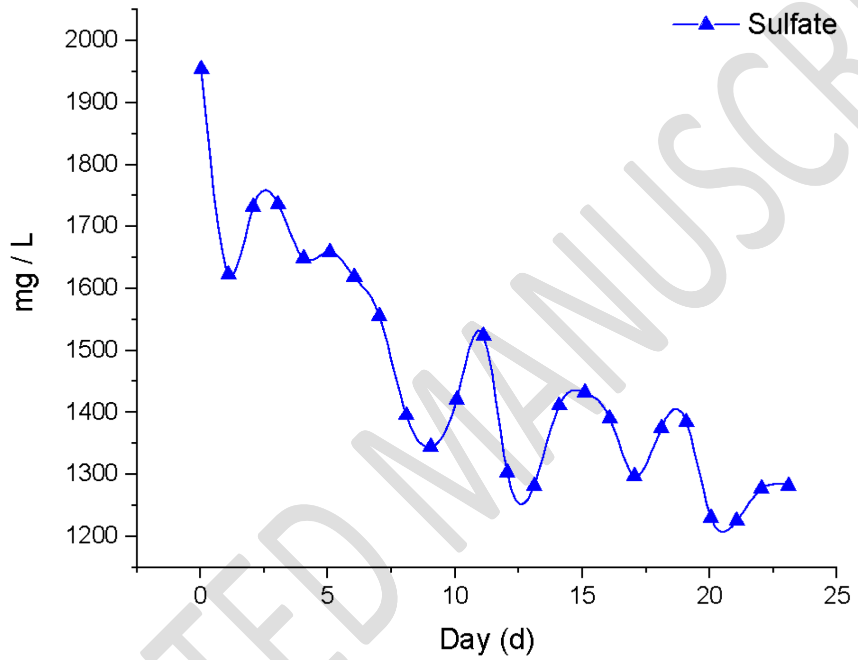

Figure 6. Sulfate Concentration Profile in the Continuous Reactor

The continuous reactor system operated for 22 days and produced a distinct response pattern compared with the batch system. Figure 7 shows the sulfate concentration in the continuous reactor. Reactor performance reflected microbial tolerance to metals, heavy metal behavior, and physicochemical processes. The K2 variation achieved the best sulfate reduction performance, indicating that Fe supported SRB activity, with Fe^{2+} showing no appreciable inhibitory effect at this concentration while promoting FeS formation and sediment stability (Hudz et al., 2011). In contrast, the K1 variation showed lower efficiency, indicating higher Mn toxicity and limitation of sulfate reduction. Under the IC_{50} and MTC variations, efficiency decreased with increasing Fe and Mn concentrations, but the decline was slower than in the batch system, suggesting stronger resistance and continuous microbial adaptation. The 100 variation showed a high sulfate decrease, but this decrease was dominated by chemical precipitation of FeS and MnS. These findings show that sulfate reduction in continuous systems occurs through both SRB activity and interactions among heavy metals, environmental conditions, and precipitation processes.

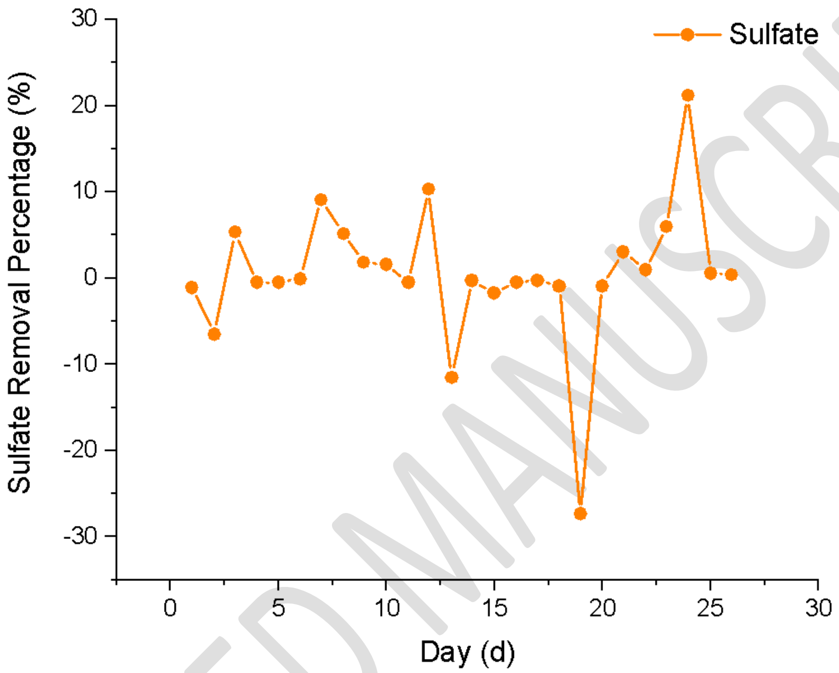

Figure 7. Sulfate Removal Percentage in the Continuous Reactor

Figure 8 shows the Percentage of Sulfate Removal in a Continuous Reactor. Compared with the batch reactor, the continuous system maintained a more controlled sulfate removal pattern despite changes in metal loading. This behavior may be associated with the continuous input–output regime, which provides a different exposure pattern to metal stress than the batch system. However, accumulation of FeS-containing sediment may create hydraulic limitations if excessive precipitation occurs. Therefore, although continuous operation may provide greater operational resilience, long-term application requires control of metal accumulation and sediment buildup (Shahsavari et al., 2015; Zhao et al., 2005). The sulfate removal percentage tended to remain above zero under Fe-dominant conditions, indicating that SRB survived better under these conditions, while iron sulfide minerals, including FeS and pyrite (FeS_2), helped maintain sediment stability (Chen et al., 2005). However, FeS formation at elevated Fe concentrations can lead to excessive sediment accumulation that may obstruct reactor flow by altering hydraulic characteristics (Liu et al., 2017). Mn removal was less efficient because Mn exerts stronger toxicity on SRB and forms highly soluble MnS, which is less prone to precipitation and contributes only marginally to sedimentation and sediment stabilization (Wan et al., 2023).

The results indicate that continuous operation provided a more stable response to changing metal conditions rather than universally higher removal performance than batch operation. This finding suggests that reactor configuration should be selected based on treatment objectives, metal loading, hydraulic conditions, and long-term operational stability requirements. The sediment-stabilizing function of Fe occurs through iron sulfide production, which improves physical stability in the system. However, Fe concentration must remain within acceptable limits to prevent sediment buildup and flow problems. Mn requires strict control because it has stronger toxic effects on SRB and does not substantially support sediment stabilization. The continuous reactor system showed better capacity to handle heavy metal fluctuations, but operational success depends on implementing metal control strategies that maintain system stability and sulfate reduction performance.

3.6. Comparison of Sulfate Reduction Performance in Batch, Intermittent, and Continuous Reactors in Relation to Treatment Stability and Efficiency

The comparison presented in this section integrates experimental results obtained in the present study with data reported in previous studies. Batch and continuous reactor results were obtained experimentally in this study, whereas intermittent reactor data were compiled from relevant literature because an intermittent reactor was not operated experimentally in the present work. Therefore, the comparison is intended to provide a broader assessment of reactor operation modes rather than a direct experimental comparison under identical operating conditions. The selection of reactor design is a crucial decision in SRB-based AMD treatment because each reactor type operates differently and affects system performance. Water movement, residence time, and interactions among microorganisms, substrates, and sulfate determine sulfate reduction effectiveness, SRB stability in sediment and biomass, and system tolerance to changes in organic matter and flow rate (Xue et al., 2023). This discussion evaluates standard reactor designs based on sulfate reduction rate, COD reduction, and removal efficiency. Selecting an appropriate reactor is essential because these systems operate under changing environmental conditions and must handle different wastewater characteristics. This assessment uses field application considerations to evaluate each reactor design because reactor selection strongly influences implementation success. Operational environments require reactor systems that can function under changing process conditions and variable wastewater compositions.

The batch reactor was operated for two weeks at laboratory scale under various COD/SO_4^{2-} ratios and heavy metal levels to assess SRB sulfate reduction performance. The operation revealed three main stages. The first phase showed substantial sulfate reduction as SRB performed metabolic activity and sulfate was captured by sediment and biomass through adsorption. In the middle phase, microorganisms showed variable sulfate reduction rates while adapting to environmental conditions and responding to substrate fluctuations and metal toxicity. The final phase reached a stable condition as sulfate reduction rates stabilized. The batch reactor achieved peak performance with a sulfate reduction rate of 35.7 mg/L·day and a sulfate removal efficiency of 99.07%. However, it was less suitable for extended AMD treatment under variable wastewater volumes. This finding is consistent with Magowo et al. (2020), who reported 92.63% sulfate reduction under batch operation. Although batch systems can achieve high sulfate reduction rates, actual performance may be affected by polysulfide interference that is not fully captured in standard measurements (Sun et al., 2020; Sun et al., 2018).

Intermittent reactors, such as SBRs, operate in scheduled cycles that include filling, reaction, settling, and discharge stages. These systems allow operators to control reaction time more flexibly than batch reactors. Treatment flexibility is achieved because operators can modify the duration of different process stages, including filling, reaction, settling, and decantation (Mello, 2019). SBR operation requires a waiting period between cycles to shift between modes, such as from reaction to settling. This configuration enables separation between treated water and sludge before the next operating cycle begins (Mello, 2019). Intermittent reactors showed a sulfate reduction rate of 19.9 mg/L·day and the highest COD reduction rate of 16.64 mg/L·day. Intermittent operation enhanced the use of organic substrates by SRB and other anaerobic microorganisms for metabolic processes and biomass formation. The intermittent reactor achieved a removal efficiency of 97.7%, which remained high but was slightly lower than that of the batch reactor because of inactive periods in its operational cycle. This result is consistent with Rasool et al. (2015), who reported sulfate reduction efficiencies of 82.35–98.42% in SBRs. Intermittent reactors are suitable for semi-continuous operation because flexible cycle settings allow load adjustment without impairing biomass retention or system function (Mello, 2019).

Continuous reactors such as CSTRs use vertical flow and continuous mixing to create relatively uniform reaction conditions throughout the reactor. Sulfate reduction performance may vary because of changes in HRT and influent organic compound patterns (Hessler et al., 2017). Based on operational characteristics, the continuous system performs between batch and intermittent systems in some respects, although sulfate reduction rates and COD values are not always directly comparable across studies. Several studies have demonstrated that CSTRs can achieve sulfate removal efficiencies of 92.70% (Wang et al., 2025), while other studies have reported sulfate removal efficiencies ranging from 94% to 100% (Kieu et al., 2011). The main advantages of CSTR systems include long-term operational stability, the ability to manage variable loading, and suitability for large-scale implementation. However, CSTR systems require technical control of HRT and mixing intensity, which must be carefully adjusted to optimize sulfate reduction and bacterial cell retention, particularly for SRB populations.

Batch reactors can achieve high sulfate reduction rates but lack operational flexibility, making them unsuitable for continuous field operation. Intermittent reactors provide operational control while maintaining high treatment performance, making them suitable for modular systems and medium-scale facilities. Continuous reactors are more suitable for industrial operations with large production demands and variable wastewater volumes, but they require more sophisticated process management. Reactor selection should consider removal efficiency, substrate availability and characteristics, sulfate and metal concentration fluctuations, system durability, and operational cost. Overall, SRB reactor designs can perform effectively, but AMD treatment system selection should be based on site-specific requirements and wastewater characteristics.

Table 4. Recapitulation of Sulfate and COD Removal Based on Reactor Type

Parameter	Batch Reactor	Intermittent Reactor	Continuous Reactor
Sulfate Reduction Rate (mg/L·day)	35.7	19.9	34.8
COD Reduction Rate (mg/L·day)	9.57	16.64	28.6
Removal Efficiency (%)	99.07	97.7	99.8

Note: Batch and continuous reactor data were obtained from the present experimental study, whereas intermittent reactor data were compiled from previous studies. Therefore, the values are intended for comparative interpretation and should not be considered results obtained under identical experimental conditions.

4. CONCLUSION

The findings of this study indicate that, under the tested experimental conditions, gradual acclimatization provided more stable sulfate reduction performance than shock acclimatization. The progressive increase in sulfate concentration was accompanied by steady sulfate reduction and the development of denser sediment, suggesting improved system stability during acclimatization. Gradual acclimatization supported continuous SRB activity, sulfide formation, and the development of compact and durable sediment. In contrast, shock acclimatization produced an initial increase in performance but did not sustain stable treatment because metabolic stress contributed to SRB inhibition, potential cell death, and brittle sediment formation. These results indicate that gradual acclimatization is preferable to shock loading because SRB require adaptation before becoming fully active under high sulfate concentrations. The presence of Fe and Mn played an important role in sulfate reduction performance and sediment stability. Fe was less inhibitory than Mn and supported better sulfate reduction performance. Mn had a stronger negative effect on SRB activity because of its greater toxicity and limited contribution to stable sulfide compound formation. Sulfate reduction depended on both SRB biological activity and chemical precipitation, which produced metal sulfide compounds such as FeS and MnS. Overall, Mn showed higher toxicity than Fe.

Batch reactors achieved high sulfate reduction efficiency but were less adaptable and less suitable for continuous field operation. Intermittent reactors provided better process control and waste removal, making them suitable for modular systems and medium-scale industrial facilities. Continuous reactors were more suitable for large-scale operation and fluctuating influent conditions, although they require more complex process monitoring and control. Reactor configuration should therefore be selected based on removal efficiency, substrate availability, sulfate and metal concentrations, operational cost, and system sustainability. No single reactor configuration outperforms all alternatives; selection must be tailored to site-specific requirements, wastewater characteristics, operational objectives, and scale of application.

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