



Effect of carbon source and metal toxicity for potential acid mine drainage (AMD) treatment with an anaerobic sludge using sulfate-reduction

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ABSTRACT


This study compares sulfate-reduction performance in an anaerobic sludge with different carbon sources (ethanol, acetate, and glucose). Also, the toxic effect of copper was evaluated to assess its feasibility for possible acid mine drainage (AMD) treatment. Serological bottles with 1.5 g VSS/L and 150 mL of basal medium (0.67 g COD/g SO_4^{2-} at a 7–8 pH) were used to determine the percentage of electron equivalents, maximum specific methanogenic (SMA), and sulfide generation activities (SGA). The copper effect was evaluated in a previously activated sludge in batch bioassays containing different concentrations of copper (0–50 mg/L, 3 gVSS/L, and 150 mL of basal medium (0.67 g COD/g SO_4^{2-}). Carbon source bioassays with glucose obtained the best results in terms of the SGA ($1.73 \pm 0.34 \text{ mg S}^{2-}/\text{g VSS}\cdot\text{d}$) and SMA ($10.41 \text{ mg COD-CH}_4/\text{g VSS}\cdot\text{d}$). The electron flow in the presence of glucose also indicated that $21.29 \pm 5.2\%$ of the metabolic activity of the sludge was directed towards sulfidogenesis. Copper toxicity bioassays indicated that a considerable decline in metabolic activity occurs above 10 mg/L. The 20%IC, 50%IC, and 80%IC were 4.5, 14.94, and 35.31 mg Cu/L. Compared to the other carbon sources tested, glucose proved to be a suitable electron donor since it favors sulfidogenesis. Finally, copper concentrations above 15 mg/L inhibited metabolic activity in the toxicity bioassays.


Key words | electron donors, heavy metals, inhibitory concentration, microbial competition, percentage of electron equivalents, sulfate-reduction

HIGHLIGHTS

- The influence of organic matter degradation and copper toxicity was assessed.
- Potential application of sulfate reducing bacteria (SRB) for acid mine drainage treatment was evaluated.
- The highest sulfidogenic activity was observed with glucose as carbon source.
- High copper concentration impacts sulfate removal and sulfide production.

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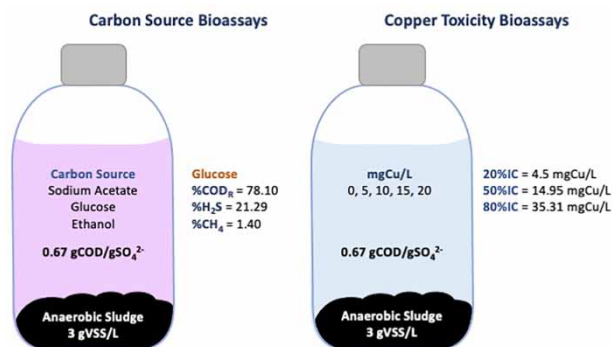
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GRAPHICAL ABSTRACT



INTRODUCTION

Acid mine drainage (AMD) is generated by physical, chemical, and biological interactions present in abandoned and active mines when oxygen and water interact with sulfur minerals, such as pyrite and marcasite (Gao *et al.* 2019). The result is a leached agent that contains acid pH, high quantities of sulfuric acid, and dissolved metals, such as Ag, As, Cu, Hg, Mn, Mo, Cr, Cd, Zn, Pb, among others (Costa *et al.* 2017). The composition of AMD varies depending on the geology of mine sites or sources. A typical AMD is highly acidic (pH 2.3), high in sulfates (2,853–3,622 mg/L), with reported concentrations of 460 mg/L of Zn, 400–2,135 mg/L Fe, and 0.3–49 mg/L of Mn, Co, Cd, Ni, and As. AMD can cause environmental degradation, water and soil contamination, severe health impact and biodiversity loss (Kefeni *et al.* 2017). Heavy metals can accumulate in water, soils, and the food chain, resulting in the death of plants or animals. For humans, exposure to high levels can lead to serious chronic diseases. In the case of sulfate, it can disrupt the natural sulfur cycle (Guo *et al.* 2017).

Therefore, AMD treatment is essential to prevent contamination, even if it can be complex and expensive. For example, physicochemical treatments, like ion exchange and sorption, often require high-cost chemicals, need solid-liquid separation, or require sludge disposal (Kumar *et al.* 2018). On the other hand, sulfate reducing bacteria (SRB) use sulfate as a terminal electron acceptor during the metabolism of organic matter to transform it into sulfide, which reacts with dissolved metals forming metal-sulfide precipitates (Yim *et al.* 2015). Metal precipitation as sulfides allows the recovery and recycling of valuable metals over a wide range of pH; besides, metal sulfides are dense, have lower solubilities

than hydroxides, and have good settling properties, allowing their removal from the aqueous phase (Xu & Chen 2020).

Anaerobic reactors are often used for AMD treatment, using sulfate removal and metal precipitation through biological methods. Heavy metal removal has been studied in various bioreactor configurations, like continuous stirred tank reactors (CSTR), up-flow anaerobic sludge blanket reactor (UASB), fixed bed reactors (FBR), and permeable reactive barriers (PRB) (Xingyu *et al.* 2013). Lately, these bioreactors have been designed to efficient natural microbial sulfate-reduction with major process control. SRB activity is influenced by biochemical and operational parameters, including substrate use, pH, dissolved oxygen, temperature, and hydraulic retention time (HRT) (Yim *et al.* 2015). A range of factors are critical for combined treatment systems, including COD/SO₄²⁻ ratios, mixed water chemistry, like pH and ion and cation concentrations (SO₄²⁻, CO₃²⁻, PO₄³⁻), microbiological diversity, and reactor configuration (Deng *et al.* 2016).

Since AMD has low organic matter concentrations, a carbon source is often needed to promote sulfate-reduction. The effectiveness of the process can be affected by the carbon source employed (Zhang & Wang 2014). SRB can utilize a wide range of substrates as electron donors that are generally organic compounds or hydrogen (Sánchez-Andrea *et al.* 2012). SRB can be subdivided into incomplete and complete oxidizers. Incomplete oxidizing SRB can partially oxidize organic compounds like lactate, pyruvate, malate, and succinate to acetate and CO₂, while complete oxidizers can oxidate acetate to CO₂ (Menon & Voordouw 2018). AMD treatment in bioreactors using anaerobic SRB is

possible, but its effectiveness depends on the organic substrates selected to feed the bacteria.

AMD contains a significant number of toxic compounds that can inhibit bacteria development. Occasionally if SRB has not been exposed to toxic metals, they can present a deficiency in their tolerance. At high metal concentrations, sulfide production sometimes is not an effective mechanism for metal decontamination (Jin *et al.* 2007). SRB have been reported to be inhibited at 2–50 mg Cu/L, 13–40 mg Zn/L, 75–125 mg Pb/L, 4–54 mg Cd/L, and 10–20 mg Ni/L (Deng *et al.* 2016). Microorganism metal resistance depends on the mobility, bioavailability, and toxicological effect of the metal, and varies with the species and their capacity of developing specific resistance mechanisms, like permeability barriers, intracellular sequestration, and enzymatic detoxification (Kieu *et al.* 2011). Hence, metal interaction with microorganisms is an important factor to consider when designing a biological treatment. In that sense, the objective of this study is to compare sulfate reduction performance with different carbon sources, ethanol, acetate, and glucose, for SRB activity in an anaerobic sludge. Additionally, the toxicity effect of copper towards an anaerobic sludge was evaluated to assess its feasibility for possible AMD treatment.

MATERIALS AND METHODS

Sludge

An anaerobic sludge was obtained from a full-scale anaerobic reactor from a brewery company. The ratio of VSS/TSS ratio was 45.89%. This sludge was selected given bacteria from brewery wastewater treatment can tolerate high concentrations of sulfate. For copper toxicity bioassays, this sludge was adapted to sulfate-reducing activity in a CSTR reactor with no oxygen supply, acid pH (5), and a 0.67 g COD/g SO_4^{2-} ratio.

Culture media

The culture media employed for the carbon source and copper toxicity bioassays contained (in mg/L): NH_4Cl (1,045), KCl (270), KH_2PO_4 (170), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (185), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (50), NaHCO_3 (2,000), yeast extract (20), anhydrous sodium sulfate (4,416), and trace element solution (1 mL/L). The trace element solution contained (in mg/L): $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$ (2,000), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (500), $\text{EDTA} \cdot 2\text{H}_2\text{O}$ (500), H_3BO_3 (50), AlCl_3 (50), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (50), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (50), Na_2SeO_3 (100), $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (50), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$

(50), resazurin (200), and 36% HCl (1 mL/L). To meet a 0.67 g COD/g SO_4^{2-} ratio glucose (1.88 g $\text{C}_6\text{H}_{12}\text{O}_6/\text{L}$), sodium acetate (4.8602 g $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}/\text{L}$), and ethanol (1.20 mL $\text{C}_2\text{H}_5\text{OH}/\text{L}$) were added as carbon source.

Analytic methods

Methane generation was determined by sodium hydroxide displacement (NaOH 3%) in an inverted column. Chemical oxygen demand was determined using a HACH kit. Sulfide was analyzed by colorimetry by the methylene blue method (4,500 S^{2-} D). Sulfate was determined by barium chloride precipitation, described in the turbidimetric method (4,500 SO_4^{2-} E). Volatile suspended solids (VSS), total suspended solids (TSS), sulfate, and sulfide analysis were performed following standardized methods (APHA 2012). Copper concentrations were analyzed using atomic absorption spectroscopy in a Perkin Elmer Analyst 400 with HNO_3 (3%).

Carbon source bioassays

Specific methanogenic activity (SMA), sulfide generation activity (SGA), and percentage of electron equivalents were evaluated using sodium acetate, glucose, and ethanol, as the carbon source in batch digesters. Assays were conducted in triplicates using 160 mL serological bottles sealed with butyl rubber stops and aluminum crimp seals. Bottles contained 3 g VSS/L of anaerobic sludge and 120 mL of basal mineral medium (0.67 g COD/g SO_4^{2-} ratio), with a pH of 7–8. Flasks without sludge were used as abiotic controls. All bioassays were carried in triplicate and incubated at 32 °C. Bioassays lasted 50 days, organic matter consumption was determined once the assay finished, sulfide production three times a week, and methane generation daily.

Specific methanogenic (mg CH_4 -COD/kg VSS·d) and sulfide generation (mg S^{2-} /kg VSS·d) was calculated using the slope of sulfide and cumulative methane production versus time (d), as described in the following equations:

$$\text{SMA} \left(\frac{\text{gCOD} - \text{CH}_4}{\text{gVSS} \cdot \text{d}} \right) = \text{Slope} \left(\frac{\text{Methane Production}}{\text{gVSS} \cdot \text{d}} \right) \quad (1)$$

$$\text{SGA} \left(\frac{\text{g COD} - \text{S}^{2-}}{\text{gVSS} \cdot \text{d}} \right) = \text{Slope} \left(\frac{\text{Sulfide Generation}}{\text{gVSS} \cdot \text{d}} \right) \quad (2)$$

The percentage of electron equivalents used for methanogenic and sulfidogenic activities was calculated with Equations (3) and (4), as described by Sierra-Alvarez *et al.*

(2007).

$$\% \text{CH}_4 - \text{COD} = 100 \times \frac{M \cdot F_m}{\text{COD}_R} \quad (3)$$

$$\% \text{H}_2\text{S} - \text{COD} = 100 \times \frac{S \cdot F_s}{\text{COD}_R} \quad (4)$$

where: M = methanogenesis (g $\text{CH}_4/\text{L}\cdot\text{d}$); S = sulfide generation (g $\text{S}^{2-}/\text{L}\cdot\text{d}$); COD_R = organic matter removal rate (g $\text{COD}/\text{L}\cdot\text{d}$); and stoichiometric ratios $F_m = 4$ g $\text{CH}_4\text{-COD}/\text{g CH}_4$; and $F_s = 2$ g $\text{S}^{2-}\text{-COD}/\text{g S}$.

Copper toxicity bioassays

Copper toxicity assays were conducted in duplicates using glass serological flasks supplemented with 150 mL of medium (0.67 g $\text{COD}/\text{g SO}_4^{2-}$ ratio), glucose as carbon source, and 3 gVSS/L of sludge. The anaerobic sludge was preactivated with glucose and adapted to acidophilic conditions (pH 5) in a CSTR reactor, with an operating volume of 1.5 L, for 5 days prior to the assay. The desired amount of copper (Cu^{2+}) was added to flasks using acidified stock solutions from a 1,000 mg/L stock. The tested concentrations were 0, 5, 10, 15, 20, and 50 mg/L. The pH, g $\text{COD}/\text{g SO}_4^{2-}$ ratio, and copper concentrations were determined based on typical AMD characteristics. Flasks lacking sludge served as abiotic control. Additionally, an uninhibited control lacking copper was incubated. All flasks were sealed with butyl rubber stoppers and aluminum crimp seals.

Toxicity assays lasted 48 h, sulfide production and glucose and sulfate consumption were determined every 6 h, and copper removal every 24 h. The initial concentrations of copper causing 20%, 50%, and 80% reduction in the glucose consumption rate compared to an uninhibited control were referred to as 20%IC, 50%IC, and 80%IC. These values were calculated by interpolating in graph plotting the inhibition observed (%) as a function of the inhibitor concentration. The reported inhibitory concentrations are average values of duplicate assays.

RESULTS AND DISCUSSION

Carbon source bioassays

SMA and SGA in the presence of sodium acetate, glucose, and ethanol are shown in Figure 1. The figure illustrates

similar methane production with glucose and sodium acetate. In the presence of glucose, there was methanogenic activity in the abiotic control; this could have happened because the assay was not carried out in sterile conditions. SMA activities were significantly lower than those reported in literature, which can be attributed to sulfate inhibition, causing sulfide production, by sulfate-reduction, which is toxic to methane-producing bacteria (Sinbuathong *et al.* 2007). The origin of the sludge can be responsible for the high methanogenic activity in the presence of ethanol since it presented the highest SMA but the lowest SGA. The sludge was previously used in an anaerobic bioreactor used for COD removal from wastewater in a brewery industry, causing a more feasible alcohol consumption.

On the other hand, in the presence of glucose, the sludge presented the highest sulfide generating activity. The initial carbon source concentration was 2 g COD/L ; therefore, the substrate was not a limiting factor for methanogenesis or sulfidogenesis to take place. As the substrates were consumed, methane and sulfide production leveled off, reaching a peak production of methane and sulfide at 30 days for all carbon sources (Figure 1(a) and 1(b)).

Given that the anaerobic sludge is a microbial consortium, and the carbon source selected can influence microbial group development, competition over substrates is an important parameter to take into consideration. Table 1 shows substrate consumption, specific methanogenic and sulfide generating activities, and the percentage of electron equivalents. Substrate usage ($\% \text{COD}_{\text{in}}$) was similar for all the electron donors. The percentage of electron equivalents showed that sulfate-reducing activity predominated over methanogenic activity.

Results demonstrate that sulfide production was influenced by the different electron donors employed. Sulfide generating activity in the presence of ethanol was less than half compared to acetate and glucose. In terms of the electron flow, ethanol presented the lowest percentage towards sulfidogenesis (7.17%). The low SRB activity in the assays can be explained by acetate accumulation due to incomplete oxidation by SRB during sulfidogenesis (Bertolino *et al.* 2014). Acetate production during sulfate-reduction can be inconvenient given that SRB cannot oxidize acetate completely, leading to cell disruption, even with excess sulfate (Cao *et al.* 2012; González-Paz *et al.* 2020). Studies have pointed out that acetate was the least suitable substrate to accomplish SRB activity when tested alongside lactate, molasses, methanol, and ethanol; molasses, on the other hand, can be economically and regulatory favorable (Geets *et al.* 2006).

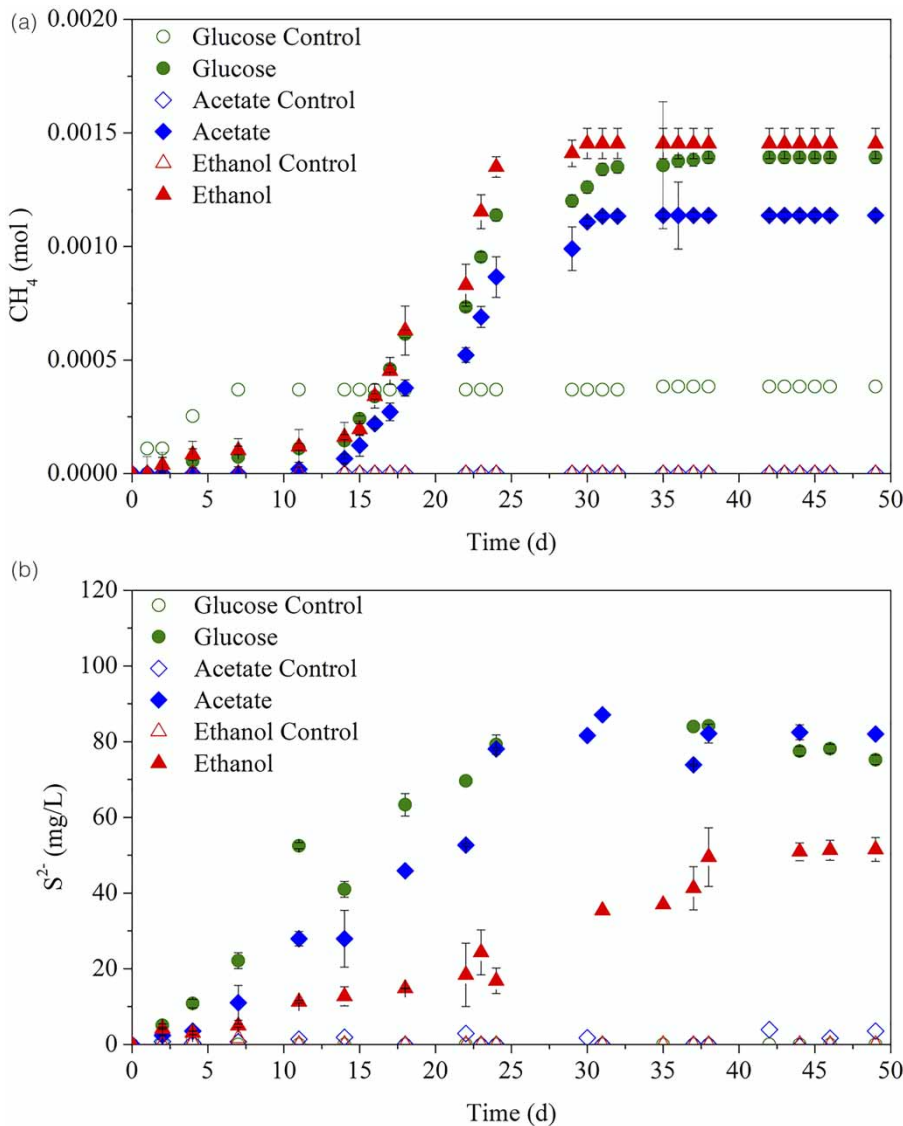


Figure 1 | Specific methanogenic (a) and sulfide generating (b) activities in batch bioassays in the presence of sodium acetate, glucose, and ethanol as electron donors (0.67 g COD/g SO₄²⁻, pH 7–8).

Table 1 | Average substrate consumption and specific methanogenic and sulfide generation activities for the anaerobic sludge in the presence of sodium acetate, glucose, and ethanol

	Methanogenic Activity mg COD-CH ₄ /g VSS•d	Sulfide Generation Activity mg S ²⁻ /g VSS•d	%COD _{in}		
			COD	H ₂ S	CH ₄
Glucose	10.41 ± 1.58	1.73 ± 0.34	78.10 ± 3.1	21.29 ± 5.2	1.40 ± 0.26
Sodium Acetate	11.25 ± 1.53	1.02 ± 0.18	80.88 ± 8.8	15.06 ± 6.4	1.19 ± 0.14
Ethanol	19.90 ± 1.40	0.43 ± 0.01	85.42 ± 1.31	7.17 ± 1.75	2.40 ± 0.60

Although glucose presented the least COD consumption, it showed the highest electron flow toward sulfidogenesis. Cao *et al.* (2012) tested different electron

donors for SRB. In the system using glucose, the remnant COD was in the form of lactic acid, butanedioic acid, formic acid, and acetic acid in small amounts.

More complex electron donor molecules allow higher tolerance of SRB to hydrogen sulfide toxicity, resulting in less inhibition to SRB. Glucose can be a reasonable electron donor for SRB if cost, handling, and purchase are not a problem. Like glucose and molasses, complex compounds can be an alternative since they can degrade into other compounds (e.g., lactate, ethanol, VFAs, CO_2 , H_2), which can be utilized by different types of SRB groups (Wakeman *et al.* 2010). Besides, glucose degradation to other compounds can promote different microorganism species growth, originating syntrophic relationships between SRB and other microorganisms, granting process stability.

Copper toxicity bioassays

The anaerobic biomass was screened based on their metal, sulfate, and COD removal, evaluating the effect of the addition of copper on sulfate and COD reduction. Figure 2 shows glucose consumption (Figure 2(a)), sulfate removal (Figure 2(b)), sulfide production (Figure 2(c)), and copper removal (Figure 2(d)) for the assays in the presence of copper. After 48 h, glucose usage for concentrations of 0 and 5 mg Cu/L was nearly 100%, contrary to the concentrations of 20 and 50 mg Cu/L where less than 5% was used (Table 2). This indicates low metabolic activity for concentrations above 10 mg Cu/L. Based on the glucose consumption rate, the inhibitory

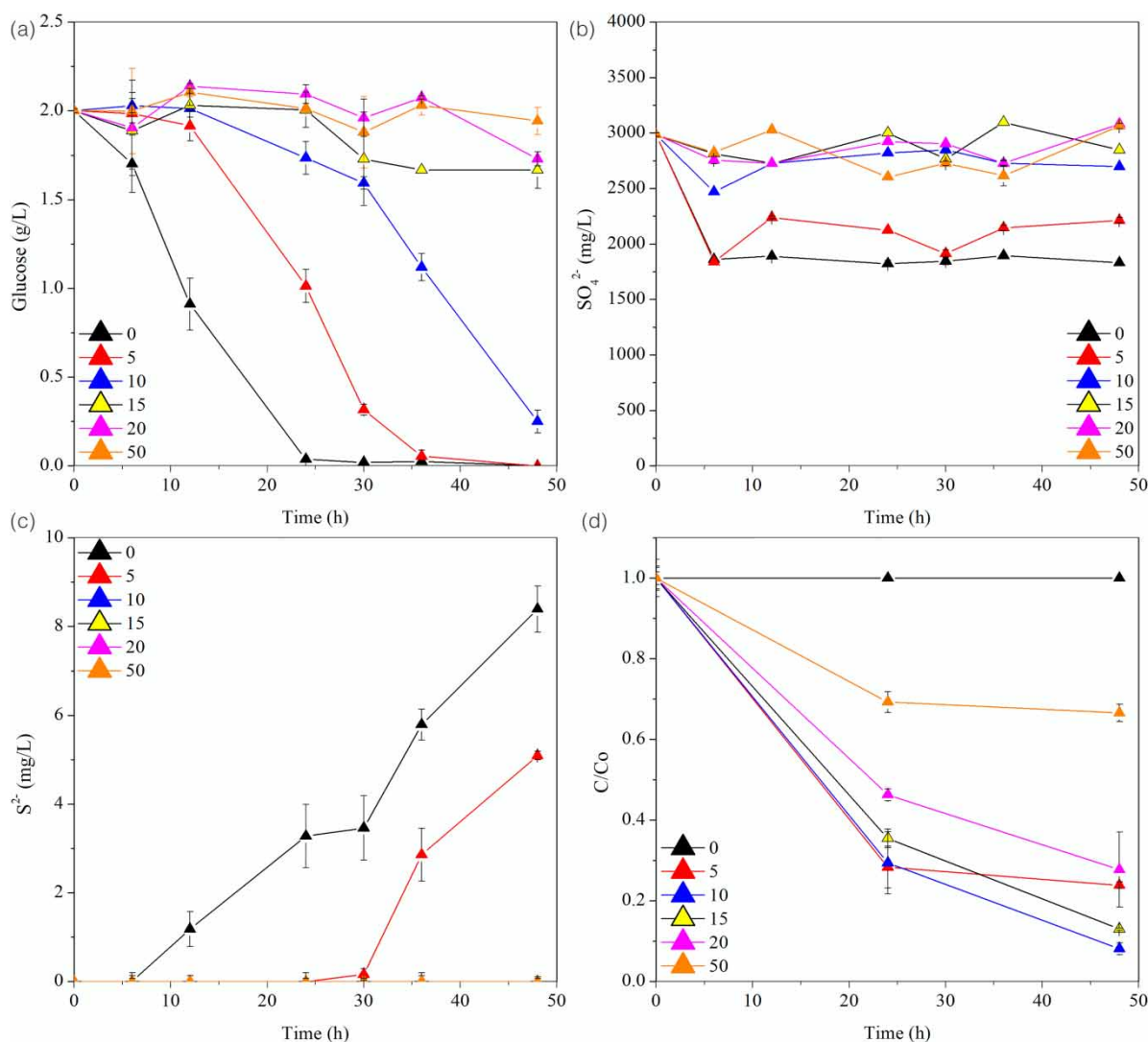


Figure 2 | Glucose consumption (a); sulfate removal (b); sulfide production (c); and copper removal in batch bioassays in the presence of glucose (0.67 g COD/g SO_4^{2-} , pH 5) (d), and different concentrations of copper (0, 5, 10, 15, 20 and 50 mg/L).

Table 2 | Average glucose, sulfate, sulfide, and copper concentrations attained after 48 h in copper toxicity bioassays

Initial Cu (mg/L)	Glucose Consumption Efficiency (%)	Sulfate Consumption Efficiency (%)	Sulfide Generation (mgS ²⁻ /L)	Copper Removal Efficiency (%)
0	100 ± 0.06	45.51 ± 9.67	8.93 ± 1.77	0 ± 0.0
5	100 ± 0.109	34.75 ± 12.60	5.09 ± 0.92	76.11 ± 1.16
10	87.51 ± 0.0	9.62 ± 17.44	1.12 ± 0.0	91.85 ± 1.78
15	9.59 ± 0.0	4.55 ± 8.14	ND	86.96 ± 0.66
20	4.88 ± 2.2	ND	ND	72.27 ± 0.83
50	2.83 ± 0.3	ND	ND	33.45 ± 0.0

concentrations were 4.5, 14.94, and 35.31 mg Cu/L, for 20% IC, 50%IC, and 80%IC, respectively.

Copper addition had a negative effect on sulfate-reduction since sulfate-reduction efficiency decreased as the metal concentration increased. The highest sulfate removal was attained in assays containing 5 mg Cu/L (34.75 ± 12.6%) stabilizing after 24 h. In assays containing concentrations of 10 mg Cu/L, sulfate removal efficiency declined significantly, dropping to 10.4 ± 0.26% (Figure 2(b)). With initial Cu concentrations of 20 and 50 mg/L, no significant sulfate removal was observed. A comparison study between WWTP and lab-scale reactor sludges showed lower sulfate removal (55%) in the presence of individual metals like cadmium, copper, nickel, and lead with initial concentrations of 10 and 50 mg/L (Kiran *et al.* 2015). This demonstrated that even low concentrations of metals could have a negative impact on sulfate-reduction. Also, sulfide production was severely affected by copper presence. No significant sulfide production was observed for concentrations above 5 mg Cu/L (Figure 2(c)).

Figure 2(d) shows copper removal during metal toxicity assays. A considerable decline in the soluble copper concentrations during the assays can be observed. Past 48 h, the highest copper removal was attained for a concentration of 10 mg Cu/L (91.85 ± 1.78%). Although copper removal percentages declined with copper concentration increase, still a 33.43 ± 2.86% removal was reached for a concentration of 50 mg Cu/L, representing the assay with the lowest removal rate (Table 2).

Sulfate-reducing activity in the assays was severely affected by copper presence. However, glucose consumption (in Figure 2(a)) suggests the growth of other microorganisms present in the assays, at least for lower concentrations of the metal. Since there is no substantial metabolic activity for higher copper concentrations regarding glucose and sulfate removal, and sulfide production, the decrease in the metal concentration cannot be only attributed to metal

precipitation as metal sulfides. Metal ions can interact with other compounds found in anaerobic sulfate-reducing systems, such as acetate, phosphates, carbonates, or bind to the cellular wall or extracellular polymeric substances (EPS) (Hwang & Jho 2018; Costa *et al.* 2021). A similar scenario could have happened in this study, explaining the partial reduction in Cu concentration by biosorption, aside from chemical precipitation. Although copper removal by precipitation is preferred, it has a low solubility product with sulfide than other metals (Kiran *et al.* 2015).

CONCLUSIONS

The purpose of this study was to evaluate simple carbon sources like glucose. Compared to the other carbon sources tested in this study, glucose proved to be an efficient substrate that can be employed in an anaerobic process and biogenic sulfide production for AMD treatment. Organic matter degradation into intermediate compounds by other bacteria present in the sludge can be used by SRB, allowing synergy between the microorganisms present in the sludge. Therefore, the selection of a non-restrictive carbon source is preferred for wastewater treatment.

On the other hand, the sludge was affected by copper in the conducted assays. Sulfide precipitation cannot be assumed, but precipitation and adsorption can be accounted for copper removal from the assays. This study indicates that the anaerobic sludge can tolerate relatively high inlet concentrations of copper; therefore, it can be used in sulfate-reducing technologies as an alternative for AMD treatment. Nonetheless, the tolerance or toxic effect of a real AMD solution should be analyzed for this particular sludge before analyzing the behavior of the sludge in a lab-scale bio-reactor. This would be the next step to determine if the sludge can be employed for full-scale AMD treatment using sulfate-reduction.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper.

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