

The effect of adapting cellulose degrading microorganisms to 25°C providing energy sources for biological sulphate removal

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ABSTRACT

Vast volumes of acid mine drainage (AMD) is still being generated in South Africa, due to decant from both active and closed mines. Research to find a cost effective, environmentally friendly treatment system to reduce the salinity and to neutralise the acidity of AMD is ongoing. The study presented here showed that high sulphate removal efficiencies were achieved applying the biological treatment technology, thereby using the degradation products of grass-cellulose as the carbon and energy sources. The process was conducted at 25°C, as opposed to 37°C described previously, using a one-stage hybrid reactor system, treating both sulphate-rich synthetic feed water and pre-treated AMD. The results showed that the fermentation microbes, originating from rumen fluid derived from cattle, could generate the carbon and energy sources for the sulphate reducing bacteria at 25°C. When comparing the results obtained at 25°C to those obtained at 37°C, it was observed that these were similar. However, at the higher temperature a faster flow-rate to the reactor was possible. The findings implied that the biological sulphate removal system described can be operated more economically at 25°C than at 37°C, as heating the reactor system can be omitted.

Keywords: Ambient temperature, biological sulphate removal, one-stage hybrid reactor system, fermentation microbes, pre-treated mine water

INTRODUCTION

Globally, research is focussed on the treatment of acid mine drainage (AMD), which is continuously generated, even at closed mining sites (Younger & Wolkesdorfer, 2004). In South Africa, coal mining still forms an important part of the local economy. The characteristics of AMD are high concentrations of sulphate and heavy metals, principally iron, and a low pH. The advantages of biological treatment of AMD are three-fold: 1) sulphate reduction, 2) pH increase through alkalinity production and 3) metal-sulphide precipitation, since sulphide is the reduction product of biologically reduced sulphate. The metal-sulphide precipitates have low solubility (Stumm & Morgan, 1996), which makes the biological sulphate removal technology a feasible

option for simultaneous metal and sulphate removal from waste waters (Freeman *et al.*, 2008). In order to achieve biological sulphate reduction, an electron donor is needed, while sulphate is the electron acceptor. Many carbon and energy sources have been tested, e.g. volatile fatty acids (VFA), such as propionic and butyric acids (Colleran *et al.*, 1995), ethanol (De Smul *et al.*, 1997; Greben *et al.*, 2000) and methanol, for which the operating temperature can be mesophilic (Weijma, 2000) or thermophilic (Davidova & Stams, 1996). For sustained sulphate reduction and maintenance of the biomass, the feed water COD/SO₄ ratio should be 1 (Rinzema & Lettinga, 1988). Thus, when the sulphate concentration in AMD amounts to ca. 2.5 g/L and when 10 ML/day AMD needs to be treated, 25 tons/day of COD is required. From this calculation, it is evident that the carbon and energy source can add considerably to the operating costs. Therefore, the use of the degradation products of grass cuttings, a potential waste product, is an attractive alternative (Greben *et al.*, 2007; 2008a).

Previous studies, conducted at the CSIR, South Africa, demonstrated that biological sulphate reduction using degradation products of grass cuttings as the carbon and energy sources resulted in sustained sulphate reduction from sulphate-rich synthetic and pre-treated mine water (Greben *et al.*, 2007; 2008a). A sulphate removal efficiency of ca. 90% was achieved when treating sulphate-rich synthetic feed water and ca. 80% treating pre-treated mine water, using a one-stage anaerobic hybrid reactor at 37°C. The results furthermore indicated that a close syntrophy existed between the fermentation and sulphate-reducing bacteria (SRB), for which the one-stage hybrid reactor is ideally suited. Most anaerobic hybrid reactors are a combination of Upflow Anaerobic Sludge Bed (UASB) and Anaerobic Filter (AF) reactors, thereby promoting the advantages of both reactor systems (Buyukkamaci & Filibeli, 2002). Operating a hybrid reactor provides suspended growth in the sludge layer and biofilm formation on the packing material, which prevents washout of biomass. Several reactor designs have been developed for biological sulphate reduction e.g. the Upflow Anaerobic Sludge Bed (UASB)

reactor (Lettinga et al., 1980), the Fluidized Bed (FB) reactor (Iza, 1991) and the Anaerobic Filter (AF) (Young & McCarty, 1969). In previous studies, a reactor temperature of 37-39°C was maintained to accommodate the microbes originating from rumen fluid. However, full-scale treatment of 1-10 ML/d mine water at elevated temperatures is not practical, since heating vast volumes of mine effluent to 37-39°C will increase operating costs substantially.

The aim of this study was therefore to investigate whether sustained biological sulphate removal can be obtained, operating a continuously fed hybrid reactor at an ambient temperature of 25°C, treating sulphate-rich, synthetic and pre-treated mine water. The envisaged challenge of this study was to adapt rumen microorganisms to a lower temperature than these microbes normally function at in ruminants.

MATERIALS AND METHODS

Feed water

Initially, synthetic sulphate-rich feed water was used, which later changed to pre-treated, sulphate-rich mine water, consisting of one part AMD and one part reactor effluent after biological sulphate reduction. The pre-treatment of AMD resulted in a higher pH and in metal removal, due to the alkalinity and sulphide concentrations, respectively, in the effluent after biological treatment. This prevented the metals from entering the bio-reactor while the precipitated metals can be generated for re-use. The AMD was obtained from a closed coal mine in the Witbank area, South Africa. The feed rate of 5 L/d resulted in a HRT of 2.4 d. Micronutrients were added (1 ml/L feed-water) to the synthetic feed water (Greben et al., 2008a).

Reactor configuration, biomass and carbon and energy source

A one-stage anaerobic (the dissolved oxygen concentration (DO) measured 0 mg/L) reactor system, HFS, (Volume: 20 L and Active Volume 12 L) was operated (Figure 1). The lower part of the reactor contained ceramic rings for biofilm formation for the SRB, thus preventing microbial wash-out. The SRB were obtained from a biological sulphate removal demonstration plant (Witbank, South Africa). The upper part of the reactor contained grass cuttings to which sieved (mesh 10x10 mm) rumen inoculum (RI), obtained from an abattoir (Witbank, South Africa) was added. Grass cuttings (Kikuyu grass) were used as the source of cellulose, from which the fermentation products served as the carbon and energy source in the reactor. Kikuyu grass cuttings were obtained from the CSIR, Pretoria,

garden service. The grass cuttings (size 10-20 mm) were collected and stored at 4°C. The mass of the grass in this paper refers to air-dried grass. Grass consists of 52% water, 14% cellulose and 28% hemicellulose. The lignin content is 5%, and the ash represents 0.5% (Sonakya et al., 2003). The empirical COD of grass was found to be 1 g O₂/g.

Experimental

The feed water entered HFS at the top (Figure 1). A recycle stream (40 L/d) was installed from the fermentation part to the top of the reactor, for improved reactor mixing purposes. The effluent left HFS at the bottom. HFS was operated at 25°C, through water recycling from a water bath (25°C) entering a water jacket surrounding the reactor. There were three different experimental periods when feeding synthetic feed water. The periods were determined by the addition of GC and microorganisms. To maintain a high COD concentration in the reactor, 150 GC were added weekly. Feeding pre-treated mine water resulted in a further three experimental periods and similarly 150 g grass-cuttings were added weekly during the total experimental period of 156 days.

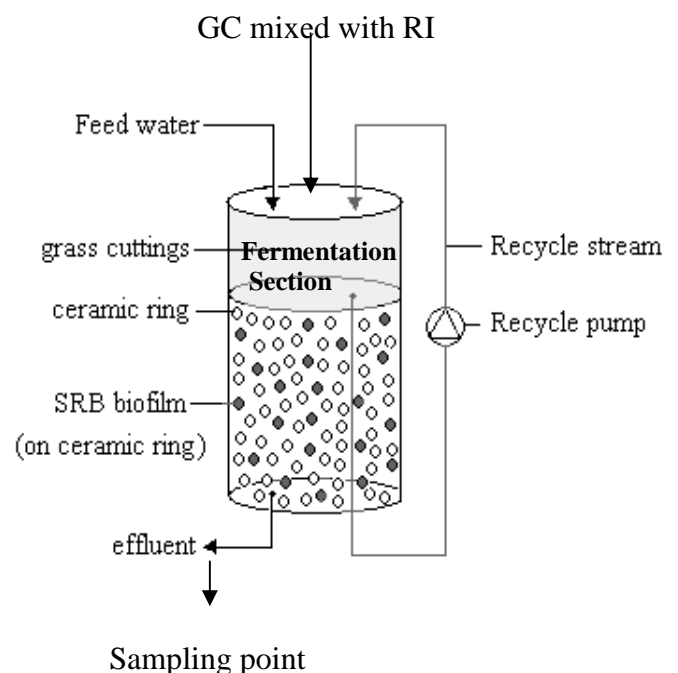


Figure 1. Schematic overview of HFS reactor system.

Sampling

Daily samples (weekends excluded) were taken from the reactor effluent (sampling point: Figure 1). The results presented in the graphs relate to

the daily sampling, while the data in the tables reflect the average results of the analyses of the daily samples.

Analytical methods

Determinations of sulphate, COD, pH, mixed liquor suspended solids (MLSS) and volatile suspended solids (VSS) were carried out according to standard analytical procedures (*Standard Methods* APHA, 1985). With the exception of the MLSS, VSS and sulphide, all analyses were carried out on filtered samples (Whatman #1). The COD samples were pre-treated to eliminate the sulphide contribution to the COD concentration. All VFA analyses were done using a gas chromatograph (Hewlett Packard. HP 5890 Series II) equipped with a flame ionisation detector (FID). The column used was a HP-FFAP, 15 m x 0.53 mm, 1 micron. The GC/FID programme can be summarized as follows: initial oven temperature 30°C, for 2 min., temperature programmed to increase thereafter from 80 °C to 200 °C at 25 °C/min, with temperature hold for 1 min at 200 °C, FID temperature 240 °C. The carrier gas (N₂) flow rate was set at 1 ml/min.

RESULTS

OPERATION OF HFS IN CONTINUOUS MODE AT 25 °C FEEDING SYNTHETIC FEED WATER

Sulphate and COD concentrations

The sulphate concentrations in the feed- and treated water and the COD concentration in the treated water during the total period of 135 days are presented in Figure 2. Initially, the COD concentration (up to Day 40) in the treated water was generally >1000 mg/L, resulting in a high sulphate removal. The relationship between the COD and SO₄ concentrations in the reactor can be observed from Figure 2: When the COD concentration was high, the sulphate concentration was low. Thus it was important to maintain a high reactor COD concentration, achieved by adding GC and by maintaining healthy cellulose degrading microbe communities. The initial high COD concentration was ascribed to the addition of 300 g GC, SRB and RI at the start of Period 1 and 150 g GC, SRB and RI at the start of Period 2. The high COD concentration was the result of fermentation by means of the cellulose degrading microorganisms. It was hypothesized that the fermentation microorganisms and the SRB work in close syntrophy: The available energy delivered by the fermentative microbes, e.g. hydrogen, is utilised by the SRB in the presence of SO₄ as soon as it is produced. The SRB keep the hydrogen partial pressure low

(Visser, 1995). Oude Elferink *et al.*, (1994) showed that the hydrogen utilizing SRB (HSRB) gain more energy from the consumption of molecular hydrogen, have a higher substrate affinity, growth rate and cell yield than for instance the hydrogen utilizing methanogenic bacteria (HMB). These authors suggested that in the presence of sulphate, compounds, such as alcohols, lactate, propionate and butyrate, may be oxidized directly by the SRB without the intermediate formation of hydrogen. As soon as the SRB utilise the energy produced by the fermenting organisms, the fermenters respond by producing more VFA and hydrogen. The VFA degradation proceeds via β-oxidation, producing acetate and hydrogen (Sousa *et al.*, 2008).

The SO₄ and COD concentrations during Period 3 (days 35-135) showed that the feed SO₄ concentration became more stable after Day 75 at approximately 2 700 mg/L. At the same time, the residual COD concentration in the reactor decreased from >1 000 mg/L to <1 000 mg/L, which resulted in an effluent SO₄ concentration of >500 mg/L. The higher inflow SO₄ concentration required additional COD for the SO₄ reduction, which subsequently resulted in a lower COD concentration in the treated water. Although 150 g GC were added weekly, it was a challenge to keep the COD concentration in the reactor at sufficiently high concentrations for a high SO₄ removal efficiency. To maintain a high reactor COD concentration, a high efficiency of the degrading microbes is required. However, a reactor temperature of 25°C may be limiting the microbial activity of RI.

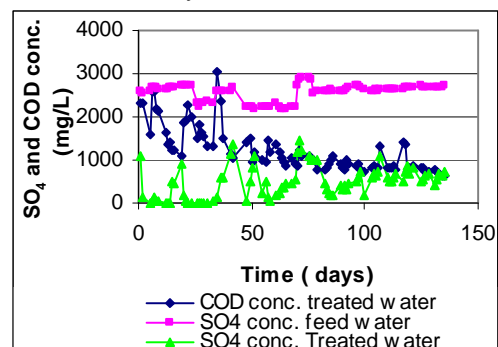


Figure 2. The SO₄ and COD concentrations in the feed and in the treated water of HFS, feeding synthetic feed water

The average SO₄ removal efficiency for the 135 day period was 82% (Figure 3). The initial high sulphate removal efficiency could not be maintained until ca. Days 80-100, whereafter the SO₄ removal stabilised at an average of >80%, but then decreased from Day 100 to Day 135 to between 70-85%.

At the start of Periods 1 and 2, the reactor was supplemented with RI and SRB, but no microorganisms were added at the start of Period 3. Empirical data obtained from experiments operating stirred batch reactors, showed that microbial supplementation, especially of SRB, enhanced the sulphate reduction (Greben *et al.*, 2008b). This finding was in agreement with the results shown by Oude Elferink *et al.*, (1994) and Harmsen (1996). Additional stirred batch reactor tests linked the microbial information applying t-RLFP (a molecular biology technique) with biotechnological findings. The results from those studies showed a high diversity of cellulose degraders when high levels of substrate (GC) were present in the reactors, while a relative low diversity of SRB was observed (Burke *et al.*, 2008). The latter finding was confirmed by Professor Peter Rose (personal communication, 2008), operating a demonstration-scale, biological sulphate removal reactor at Grootvlei Mine, Springs, South Africa (Greben, 2008a).

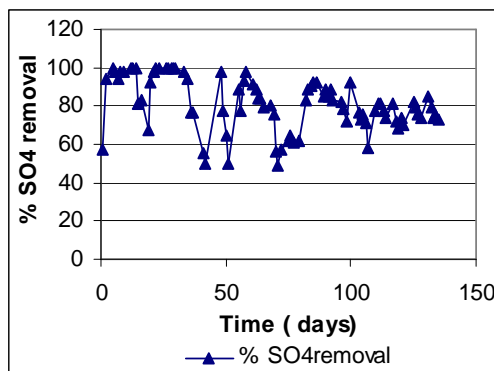
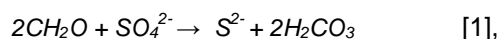


Figure 3. Sulphate removal (%) from synthetic feed water

OPERATION OF HFS IN CONTINUOUS MODE AT 25 °C FEEDING PRE-TREATED MINE WATER

Sulphate removal as function of COD concentration

Figure 4 shows the SO_4 concentrations in the feed and treated water and the COD concentration in the treated water during the experimental period of 156 days. The SO_4 concentration in the feed water fluctuated, due to the changing SO_4 concentration in the AMD as well as the mixing of the AMD with the effluent water from the reactor. The average feed water SO_4 concentration was 2 435 mg/L and 348 mg/L in the treated water. This efficient SO_4 removal result was ascribed to the relatively high reactor COD concentration (1009 mg/L), maintained by the weekly addition of 150 g GC (Equation 1):



where $2\text{CH}_2\text{O}$ represents the carbon and energy source.

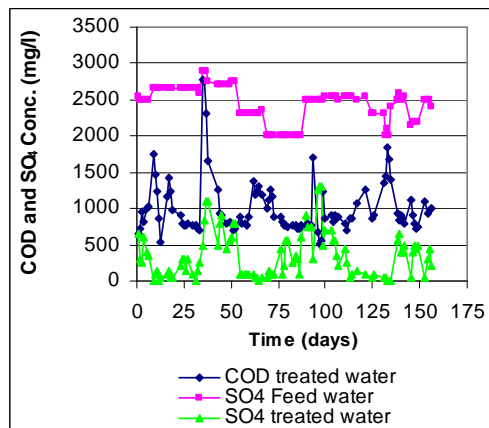


Figure 4. The sulphate concentration in feed and treated water and COD concentration in treated water, feeding pre-treated mine water

The sulphate removal efficiency in the hybrid reactor while treating pre-treated AMD showed the average sulphate removal at 86% (Figure 5), which was slightly higher than the removal feeding synthetic feed water (82%).

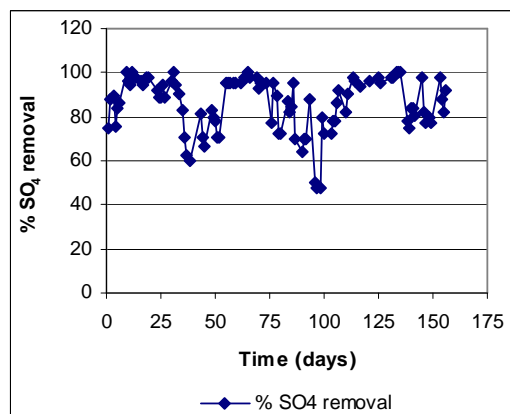


Figure 5. The sulphate removal efficiency (%) feeding pre-treated mine water

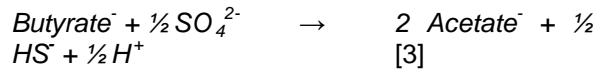
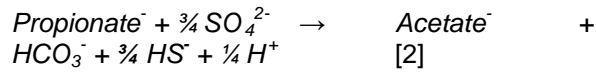
REACTION PARAMETERS

The average SO_4 removal data and the average COD concentrations as well as other operational parameters obtained from the studies treating synthetic feed water and pre-treated mine water, are presented in Table 3. It was observed that the results for both feeding regimes were similar and were associated with a stable reactor operation.

Volatile Fatty Acids (VFA)

The VFA concentrations in the treated water, especially the propionic and butyric acid concentrations were low, while the residual acetate concentration was higher at 108 and 70 mg/L, feeding synthetic feed water and pre-treated mine water, respectively. The higher C2

acid concentrations were explained by the fact that acetate is formed when propionic and butyric acids are utilised for biological sulphate reduction. Furthermore, the lower C3 and C4 acid concentrations in the treated water were ascribed to the utilisation of these energy sources for the sulphate reduction (Equations 2 and 3):



SRB show a preference for propionate and butyrate before acetate as substrate (Visser, 1995). Acetate is often the limiting factor in sulphate-reducing reactors (Lens *et al.*, 1998; Vallero *et al.*, 2003 and Greben *et al.*, 2004). However, empirical data from HFS appeared to indicate that acetate is utilised by the SRB, most likely when no other carbon source is available. Hulshoff Pol *et al.*, (1998) indicated that the HSRB require acetate as the carbon source. During the degradation of cellulosic material, hydrogen is produced by the RI and immediately utilised by the HSRB, for which acetate is required as the carbon source.

Reactor parameter ratios

The experimental $\text{S}^{2-}_{\text{produced}}/\text{SO}_{4\text{removed}}$ when feeding synthetic feed water was 0.21, while it was 0.20 when feeding pre-treated mine water. Although the theoretical $\text{S}^{2-}_{\text{produced}}/\text{SO}_{4\text{removed}}$ ratio is 0.33, the experimentally obtained ratios are mostly ± 0.20 , due partly to sulphide to sulphur oxidation, to other intermittent sulphur species and to the formation of H_2S gas. The experimental $\text{Alkalinity}_{\text{produced}}/\text{SO}_{4\text{removed}}$ ratio, was 1.12 feeding synthetic feed water, while the ratio was 1.05, close to the theoretically value of 1.04, feeding pre-treated mine water. These results showed that alkalinity was produced according Reaction [1]. The $\text{GC}_{\text{added}}/\text{SO}_{4\text{removed}}$ ratio (Table 3) showed that 1 g GC led to the removal of 0.49 g SO_4 feeding pre-treated mine water. This result was marginally more favourable than when feeding synthetic feed water, where 1 g GC removed an average of 0.41 g SO_4 .

Table3. Experimental data obtained from HFS, feeding synthetic feed water and pre-treated mine water

| Parameter | Synthetic feed water | Pre-treated AMD |
|--|----------------------|-----------------|
| Residual COD (mg/L) | 1 228 | 1 009 |
| SO_4 removed (g/d) | 12.1 | 10.4 |
| SO_4 removed (%) | 82 | 86 |
| S^{2-} produced (mg/L) | 441 | 536 |
| S^{2-} -produced/ SO_4 removed ratio | 0.21 | 0.20 |
| Alkalinity produced (mg/L) | 2 351 | 2 070 |
| Alk produced/ SO_4 removed ratio | 1.12 | 1.05 |
| pH feed water | 6.39 | 5.14 |
| pH treated water | 7.20 | 6.97 |
| Acetate | 108 | 70 |
| Propionate | 21 | 14 |
| Butyrate | 11 | 11 |
| g SO_4 / 1g GC removed | 0.41 | 0.49 |
| Redox Potential (mV) | -165 | -168 |
| $\text{NO}_3\text{-N}$ in treated water (mg/L) | 93 | 29 |
| $\text{NH}_3\text{-N}$ in treated water | - | 98 |
| PO_4 in treated water (mg/L) | 133 | 138 |
| Reactor VSS (g/L) | 2.5 | 0.8 |
| VSS in treated water (g/d) | 4.1 | 1.6 |

Since the COD concentration provided by 1 g/L GC is 1000 mg/L, this experimentally obtained ratio translated to a COD/ SO_4 ratio of 2:1, which is higher than the theoretical COD/ SO_4 ratio of 0.67. Thus, not all COD provided by the GC can be utilised by the fermentation bacteria to provide the carbon and energy required by the SRB. This finding can be ascribed to the fact that mainly cellulose and hemicellulose are degraded by the microorganisms. Present research studies concentrate on improving the $\text{GC}_{\text{added}}/\text{SO}_{4\text{removed}}$ ratio by adapting the fermenting microbes to less substrate.

Volatile Suspended Solids concentration

The reactor VSS concentrations were 2.5 g/L feeding synthetic feed water and 0.8 g/L feeding pre-treated mine water, respectively. The first result was explained by the high concentration of organic matter added to the reactor at the beginning of the experimental periods, in the form of GC, SRB (VSS of 5 g/L and 3.6 g/L, Table 1) and RI (VSS of ca. 39 g/L and 25 g/L, Table 1), which partly washed out and was subsequently measured in the outflow (4.1 g/d). When feeding pre-treated mine water, the VSS concentrations decreased, because during this experimental period, the SRB had formed a biofilm on the ceramic rings and the RI had attached to the GC, which consequently resulted in a lower VSS concentration in the effluent (1.6 g/d), since washout was thus prevented.

pH

During both experimental periods, the reactor pH was manually controlled at 6.6-6.9, to accommodate the rumen microorganisms. Since the reactor pH increased due to the SO₄ removal process, this was attained by controlling the pH of the synthetic feed water. The pH, feeding pre-treated mine water varied from 5.14 in the feed to 6.97 in the treated water.

Nutrients

Due to the anaerobic degradation of GC, macro nutrients, such as nitrate (NO₃⁻-N), ammonia (NH₃-N) and phosphate (PO₄³⁻-P) are released, which are used by the microorganisms for their sustainability. Most rumen bacteria obtain their energy for growth through anaerobic fermentation of carbohydrates, while nitrogen is utilised for cell structure. Generally, bacteria use carbon 35-30x faster than they use nitrogen. This implies that the C/N ratio should be in the order of (25-30):1 (Polprasert, 2007). The NO₃-N, NH₃-N and the PO₄-P concentrations in the treated water are listed in Table 3. The nutrient concentrations in the treated water were too high for discharge. However, if the treated water, after sulphate reduction, is used for land irrigation, to grow grass and/or other crops, the increased nutrient and COD concentrations in the treated water can act as fertiliser and soil conditioner, respectively.

Metal removal

Samples were taken from the AMD, as obtained from the mine, from the pre-treated AMD and from the effluent after sulphate removal in HFS. The results are presented in Table 4, where it can be seen that, except for the chromium (Cr) concentration, all metals were removed to levels below 1mg/L, except for manganese (Mn), which decreased from 9 mg/L to 1 mg/L. The iron

concentration of 11 mg/L in the AMD decreased to 0.33 mg/L, already due to AMD pre-treatment. The zinc (Zn) concentration decreased from 1.4 mg/L in the AMD to 0.28 mg/L after pre-treatment and decreased further to values < 0.04 mg/L after the sulphate removal in HFS. The average lead (Pb) concentration remained similar during the sulphide precipitation process.

Table 4. Metal removal from AMD during pre-treatment and SO₄ removal

| Metal (mg/L) | AMD | Pre-treated AMD | HFS Treated water |
|--------------|------|-----------------|-------------------|
| Cr | 0.1 | 0.09 | 0.11 |
| Fe | 11 | 0.33 | 0.3 |
| Pb | 0.25 | 0.34 | 0.22 |
| Mn | 9 | 8 | 1 |
| Ni | 0.53 | 0.4 | 0.09 |
| Zn | 1.4 | 0.28 | < 0.04 |

Comparison of the SO₄ removal results at 25°C and 37-39°C (Greiben *et al.*, 2008a).

When feeding synthetic feed water, operating HFS at 37-39°C, the percentage sulphate removal efficiency was 86% and the SO₄ removal rate was 11.5 g/d. At 25°C this was 82% and 12.1 g/d, respectively. Feeding pre-treated AMD, these values were 67% and 29 g/d at 37-39°C and 86% and 10.4 g/d at 25°C. The higher SO₄ removal rate at 37-39°C feeding pre-treated AMD was explained by the higher feed-rate (30 L/d at 37-39°C compared to 5 L/d at 25°C). From these results it was concluded that high sulphate removal efficiencies can be achieved at 25°C, however, the higher sulphate removal rate at 37-39°C, was due to a higher microbial activity. The limiting degradation rate operating at 25°C is determined by the activity of the fermentative microorganisms. Generally, the microbial reaction rate halves by every 10°C decrease in temperature. The growth and survival of organisms is influenced by temperature, e.g. enzymatic reactions in the cell proceed faster at a higher temperature (Madigan *et al.*, 1997). This observation implied that although the AMD to be treated does not require heating, a lower flow-rate is advisable at 25°C due to the slower microbial fermentation process.

CONCLUSIONS

The results of this study showed that the fermentation microorganisms could adapt to degrade GC at 25°C since high sulphate removal efficiencies were achieved feeding synthetic feed water (82%) and pre-treated mine water (86%). The high sulphate removal efficiency was directly

related to the COD concentration in the reactor. It was observed that the performance during the 135 day and 156 day experimental periods, respectively, was stable. When comparing the treatment of synthetic sulphate-rich water to treatment of pre-treated mine water, most resulting parameters were similar. The ratio $GC_{used}/SO_{4removed}$ was 0.41 feeding synthetic water, which improved to 0.49 when pre-treated mine water was used. This result implied that for every gram sulphate removed two grams of grass were utilised. Using the degradation products of GC and RI is a promising option for treating high sulphate concentrations in mine and other sulphate-rich, industrial wastewaters. The advantage is that grass can be grown at mining sites, using the treated water for irrigation, since this water contains nutrients (NO_3-N and PO_4-P) as degradation products of GC, while the residual carbon is beneficial as soil improver.

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