Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/watres

Simultaneous biodesulphurization and denitrification using an oil reservoir microbial culture: Effects of sulphide loading rate and sulphide to nitrate loading ratio

Shijie An [a], *Kimberley Tang* [b], *Mehdi Nemati* [b,*]

[a] *Division of Environmental Engineering, University of Saskatchewan, 57 Campus Drive, Saskatoon, Canada*
[b] *Department of Chemical Engineering, University of Saskatchewan, 57 Campus Drive, Saskatoon, SK S7N 5A9, Canada*

ARTICLE INFO

Article history:
Received 15 April 2009
Received in revised form
12 October 2009
Accepted 29 October 2009
Available online 4 November 2009

Keywords:
Oil reservoir souring
Sour waters
Desulphurization
Denitrification
Continuous bioreactor
Loading rate
Removal rate

ABSTRACT

Biooxidation of sulphide under denitrifying conditions is a key process in control of souring in oil reservoirs and in treatment of gas and liquids contaminated with sulphide and nitrate. In this work, biooxidation of sulphide was studied using a representative culture originated from an oil reservoir. Effects of sulphide concentration, sulphide to nitrate molar ratio, and loading rates of sulphide and nitrate on their removal rates and composition of the end products were investigated. In the batch system sulphide removal rate passed through a maximum as sulphide concentration was increased from 2.1 to 16.3 mM, with the highest rate (2.06 mM h^{-1}) observed with 10.7 mM sulphide. Nitrate removal was coupled to sulphide oxidation and the highest removal rate was 1.05 mM h^{-1}. In the continuous bioreactors fed with 10 and 5, 15 and 7.5, and 20 and 10 mM sulphide and nitrate, cell wash-out occurred as dilution rate was increased above 0.15, 0.13 and 0.08 h^{-1}, respectively. Prior to cell wash-out linear increases in sulphide and nitrate removal rates were observed as loading rate was increased. The highest sulphide and nitrate removal rates of 2.0 and 0.92 mM h^{-1} were obtained in the bioreactor fed with 15 mM sulphide and 7.5 mM nitrate at loading rates of 2.1 and 0.93 mM h^{-1}, respectively. Short residence times and high sulphide to nitrate ratios promoted the formation of sulphur, a desired end product for ex situ treatment of contaminated streams. Combination of long residence times and low sulphide to nitrate ratios, which favours formation of sulphate, is the suitable strategy for in situ removal of H_2S from oil reservoirs.

1. Introduction

Microbial production of H_2S (souring) is often observed in the offshore and onshore oil reservoirs which are subjected to water flooding for secondary production of oil. Sulphate-reducing bacteria (SRB) are responsible for souring of oil reservoirs (Nemati et al., 2001a). The produced H_2S contaminates oil, gas, and the co-produced water. The toxic and corrosive nature of H_2S, potential for the emission of SO_2 upon combustion of oil and gas, and the need for sustainable use of water have prompted the oil industry to use various ex situ and in situ strategies to control H_2S-associated problems. Treatment of the contaminated fluids through physicochemical processes such as Claus, Alkanolamine, Lo-Cat and Holmes-Stretford (McComas and Sublette, 2001) or biological processes relying on sulphide-oxidizing bacteria are typical

* *Corresponding author*. Tel.: +1 306 966 4769; fax: +1 306 966 4777.
E-mail address: mehdi.nemati@usask.ca (M. Nemati).
0043-1354/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.watres.2009.10.037

examples of ex situ approaches. The physicochemical processes are energy and cost intensive and are feasible for the treatment of large streams containing high levels of sulphide. The biological processes by contrast operate at ambient pressure and temperature without the need for expensive catalysts. Moreover, biological processes are effective for the removal of low levels of sulphide such as those usually present in the contaminated co-produced waters and biogas (Tang et al., 2009).

Removal of sulphate from the water prior to injection, application of biocides, and addition of metabolic inhibitors such as nitrite and molybdate are common in situ approaches used to suppress the sulphate-reducing bacteria (SRB) and to control the reservoir souring (Nemati et al., 2001b; Thorstenson et al., 2002; Kodama and Watanabe, 2003; Greene et al., 2006). Banking on the fact that oil reservoirs harbour a diverse microbial population, a biological approach relying on the manipulation of the resident microbial community through addition of nitrate has been developed to tackle reservoir souring. The underlying principle of this approach is the stimulation of resident heterotrophic nitrate-reducing bacteria (h-NRB) and autotrophic sulphide-oxidizing species (NR-SOB). In the presence of nitrate, h-NRB out-compete SRB for the common electron donor, thus biogenic formation of sulphide is eliminated. This is possible due to the fact that nitrate reduction is thermodynamically more exergonic than sulphate reduction (Eckford and Fedorack, 2002). NR-SOB use sulphide as the energy source, converting it to sulphur or sulphate thereby decreasing the level of sulphide already present in the system. The effectiveness of nitrate amendment has been demonstrated in model laboratory systems (Myhr et al., 2002; Okabe et al., 2002; Hubert et al., 2003; Kodama and Watanabe, 2003; Kjellerup et al., 2005; O'Reilly and Colleran, 2005; Hubert and Voordouw, 2007; Kaster et al., 2007) and in the onshore and off shore reservoirs (Telang et al., 1997; Larsen et al., 2004). Field testing conducted in the Coleville oil field, Saskatchewan, Canada in 1996 is one of the earliest attempts in which 6.26 mM ammonium nitrate was added to the injection water over a period of 50 days. During this trial sulphide was not detected in one of the two injectors employed, and sulphide content of co-produced water from two adjacent producing wells decreased by 50–60% (Telang et al., 1997). In a follow-up study with microbial cultures enriched from the Coleville oil field, Nemati et al. (2001a) reported that the addition of nitrate and an NR-SOB enrichment to an SRB consortium inhibited the production of sulphide and led to removal of the present sulphide. Although nitrate-mediated control of souring has been studied extensively, information on biooxidation of sulphide by a representative culture from an oil reservoir is limited and the underlying principles are not well understood.

In the present work a microbial culture enriched from the produced water of the Coleville oil field has been used to study the biooxidation of the sulphide under denitrifying conditions. The effects of sulphide concentration and the ratio of sulphide to nitrate concentrations on the kinetics and composition of the end products have been investigated in the batch system. The potential of this culture for ex situ treatment of contaminated waters has been further assessed in continuous bioreactors, with the focus being on the effects of sulphide and nitrate loading rates on the removal of sulphide and nitrate and composition of the end products.

2. Materials and methods

2.1. Microbial culture and medium

A mixed culture enriched from the produced water of the Coleville oil field, Saskatchewan, Canada was used in this work using Coleville Synthetic Brine (CSB) as growth medium (Nemati et al., 2001a). To enrich this culture two serum bottles (125 mL) were charged with 100 mL of CSB medium. The medium was purged with N_2 for 5 min. The bottles were then sealed with a rubber septum and aluminium cap, and autoclaved for 30 min at 121 °C. After reaching room temperature, filter-sterilized solution of Na_2S (1 M) was added to these bottles to achieve sulphide concentration of 5 mM. The pH was adjusted to 6.8–7.2 using 4 M HCl. The bottles were then inoculated by Coleville-produced water (25% v/v) and maintained at room temperature (22–23 °C). The established culture was used as inoculum (10% v/v) in the subsequent bi-weekly sub-culturings (average protein concentration of the stock cultures: 22.9 ± 1.9 mg L^{-1}). Reverse sample genome probing of the Coleville enrichment has shown that *Thiomicrospira* sp. CVO is the main member and *Desulfovibrio* sp., *Desulfomicrobium* sp., *Desulfobulbus* sp., *Bacillus* sp., *Vibrio* sp., *Pseudomonas* sp., *Eubacterium* sp., *Serratia* sp. and *Yersinia* sp. are present in smaller proportion (Nemati et al., 2001a), although we did not reconfirm this in the present study.

2.2. Batch experiments

The effect of sulphide initial concentration on sulphide biooxidation and denitrification was investigated in serum bottles containing 100 mL sterilized CSB medium with 10 mM nitrate. Filter sterilized Na_2S solution (1 M) was added to these bottles to achieve the desired sulphide concentrations, and pH was adjusted to 6.8–7.2. Bottles were inoculated with 10 mL (10% v/v) of a 3-day-old enrichment culture (5.5 ± 0.8 mg protein L^{-1}). Sulphide concentrations in these bottles were determined immediately and were 2.1, 6.3, 10.7, 16.3 and 21 mM. The bottles were maintained at room temperature (22–23 °C) and sampled regularly. Sulphide concentration was determined immediately after sampling. The remaining portion of the sample was centrifuged for 5 min at $9180 \times g$ and the supernatant was preserved in a freezer (−75 °C) for further analysis. Thiosulphate, sulphate, nitrate, nitrite and acetate concentrations were determined in these samples. The effect of sulphide to nitrate molar concentrations ratio (0.4 to 3.5) on sulphide biooxidation, denitrification and composition of the end products was determined by conducting additional experiments in serum bottles containing 100 mL sterilized CSB with either 1.25, 2.5, 5.0, 7.5, 10 or 15 mM nitrate. The initial sulphide concentration in all bottles was adjusted to 6–6.5 mM and pH was set at 6.8–7.2. A 3-day-old enrichment culture was used as the inoculum (10% v/v). The exact sulphide to nitrate ratios after adjustment of pH and inoculation were 3.13, 2.39, 1.19, 0.85, 0.58 and 0.41. All other conditions and monitoring approaches were similar to those

described earlier. All batch experiments were carried out in duplicate. Control runs (in duplicate) were conducted under similar conditions without inoculation.

2.3. Continuous experiments

2.3.1. Effects of sulphide concentration and loading rate

The effects of sulphide concentration and loading rate on sulphide removal and denitrification were investigated in three identical continuous bioreactors (Fig. 1). Each system consists of a glass stirred tank bioreactor (working volume: 230 mL) and a magnetic stirrer. CSB medium containing sulphide and nitrate at the designated concentrations was pumped into each bioreactor continuously using a variable speed peristaltic pump. Effluent was transferred into a container through an overflow tube. CSB medium was prepared in a 2-L glass flask and autoclaved for 30 min at 121 °C. Once cooled to room temperature, the medium was purged with filter sterilized nitrogen for 30 min. Sulphide stock solution (1 M) was then added to achieve the desired concentration and pH was adjusted to 6.8–7.2. The medium was then transferred from the flask to a sterile collapsible medium bag (2 L) connected to each bioreactor by introducing pressurized sterilized nitrogen gas into the flask. Use of collapsible bags allowed proper operation of the pumps and maintenance of the anaerobic conditions.

Each bioreactor was charged with CSB medium containing 5 mM sulphide and 10 mM nitrate, and inoculated with 30 mL of a 3-day-old enrichment culture. Once complete removal of sulphide was achieved, CSB medium containing either 10 and 5 mM or 15 and 7.5 mM or 20 and 10 mM sulphide and nitrate, respectively, was pumped into the bioreactor at a flow rate of 0.2–0.5 mL h^{-1}. The flow rate was increased stepwise until cell wash-out occurred. At each flow rate sufficient time was given for the establishment of steady-state conditions (3–5 residence times). Steady-state conditions were assumed when 100% removal of sulphide was achieved or when residual sulphide and nitrate concentrations changed less than 10% over 3 days. The experiments were carried out at room temperature (22–23 °C). Concentrations of sulphide, sulphate, thiosulphate, nitrate, nitrite, and acetate were determined daily. Protein concentration and pH were determined at each flow rate at steady-state conditions. Experiments with medium containing 20 mM sulphide and 10 mM nitrate were repeated to ensure the reproducibility. Concentrations of various ions in the feed were also monitored regularly.

2.3.2. Effect of sulphide to nitrate loading rates ratio

Two additional runs aiming to identify the effect of sulphide to nitrate loading ratio were conducted. The bioreactors were run batch-wise, initially following the exact procedure described earlier. Upon complete removal of sulphide, medium was pumped into the bioreactors at constant flow rates of 3.45 and 17.25 mL h^{-1}, corresponding to dilution rates of 0.015 and 0.075 h^{-1}, respectively. CSB media containing approximately 15 mM sulphide and various concentrations of nitrate corresponding to sulphide to nitrate ratios of 0.10, 0.26, 0.55, 0.96, 2.13 and 3.39 were then tested. With each medium sufficient time was given for the establishment of steady-state conditions, then the next medium was tested. The experiments were carried out at room temperature. Concentrations of various ions were determined on a regular basis. Operating the bioreactors at different dilution rates (low and high) while varying the ratio of sulphide to nitrate loading rates allowed us to verify the effect of available nitrate, as well as the dilution rate (residence time) on the composition of the end products.

2.4. Analytical procedures

Concentration of sulphide was determined using a spectrophotometric method described elsewhere (Cord-Ruwisch, 1985). Concentrations of other ions were determined using a Dionex Ion chromatograph (ICS-2500) with a thermal conductivity detector (CD25A), an IonPac CG5A guard column and an IonPac CS5A analytical column (Dionex Corporation, Sunnyvale, CA, USA). The eluent was 1.0 mM KOH at a flow rate of 1.5 mL h^{-1}. The system was calibrated using standard solutions of each ion at concentrations in the range 5–20 mg L^{-1}. A Coomassie Plus Bradford assay kit was used to determine the protein concentration (Pierce, Rockford, IL, USA). The liquid sample (0.5–1 mL) was first sonicated for

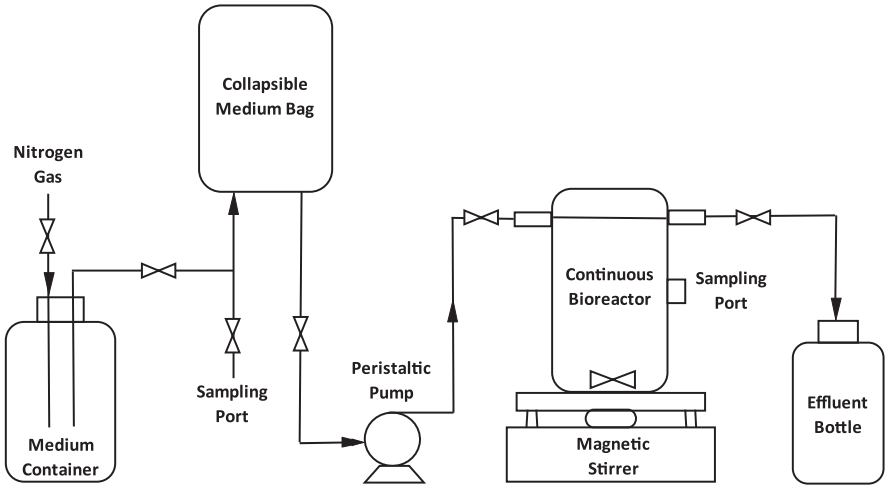

Fig. 1 – Schematic of the experimental system used for continuous biooxidation of sulphide and denitrification.

2 min at 10 W using a Branson 450 Sonifier (Branson Ultrasonics, Dansbury, CT, USA). The sample was then mixed with the assay reagent and the absorbance of the resulting mixture was measured at 595 nm using a UVmini-1240 spectrophotometer (Shimadzu, Japan). A calibration curve based on the standard solutions of bovine serum albumin was used to determine the protein concentration.

3. Results

3.1. *Batch experiments*

Fig. 2 (left panels) presents selected results obtained in the batch experiments aiming to verify the effects of sulphide initial concentration. With 2.1, 6.3 and 10.7 mM sulphide, the lag phase was relatively short but an extended lag phase of 240 h was observed with 16.3 mM sulphide. In all cases biooxidation occurred in two distinct stages. First, sulphide concentration decreased continuously but sulphate concentration remained constant. Once concentration of sulphide reached to a low level, a continuous but slow increase in concentration of sulphate was observed. During this phase the turbidity of the liquid decreased and in most cases the liquid eventually became clear, an indication of oxidation of sulphur to sulphate. Sulphide oxidation was accompanied by denitrification, which also occurred in two distinct phases, coinciding with removal of sulphide (oxidation of sulphide to sulphur) and production of sulphate (oxidation of sulphur to sulphate), with slower nitrate removal rates observed during

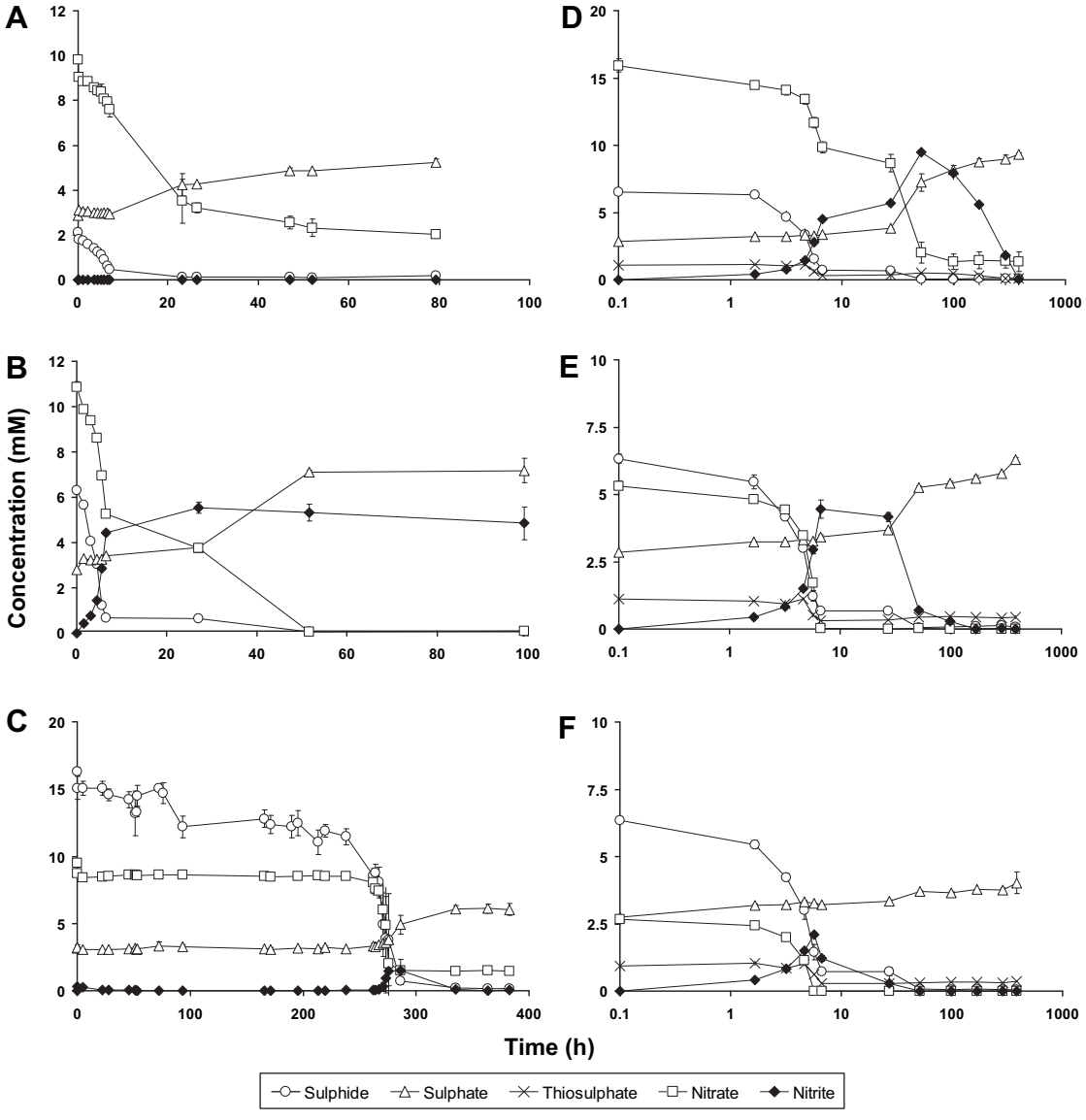

Fig. 2 – Profiles of sulphide, sulphate, thiosulphate, nitrate and nitrite concentrations during the biooxidation of sulphide under various conditions. Left panels: sulphide concentrations of 2.1 mM (A), 6.3 mM (B), 16.3 mM (C), all with 10 mM nitrate. Right panels: 6–6.5 mM sulphide and various nitrate concentrations corresponding to sulphide to nitrate ratios of 0.41 (D), 1.19 (E) and 2.39 (F). Data points represent average concentration based on the results of the duplicate runs, and error bars represent the corresponding standard deviations.

the second phase. Except for the culture containing 2.1 mM sulphide, reduction of nitrate led to production of nitrite. With 6.3 mM sulphide, concentration of nitrite decreased slowly from an initial value of 5.5 to 4.8 mM at the end of the experiment. With 10.7 and 16.3 mM sulphide, the levels of produced nitrite were 4 and 1.5 mM, respectively and nitrite was utilized as biooxidation proceeded. A small amount of thiosulphate (0.5–1 mM) was detected in the samples taken immediately after the inoculation. It seems that thiosulphate was not an intermediary product as the level of present thiosulphate remained unchanged (data not shown). A slight decrease of 0.5 mM in concentration of acetate was observed at the end of experimental runs (data not shown). However, this decrease could be due to measurement error as the standard deviation for acetate concentration was 0.5–0.6 mM. It seems that under the applied conditions bacteria relied on the carbonate as the sole carbon source. With 21 mM sulphide, apart from an initial decrease of 2 mM, potentially due to spontaneous chemical oxidation, sulphide concentration remained constant even after a prolonged period of monitoring. Nitrate concentration also remained constant and no nitrite was detected, indicating that bacteria were not active.

Sulphide removal rates (slope of the linear part of the concentration profiles) in the cultures initially containing 2.1, 6.3, 10.7, and 16.3 mM sulphide were 0.21, 0.95, 2.06 and 0.59 mM h^{-1}, respectively. The extended lag phase and lower oxidation rate obtained with 16.3 mM sulphide, and inability of bacteria to oxidize 21 mM sulphide indicate sulphide inhibition effect. The nitrate removal rates in the cultures containing 2.1, 6.3, 10.7, and 16.3 mM sulphide were 0.25, 0.78, 1.05 and 0.40 mM h^{-1}, respectively. With 2.1 and 6.3 mM sulphide, nitrate removal rates of 0.03 and 0.12 mM h^{-1} observed during the production of sulphate, respectively. With 10.7 and 16.3 mM sulphide, nitrate was used completely during the removal of sulphide, and produced nitrite served as electron acceptor during the production of sulphate, with the nitrite reduction rates being 0.05 and 0.03 mM h^{-1}, respectively. In the control systems a small portion of sulphide (8–15%) was oxidized in the first few hours, possibly due to spontaneous chemical oxidation of sulphide. Following this, sulphide concentration remained constant. Nitrate reduction did not take place and nitrite was not detected.

Selected results representing the effect of sulphide to nitrate ratio are shown in Fig. 2 (right panels). Choosing an initial sulphide concentration of 6–6.5 mM allowed frequent sampling and close monitoring of the reactions during the first stage of biooxidation. With ratios in the range 0.4 to 2.4 sulphide, removal rates were in the range 0.88–0.95 mM h^{-1}. With a ratio of 3.1 a residual sulphide concentration of 0.5–1 mM and a lower rate of 0.76 mM h^{-1} was observed, likely due to lack of sufficient nitrate. In all cases nitrate reduction and concomitant production of nitrite occurred during the removal of sulphide. The reduction rate of nitrate decreased from 0.95 to 0.54 mM h^{-1} as sulphide to nitrate ratio increased from 0.4 to 3.1. For ratios in the range 0.4–0.8, reduction of nitrate continued during the production of sulphate, though at much slower rates of 0.18 and 0.06 mM h^{-1}, respectively. With ratios in the range 0.4–1.1 higher levels of nitrite were produced, as expected. In all cases nitrite was eventually reduced but only after complete utilization of nitrate, indicating that the nitrate was the preferred electron acceptor for the bacteria. In the control systems an initial decrease in sulphide concentration was observed. Nitrate concentration remained unchanged and nitrite was not detected.

Biooxidation of sulphide under denitrifying conditions occurs through a number of pathways, resulting in formation of sulphur, sulphate, nitrite and nitrogen gas as outlined below (Cardoso et al., 2006, Gadekar et al. 2006):

$$S^{2-} + NO_3^- + 2H^+ \rightarrow S^o + NO_2^- + H_2O \quad (1)$$

$$S^{2-} + 0.4NO_3^- + 2.4H^+ \rightarrow S^o + 0.2N_2 + 1.2H_2O \quad (2)$$

$$1.5S^{2-} + NO_2^- + 4H^+ \rightarrow 1.5S + 0.5N_2 + 2H_2O \quad (3)$$

$$S^{2-} + 4NO_3^- \rightarrow SO_4^{2-} + 4NO_2^- \quad (4)$$

$$S^{2-} + 1.6NO_3^- + 1.6H^+ \rightarrow SO_4^{2-} + 0.8N_2 + 0.8H_2O \quad (5)$$

The following reactions describe the common pathways for oxidation of sulphur to sulphate:

$$S^o + 3NO_3^- + H_2O \rightarrow SO_4^{2-} + 3NO_2^- + 2H^+ \quad (6)$$

$$S^o + 1.2NO_3^- + 0.4H_2O \rightarrow SO_4^{2-} + 0.6N_2 + 0.8H^+ \quad (7)$$

$$S^o + 2NO_2^- \rightarrow SO_4^{2-} + N_2 \quad (8)$$

Results of our batch study showed that sulphide was first converted to sulphur or other intermediary compounds. In all cases including control systems, over the first 1–2 h a slight decrease in sulphide concentration and an increase in sulphate concentration (0.5–1 mM) were observed, likely due to spontaneous oxidation of sulphide. Apart from this there was no increase in concentration of sulphate until sulphide concentration decreased to zero. Following this, oxidation of intermediary sulphur compounds, especially elemental sulphur, caused a continuous increase in sulphate concentration. It was attempted to determine the level of suspended solids as an indication of the present sulphur, but the limited volume of sample taken from the cultures did not allow for accurate quantifications. The turbidity of the cultures during the first stage of sulphide oxidation, and decrease in this turbidity during the production of sulphate especially in the cultures with low sulphide to nitrate ratio, are strong indications that the sulphur was the main intermediary product. Reduction of nitrate led to accumulation of nitrite, which was utilized after exhaustion of nitrate. The observed patterns overall indicate that under the conditions investigated in these experiments reaction 1 was the first step in sulphide biooxidation at ratios in the range 0.4 to 1.2, while at ratios of 2.4 and 3.1, due to lack of sufficient nitrate both reactions 1 and 3 were involved. Furthermore, depending on the ratio of sulphide to nitrate, oxidation of the produced sulphur proceeded through various combinations of reactions 6, 7 and 8. At the lowest ratio of 0.41 reactions 6 and 8 were involved, with ratios of 0.58 and 0.85 all three reactions were involved, and with ratios of 1.2 and 2.4 only reaction 8 was involved. At the highest ratio of 3.1, due to lack of sufficient nitrate, oxidation of sulphur to sulphate did not take place.

Fig. 3 shows the effect of sulphide to nitrate ratio on the percentage of sulphide converted to sulphate. At the lowest ratio of 0.2 sulphate was the sole end product, and as this ratio was increased the level of produced sulphate decreased. With the highest ratio of 3.1 only 4.4% of sulphide was converted to sulphate. Due to involvement of several reactions in the overall process, it is rather difficult to determine the theoretical percentage of sulphate as expected from the stoichiometry.

3.2. Continuous experiments

3.2.1. Effects of sulphide initial concentration and loading rate

The continuous bioreactors were run with media containing 9.62 ± 1.05, 14.42 ± 1.32 and 19.07 ± 1.08 mM sulphide, and a constant sulphide to nitrate ratio of 2. A typical set of data obtained in the continuous bioreactors is shown in Fig. 4. Thiosulphate at concentrations of 1.29 ± 0.43, 2.31 ± 0.88 and 3.71 ± 0.96 mM were present in these media, respectively. Thiosulphate must have formed as a result of spontaneous oxidation of sulphide, as thiosulphate is not an ingredient of the CSB medium. In the bioreactor operated with lowest sulphide concentration, residual sulphide and nitrate concentrations were close to zero for the dilution rates up to 0.15 h^{-1}. Further increase of dilution rate caused a sudden increase in concentrations of both ions, and complete cell wash-out occurred at $D = 0.17$ h^{-1}. No nitrite was detected in the bioreactor over the entire range of applied dilution rates, neither during the transition nor after establishment of steady-state conditions. At low dilution rates thiosulphate concentration was close to that in the medium. However, as higher dilution rates were applied thiosulphate concentration decreased to around 0.5 mM, indicating oxidation of thiosulphate. Sulphate concentration was around 6.8 mM for dilution rates in the range 0.006–0.012 h^{-1} and decreased continuously as dilution rate was increased. This coincided with increase in turbidity and presence of sulphur particles, an indication that at lower dilution rates (higher residence times) most of the sulphide was converted to sulphate and at higher dilution rates (shorter residence times) sulphur was likely the main product. No obvious trend in acetate concentration was established, and its average value was close to that in the feed. Protein concentration fluctuated in the range 15–38 mg protein L^{-1} (average concentration 24.4 ± 8.7) for dilution rates up to 0.15 h^{-1} which then dropped to zero when dilution rate increased to 0.17 h^{-1}. Environmental pH fluctuated in the range 7.6–7.9. The concentration profiles in the bioreactors operated with higher feed sulphide concentrations were similar to those in the previous run (data not shown). With 14.42 ± 1.32 and 19.07 ± 1.08 mM sulphide, cell wash-out occurred at a dilution rates around 0.13 and 0.08 h^{-1} and average protein concentrations were 60.5 ± 23.3 and 38.1 ± 16.7 mg L^{-1}, respectively. In both bioreactors pH was in the range 7.4–8.0.

In all bioreactors before cell wash-out sulphide removal percentage was in the range 94–98%, and a linear dependency between sulphide removal rate and loading rate was observed (Fig. 5, left panel). The rates calculated for each case interestingly fall on the same line, with the slope of this line being equal to the average sulphide removal percentage of 96%. The maximum sulphide removal rates in the bioreactors

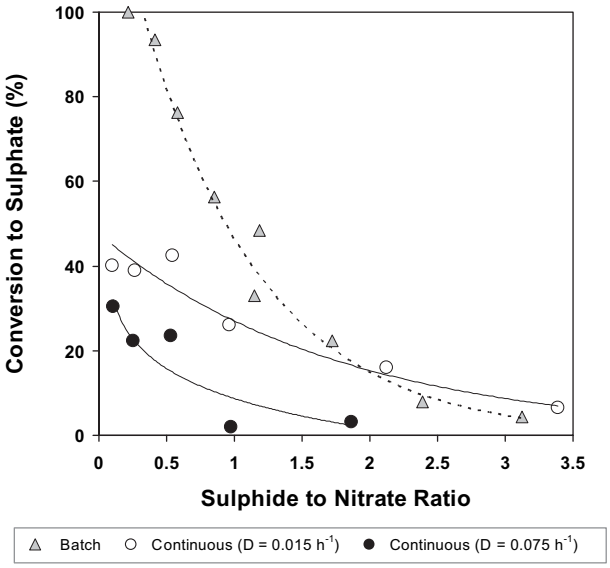

Fig. 3 – Percentage of sulphide converted to sulphate as a function of sulphide to nitrate ratio in the batch and continuous systems.

operated with 9.62 ± 1.05, 14.42 ± 1.32 and 19.07 ± 1.08 mM sulphide were 1.41, 2.02 and 1.47 mM h^{-1} (1.85, 1.07, 1.23 mg S^{2-} mg protein^{-1} h^{-1}) and observed at the loading rates of 1.46, 2.1 and 1.51 mM h^{-1} (residence times of 6.7, 7.7 and 12.5 h), respectively. Prior to cell wash-out nitrate removal percentage was in the range 98–100% and the linear dependency between the nitrate removal and loading rates was observed, with the slope of this line being 99% (Fig. 5, right panel). The maximum nitrate removal rate for the bioreactors operated with 9.62 ± 1.05, 14.42 ± 1.32 and 19.07 ± 1.08 mM sulphide were 0.72, 0.92 and 0.75 mM h^{-1} (1.86, 0.96, 1.24 mg NO_3^- mg protein$^{-1}h^{-1}$) and observed at nitrate loading rates of 0.75, 0.93 and 0.75 mM h^{-1}, respectively. The calculated molar removal rates of sulphide in all cases were almost twice of the nitrate removal rates. Considering that the ratio of sulphide to nitrate loading rates was also close to 2 and that the dependencies of the removal rate on the loading rate for both sulphide and nitrate were identical, one can conclude that under applied conditions reduction of nitrate was coupled to oxidation of sulphide and acetate did not contribute to this process.

The effect of sulphide loading rate (residence time) on the extent of produced sulphate is shown in Fig. 6. It can be seen that the increase of the loading rate (decrease of the residence time) decreased the percentage of sulphide converted to sulphate, regardless of feed sulphide concentration. The highest conversion of 41% was observed at a loading rate of 0.012 mM h^{-1}, and with loading rates above 1.5 mM h^{-1} only a small portion of sulphide (0–7%) was converted to sulphate. This was in agreement with batch results in which oxidation rate of sulphur to sulphate was much slower than oxidation of sulphide to sulphur, and confirmed that the residence time (loading rate) can be used to control the composition of end products.

3.2.2. Effect of sulphide to nitrate loading rates ratio

In the bioreactor operated at a dilution rate of 0.015 h^{-1} for the entire range of applied ratios (0.1–3.39) residual sulphide

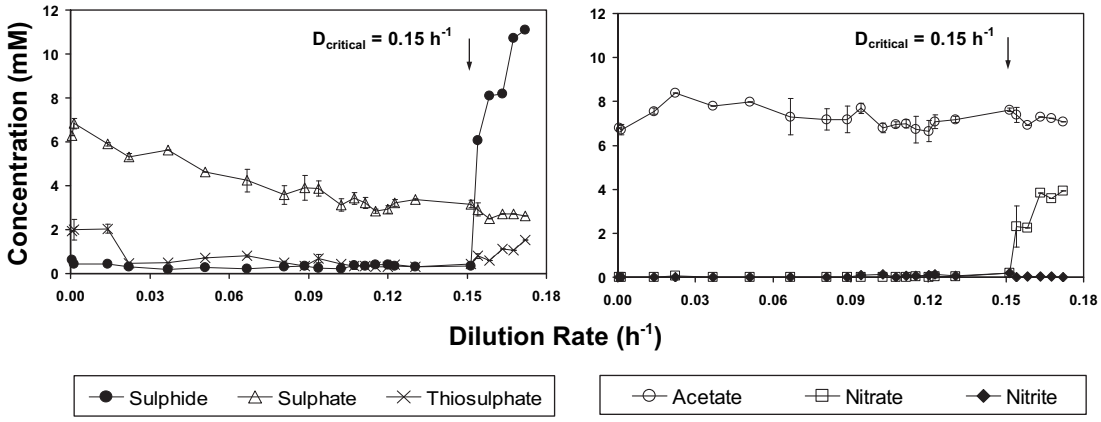

Fig. 4 – Steady-state concentrations of sulphide, sulphate, thiosulphate, nitrate, nitrite and acetate in the continuous bioreactor operated with medium containing 9.62 ± 1.05 mM sulphide with a sulphide to nitrate ratio of approximately 2. Data points represent average concentration for the samples taken in two or three consecutive days following the establishment of steady state at each flow rate, and error bars represent the associated standard deviation.

concentration was 0.3–0.6 mM (conversion: 98–100%). Thiosulphate concentration was also relatively constant and close to that in the feed. Sulphate concentration, however, increased as the ratio of sulphide to nitrate was decreased from 3.39 to 0.55 (data not shown). Residual nitrate (89 mM) was only present at the lowest ratio of 0.1, which corresponded to the highest level of nitrate in the feed (152 mM). Accumulation of nitrite was only observed at 0.25 and 0.1 ratios. Contrary to the previous runs, acetate concentration decreased as the ratio of sulphide to nitrate loading rates was decreased, and was zero for 0.55, 0.26 and 0.1 ratios. This, together with utilization of nitrate at a level higher than that required for complete oxidation of sulphide to sulphate, signified a shift in metabolism of microbial population toward the utilization of acetate as an electron donor during the reduction of nitrate. It should be pointed out that with sufficient sulphide, acetate was not a preferred electron donor and was utilized only after the exhaustion of sulphide. One should bear in mind that acetate concentration in the feed was around 10 mM and acetate was likely a limiting factor in reduction of excess nitrate.

The response observed in the bioreactor operated at a dilution rate of 0.075 h^{-1} was somewhat different. The most notable difference occurred at a sulphide to nitrate ratio of 3.5 in which cell wash-out occurred after 1 day of operation. It seems that combination of a short residence time and absence of the sufficient electron acceptor hampered the growth and cell activity and led to their wash-out. With other applied ratios, 96–98% of sulphide was removed. Higher concentrations of sulphate were observed as the ratio of sulphide to nitrate was increased, but for the similar ratios the level of produced sulphate at a dilution rate of 0.075 h^{-1} was lower than that at 0.015 h^{-1}, likely due to a shorter residence time in the former case. Residual nitrate concentrations of 7.3, 9.9, 30.8 and 126.1 were measured at the ratios of 1, 0.5, 0.25 and 0.1, respectively. Removal of nitrate only occurred during the

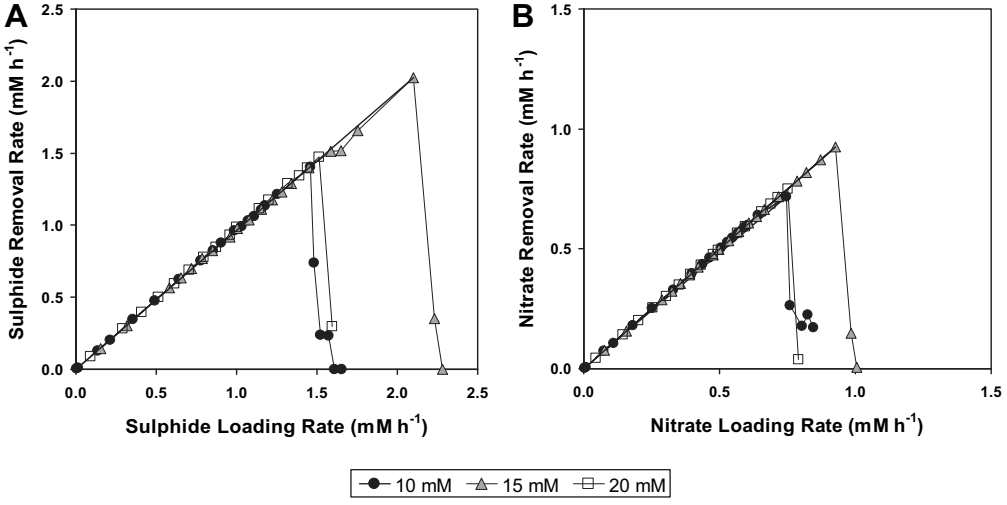

Fig. 5 – Dependency of the removal rate on the loading rate for sulphide (A) and nitrate (B) in the continuous bioreactors. Feed sulphide concentrations are given in the legend.

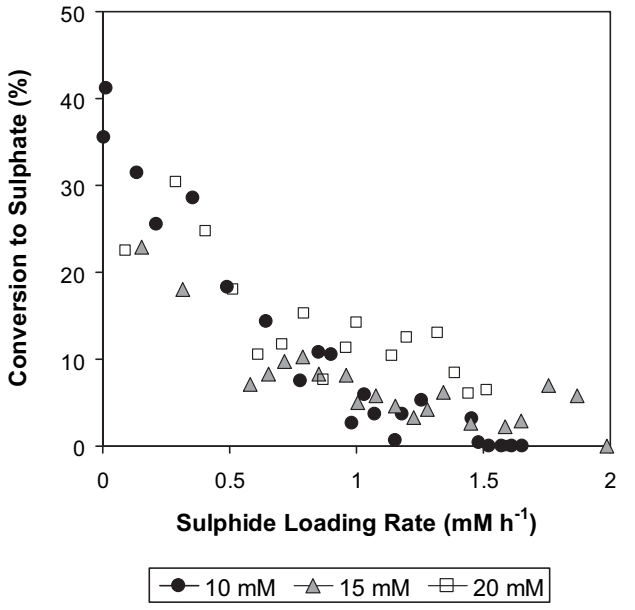

Fig. 6 – Effect of sulphide volumetric loading rate (residence time) on the extent of sulphide converted to sulphate in the continuous bioreactors. Feed sulphide concentrations are given in the legend.

oxidation of sulphide and acetate was not used for nitrate reduction, even after exhaustion of sulphide. The reason for this observation is not clear, and further work with this culture is required to verify the underlying principles of heterotrophic nitrate reduction. The average sulphide loading rates in the bioreactors operated at 0.015 and 0.075 h^{-1} were 0.23 and 1.12 mM h^{-1}, with the corresponding sulphide removal rates being 0.22 and 1.08 mM h^{-1}, respectively. Nitrate removal was in the range 98–100% for loading rates up to 0.86 mM h^{-1} and decreased continually as loading rate was increased further (Fig. 7, left panel). The linear dependency of nitrate removal rate on its loading rate was observed for loading rates up to 4.34 mM h^{-1} (Fig. 7, right panel), with highest removal rate being 2.03 mM h^{-1}. The increase in volumetric loading rate to 11.24 mM h^{-1} led to a lower removal rate of 1.79 mM h^{-1}. It seems that application of higher concentrations of nitrate in the feed and consequential increases in nitrate loading rate could lead to higher removal rates, and that nitrate at high concentrations would not impose a strong inhibitory effect.

The variation in percentage of sulphide converted to sulphate in the bioreactors operated at 0.015 h^{-1} (residence time: 66.7 h) and 0.075 h^{-1} (residence time: 13.3 h) are included in Fig. 3. Consistent with the pattern observed in the batch system, decreases in the ratio of sulphide to nitrate caused the conversion of a larger portion of the sulphide to sulphate in both bioreactors. For similar ratios, however, this conversion was higher in the bioreactor operated at a lower dilution rate of 0.015 h^{-1}, which reconfirmed the determining role of the residence time on the composition of the end products.

4. Discussion

Bacterial oxidation of sulphide is instrumental in biotreatment of acid mine drainage, in situ removal of H_2S from the oil reservoirs, desulphurization of the natural gas and biogas, and in treatment of sour waters that are produced during the enhanced oil recovery. Sulphide biooxidation is carried out by both photoautotrophic and chemolithotrophic sulphide oxidizers. The energy intensiveness and challenges in effective supply of light energy are major impediments in utilization of phototrophs (Tang et al., 2009). Chemolithotrophic sulphide oxidizers use oxygen (aerobic species) or nitrate (anaerobic species) as terminal electron acceptors during the oxidation of sulphide (Tang et al., 2009). Chemolithotrophic oxidation of sulphide in the presence of oxygen has been the subject of a number of studies aiming to improve the removal rate of sulphide and maximizing the production of sulphur (Annachhatre and Suktrakoolvait, 2001; Alcantara

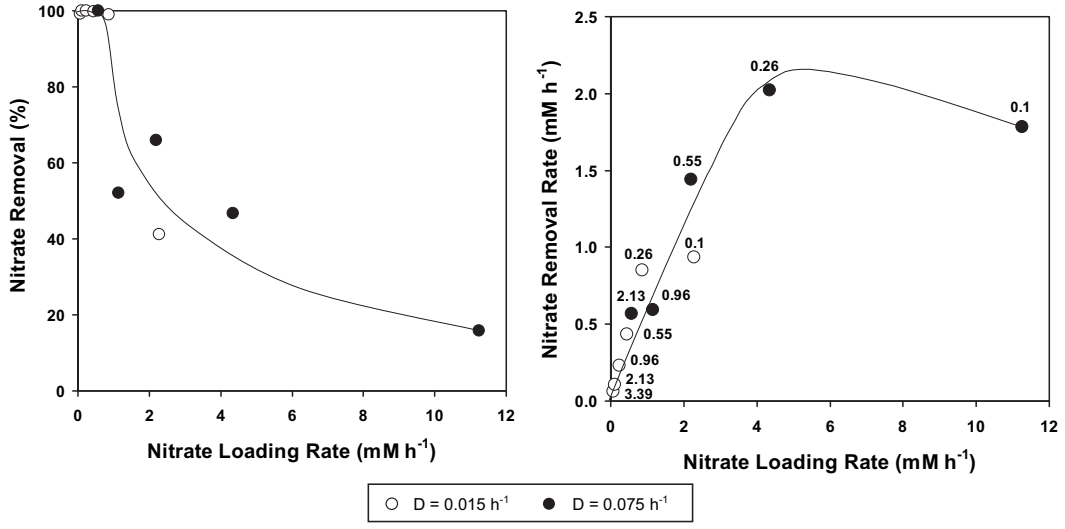

Fig. 7 – Nitrate removal percentage and removal rate a function of loading rate for bioreactors operated at 0.015 and 0.075 h^{-1} and various sulphide to nitrate ratios. Numbers above the data points in the right panel are the applied ratios.

et al., 2004; Ng et al., 2004; Duan et al., 2005; Lee et al., 2006; Datta et al., 2007; van der Zee et al., 2007). The required energy for the aeration and safety risk associated with treatment of gaseous stream under an oxygen-rich environment are some of the main drawbacks of this approach. Anaerobic option eliminates the aeration cost and the safety concerns, and could be used for simultaneous desulphurization and denitrification. The renewed interest in anaerobic sulphide biooxidation also stems from its instrumental role in control of souring in onshore and offshore oil reservoirs. Using a pure culture of *Thiomicrospira* CVO, Gadekar et al. (2006) reported sulphide removal rates of 0.50, 0.57, 0.70 and 0.43 mM h^{-1} for initial sulphide concentrations of 6.3, 8.7, 12.8 and 16.2 mM, respectively. Although applied concentrations are not exactly the same as those in the present work, sulphide removal rates achieved by the Coleville enrichment are higher than those reported for *Thiomicrospira* strain CVO, an indication that the other members of this consortium may have had a positive impact on sulphide removal. Simultaneous oxidation of sulphide, sulphur and thiosulphate coupled to nitrate reduction was investigated by Cardoso et al. (2006) in a batch system. Increases in initial sulphide concentration from 2.5 to 10 mM decreased sulphide and nitrate removal rates by almost 21-fold. For sulphide to nitrate ratios of 0.62 and 2.5, sulphate and sulphur were reported as the main end products, respectively. Despite the differences in the origin and make-up of the cultures, findings of the present work are in agreement with those reported by Cordoso et al. For instance, in the present work for a sulphide to nitrate ratio of 0.62 around 76% of the sulphide was converted to sulphate, while at a ratio of 2.5 the conversion to sulphate was only 10%. Similarly, the inhibitory effect of sulphide was also observed in the present work, although at a much higher concentration of 16.3 mM.

Reyes-Avila et al. (2004) studied the removal of acetate, nitrate and sulphide in a continuous bioreactor. The loading rates of acetate and nitrate were maintained at 0.50 and 0.59 mM h^{-1}, respectively, while sulphide loading rate was increased in the range 0.05–0.38 mM h^{-1}. Nitrate removal efficiencies close to 100% were observed for the entire range of sulphide loading rates, while sulphide removal percentage increased from 24% to 98% when sulphide loading rate was increased. The maximum nitrate and sulphide removal rates were reported as 0.59 and 0.37 mM h^{-1}, respectively. In spite of maintaining a low sulphide to nitrate ratios (0.64 or lower), sulphur was the main end product. At the highest sulphide loading rate only 65% of the acetate was used, while in the absence of sulphide 94% of acetate was utilized. De Gusseme and De Schryver, 2009) studied simultaneous oxidation of sulphide and reduction of nitrate with a microbial culture dominated by *Arcobacter* sp. in a continuous bioreactor. Complete removal of sulphide, nitrate and acetate at respective concentrations of 100, 17.5 and 87.5 mg L^{-1} was achieved at a residence time of 1 day, suggesting that both chemolithotrophic and heterotrophic nitrate reductions occurred in the reactor. The highest sulphide removal rate observed in this work was around 52 mg sulphide $(gVSS\text{-}h)^{-1}$. Sulphate formed 19% of the final product with elemental sulphur or polysulfide being the remaining 81%. This was expected, considering that the molar ratio of sulphide to nitrate was around 2.6. Using *Thiobacillus denitrificans* Wang et al. (2005) investigated simultaneous desulphurization and denitrification in a continuous bioreactor. At sulphide and nitrate loading rates of 0.78 and 0.31 mM h^{-1}, 75% sulphide and 90% nitrate were removed, respectively and the corresponding removal rates were 0.58 and 0.28 mM h^{-1}. Vaiopoulou et al. (2005) operated a continuous bioreactor with sulphide and nitrate loading rates of 0.61 and 0.84 mM h^{-1}, respectively and reported the complete removal of both ions. Under applied conditions sulphate was the sole end product. McComas and Sublette, 2001) used a mixture of *Thiomicrospira* CVO, *Arcobacter* FWKO B and Coleville brine enrichment in a fed batch bioreactor, and reported complete oxidation of 10,000 ppmv gaseous H_2S a in less than 3 s. The maximum removal rate obtained in this work was 2.9–3.1 mmol H_2S $(g\ biomass\text{-}h)^{-1}$. Gadekar et al. (2006) used *Thiomicrospira* sp. CVO in a continuous bioreactor fed with 17.8 ± 0.8 sulphide and 10 mM nitrate. The maximum sulphide removal rate of 2.4 mM h^{-1} was obtained at a loading rate of 3.2 mM h^{-1}. Under these conditions only 76% of sulphide was removed and sulphur was the main end product.

A close examination of the literature reveals that the results of the present work are in agreement with those reported earlier, and imply that higher ratios of sulphide to nitrate favour the dominance of sulphur as the end product. The only exception is the data presented by Reyes-Avila et al. (2004) in which despite maintaining a low ratio of 0.64, sulphur was the main end product. This unexpected result could be attributed to utilization of nitrate for simultaneous oxidation of acetate and sulphide by the bacteria and that the available nitrate was partly used for the conversion of sulphide to sulphur. A comparison of the data also indicates that the sulphide removal rates obtained with *Thiomicrospira* CVO or Coleville enrichment are at least threefold higher than those reported with other microbial cultures, while nitrate removal rates are at comparable levels. The maximum sulphide removal rate obtained with the Coleville enrichment in the continuous system (2.1 mM h^{-1}) was slightly lower than 2.4 mM h^{-1} reported for *Thiomicrospira* strain CVO. However, the corresponding removal percentage with Coleville enrichment was 96% as opposed to 76% for *Thiomicrospira* CVO.

The main bacterial component of the Coleville enrichment (*Thiomicrospira* CVO) is capable of both autotrophic and heterotrophic growth with CO_2 or acetate as carbon source, respectively (Gevertz et al., 2000). Our current results, however, indicate that in the presence of sulphide or sulphur, CO_2 serves as the sole carbon source and acetate is not utilized. Furthermore, heterotrophic reduction of nitrate occurs only after complete oxidation of sulphide. This is in contrast with the findings of De Gusseme and De Schryver, 2009) in which simultaneous sulphide and acetate removals and denitrification were reported. As suggested by De Gusseme et al., activity of both h-NRB and NR-SOB could have led to simultaneous elimination of sulphide, acetate and nitrate in their study, while with the Coleville enrichment a shift in microbial metabolism as a result of exhaustion of sulphide may be responsible for heterotrophic denitrification. It should also be pointed out that the highest nitrate removal rate reported in the present work may not represent the ultimate potential of the Coleville enrichment, as these rates have been achieved in the absence of sufficient electron donor. Further

work, therefore, is needed to verify the ultimate potential of the Coleville enrichment and to elucidate the biological aspects of the denitrification process by this culture.

5. Conclusions

The results of the present study have important implications for the in situ removal of sulphide from the oil reservoirs and ex situ treatment of sulphide-contaminated waters. Formation of sulphur in oil reservoirs is not desirable due to potential plugging of the oil-bearing strata with sulphur, and severe corrosion in the production and distribution facilities. Thus, adjusting the ratio of sulphide to nitrate at a low level and maintaining a sufficiently long residence time would be the appropriate strategy for the in situ removal of H_2S. Equally important is the injection of nitrate at the early stages of the souring, otherwise the inhibitory effect of sulphide would hinder the bacterial activity and effectiveness of the nitrate amendment. An ex situ treatment process, by contrast, aims to maximize the production of the sulphur and to remove it from the contaminated stream permanently. Therefore, in this case application of a high sulphide to nitrate ratio and short residence times (high loading rates) would be beneficial. The dominance of sulphur as the main end product at high volumetric loading rates (short residence times) that coincide with the highest removal rates is an important characteristic which should be exploited in the design and operation of an ex situ treatment process.

Acknowledgements

This work was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada (NSERC). The authors would like to thank Richard Blondin for his technical assistance in maintenance of the ion chromatograph and analysis of the samples.

REFERENCES

Alcantara, S., Velasco, A., Muñoz, A., Cid, J., Revah, S., Razo-Flores, E., 2004. Hydrogen sulfide oxidation by a microbial consortium in a recirculation reactor system: Sulfur formation under oxygen limitation and removal of phenols. Environ. Sci. Technol 38, 918–923.

Annachhatre, A.P., Suktrakoolvait, S., 2001. Biological sulphide oxidation in a fluidized bed reactor. Environ. Technol 22 (6), 661–672.

Cardoso, R.B., Sierra-Alvarez, R., Rowlette, P., Flores, E.R., Gomez, J., Field, J.A., 2006. Sulfide oxidation under chemolithoautotrophic denitrifying conditions. Biotechnol. Bioeng 95, 1148–1157.

Cord-Ruwisch, R., 1985. A quick method for determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. J. Microbiol. Methods 4, 33–36.

Datta, I., Fulthorpe, R.R., Sharma, S., Allen, D.G., 2007. High-temperature biotrickling filtration of hydrogen sulphide. Appl. Microbiol. Biotechnol 74, 708–716.

De Gusseme, B., De Schryver, P., De Cooman1, M., Verbeken, K., Boeckx, P., Verstraete, W., Boon, N., 2009. Nitrate-reducing, sulfide-oxidizing bacteria as microbial oxidants for rapid biological sulide removal. FEMS Microbiol. Ecol 67, 151–161.

Duan, H., Koe, L.C.C., Yan, R., 2005. Treatment of H_2S using a horizontal biotrickling filter based on biological activated carbon: reactor setup and performance evaluation. Appl. Microbiol. Biotechnol 67, 143–149.

Eckford, R.E., Fedorack, P.M., 2002. Chemical and microbiological changes in laboratory incubations of nitrate amendment ''sour'' produced waters from three western Canadian oil fields. J. Ind. Microbiol. Biotechnol 29, 243–254.

Gadekar, S., Nemati, M., Hill, G.A., 2006. Batch and continuous biooxidation of sulphide by *Thiomicrospira* sp. CVO: Reaction kinetics and stoichiometry. Water Res. 40, 2436–2446.

Gevertz, D., Telang, A.J., Voordouw, G., Jenneman, G.E., 2000. Isolation and characterization of strains CVO and FWKO B, Two novel nitrate-reducing, sulfide-oxidizing bacteria isolated from oil field brine. Appl. Environ. Microbiol. 66, 2401–2501.

Greene, E.A., Brunelle, V., Jenneman, G.E., Voordouw, G., 2006. Synergistic inhibition of microbial sulfide production by combinations of the metabolic inhibitor nitrite and biocides. Appl. Environ. Microbiol. 72 (12), 7897–7901.

Hubert, C., Voordouw, G., 2007. Oil field souring control by nitrate-reducing *Sulfurospirillum* spp. that outcompetes sulfate-reducing bacteria for organic electron donors. Appl. Environ. Microbiol. 73 (8), 2644–2652.

Hubert, C., Nemati, M., Jenneman, G.E., Voordouw, G., 2003. Containment of biogenic sulfide production in continuous up-flow packed-bed bioreactors with nitrate or nitrite. Biotechnol. Prog 19 (2), 338–345.

Kaster, K.M., Grigoriyan, A., Jenneman, G.E., Voordouw, G., 2007. Effect of nitrate and nitrite on sulfide production by two thermophilic, sulfate-reducing enrichments from an oil field in the North Sea. Appl. Microbiol. Biotechnol 75, 195–203.

Kjellerup, B.V., Veeh, R.H., Sumithraratne, P., Thomsen, T.R., Buckingham-Meyer, K., Frølund, B., Sturman, P., 2005. Monitoring of microbial souring in chemically treated, produced-water biofilm systems using molecular techniques. J. Ind. Microbiol. Biotechnol 32, 163–170.

Kodama, Y., Watanabe, K., 2003. Isolation and characterization of a sulfur-oxidizing chemolithotroph growing on crude oil under anaerobic conditions. Appl. Environ. Microbiol. 69 (1), 107–112.

Larsen, J., Rod, M.H., Zwolle, S., 2004. Prevention of reservoir souring in the Halfdan field by nitrate injection. Corrosion 2004, Proceedings of 59th annual conference, Paper No. 04762.

Lee, E.Y., Lee, N.Y., Cho, K.S., Ryu, H.W., 2006. Removal of hydrogen sulfide by sulfate-resistant *Acidithiobacillus thiooxidans* AZ11. J. Biosci. Bioeng 101, 309–314.

McComas, C., Sublette, K.L., 2001. Characterization of a novel biocatalyst for sulfide oxidation. Biotechnol. Prog 17, 439–446.

Myhr, S., Lillebø, B.L.P., Sunde, E., Beeder, J., Torsvik, T., 2002. Inhibition of microbial H_2S production in an oil reservoir model column by nitrate injection. Appl. Microbiol. Biotechnol 58, 400–408.

Nemati, M., Jenneman, G.E., Voordouw, G., 2001a. Mechanistic study of microbial control of hydrogen sulfide production in oil reservoirs. Biotechnol. Bioeng 74 (5), 424–434.

Nemati, M., Mazutinec, T., Jenneman, G.E., Voordouw, G., 2001b. Control of biogenic H_2S production by nitrite and molybdate. J. Ind. Microbiol. Biotechnol 26, 350–355.

Ng, Y.L., Yan, R., Chen, X.G., Geng, A.L., Gould, W.D., Liang, D.T., Koe, L.C.C., 2004. Use of activated carbon as a support medium for H_2S biofiltration and effect of bacterial immobilization on available pore surface. Appl. Microbiol. Biotechnol 66, 259–265.

O'Reilly, C., Colleran, E., 2005. Toxicity of nitrite toward mesophilic and thermophilic sulphate-reducing methanogenic and syntrophic populations in anaerobic sludge. J. Ind. Microbiol. Biotechnol 32, 46–52.

Okabe, S., Santegoeds, C.M., De Beer, D., 2002. Effect of nitrite and nitrate on in situ sulfide production in an activated sludge

immobilized agar gel film as determined by use of microelectrodes. Biotechnol. Bioeng 81 (5), 570–577.
Reyes-Avila, J., Razo-Flores, E., Gomez, J., 2004. Simultaneous biological removal of nitrogen, carbon and sulfur by denitrification. Water Res. 38, 3313–3321.
Tang, K., Baskaran, V., Nemati, M., 2009. Bacteria of the sulphur cycle: An overview of microbiology, biokinetics and their role in petroleum and mining industries. Biochem. Eng. J. 44, 73–94.
Telang, A.J., Ebert, S., Fought, J.M., Westlake, D.W.S., Jenneman, G. E., Gevertz, D., Voordouw, G., 1997. Effect of nitrate injection on the microbial community in an oil field as monitored by reverse sample genome probing. Appl. Environ. Microbiol. 63 (5), 1785–1793.
Thorstenson, T., Boedtker, G., Sunde, E., Beeder, J., 2002. Biocide replacement by nitrate in seawater injection systems. Corrosion 33, 1–10.
Vaiopoulou, E., Melidis, P., Aivasidis, A., 2005. Sulfide removal in wastewater from petrochemical industries by autotrophic denitrification. Water Res. 39, 4101–4109.
van der Zee, F.P., Villaverde, S., Garcia, P.A., Fdz-Polanco, F., 2007. Sulfide removal by moderate oxygenation of anaerobic sludge environments. Bioresour. Technol 98 (3), 518–524.
Wang, A.J., Du, D.Z., Ren, N.Q., van Groenestijn, J.W., 2005. An innovative process of simultaneous desulfurization and denitrification by *Thiobacillus denitrificans*. J. Environ. Sci. Health 40 (10), 1939–1949.