Accumulation and inhibitory effects of acetate in a sulphate reducing *in situ* reactor for the treatment of an acidic pit lake

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Abstract

An ethanol-fed sulphate reducing *in situ* bioreactor for the treatment of acidic mining impacted lakes is currently under development. The reactor comprises a straw filled fixed bed reactor which is installed vertically in an enclosure in the lake. In a laboratory study it was tested which microbial processes take place in these reactors and whether elevated concentrations of acetate had an inhibitory effect on sulphate reduction.

In batch assays ethanol was exclusively oxidised to acetate via sulphate reduction. Complete oxidation of acetate as well as methanogenesis were not observed. Acetate inhibited sulphate reduction at concentrations above 15 mmol L^{-1}. The process performance, however, is unlikely to be inhibited by acetate since the highest concentration ever measured in the *in situ* reactors was below 10 mmol L^{-1}. High acetate concentrations in the efflu-

ent, however, are not acceptable both from an economically and environmental point of view.

1 Introduction

Lakes affected by acid mine drainage are a major environmental problem in many mining areas [11]. Such lakes are characterized by a low pH (usually between two and three) and high concentrations of iron and sulphate. There is currently no method available for the successful treatment of existing acidic pit lakes.

Strategies for the treatment of acid mine drainage include passive techniques like constructed wetlands, anoxic limestone drains or reactive barriers [10], [28] or "active" reactor systems. Sulphate reducing bioreactors are a promising method for the treatment of acid mine drainage [15], [20], [21]. The use of sulphate reducing bacteria (SRB) has the advantage that acidity, sulphate and iron are removed from the water in one process. Existing studies concentrate on laboratory or ex-situ systems. Fixed bed reactors [7], [8], fluidized bed reactors [16] and upflow anaerobic sludge blanket (UASB) reactors [29] were tested. Electron donors used include ethanol, [16], [17], [27] methanol [31], [12], lactate [7], [16], H_2/CO_2 [8] or complex organic substrates [2], [6], [14]. There are contradictory results regarding the utilisation of acetate in sulphate reducing bioreactors. In some cases acetate was successfully used [4], [5], [29], in other cases acetate was not used by SRB [18], [27], or acetate utilisation was the rate limiting step of organic matter degradation [17], [22], [35]. It is well known that acetate oxidising SRB are more difficult to grow than uncomplete oxidising SRB and that they are more sensitive to culture conditions [34].

We are currently developing an *in situ* reactor system for the treatment of acidic mining lakes [19], [23]. The system comprises an ethanol-fed fixed bed reactor which is swimming in an enclosure in the lake. Bottom water is pumped through the reactor, and ethanol is continuously fed as substrate for the microbial processes. First results with a pilot system showed that it was possible to run the sulphate reducing process under field conditions.

There are, however, a number of unresolved problems. During the start up phase vigorous gas production caused buoyancy problems. It is not clear whether methanogenesis contributes to ethanol consumption and gas development. During the degradation of ethanol high amounts of acetate and H_2S accumulated in the system. Acetate is a potential inhibitor of microbial sulphate reduction [1], [13]. At low pH it acts as an uncoupler of

the cell membrane potential. The release of acetate into the lake would lead to undesired eutrophication problems. The goal of the present study was to identify the active metabolic processes in the reactors and to evaluate possible inhibitory effects of acetate on the sulphate reducing process.

2 Material and Methods

2.1 *In situ* reactors

Technical details about the *in situ* reactors are described in [23]. Basically the reactors are straw filled cylinders (1.6 m diameter, 3.5 m height) which are suspended in an enclosure (30 m diameter) in the Mining Lake 111 (ML111) in the Lusatian Mining District in Germany. There are 8 such reactors of which each four are run parallel. The water can be circulated by pumping water from the bottom of the reactors to a mixing pot, where it can be mixed with organic substrate (ethanol). The water is then fed back into the reactors. In order to facilitate the establishment of SRB the pH in the reactors was initially increased by adding 50 kg of Carbokalk to each of the reactors. Carbokalk is a by-product of sugar production and contains both lime and organic and inorganic nutrients [9].

During the start-up phase in 2001 the reactors were run as a closed circulating system. During that phase an active sulphate reduction was established as could be seen by a decrease of sulphate and the buildup of free H_2S. In the following time the reactors were operated with changing loadings of acidic lake water. Continuous technical modifications and changing operating parameters hampered the quantitative interpretation of process performance. It was, however, possible to take samples of the reactor fluid for analysis and for laboratory experiments. Samples were taken on two occasions (22.10.2002, 17.12.2002) from the inflow mixing pots of the reactors, where circulating reactor fluid is mixed with ethanol before it is fed back into the reactors.

2.2 Laboratory experiments

In laboratory experiments the reaction pathway of ethanol consumption and the effect of elevated concentrations of acetate was investigated. Water from the mixing pot of the reactors was sampled and immediately filled into glass flasks (116 ml) avoiding any gas bubbles. In a first experiment the consumption of ethanol was investigated. The flasks of that experiment were incubated without further additions.

In a second experiment the effect of elevated concentrations of acetate was investigated. Since the ethanol concentration in the reactors was very low at that

time, ethanol (7.5 mmol L^{-1}) was added to the flasks with the exception of an ethanol free control. Different concentrations of acetate where adjusted by adding sodium acetate to the flasks prior to filling in the reactor fluid. Each treatment was done in four replicates.

The flasks which contained magnetic stir bars were incubated on a magnetic stirrer at 30° C in the dark. The high incubation temperature was used in order to get measurable concentration changes in a shorter time. Periodically samples where taken by sterile 5 ml syringes, displacing the sample volume with N_2 gas.

Chemical analyses were carried out as described in [19]. Organic acids and ethanol where analysed by HPLC using a AMINEX®HPX-87H Column (BIORAD), 0.005 mol L^{-1} H_2SO_4 as eluent and a diode array detector combined with a refractory index detector. CH_4 and CO_2 were analysed by gas chromatography using a HaySep column, H_2 as carrier, methanizer and FID. Sulphide was determined by polarography (Radiometer MDE 150). MPN counts of different bacteria were carried out in microplates using standard media [33]. Thermodynamic calculations were carried out using the tables of [30].

3 Results

The composition of the water inside the reactors compared to the untreated lake water (Table 1) shows that sulphate reduction was active in the reactors. The sulphate concentration was lowered and free sulphide was detected.

Table 1. Composition of untreated lake water and the water inside the *in situ* reactors at the time of sampling [mmol L^{-1}].

	lake water (19.11.2002)	Reactor sampling 1 (22.10.2002)	Reactor sampling 2 (17.12.2002)
pH	2.6	5.83	6.05
SO_4^{2-}	13.3	5.6	6.6
Sulphide	Bd	1.37	0.41
Fe^{2+}	0.02	Bd	0.2
Fe^{3+}	2.6	Bd	Bd
DIC	Nd	6.1	9
ethanol	Bd	17.6	1.2
acetate	Bd	0.5	0.4
CH_4	Bd	0.04	0.03

Nd = not determined, sulphide= $H_2S + HS^- + S^{2-}$, Bd = below detection limit, DIC = dissolved inorganic carbon

The occurrence of free H_2S and the low concentration of ferrous iron suggest that the precipitation of iron sulphides was limited by iron. Thus, the

reactors were iron-limited rather than limited by the rate of sulphate reduction. Accordingly the sulphide concentration was lower at the second sampling date, when ferrous iron was detectable. Differences between the two sampling dates demonstrate the unstable conditions in the reactors which were due to technical modifications and changing operating parameters. Common to both samplings were acetate concentrations around 0.5 mmol L^{-1}. The highest acetate concentration measured in the reactors during their time of operation was 9 mmol L^{-1} on 18.6.2002 (not shown).

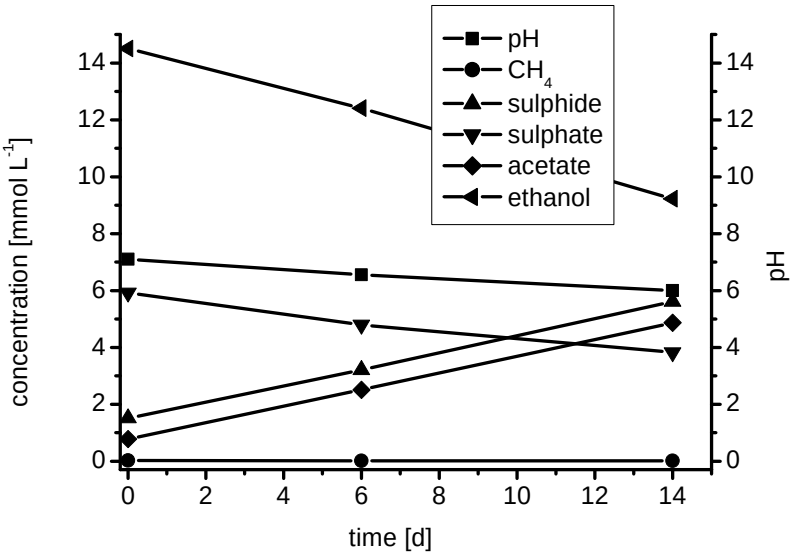

Fig. 1.: Concentration changes of metabolites (batch experiment 1, mean of duplicates).

The first batch incubation experiment showed that sulphate and ethanol were consumed while sulphide and acetate were produced (Fig. 1). Methane was not produced and the pH slightly dropped from 7 to 6, probably because of acetate production. The almost complete conversion of ethanol to acetate via sulphate reduction was confirmed by the second experiment. The formation rates of the different compounds (Table 2) suggest the reaction of ethanol consumption given in equation 2.

Table 2. Formation rates of different reactants in batch experiments [µmol L^{-1} d^{-1}]. The concentration of ethanol was 14 (experiment 1) and 7 mmol L^{-1} (experiment 2). Rates were calculated from the initial linear concentration change in batch assays.

	Experiment 1 [µmol L^{-1} d^{-1}]	Experiment 2 [µmol L^{-1} d^{-1}]
SO_4^{2-}	-149	-59
Sulphide	154	Nd
Ethanol	-379	-137
Acetate	292	105
CH_4	Bd	Nd

Nd = not determined, Bd = below detection limit

The low concentrations of CH_4 in the reactor fluid (Table 1) as well as in the batch experiments (Fig. 1, Table 3) demonstrate that methanogenesis was not competitive in the reactors.

Table 3. Concentration of dissolved CO_2 and CH_4 at the end of the batch incubation experiment 2 after 94 days. Mean and standard deviation of four replicates.

Initial acetate concentration [mmol L^{-1}]	CO_2 [µmol L^{-1}]	CH_4 [µmol L^{-1}]
0.3	63 ± 2	0.72 ± 0.10
3.9	54 ± 11	0.80 ± 0.05
15.2	14 ± 11	2.43 ± 1.24
94	2198 ± 1355	78.3 ± 8
915	823 ± 96	78.5 ± 9.8

MPN counts proved the presence of high numbers of sulphate reducing and sulphur oxidising bacteria ($8.5 \pm 5 \times 10^6$ and $5 \pm 2.5 \times 10^5$ cells ml^{-1}, respectively) while direct iron reducers (86 ± 58 and 435 ±126 cells ml^{-1} for acidophiles and neutrophiles) or iron oxidising bacteria (236 ± 159cells ml^{-1}) were hardly present. This is consistent with the observation of iron limitation inside the reactors. The specific rate of sulphate reduction was 6.9×10^{-15} mol $cell^{-1}$ d^{-1} which is in the range of known pure culture values [32]. At concentrations higher than 94 mmol L-1 acetate nearly completely inhibited sulphate reduction while concentrations below 15 mmol L-1 had no or even a slight stimulating effect (Table 4).

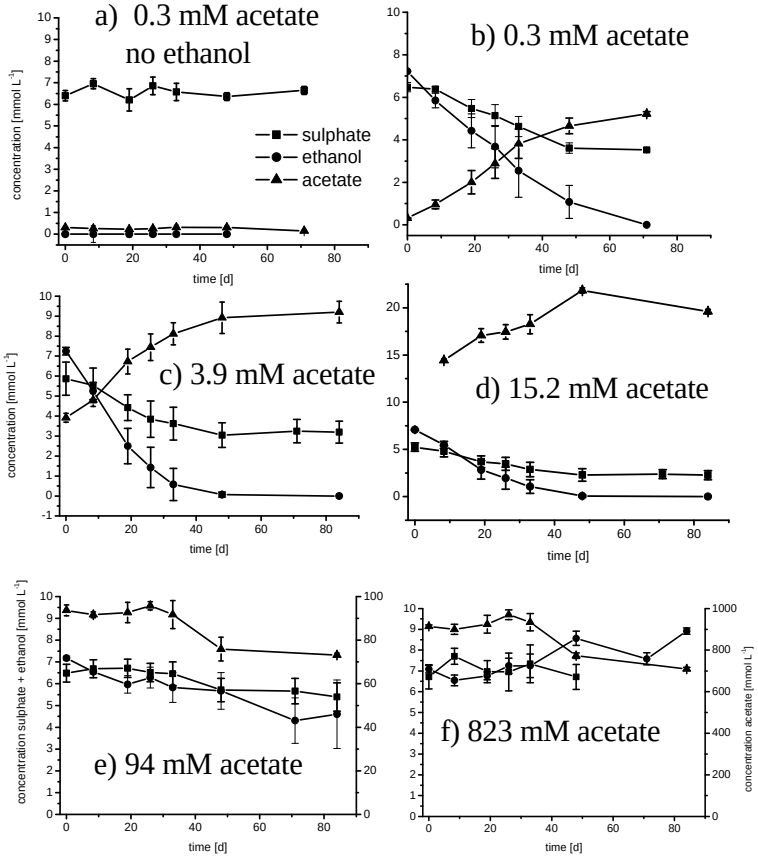

Fig. 2.: Concentration changes of sulphate (■), ethanol (●) and acetate (∅) in batch experiment 2. Points are means of 4 replicates. Treatments b to f were initially suppplemented with 7.5 mmol L^{-1} ethanol. Note different scales for acetate in e and f.

The time course of the experiment showed that sulphate removal was directly coupled to ethanol consumption (Fig. 2). Without the addition of ethanol there was no sulphate reduction (Fig. 2a). When ethanol was depleted, the consumption of sulphate stopped even in the presence of acetate (Fig. 2c,d). In the high acetate treatments (i.e. 94 and 915 mmol L^{-1}) a slight consumption of acetate started after a lag phase of about 3 weeks (Fig. 2e, f). The high concentrations of CO_2 in these treatments at the end

of the experiment (Table 3) further demonstrate the development of acetate oxidising micro-organisms at very high acetate concentrations.

Table 4. Sulphate reduction rate at different concentrations of acetate.

Acetate [mmol L^{-1}]	Sulphate reduction rate [µmol L^{-1} d^{-1}]	[% of control]
0.3 (= control)	59	100
3.9	74	125
15.2	73	124
94	2	3
915	5	8

4 Discussion

Ethanol can be oxidised by SRB either completely to CO_2 (eq. 1) or incompletely to acetate (eq. 2). Oxidation of acetate is also possible (eq. 3).

$$CH_3CH_2OH + 1.5\ SO_4^{2-} \rightarrow 1.5\ HS^- + 2\ HCO_3^- + H_2O + 0.5\ H^+ \quad (\Delta G_0' = -114\ kJ) \qquad (1)$$

$$2\ CH_3CH_2OH + SO_4^{2-} \rightarrow 2\ CH_3COO^- + HS^- + H^+ + 2\ H_2O \quad (\Delta G_0' = -133\ kJ) \qquad (2)$$

$$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2\ HCO_3^- \quad (\Delta G_0' = -48\ kJ) \qquad (3)$$

Our results clearly demonstrate that sulphate reducing bacteria that oxidize ethanol completely (to CO_2) were not active in our reactors. It is an unresolved problem why acetate oxidation occurred in some SRB reactors [29], [17] while it did not occur in other studies ([18], [27], [26], this study). There should be no reason for acetate-consuming SRB to be absent when the pH is near neutral, sulphate is present in excess and competing microbial processes like methanogenesis are not taking place [21]. A syntrophic association of *Geobacter*-like organisms and partner bacteria would allow *Geobacter* to oxidize acetate under iron-limiting conditions [3]. Probably such an association contributed to acetate oxidation in the high-acetate treatments. However, MPN counts of *Geobacter*-like organisms were rather low (86 ± 58 cells ml^{-1}). We assume that under the conditions in our reactors (no ferric iron, no nitrate, low temperature) syntrophic processes utilising acetate were not relevant.

One might argue that we used an inappropriate inoculum. In microcosm experiments with lake water and sediment acetate was not very effective in stimulating microbial alkalinity production [9]. Besides the SRB already

present in the Carbokalk, we inoculated the reactors with sediment from ML111. One out of five strains of SRB isolated from the sediment of ML111 was able to use acetate [25]. Therefore, although SRB that oxidize ethanol to CO_2 are probably not very abundant in ML111 they are very likely to be present.

The choice of lake sediment as inoculum might have the additional advantage that methanogenesis (which was never observed in ML111, Koschorreck unpublished) was nearly completely excluded from the reactors. The development of acetate consumption in the high acetate treatments further shows that at least some acetate using SRB were present in the reactors. The conditions in the reactors were obviously highly selective for SRB that incompletely oxidise ethanol and it is questionable if another inoculum would have led to the enrichment of acetate-consuming SRB. An alternative strategy might be the use of acetate instead of ethanol during the start up phase of the reactor in order to establish an acetate consuming microbial community. The development of acetate oxidising SRB, however, can be very slow. An increase of acetotrophic sulphate reduction of only 13.5 % in 138 days has been observed [22]. Assuming such a slow growth the SRB would surely be overgrown by ethanol utilizers as soon as the substrate is changed to ethanol.

It is an interesting question why acetate oxidising SRB only developed in the high acetate treatments. One could argue that the energy yield of acetate oxidation is too low at low acetate concentrations. We calculated the Gibbs free energy of eq. 3 for the conditions in experiment 2 (Fig. 3). Although the ΔG increases with increasing acetate concentration the energy yield at low acetate concentrations should be high enough to support the growth of SRB. Thermodynamic calculations also show that ethanol oxidation to acetate was always energetically favourable compared to acetate oxidation. Only in the extreme case of ethanol concentrations below 1 µmol L^{-1} and acetate above 0.2 mol L^{-1} acetate oxidation becomes energetically favourable.

It has been reported that methanogens are more sensitive to H_2S than SRB and that acetate utilizing SRB are more susceptible to sulphide inhibition than other SRB [5], [24], [35]. We have no data to indicate that sulphide concentrations up to 6 mmol L^{-1} were inhibitory to sulphate reduction. It is, however, possible that the high sulphide concentrations in the reactors selectively inhibited acetate oxidising SRB. In an ethanol-fed expanded granular sludge bed (EGSB) reactor the complete oxidation of ethanol could be established, after the concentration of H_2S was reduced either by stripping of H_2S or pH increase [4].

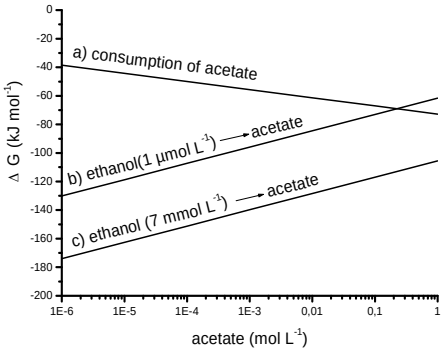

Fig. 3.: Free energy change (ΔG) of (a) acetate oxidation and (b+c) ethanol conversion to acetate for two different concentrations of ethanol at different concentrations of acetate in batch experiment 2.

Acetate oxidation by SRB was the most sulphide-sensitive step in the anaerobic degradation of propionate [24]. We might achieve oxidation of acetate if we could overcome iron limitation in our reactors and get a near quantitative precipitation of sulphides. On the other hand it might be favourable to keep some free H_2S in the reactor in order to suppress methanogenesis. It remains to be investigated which concentration of sulphide is optimal to inhibit methanogenesis but not the oxidation of acetate by SRB.

Acetate was inhibitory to sulphate reduction at high concentrations. The mechanism of this inhibition, however, is unclear in our case. Acetate is assumed to be inhibitory at low pH since the unprotonated form diffuses across the cytoplasmic membrane and acts as an uncoupler [1]. This might be relevant at the inflow of the *in situ* reactor where acidic lake water enters the system. Since the pH in our batch experiment was above 6 at every sampling occasion, the mechanism of acetate inhibition must be different. Acetate was added to the assays as sodium salt. Thus, the sodium concentration in the high acetate treatments exceeded that of seawater. We can not exclude that not acetate but sodium caused the inhibitory effect at such high concentrations [26].

Our observations have important consequences for the future development of *in situ* reactors for the treatment of acidic pit lakes:

Toxicity of acetate appears not to be a major problem with this system. The highest acetate concentration measured in our reactors was much lower than a potential inhibitory concentration. In the reactors the build-up of acetate never led to inhibition of the sulphate reduction rate.

The loss of acetate from the reactors is economically undesirable since the reducing power supplied by the ethanol is only partly used. When ace-

tate is not further oxidised 3 times as much ethanol is needed to achieve the same amount of sulphate reduction.

The organic carbon-rich effluent will promote the possibility of lake eutrophication. It has been suggested to add small amounts of alternative electron acceptors (e.g. O_2) to remove acetate from the effluent [21]. We expect that in our case free H_2S rather than acetate would be oxidised by an additional electron acceptor. In a previous experiment with a small pilot *in situ* reactor, sulphide and dissolved organic carbon in the effluent led to anoxic conditions and a buildup of H_2S in the water column of the enclosure [19]. After mixing of the lake in autumn all the accumulated H_2S was reoxidised.

Competition between methanogens and SRB is a common problem in anaerobic treatment of wastewater. The oxidation of ethanol by methanogens would not lead to an alkalinity gain and the electrons would have been wasted. In our reactors competing processes were not a problem and ethanol was nearly completely used by SRB.

5 Conclusions

Our results provide both positive and negative aspects for future improvement of *in situ* reactors of acidic mining lakes:

Positive is that no other processes compete with SRB for ethanol and no gas development occurred after the start up phase. Negative are high concentrations of organic carbon in the effluent and incomplete usage of ethanol. It remains an open question why acetate is not used by SRB and how acetate oxidation can be stimulated in order to improve the reactor performance.

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