Simultaneous carbon and sulphur substrates removal by *Desulfobacter postgatei* dominated biofilms in anaerobic packed reactors

Pavlina Kousi\textsuperscript{1*}, Emmanouella Remoundaki\textsuperscript{1}, Artin Hatzikioseyian\textsuperscript{1} and Marios Tsezos\textsuperscript{1}

\textsuperscript{1}Laboratory of Environmental Science and Engineering, School of Mining and Metallurgical Engineering, National Technical University of Athens. Heroon Polytechniou 9, 15780 Zografou, Greece.  
*pkousi@metal.ntua.gr

\textbf{Abstract} Sulphate-reducing bioreactors may be promising alternative treatment schemes for a broad range of industrial wastewater where sulphate reduction, wastewater neutralisation, soluble metal species sequestering and easier sludge management may be simultaneously addressed and achieved. This study presents an extensive experimental work, aiming at the optimisation of the efficiency of such reactors in terms of simultaneous minimisation of sulphate and organic content in the effluent. This work involved the set-up and monitoring of batch upflow packed-bed sulphate-reducing bioreactors for the treatment of synthetic, acidic (pH 3-3.5) solutions containing divalent iron (100 mg/L), zinc (100 mg/L), copper (100 mg/L), nickel (100 mg/L) and sulphate (1,800 mg/L), using acetate, ethanol and lactate as sole electron donors at various carbon:sulphur ratios. The reactors were inoculated with an ethanol-grown microbial consortium, which was dominated by *Desulfobacter postgatei*, an acetate-utilising species. The results obtained are comparatively discussed in terms of sulphate reduction and organic substrate utilisation, focusing on the attainment of minimum sulphate and organic content in the effluent.

\textbf{Keywords:} sulphate reduction, acetate oxidation, ethanol oxidation, lactate oxidation, carbon:sulphate ratio, residual organic content minimisation

\textbf{Introduction}  
Sulphate-reducing bacteria (SRB) (Postgate 1979) are anaerobic microorganisms that are widespread in anoxic habitats, where they use sulphate as terminal electron acceptor for the degradation of organic compounds resulting in the production of sulphide. It has been estimated that sulphate reduction can account for more than 50% of the organic carbon mineralisation in marine sediments which indicates the importance of sulphate reducers in both the sulphur and carbon cycle (Muyzer and Stams 2008). Moreover, the interactions between dissimilatory sulphate-reducing bacteria and metal ions have been studied since the first half of the 20th century (Hockin and Gadd 2007). As SRB have the apparently unique potential to simultaneously remove sulphate, organic content, metals and acidity from aqueous media, various processes have developed from isolating useful organisms from the environment and engineering conditions to promote their growth and activity in controlled systems (Johnson 2000). As a result, results from pilot-scale reactors were reported in the
early 90’s (Dvorak et al. 1992) and two major patented biotechnology processes emerged in the late 90’s (Rowley et al. 1997; Boonstra et al. 1999).

In bioremediation processes based on microbially-mediated sulphate reduction, sulphate reducing bacteria obtain energy for cell synthesis and growth by coupling the oxidation of organic substrates or molecular hydrogen (H₂), under anaerobic conditions, to the reduction of sulphate (SO₄²⁻) to sulphide (H₂S, HS⁻) (Rabus et al. 2007). The generated sulphide may then react, either in situ or ex situ (Kaksonen and Puhakka 2007), with divalent metal ions, which are then sequestered from wastewater as insoluble metal sulphides, in the form of various mineral phases (Herbert et al. 1998), producing sludge of better qualities compared to conventional chemical treatment (Kaksonen and Puhakka 2007). Sulphide and bicarbonate ions, which are formed during sulphate reduction and carbon source oxidation, equilibrate into a mixture of H₂S, HS⁻, S²⁻, CO₂, HCO₃⁻ and CO₃²⁻, which buffers the solution pH around neutral to slightly alkaline values (Dvorak et al. 1992). Thus, sulphate-reducing bioreactors may be promising alternative treatment schemes for a broad range of industrial wastewater where sulphate reduction, wastewater neutralisation, soluble metal species sequestering and easier sludge management may be simultaneously addressed and achieved.

However, the dissolved organic carbon content of most industrial wastewater is very low, usually < 10 mg/L (Johnson 2000). Therefore, addition of a suitable carbon source and electron donor for sulphate reduction is necessary to promote biogenic sulphide production. The preferred carbon sources for SRB are low molecular-weight compounds such as organic acids (e.g. lactate, pyruvate, formate and malate), fatty acids (e.g. acetate) and alcohols (e.g. ethanol, propanol, methanol and butanol) (Liamleam and Annachhatre 2007). Nevertheless, several other materials have been examined as alternative, cost-effective electron donors for the SRB-based sulphate reduction, such as hydrogen (coupled with CO or CO₂ as carbon source) (van Houten et al. 1994; Foucher et al. 2001) and various organic materials, such as molasses (Annachhatre and Suktrakoolvait 2001) or wastes (Boshoff et al. 2004).

The carbon source/electron donor is clearly an influential variable in the design of a full-scale industrial process for two main reasons: (a) it determines the composition of mixed microbial communities and the growth rate of the involved bacterial species and (b) it is a significant parameter for the economics of the process. The latter may easily be understood as the carbon source is not only an essential and often costly material to be added in the reactor inflow, but it also determines the nature and quantity of the organic substances residing in the reactor outflow as residual organic content demanding further treatment. For these reasons, the present study focuses on the experimental testing of three simple carbon sources/electron donors, namely lactate, ethanol and acetate, aiming to examine their suitability for the maintenance of a sulphate-reducing community, successful sulphate reduction and minimisation of the residual organic content.
The oxidation of lactate, which is a relatively common substrate, is a two-stage process as it proceeds via the oxidation of the intermediately-produced acetate. Thus, depending on the SRB species, lactate can be incompletely oxidized to acetate (reaction (1)) or completely oxidized to CO₂ (reaction (2)) via reaction (3) (Rabus et al. 2007).

\[
2CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2CH_3COO^- + 2HCO_3^- + H_2S \quad (1)
\]
\[
2CH_3CHOHCOO^- + 3SO_4^{2-} \rightarrow 6HCO_3^- + 3H_2S \quad (2)
\]
\[
CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^- \quad (3)
\]

Ethanol is proposed as carbon source/electron donor for sulphate-reducing bacteria for reasons such as ease of availability and low cost. Moreover, White and Gadd demonstrated that ethanol was more effective in stimulating sulphide production than lactate which, however, produced the greatest biomass (White and Gadd 1996). Ethanol, like lactate, can be incompletely oxidized to acetate (reaction (4)) or completely oxidized to CO₂ (reaction (5)), depending on the SRB species (Rabus et al. 2007):

\[
2CH_3CH_2OH + SO_4^{2-} \rightarrow 2CH_3COO^- + H_2S + 2H_2O \quad (4)
\]
\[
2CH_3CH_2OH + 3SO_4^{2-} \rightarrow 4HCO_3^- + 3HS^- + 2H_2O + H^+ \quad (5)
\]

Acetate oxidation (reaction (3)), which depends on the presence of acetate-utilising SRB in the microbial consortium, has been proven the critical step, as it controls the generation of alkalinity and the residual organic content of the effluent. Acetate may also inhibit sulphate reduction at high concentration and low pH (Reis et al. 1990), being highly toxic to SRB in undissociated forms (Johnson et al. 2006). Thus, in order to avoid acetate accumulation, efforts have been made towards engineering biosulphidogenic systems by enriching cultures with acetate-utilising bacteria (Nagpal et al. 2000) or by developing syntrophic sulphidogenic microbial consortia (Johnson et al. 2006) or by even supplying alternative electron acceptors, such as oxygen or nitrate (Lens et al. 2000). Therefore, acetate oxidation is considered a key factor for the optimisation of the entire process, since it determines the neutralisation potential of the acidic feeding solution and the effluent organic carbon content.

The present paper presents an extensive experimental work and discusses the utilisation potential of acetate by a characterized sulphate-reducing biofilm in packed reactors running at different carbon/sulphate ratios, focusing on the minimisation of residual organic content in the effluent. This work represents an optimisation effort for a microbially-mediated process which has been proven efficient (Kousi et al. 2007) for the sequestering of metals from acidic metal-bearing wastewater.
Materials and methods

Reactors
The sulphate-reducing fixed-bed reactors were PVC tubes which were filled with porous, sintered-glass pipes (length: 1.5 cm; wall thickness: 5 mm; specific surface: 1,200 m²/L – SintoMec®, JBL Germany). The reactors were inoculated by sufficient support material with already grown bacterial biomass and operated in upflow, batch mode at constant room temperature (~25 °C). The experimental set-up is described in detail in previous published work (Kousi et al. 2011a; Kousi et al. 2011b).

The synthetic feeding solutions were a modified variation of Postgate’s medium (DSMZ 2005), using lactate, ethanol and acetate as sole carbon sources/electron donors at different carbon/sulphate ratios:

a. Lactate was substituted with ethanol and acetate for the ethanol- and acetate-fed reactors, maintaining the organic surplus of the original Postgate’s medium (i.e. 58% over stoichiometry) to ensure non substrate limiting conditions (Case A).

b. The ethanol concentration in the feeding solution was reduced to the stoichiometrically required (reaction (5)) for the complete reduction of the sulphate content of the medium (Case B).

c. The acetate concentration in the feeding solution was adjusted to 20% over stoichiometry (reaction (3)) for a given sulphate content (Case C).

The feeding solutions also contained divalent iron (100 mg/L, added as FeSO₄·7H₂O), zinc (100 mg/L, added as ZnCl₂), copper (100 mg/L, added as CuCl₂·2H₂O), nickel (100 mg/L, added as NiSO₄·6H₂O) and sulphate (1,800 mg/L, added as Na₂SO₄, MgSO₄·7H₂O, FeSO₄·7H₂O and NiSO₄·6H₂O). The pH of the feeding solutions was adjusted to 3-3.5 by addition of HCl (Merck, analytical grade).

Biofilm
The biofilm established on the support material involved a mixed sulphate-reducing bacterial community, which was largely dominated (83% of the clones) by dsrAB sequences closely related (92-94% similarities) to that of Desulfobacter postgatei (Kousi et al. 2011a).

This community developed after adaptation of a lactate-fed sulphate-reducing consortium to ethanol. The lactate-fed reactor was initially inoculated with sludge from the anaerobic digestion tank of a wastewater treatment plant (Municipal Wastewater Treatment Plant, Metamorphosi, Greece). The microbial characteristics of both consortia are presented comparatively in Table 1.

The ethanol-fed community was less diverse than the community originating from the reactor fed with lactate medium, which is a common substrate to many sulphate reducers. This result
shows that the use of ethanol as carbon source/electron donor limited the growth of certain species present in the inoculum, maintaining a less diverse sulphate-reducing community. Concerning *Desulfobacter postgatei*, which degrades acetate (Widdel and Pfennig 1981; Ingvorsen *et al.* 1984) as all other species belonging to the *Desulfobacter* genus (Rabus *et al.* 2007), has demonstrated higher specific growth rate (in relation to *Desulfovibrio baculatus* and *Desulfobulbus propionicus*) in the presence of excess ethanol and sulphate (Laanbroek *et al.* 1984). Therefore, the substrate change, from lactate to ethanol, may have favoured the growth of this specific species which, also due to its ability to completely oxidise ethanol, finally dominated the sulphate-reducing community in the reactor.

**Table 1** Bacterial community characterisation

<table>
<thead>
<tr>
<th>Molecular CE-SSCP fingerprints of 16S rRNA genes</th>
<th>Lactate-fed community</th>
<th>Ethanol-fed community</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identified species</td>
<td><em>Desulfobacter postgatei</em> 2 <em>Desulfovibrio</em> species 2 <em>Desulfobulbus</em> species 2 <em>Desulfomicrobium</em> species</td>
<td><em>Desulfobacter postgatei</em> 2 <em>Desulfovibrio</em> species (similar to <em>Dvb. fructosovorans</em>, <em>Dvb. carbinolicus</em> &amp; <em>Dvb. burkinensis</em>) 1 <em>Desulfomicrobium</em> species</td>
</tr>
<tr>
<td>GenBank accession no.</td>
<td>EF645664 - EF645676</td>
<td>HQ640652 - HQ640659</td>
</tr>
<tr>
<td>Reference</td>
<td>(Remoundaki <em>et al.</em> 2008)</td>
<td>(Kousi <em>et al.</em> 2011a)</td>
</tr>
</tbody>
</table>

**Parameter monitoring**

The liquid phase parameters were monitored systematically after the establishment of the biofilm on the support material of the reactor. Sampling was performed at the feeding bottle each time the solution was renewed as well as at the reactor outlet at regular intervals (Kousi *et al.* 2011a; Kousi *et al.* 2011b). Solution pH, sulphate, total organic carbon (TOC), ethanol, acetate and lactate content were determined as already described in detail (Kousi *et al.* 2011b).
Results and discussion

Case A: carbon source:sulphate ratio at 58% over stoichiometry

Comparing the neutralisation results of the feeding solution (initial pH: 3.5) for the acetate-fed reactor (reactor [A]), the ethanol-fed reactor (reactor [E]) and the lactate-fed reactor (reactor [L]), it was found that, at the end of each cycle, pH always reached a value of 6.80 in reactor [A] (Figure 1(a)) and 7.10 in reactors [E] (Figure 1(c)) and [L] (Figure 1(e)), as a result of the alkalinity generated during the SRB metabolism. Sulphidogenesis is, therefore, possible at low initial pH values; thus, confirming the viability and the efficiency of the process when the mixed sulphate-reducing community is in direct contact with acidic wastewater (Elliott et al. 1998; Johnson et al. 2006; Kimura et al. 2006).

Sulphate was reduced by 97% in reactors [E] (Figure 1(c)) and [L] (Figure 1(e)) and by 80% in reactor [A] (Figure 1(a)). This finding is in agreement with the relative results regarding the utilisation of the total organic carbon available in the feeding media. It is shown that organic content was oxidized by 95% in reactors [E] and [L] and by 85% in reactor [A]. Moreover, Figure 1(d) and Figure 1(f), presenting ethanol and lactate oxidation respectively, show that the intermediately produced acetate was oxidised to less than 100 mg/L (equivalent TOC < 40 mg/L) in both reactors [E] and [L] after 30 hours of operation. This strongly confirms the capacity of the established biofilm to oxidise the intermediately produced acetate.

Regarding the experimental molar substrate:sulphate ratio reported in Table 2, all ratios are higher than the theoretical values. This is due to the fact that part of the carbon source is utilised for culture maintenance. Moreover, these carbon-rich conditions may have favoured the growth of other, non-sulphate-reducing, anaerobic bacterial strains. Thus, given the low growth rate of acetotrophs, part of the initial lactate, ethanol and acetate content may have been degraded via different metabolic pathways.

Initial sulphate reduction rates were calculated based on the reduction of the available sulphate content within 2 hours after the commencement of the experiments. It was found that sulphate was reduced at a rate of 5.3 g/Ld in reactor [A], 7.2 g/Ld in reactor [E] and 7.4 g/Ld in reactor [L] (Kousi et al. 2011b). These calculated sulphate reduction rates are comparable to the findings reported for a previously run lactate-fed reactor (Kousi et al. 2007), operating continuously at HRT = 9 h, where the microbial community was more diverse (Remoundaki et al. 2008).
Figure 1 Sulphate reduction, total organic carbon degradation and pH variation (inset) for the acetate-fed reactor (a), the ethanol-fed reactor (c) and the lactate-fed reactor (e); acetate oxidation for the acetate-fed reactor (b), ethanol/acetate oxidation for the ethanol-fed reactor (d) and lactate/acetate oxidation for the lactate-fed reactor (f) for Case A scenario
Table 2 Utilisation of organic substrate for sulphate reduction

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Substrate degraded (mM)</th>
<th>Sulphate reduced (mM)</th>
<th>Experimental molar substrate:sulphate ratio</th>
<th>Stoichiometric molar substrate:sulphate ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate-fed</td>
<td>21.6</td>
<td>15.0</td>
<td>1.44</td>
<td>1.00</td>
</tr>
<tr>
<td>Ethanol-fed</td>
<td>16.9</td>
<td>19.2</td>
<td>0.88</td>
<td>0.67</td>
</tr>
<tr>
<td>Lactate-fed</td>
<td>22.2</td>
<td>18.6</td>
<td>1.19</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Case B: ethanol:sulphate ratio at stoichiometry

The initial pH of the fresh feeding solution was 3-3.5, whereas pH at the end of the cycle always reached a value of about 7.5 (Figure 2(a)), as a result of the alkalinity generated during the SRB metabolism; thus, confirming that the metabolic process of the bacterial community was not adversely affected nor inhibited by the initial low pH.

The data depicted in Figure 2(a) show that sulphate was reduced by 85-90% although there is minimum residual TOC in the treated solution. Similar results have been published by de Smul et al. for an ethanol-fed EGSB reactor operating at near stoichiometry at HRT of 3 h (de Smul et al. 1997) whereas Kaksonen et al. showed that the stoichiometric ratio was adequate to attain around 80% of sulphate reduction with an initial sulphate concentration of 2,000 mg/L in a fluidized-bed reactor running at HRT of 20 h (Kaksonen et al. 2004).

The biofilm studied in the present work is largely dominated by Desulfobacter postgatei. Therefore, according to Laanbroek et al. (Laanbroek et al. 1984), approximately 12.5% of the added organic material is utilised in bacterial growth processes, resulting in a TOC deficit for sulphate reduction which is reflected in the percentage of sulphate that remain in solution. This rationale leads to the calculation of a corrected molar ethanol:sulphate ratio to attain complete ethanol oxidation and sulphate reduction in the process presented in this study. This revised stoichiometric ratio is found to be 0.75 and is also confirmed (via the resulting TOC deficit) by our experimental results shown on Figure 2(a), where sulphate is reduced by 85-90% and no residual TOC is determined at the end of each experimental cycle.

Case C: acetate:sulphate ratio at 20% over stoichiometry

Compared to the findings after the application of Case A scenario on the acetate-fed reactor (acetate:sulphate ratio = 1.58, Figure 1(a) and Figure 1(b)), the results obtained for acetate:sulphate ratio equal to 1.2 (i.e, 20% higher than the stoichiometrically required shown on Table 2) demonstrate a significant difference in the final sulphate content (Figure 3(a)) despite the attained solution neutralisation (Figure 3(a, inset)) and although acetate, and subsequently organic carbon, degradation proceeds to comparable levels (Figure 3(b)). Thus, the experimental molar acetate:sulphate ratio is determined to 1.85, namely 28% higher than
the value calculated based on the Case A results, meaning that more acetate is utilised to reduce sulphate.

Apparently, acetate at the applied ratio is not sufficient for the reduction of the given sulphate content, which may be attributed to the slow acetate degradation by sulphate reducers. Therefore, despite the proven acetate-utilising capacity of the biofilm and assuming negligible the possible inhibition effect of acetate in the low-pH range on the activity of SRB, the use of acetate as carbon source/electron donor seems to necessitate an even higher excess over the stoichiometrically required concentration to simultaneously achieve desirable levels of both sulphate and organic carbon in the reactor effluent.

Figure 2 (a) sulphate reduction, total organic carbon degradation and pH variation (inset) and (b) ethanol/acetate oxidation for Case B scenario

Figure 3 (a) sulphate reduction and pH variation (inset) and (b) acetate oxidation for Case C vs. A scenario
Conclusions
This paper presents a data set, complementary to previous experimental work, in order to conclude and confirm significant design parameters of a simple, sulphate-reducing, packed-bed upflow reactor for biological wastewater treatment.

The successful growth and maintenance of a biofilm dominated by *Desulfobacter postgatei*, a species which is characterised by its acetate-utilising capacity, was the prerequisite for the further optimisation of a biological sulphate-reducing process proven efficient for neutralising acidic wastewater (initial pH ranging from 3 to 3.5) as well as for quantitatively sequestering soluble metal species. The referred optimisation focused on the simultaneous removal of the sulphate and organic carbon content.

For this purpose, three carbon sources (lactate, ethanol and acetate) have been comparatively studied at different carbon to sulphate ratios, referring to the stoichiometry of the relative reactions of complete oxidation. According to the results presented in this paper, ethanol seems to be the more promising carbon source/energy donor among the three tested as it combines:

(a) lower cost compared to lactate.
(b) selective and long-lasting conservation of an active sulphate-reducing biofilm: the ethanol-fed reactor run efficiently for over 2 years.
(c) simultaneous and efficient sulphate and organic carbon removal: sulphate reduction rate was experimentally determined to about 7 g/Ld whereas the residual organic carbon in the treated solution was determined at a level < 40 mg/L.
(d) requirement for a slight excess over stoichiometry for the reduction of a given sulphate content: the calculated minimum molar ethanol:sulphate ratio to attain complete ethanol oxidation and sulphate reduction was calculated to 0.75.

Acknowledgments
This work was carried out in the frame of BioMinE (European project contract NMP1-CT-500329-1). The authors acknowledge the financial support given to this project by the European Commission under the Sixth Framework Programme for Research and Development.

References


