Article V: Chemical characterisation of natural organic substrates for biological mitigation of acid mine drainage

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O. Gibert, J. de Pablo, J.L. Cortina, C. Ayora.
CHEMICAL CHARACTERISATION OF NATURAL ORGANIC SUBSTRATES FOR BIOLOGICAL MITIGATION OF ACID MINE DRAINAGE

Oriol Gibert 1, Joan de Pablo 1,2,*, José Luis Cortina 1, Carlos Ayora 3

1 Departament d'Enginyeria Química, ETSEIB, Universitat Politècnica de Catalunya, 08028 Barcelona, Spain.
2 Departament d'Enginyeria Química, Àrea de Tecnologia Ambiental, Centre Tecnològic de Manresa, Universitat Politècnica de Catalunya, 08240 Manresa, Spain.
3 Institut de Ciències de la Terra Jaume Almera, CSIC, 08028 Barcelona, Spain

* Corresponding author phone: 93 4016557; fax: 93 4015814; e-mail: joan.de.pablo@upc.es

Abstract: The current approach of the biological treatment of acid mine drainage by means of a passive remediation system involves the choice of an appropriate organic substrate as electron donor for sulphate reducers. Nowadays this selection is one of the critical steps in the performance of such treatment, as this depends to a great extent on the degradability of the organic substrate. Thus, a prior characterisation of the organic substrate predicting its biodegradability would be desirable before embarking on an extensive large-scale application. The aim of this study was to correlate the chemical composition (lignin content) of four different natural organic substrates (compost, sheep and poultry manures, oak leaf) and their capacity to sustain bacterial activity in an attempt to predict biodegradation from chemical characterisation. Results showed that the lower the content of lignin in the organic substrate, the higher its biodegradability and capacity for developing bacterial activity. Of the four organic materials, sheep and poultry manures and oak leaf evolved reducing conditions and sustained active sulphidogenesis, which coupled with the decrease in sulphate concentration indicated bacterial activity. Sheep manure was clearly the most successful organic material as electron donor (sulphate removal ≥99%), followed by poultry manure and oak leaf (sulphate removal of 80%). Compost appeared to be too poor in carbon to promote SRB activity by itself. Column experiments emphasised the importance of considering the residence time as a key factor in the performance of continuous systems. With residence time of 0.73 days, sheep manure did not promote sulphidogenesis. However, extending residence time to 2.4 and to 9.0 days resulted in an increase in the sulphate removal to 18% and 27%, respectively.
**Key words**: acid mine drainage, biodegradable fraction, lignin, organic substrate characterisation, permeable reactive barrier, sulphate-reducing bacteria, RAPS, wetlands

**INTRODUCTION**

The exposure of the post-mining wastes from sulphidic mine activities to water and air undergoes chemical and biological oxidation processes resulting in a highly acidic leachate with substantial quantities of sulphate and heavy metal ions. These effluents are known as acid mine drainage (AMD). Fuller accounts on the genesis of AMD and the potential hazardous impact on the surrounding environment have been well documented (Younger et al., 2002). Literature offers an extensive overview of chemical and biological methods for remediating AMD (Diels et al., 2002; Gibert et al., 2002). The biological approach consists in the use of anaerobic sulphate reducing bacteria (SRB), which can reduce sulphate to sulphide by oxidising an organic carbon source. Biogenically generated sulphide easily precipitates many of the dissolved metal ions as metallic sulphides. Moreover, by mineralising the organic substrate, the overall process leads to an increase in alkalinity and the pH value of water. The generated carbonate and hydroxide anions may also contribute to metal removal (Dvorak et al., 1992).

Latterly, attention has been focused on *in-situ* passive systems, such as artificial wetlands (Karathanasis and Thompson, 1995), reducing and alkalinity producing systems (RAPS) (Hedin et al., 1994; Younger et al., 2002) and permeable reactive barriers (PRB) (Blowes et al., 2000). Laboratory-scale studies conducted to simulate these systems report residence time as a key factor in the performance of the treatment (Béchard et al. 1994; Dvorak et al., 1992; Benner et al., 2002), and low residence time or high flux rates leading to an incomplete sulphate consumption and therefore an incomplete contaminant removal.

The availability of sulphate (SO$_4^{2-}$) and organic carbon (CH$_2$O) affects the extent of microbial activity and hence biotreatment efficiency. Since AMD tends to contain high concentrations of sulphate but relatively little dissolved organic carbon (<10 mg dm$^{-3}$)(Kolmert and Johnson, 2001), the most critical limiting factor is the availability of carbon from an additional organic source, i.e., the biodegradability of this organic substrate (Waybrant et al., 1998; Castro et al., 1999). Thus, from a biotechnological point of view one primary objective is to select a suitable organic substrate to make the process efficient and economically feasible. The raw organic materials assessed in earlier research cover a wide range of agricultural and food processing wastes (Waybrant et al., 1998; Cocos et al., 2002; Chang et al., 2000; Béchard et al., 1994).
One common conclusion of the above studies is that natural complex organic substrates in continuous systems scarcely promote sulphidogenesis (Hammack and Edenborn, 1992; Dvorak et al., 1992; Gibert et al., 2003). It should be noted that SRB are able to metabolise a limited range of organic substrates, usually organic acids and alcohols (Postgate, 1979; Chang et al., 2000), and that sulphate reduction in such complex systems is achieved when SRB form part of a community of interdependent anaerobic organisms, where each single species carries out only one step in the mineralisation of the complex organic substrate (Postgate, 1979; Tsukamoto and Miller, 1999; White and Gadd, 1996). Hence, a proper cooperation in such consortia is needed. Sustained sulphidogenesis in continuous systems can also be attained by the amendment of easily assimilable electron donors, essentially small molecular weight compounds, which are readily decomposable by SRB (Hammack et al., 1994; Webb et al., 1998; Tsukamoto and Miller, 1999; Kolmert and Johnson, 2001; Steed et al., 2000). However, the continuous addition of soluble nutrients to ensure bacterial activity in field applications is not realistic because it may entail prohibitive costs.

Although it is generally assumed that the chemical composition of an organic substrate controls the patterns of its degradability, no minimum dataset to predict it has yet been established. Animal nutritionists have developed chemical and enzymatic procedures to estimate feedstuff digestibility for ruminant diets (Rahn et al., 1999; Harper and Lynch, 1981; Jung et al., 1997) and engineers have attempted to quantify the degradability of a raw organic waste in methane fermentation research (Chandler et al., 1980; Haug, 1993), but unfortunately little standard practice has been developed in environmental engineering (Prasad et al., 1999). Chemical approaches to predict the nutritive value of an organic material are classically based on the quantification of poorly digestible fractions (mostly structural organics, such as lignin and cellulose, which are resistant to microbial decomposition) and rapidly digested fractions (Chandler et al., 1980; Rahn et al., 1999; Prasad et al., 1999).

Other additional criteria have been recognised in assessing the potential of the degradability of an organic substrate, i.e. C/N ratio (Kayhanian and Tchobanoglous, 1992) or specific substances (proteins, carbohydrates, fats, etc.) (Jung et al., 1997).

Chandler and co-workers proposed a model equation to estimate the substrate biodegradable fraction (B) based on the lignin content as represented in eq 1 (Chandler et al., 1980; Haug, 1993):

\[ B = -0.028X + 0.830 \]
where the biodegradable fraction (B) is expressed on a volatile solid (VS) basis and X is the lignin content of the VS, expressed as a percent of the dry weight.

However, the results of this array of methods show that (1) they yield vastly different results and therefore constituent contents can at most be considered as proximate values, and that (2) none of these parameters alone are sufficient to predict the biodegradability of an organic substrate by micro-organisms. Thus, a routine and rigorous method of analysis for predicting substrate biodegradability remains to be developed.

The aim of the present study was to rapidly predict through chemical characterisation the digestibility of four different organic substrates as nutritive suppliers to SRB in the biological treatment of AMD. The application in this study of a chemical procedure developed by animal nutritionists is based on the fact that SRB used in this study are encountered in large numbers in ruminant animals (Postgate, 1979; Amos and Younger, 2003). The chemical characterisation of the organic substrates was then correlated with their assessment in promoting sulphate reduction from both batch and column experiments.

**MATERIALS AND METHODS**

**Substrate collection**

Four different organic materials were used in this study: municipal compost, sheep and poultry manures and oak leaf. Municipal compost was supplied by a wastewater treatment plant in Manresa, Catalonia (Spain). Animal manure and leaf material were collected from two local farms near Barcelona. All four organic substrates were considered to be potentially suitable organic materials with respect to the technico-economic characteristics (Beaulieu et al., 2000; Benner et al., 1997; Waybrant et al., 1998).

**Chemical characterisation of the organic substrates**

Representative subsamples of the four organic substrates were taken and subjected to the following analyses:

1) lignin/cellulose: lignin and cellulose contents were determined by the method of successive extractions described by Rahn and co-workers (Rahn et al., 1999). 1 g of dried sample was refluxed with 100 cm³ of a solution of 60 mM acetyltrimethyl ammonium bromide in 0.5 M
H$_2$SO$_4$ for 1 h, yielding an acid detergent fibre (ADF) composed of cellulose and lignin, which was washed with acetone, dried at 100 ºC and weighed. This remaining residue was then delignified by incubation with a 0.3 M KMnO$_4$ solution for 1.5 h, after which the remaining fraction was washed with a solution containing 0.4 M oxalic acid, 0.6 M HCl and 12 M ethanol. After being dried in a 100 ºC oven for 24 h, the residue was re-weighted. Lignin was calculated by weight difference following KMnO$_4$ extraction. The remaining residue was fired in a muffle furnace at 400 ºC for 16 h to determine ash content. Cellulose content was calculated by subtracting the weight of ash from the weight of KMnO$_4$-stable material.

2) Easily available substances (EAS): EAS fraction can be regarded as “the organic portion of a substrate that can be readily used by the microorganisms. It is, in essence, similar to the term “biodegradable portion” of a substrate (soluble sugars, starch, hemicellulose, amino acids, some proteins)” (Prasad et al., 1999). It is worth noting that not all of the constituents present in the extract EAS can actually be utilised by SRB, and consequently EAS should be taken only as an approximate value. An estimation of the actual degradable amount of carbon in the extract EAS is reported by Prasad et al. (1999). In this determination, 1 g of dried sample was washed with acetone to dissolve the lipids and resins, after which the residue was refluxed with 5% HCl to extract soluble sugars, starch, amino acids and hemi-cellulose and dried at 40 ºC overnight and weighed. The HCl-soluble constituents removed in this step were considered as the EAS fraction of the organic material and were calculated by weight difference following HCl extraction.

3) Elemental analysis: C, N, H, P, S contents were obtained by dry combustion (1000ºC) of a given mass of dry organic matter. Combustion products were separated by gas chromatography (Porapak 50/80 column, 60ºC) and quantified with a thermal-conductivity detector (CE Instruments 1108).

4) Volatile Solids (VS): VS content was determined as a needed parameter to apply the equation reported by Chandler in the estimation of the biodegradable fraction in an organic substrate. It was determined in accordance with procedures outlined in Standard Methods (APHA, 1992). A portion of the sample was dried and ashed in a muffle furnace (550 ºC) to determine the VS fraction.
**Batch experiment description**

The capacity of the four organic substrates (compost, sheep and poultry manures, oak leaf) to conduct sulphate reduction was assessed in a series of four 500 cm$^3$ glass anaerobic reactors. The mixture proportion (v/v) was 15% organic substrate, 15% creek sediment, 30% calcite and 40% quartz. Creek sediment acted as a SRB source and was collected from the anoxic zone of a local creek. The presence of SRB was indicated by a strong H$_2$S odour. No enrichment of SRB was carried out. Limestone from a quarry (2-4 mm size) was used as a neutralising agent. Quartz was included as an inert material. These components were mixed homogeneously before being placed in the reactors. After the mixtures were added, the reactors were filled with a synthetic mine water containing a sulphate concentration of 10.6 mM and a pH of 2.4 (adjusted by HCl addition). To simplify the chemical system and avoid the uncontrolled precipitation of sulphides, this synthetic water did not contain dissolved heavy metals. The solid:liquid ratio (in volume) was 1:10. The reactors were covered with an opaque material to simulate light conditions encountered in the aquifer. All experiments were conducted at room temperature (21±2ºC). The water composition (pH, Eh, SO$_4^{2-}$, S$_2^-$ determinations) was monitored throughout the experiment, which lasted 6 months. pH, SO$_4^{2-}$ and S$_2^-$ were measured as target parameters of treated water. Eh was measured as an overall redox parameter indicator of the reducing conditions of the system, which are often-cited as a minimum requirement for SRB activity to take place (Postgate, 1979; White and Gadd, 1996). The growth of the microbial community was anticipated but not measured.

**Column experiment description**

Sheep manure and compost showed the highest and lowest capacity for stimulating bacterial activity respectively, and were thus selected for column (i.d.=2.5 cm, l=25 cm, vol=123 cm$^3$) experiments. Compositions (v/v) were similar to those tested in the earlier batch experiments: 15% creek sediment, 30% organic substrate and 55% limestone. Two feed waters containing a sulphate concentration of 5.5-5.8 mM and a pH of 2.4 (adjusted by HCl addition) were prepared. As in the aforementioned batch experiments, they did not contain dissolved heavy metals. The inflowing waters were kept in a closed reservoir with a low oxygen content by bubbling nitrogen and were introduced into the columns from the bottom by means of a peristaltic pump at an average flow rate of 0.058 cm$^3$ min$^{-1}$. Taking into account the column section and the estimate filling material porosity (0.5), this flow rate equals a flux velocity of 0.34 m d$^{-1}$ (average residence time of 0.73 days). For the column with sheep manure, the flow
rate was deliberately decreased to 0.018 cm$^3$ min$^{-1}$ (average residence time of 2.4 days) after 69 days of experiment. On day 112 the flow rate was reduced again to 0.005 cm$^3$ min$^{-1}$ (average residence time of 9.0 days). All tubings and fittings were made of Teflon excluding the pump tubings, which were made of Tygon. Joints were wrapped with wax film to minimise air infiltration. Precautions against air infiltration and light exposure were undertaken as in the series of batch experiments. A follow-up of effluent composition (pH, Eh, SO$_4^{2-}$, S$^{2-}$ determinations) was carried out throughout the experiment.

Water Sampling and Analysis

Chemical parameters monitored over the experiments included pH, Eh, and SO$_4^{2-}$ and S$^{2-}$ concentrations. All pH and Eh measurements were made routinely using a HAMILTON combination pH electrode and a platinum electrode paired with an Ag/AgCl-reference electrode coupled to a CRISON GLP22 pH meter. In the batch experiments, measurements were carried out by inserting the two electrodes into the reactor, whereas in the column experiments, pH measurements were done in a sealed flow-through cell placed at the exit of the columns and Eh was measured using a tubular platinum electrode at the exit of the cell. Effluent samples were collected periodically for SO$_4^{2-}$ and S$^{2-}$ analysis. Sulphate was measured in acidified (by HCl addition) and filtered (0.22 µm) samples by liquid ion chromatography (ALLIANCE model Waters 2690) coupled to an electrical conductivity detector (Waters 996). The evolved H$_2$S was trapped in a zinc acetate solution and was analysed following the Methylene Blue method (Kubáň et al., 1992; Lawrence et al., 2000) by UV-spectrophotometry (Hewlett-Packard 8453). For H$_2$S sampling, the flow rate was accelerated for a few seconds to collect 1 ml of sample. Measurements were determined immediately after sampling to minimise H$_2$S loss by gas volatilisation.

RESULTS AND DISCUSSION

Chemical characterisation of organic substrates

The chemical characterisation of the selected organic substrates is given in Table 1. Lignin and cellulose contents ranged from 8-15% (w/w) and 7-37% respectively, sheep manure containing the lowest content of lignino-cellulosic materials, and compost and oak leaf the highest. The
content of EAS in the four organic substrates ranged between 45% for sheep manure and 26% for oak leaf.

C/N ratio (w/w), based on total dry organic matter, ranged between 17-26. Although this method of C/N computation is the most commonly used, it may not be appropriate because not all organic carbon is biodegradable and/or available for biological decomposition. Earlier studies using lactate and ammonium as readily decomposable carbon and nitrogen sources recommend a maximum C/N ratio (w/w) for growth of SRB in the range of 45 – 120. Above this threshold value, the medium is considered low in nitrogen (Okabe et al., 1992).

The biodegradability of the four organic substrates was estimated by using the lignin and volatile solids and the relationship reported by Chandler and co-workers (Chandler et al., 1980; Haug, 1993) (eq 1). As shown in Table 1, the biodegradable fraction ranged between 0.11 for compost and 0.56 for sheep manure.

<table>
<thead>
<tr>
<th></th>
<th>Compost</th>
<th>Oak leaf</th>
<th>Poultry manure</th>
<th>Sheep manure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lignin (% w/w)</td>
<td>15.34 ± 1.85</td>
<td>15.11 ± 3.35</td>
<td>10.76 ± 2.14</td>
<td>8.32 ± 1.28</td>
</tr>
<tr>
<td>Cellulose (% w/w)</td>
<td>37.21 ± 3.08</td>
<td>33.78 ± 2.12</td>
<td>24.42 ± 2.72</td>
<td>7.45 ± 1.87</td>
</tr>
<tr>
<td>EAS (% w/w)</td>
<td>33.95 ± 3.25</td>
<td>26.33 ± 2.35</td>
<td>30.13 ± 2.11</td>
<td>44.87 ± 4.13</td>
</tr>
</tbody>
</table>

**Elemental analysis (% w/w)**

<table>
<thead>
<tr>
<th>Element</th>
<th>Compost</th>
<th>Oak leaf</th>
<th>Poultry manure</th>
<th>Sheep manure</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>27.19 ± 0.17</td>
<td>43.81 ± 0.32</td>
<td>27.28 ± 0.15</td>
<td>38.65 ± 0.27</td>
</tr>
<tr>
<td>N</td>
<td>1.26 ± 0.05</td>
<td>1.67 ± 0.10</td>
<td>1.61 ± 0.07</td>
<td>1.98 ± 0.07</td>
</tr>
<tr>
<td>H</td>
<td>3.37 ± 0.25</td>
<td>5.87 ± 0.20</td>
<td>3.51 ± 0.05</td>
<td>5.26 ± 0.07</td>
</tr>
<tr>
<td>S</td>
<td>0.50 ± 0.05</td>
<td>0.14 ± 0.05</td>
<td>0.36 ± 0.02</td>
<td>1.37 ± 0.07</td>
</tr>
<tr>
<td>P</td>
<td>0.91 ± 0.02</td>
<td>1.02 ± 0.01</td>
<td>&lt;0.05</td>
<td>1.45 ± 0.08</td>
</tr>
</tbody>
</table>

| C/N ratio (w/w) | 21.58 ± 2.91 | 26.23 ± 5.03 | 16.94 ± 1.58 | 19.52 ± 2.66 |
| Volatile Solids (% w/w) | 59.32 ± 1.15 | 93.16 ± 2.90 | 70.65 ± 1.45 | 86.70 ± 2.58 |
| Biodegradable fraction (B)* | 0.10 ± 0.07 | 0.37 ± 0.05 | 0.40 ± 0.05 | 0.56 ± 0.05 |

*Uncertainty of B was calculated from the raw data (Haug, 1993) and the linear regression of eq 1.

Table 1. Chemical characterisation of the evaluated organic substrates. Contents are computed on the total mass of the dry organic matter. Biodegradable fraction has been determined on the basis of lignin and volatile solids according to eq 1.
Batch experiments

The evolution of pH, Eh, SO$_4^{2-}$ and S$_2^-$ throughout the batch experiments is shown in Figure 1.

![Graphs showing the evolution of pH, Eh, sulphate, and sulphide for different substrates over time.](image)

Fig. 1. Evolution of a) pH; b) Eh; c) sulphate and d) sulphide for compost, oak leaf, poultry manure and sheep manure during batch experiments.

Clearly, the compost reactor exhibited a behaviour different from that of the other three organic substrates. In this reactor, pH rose gradually from 2 to approx. 7 on day 60. From this day onwards, it remained unchanged throughout the experiment (Figure 1a). During the first days of the experiment no evidence of bacterial activity was observed: high positive Eh values maintained around +500 mV compared with the standard hydrogen electrode (Figure 1b), and no sulphate removal and subsequent sulphide generation were detected (Figures 1c and 1d). On day 27, 1 g dm$^{-3}$ sodium acetate and 1 g dm$^{-3}$ potassium oxalate were added to the bioreactor in order to stimulate bacterial activity. Drastic changes could be observed: redox potentials dropped to ~200 mV (Figure 1b), water acquired a strong hydrogen sulphide odour, sulphate
concentration decreased gradually from 11.0 to 4.2 mmol dm$^{-3}$ on day 123 (Figure 1c) and sulphide concentration increased to around 6 mmol dm$^{-3}$ over the same period (Figure 1d). These changes indicated that bacterial sulphate reduction took place. Sulphidogenesis was due to the acetate as a readily available carbon and energy source rather than to the oxalate, which was not able to promote SRB activity as observed in subsequent studies (data not shown). On day 124 a sulphate load was added to the initial 10.6 mmol dm$^{-3}$ (Figure 1c), which increased the sulphide generation to a maximum concentration of 6.6 mmol dm$^{-3}$ on day 175.

In contrast to compost, the three other organic substrates developed sulphate-reducing conditions without any extra addition of simple organic compounds. These conditions led to the achievement of negative redox potentials (around -200 mV) (Figure 1b), to a decrease in sulphate concentration (Figure 1c) and to the subsequent generation of sulphides (Figure 1d). Of these, sheep manure was more successful in promoting sulphidogenesis (sulphate concentrations below 0.1 mmol dm$^{-3}$ corresponding to a sulphate removal >99%) than oak leaf and poultry manure, which gradually decreased sulphate concentrations to 2.1 mmol dm$^{-3}$ (removal level 80%). These sulphate profiles were accompanied by a rapid increase in sulphide concentration from the initial undetectable levels to nearly 6.6 mmol dm$^{-3}$ for sheep manure, and to around 3 mmol dm$^{-3}$ for oak leaf and poultry manure after three months of experiment.

**Sulphur balance:** A sulphur balance throughout the experiment was roughly estimated by assuming that initial sulphur (as SO$_4^{2-}$) was converted either to S$^{2-}$ or maintained as unreacted SO$_4^{2-}$. This assumption considered that the sulphur removed in the solid phase constituted only a small percentage of the initial sulphur and that the concentration of other sulphide species in the pore water was negligible. Recovering percentages of sulphur (computed from differences between initial S –as sulphate- and final S –as generated sulphide and unreacted sulphate ) were 52% for oak leaf, 72% for acetate-amended compost and sheep manure, and 74% for poultry manure. This imbalance may be attributed to the loss of volatile H$_2$S (Elliott et al., 1998; Dvorak et al., 1992), or retentions as metallic sulphides.

**Sulphate-reduction rates:** Microbial sulphate-reduction and sulphide-generation rates are given in Table 2. They were calculated using linear least-square regression analysis of sulphate and sulphide concentration profiles in Figures 1c and 1d, ignoring early-time data, which may have been affected by acclimatisation periods and adsorption of sulphates onto ferric (oxy)hydroxides (presumably formed after the release of the acid-soluble Fe contained in sediment and compost), and late-time data, which may have been SO$_4^{2-}$ limited (Waybrant et al., 1998; Cocos et al., 2002). The selected points for this calculation were those encountered
within the linear range of 12-70 days for oak leaf and sheep manure experiments, and 20-120 days for acetate-amended compost and poultry manure. The sulphate removal rates were 0.25 mmol dm\(^{-3}\) d\(^{-1}\) for oak leaf and sheep manure and 0.08 mmol dm\(^{-3}\) d\(^{-1}\) for the acetate-amended compost and poultry manure. The sulphide-generation rate ranged between 0.03 mmol dm\(^{-3}\) d\(^{-1}\) for oak leaf and poultry manure and 0.11 mmol dm\(^{-3}\) d\(^{-1}\) for sheep manure. These rate values compare well with those reported in the literature in similar batch experiments (Table 2).

**Column experiments**

The evolution of pH, SO\(_4^{2-}\) and S\(^{2-}\) during the column experiments is presented in Figure 2. Eh was monitored only for the compost column and is not represented.

From the beginning, pH rose from 2 in the influent to around 6.3 for the compost column and around 7.7 for the sheep manure column. These pH values remained unchanged throughout the experiment (Figure 2a).

For the compost column, no reduction conditions were evident during the first 20 days: Eh remained within the range of positive values (+200 mV) and sulphide concentration was under detection limits (Figure 2c). Despite these observations, a net sulphate removal (about 50%) was observed (Figure 2b). The reason for this unexpected removal may be due to the initial sorption of sulphates onto ferric(oxy)hydroxides, which appear to be an important process as long as reducing conditions are not reached (Christensen *et al.*, 1996; Waybrant *et al.*, 1998). The formation of ferric(oxy)hydroxides is presumably due to the release of the acid-soluble Fe contained in sediments and compost. This solubilised Fe can precipitate as (oxy)hydroxide at the prevailing neutral pH. Amorphous ferric(oxy)hydroxides containing metals and sulphates have been observed ubiquitously under scanning electron microscopy in earlier studies (Gibert *et al.*, 2003). The low Fe concentrations measured in the effluent (data not shown) were consistent with the assumption of ferric(oxy)hydroxide precipitation. In the absence of SRB activity, after two weeks of experiment, 1g dm\(^{-3}\) sodium acetate and 1g dm\(^{-3}\) potassium oxalate were added to the feed water in order to stimulate bacterial activity. Changes were observed again: Eh dropped to values corresponding to sulphidogenic activity (-200 mV), sulphate reached a steady state at 3 mmol dm\(^{-3}\) (50% removal) (Figure 2b) and sulphide concentration increased to 2.5 mmol dm\(^{-3}\) (Figure 2c). It remains unclear why the phenomenon of initial SO\(_4^{2-}\) sorption occurred in the column experiments but not in the batch experiments.
Fig. 2. Evolution of a) pH; b) sulphate and c) sulphide for compost and sheep manure during column experiments. After 14 days feed water for the compost-column was supplemented with sodium acetate (1 g dm$^{-3}$). For the sheep manure-column, the flow rate was decreased on day 69 and day 112, the average residence time increasing from 0.73 days to 2.4 and 9.0 days, respectively.
For the sheep manure column no evidence of microbial sulphate reduction was observed at a residence time of 0.73 days. During the first days, effluent sulphate concentrations were even higher than those in the influent, which may be attributable to the leaching of sulphate contained in the sheep manure. Compared with batch results, the lack of sulphidogenesis can be closely attributed to kinetic factors. The flow rate was consequently reduced on day 69 to 0.018 cm$^3$ min$^{-1}$, which corresponded to a residence time of 2.4 d. A longer residence time resulted in a relatively higher sulphate reduction (18% removal) accompanied by a sulphide generation, with average sulphate and sulphide effluent concentrations of 4.8 (± 0.2) and 1.0 (± 0.1) mmol dm$^{-3}$, respectively. On day 112 the flow rate was decreased again to 0.005 cm$^3$ min$^{-1}$ (9.0 days average residence time), and a slight increase in the sulphate removal (27%) and sulphide generation was observed (average sulphate and sulphide effluent concentrations of 4.3±0.5 and 1.7±0.2 mmol dm$^{-3}$, respectively).
<table>
<thead>
<tr>
<th>System</th>
<th>Material</th>
<th>Influent $\text{SO}_4^{2-}$ (mmol dm$^{-3}$)</th>
<th>$t_R$ (days)</th>
<th>$\text{SO}_4^{2-}$ reduction rate (mmol dm$^{-3}$d$^{-1}$)$^{(1)}$</th>
<th>$\text{S}^2$ generation rate (mmol dm$^{-3}$d$^{-1}$)$^{(1)}$</th>
<th>% $\text{SO}_4^{2-}$ removed$^{(1)}$</th>
<th>Reference</th>
</tr>
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<tr>
<td>Batch</td>
<td>compost, limestone</td>
<td>10.6</td>
<td>-</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Present study</td>
</tr>
<tr>
<td>Batch</td>
<td>oak leaf, limestone</td>
<td>10.6</td>
<td>0.25</td>
<td>0.03</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batch</td>
<td>poultry manure, limestone</td>
<td>10.6</td>
<td>-</td>
<td>0.07</td>
<td>0.03</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Batch</td>
<td>sheep manure, limestone</td>
<td>10.6</td>
<td>0.25</td>
<td>0.11</td>
<td>&gt;99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batch</td>
<td>cow manure, whey</td>
<td>10.4</td>
<td>-</td>
<td>0.031-0.046</td>
<td>n.a.</td>
<td>19-27</td>
<td>Christensen et al., 1996</td>
</tr>
<tr>
<td>Batch</td>
<td>sewage sludge, leaf mulch, wood chips, sheep manure, sawdust, cellulose, creek sediment, limestone</td>
<td>12.5-47.9</td>
<td>-</td>
<td>0.233-1.641</td>
<td>n.a.</td>
<td>25-100</td>
<td>Waybrant et al., 1998</td>
</tr>
<tr>
<td>Batch</td>
<td>poultry manure, wood chips, leaf compost, oxidised mine tailings, silica sand, creek sediment, urea, limestone</td>
<td>30.6</td>
<td>-</td>
<td>0.371-1.627</td>
<td>n.a.</td>
<td>&gt;95</td>
<td>Cocos et al., 2002</td>
</tr>
<tr>
<td>Column</td>
<td>Compost$^{(3)}$, limestone</td>
<td>5.5</td>
<td>0.73</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Present study</td>
</tr>
<tr>
<td>Column</td>
<td>sheep manure, limestone</td>
<td>5.5-5.8</td>
<td>0.73</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>Bioreactor</td>
<td>mushroom compost (manure, hay straw, corn cobs, wood chips, gypsum, limestone)</td>
<td>10.4</td>
<td>5-17</td>
<td>0.214-0.333</td>
<td>0.062-0.126</td>
<td>17-20</td>
<td>Dvorak et al., 1992</td>
</tr>
<tr>
<td>Bioreactor</td>
<td>fresh alfalfa</td>
<td>10.3</td>
<td>3.5-35</td>
<td>0.632-1.707</td>
<td>n.a.</td>
<td>42-58$^{(2)}$</td>
<td>Béchard et al., 1994</td>
</tr>
<tr>
<td>Column$^{(3)}$</td>
<td>oak chips/mushroom compost/paper sludge</td>
<td>26.9</td>
<td>20</td>
<td>0.260$^{(3)}$</td>
<td>n.a.</td>
<td>50</td>
<td>Chang et al., 2000</td>
</tr>
<tr>
<td>Column</td>
<td>wood chips, leaf compost, poultry manure, urea, limestone, sand</td>
<td>15.6</td>
<td>&gt;0.5</td>
<td>&lt;4.26</td>
<td>n.a.</td>
<td>12</td>
<td>Beaulieu et al., 2000</td>
</tr>
<tr>
<td>Column</td>
<td>pyrite, silica, leaf mulch, wood chips, sawdust, sewage sludge, creek sediment, limestone$^{(5)}$</td>
<td>10.3-41.6</td>
<td>12</td>
<td>0.5-0.8</td>
<td>n.a.</td>
<td>20-60</td>
<td>Waybrant et al., 2002</td>
</tr>
<tr>
<td>Full-scale barrier</td>
<td>wood chips, limestone, silica sand</td>
<td>20.6-41.6</td>
<td>60-165$^{(6)}$</td>
<td>0.110-0.159$^{(7)}$</td>
<td>n.a.</td>
<td>30-60</td>
<td>Benner et al., 2002</td>
</tr>
</tbody>
</table>

n.a.: not available, n.d.: not detectable
(1) rate values estimated from raw data
(2) decreasing to 0 over time
(3) columns were operated after 22 weeks of batch operation
(4) values referring to the steady-state after an initial high sulphidogenesis
(5) with initial lactate addition to help bacterial acclimation and sulphate reducing conditions
(6) the two different residence times correspond to the slow flow path at bottom and top of the barrier and fast flow path in the middle of the barrier.
(7) A 30% decrease in the overall rate of sulphate reduction has been found 38 months after the barrier installation

Table 2. Summary of key parameters in both discontinuous and continuous systems based on natural organic substrates without any supplement of easily assimilable organic compounds reported in the literature
**Sulphur balance:** Based on the same assumptions as in the earlier batch experiments, a balance of total S was conducted from liquid-phase measurements related to Figures 2b and 2c. The recovering percentages of sulphur (computed from differences between the total entered S –as sulphate- and the total exited S –as generated sulphide and unreacted sulphate) were calculated throughout the experiment for compost and separately for each of the three periods of the experiment for sheep manure, i.e. for each of the three residence times evaluated during the experiment. The recovering percentages were 84% for compost, and 88% (over the residence time period of 0.73 days, excluding the initial days affected by sulphate releasing from the organic matter), 99% (residence time of 2.4 days) and 103% (residence time of 9.0 days) for sheep manure.

**Sulphate-reduction rates:** Table 2 gives the stabilised biological degradation rates for each column. These calculations were based on the difference between influent and effluent sulphate concentrations and on the residence time in the column (Benner *et al.*, 2002). For the acetate-amended compost column and a residence time of 0.73 days, sulphate and sulphide effluent concentrations of 3.14 mmol dm\(^{-3}\) and 2.40 mmol dm\(^{-3}\) resulted in a sulphate reduction rate of 3.27 mmol dm\(^{-3}\) d\(^{-1}\) and a sulphide generation rate of 3.29 mmol dm\(^{-3}\) d\(^{-1}\). For the sheep manure column, no evidence of microbial sulphate reduction was observed. However, for residence times of 2.4 and 9.0 in the sheep manure column, sulphate reduction and sulphide generation rates were 0.44 and 0.42 mmol dm\(^{-3}\) d\(^{-1}\) and 0.17 and 0.19 mmol dm\(^{-3}\) d\(^{-1}\), respectively. These rate values compare well with those reported in the literature in continuous systems with similar complex organic substrates (Table 2). It is worth noting that although longer residence times resulted in higher sulphate removals, they led to lower sulphate reducing rates. A similar pattern has been reported in earlier works (Glombitza, 2001).

**CONCLUSIONS**

The results demonstrate the feasibility of SRB for AMD treatment and highlight the importance of considering biodegradability of the organic substrate and residence time in the overall performance of the system.

In the batch experiments, compost proved to be too poor in carbon to promote and sustain SRB activity by itself. The positive response of the system to acetate amendment indicated that sulphate reduction was carbon-limited. In contrast to compost behaviour, sheep and poultry manures and oak leaf produced reducing conditions and sustained active sulphidogenesis, indicating bacterial activity. Sheep manure was clearly the most successful electron donor (sulphate removal level of >99%), followed by poultry manure and oak leaf
The capacity of the evaluated organic substrates to promote sulphate removal by SRB activity was correlated with their chemical characterisation. There seems to be a clear relationship between the lignin content and the efficiency of the organic material to decompose and sustain bacterial activity. The results show that the lower the content of lignin in the organic substrate, the higher its degradability and capacity for developing bacterial activity. This capacity for decomposition was computed in terms of biodegradable fraction and quantified using Chandler’s equation (eq 1). Sheep manure, poultry manure, oak leaf and compost presented biodegradable fractions of 0.56, 0.39, 0.38 and 0.11, respectively. Figure 3 represents the sulphate removal in batch experiments as a function of the biodegradable fraction for each organic substrate. The linear correlation suggests that the biodegradable fraction may be a useful criterion for the selection of an organic matter for AMD treatment.

Fig. 3. Sulphate removal attained in batch experiments as a function of the biodegradable fraction of the evaluated organic substrates.

These findings are consistent with other studies on the limited degradability of lignin-cellulosic substrates and on the need to use organic substrates as manure to maximise sulphidogenesis (Cocos et al., 2002). In contrast, Waybrant et al. (1998) concluded that a cellulosic material alone could sustain a satisfactory bacterial activity. It is clear that an improved methodical analysis of organic substrates is warranted to specify more accurately the characteristics of the tested organic substrates.

The column experiments emphasised the importance of considering residence time as a key factor in the performance of continuous systems. With a residence time of 0.73 days, sheep manure only marginally stimulated sulphide production. However, extending residence time to 2.4 and 9.0 days resulted in an increase in the sulphate removal to 18% and 27%, respectively. This time-dependent performance of the system has been reported in earlier studies (Dvorak et al., 1992; Benner et al., 2002) and should be borne in mind when designing a PRB.
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