

# Performance of sulphate- and selenium-reducing biochemical reactors using different ratios of labile to recalcitrant organic materials

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## ABSTRACT

Successful operation of sulphate-reducing bioreactors using complex organic materials depends on providing a balance between more easily degrading material that achieves reasonable kinetics and low hydraulic retention times, and more slowly decomposing material that sustains performance in the long term. In this study, two organic mixtures containing the same ingredients typical of bioreactors used at mine sites (woodchips, hay and cow manure) but with different ratios of wood (recalcitrant) to hay (labile) were tested in six continuous flow bioreactors treating synthetic mine-affected water containing 600 mg/L of sulphate and 15 µg/L of selenium. The reactors were operated for short (5–6 months) and long (435–450 days) periods of time at the same hydraulic retention time of 15 days. There were no differences in the performance of the bioreactors in terms of sulphate-reduction over the short term, but the wood-rich bioreactors experienced variable and sometimes unreliable sulphate-reduction over the long term. Presence of more hay in the organic mixture was able to better sustain reliable performance. Production of dissolved organic compounds due to biodegradation within the bioreactors was detected for the first 175–230 days, after which their depletion coincided with a crash phase observed in the wood-rich bioreactors only.

**Key words** | bioreactor, bioremediation, mine, organic material, selenium, sulphate-reduction

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## INTRODUCTION

Biochemical reactors (BCRs) using waste organic materials show promise as effective ways of removing contaminants, such as sulphate and metals, from mining-influenced waters (Neculita *et al.* 2007). Since they rely on natural geochemical and biological processes for effective treatment, BCRs are considered a passive technology. They are more cost-effective than active chemical and biological processes, with high-density sludge treatment costing up to 16 times more than biological passive treatment (Zinck & Griffith 2013). They can be installed in remote, inaccessible locations with little or no infrastructure, and are attractive for treating seeps disseminated throughout the mine site (Ziemkiewicz *et al.* 2003). Their most desirable characteristic is that the slow decomposition of the organics provides a sustained supply of carbon sources for sulphate-reducing bacteria and other microbes important for metal removal, allowing long-term deployment without the need for constant nutrient amendment. Sulphate-

reducers rely on microbes that hydrolyze long-chain carbohydrates and proteins to sugars and amines, and others that ferment these intermediates to the organic acids, alcohols and hydrogen that are preferred electron donors (Postgate 1984). Many other microbes supported by the decomposing complex organic material can immobilize metals via other mechanisms such as direct metal reduction, assimilation, adsorption and volatilization (Gadd 2010).

Sulphate and selenium are two emerging constituents of concern at mine sites in British Columbia, Canada. Sulphate concentrations in receiving waters with low hardness must not exceed 128 mg/L (Ministry of Environment 2013). Regulation of sulphate was a recent development at the time of writing and there was a need for cost-effective technologies. The guideline for selenium is very stringent at 2 µg/L (Ministry of Environment 2014) due to its toxicity and propensity for bioaccumulation (Lemly 1996). Although

BCRs have been researched thoroughly for their effectiveness at removal of metals such as copper and zinc (Bratkova *et al.* 2013; Zhou *et al.* 2013), little is known about their ability to remove sulphate and selenium, particularly in long-term experiments (longer than a few weeks). In this work, laboratory-scale continuous-flow BCRs were investigated as one potential technology for sulphate and selenium removal from influent typical of a mine tailings pond effluent.

Choice of which organic materials to use for mine-influenced water treatment BCRs is usually restricted to what is available close to the mine site so as to minimize transportation costs. Successful performance of the bioreactor requires a balance between readily available organic substrates that fuel kinetics for a reasonable hydraulic retention time and consistent slow degradation of the more recalcitrant components to ensure a continuous supply of electron donors over the long term. Macromolecules such as lignin and cellulose degrade slowly (Leschine 1995) and support low sulphate-reduction rates (Coetser *et al.* 2006), but they are expected to last longer (Hulshof *et al.* 2003). Materials with a high lipid or protein content (whey or alfalfa hay) achieve higher rates of sulphate-reduction (Gibert *et al.* 2004) but they may need to be replenished more often. The consensus of previous studies is that a mixture of organic materials is more effective than using only one type (Waybrant *et al.* 1998; Zagury *et al.* 2006; Greben *et al.* 2007). Few studies have been done on continuous-flow bioreactors (e.g., Costa *et al.* 2007; Pulles & Heath 2009; Song *et al.* 2012) and most were only for short periods of time (167 days, 140 days and 400 days, respectively). In particular, information on what effect the ratio of labile to recalcitrant organic materials has on the short- and long-term performance of BCRs is missing. The current study tests the hypothesis that the labile (hay) to recalcitrant (wood) ratio will affect the short- and long-term performance of the BCR. Bioreactors with more labile material are expected to perform well in the short term, but their performance might decrease when the labile carbon sources run out. Whereas BCRs with more recalcitrant material will fail sooner than the BCR with more labile material, over the long term, both bioreactors should perform similarly since they would both be fuelled by wood biodegradation. Alternatively, having more labile to recalcitrant material creates an overlap between the time the labile components are completely consumed and the time period that recalcitrant compounds are broken down to more easily degradable molecules.

## MATERIALS AND METHODS

### Design of the bioreactors

Six columns (inside diameter: 11.4 cm; length: 50.8 cm) were used as up-flow BCRs. One series of three bioreactors, BCRW1, BCRW2 and BCRW3, contained a wood-rich mixture (40%<sub>dw</sub> woodchips, 30%<sub>dw</sub> hay and 30%<sub>dw</sub> cow manure) and a second series was hay-rich, BCRH1, BCRH2 and BCRH3, contained 20%<sub>dw</sub> woodchips, 50%<sub>dw</sub> hay and 30%<sub>dw</sub> cow manure (%<sub>dw</sub> denotes percent dry weight).

### Inoculum preparation

A sulphate-reducing culture (SRC) was maintained in Postgate-B growth medium (Postgate 1984) at 32 °C. Sludge from an upflow anaerobic sludge bed reactor treating sulphate-containing waters (Li & Baldwin 2011) was used as the initial inoculum.

Cellulose-degrading cultures (CDCs) were prepared from an organic mixture (40%<sub>ww</sub> wood chips, 30%<sub>ww</sub> hay, 30%<sub>ww</sub> manure) submerged in distilled water incubated anaerobically at 32 °C. Cellulose activity was measured using the method developed by Mewis *et al.* (2011): Briefly, a 0.1 mg/mL 2,4-dinitrophenyl (DNP)-cellobiose solution was prepared from 75 mg/mL DNP-cellobiose in a dimethyl sulfoxide and lysis mix (10% Triton X-100, 100 mM Tris, 10 mM ethylenediaminetetraacetic acid (EDTA)). This was added to a sample from the cultures followed by incubation at 37 °C for 12–16 hours. Absorbance at 400 nm indicated the extent of cellulose degradation. Cultures exhibiting strong cellulose activity were used for bioreactor inoculum. The CDCs were maintained in Luria broth (Atlas 2004) with fresh organics at 32 °C until needed.

Bioreactor inoculum was prepared in an anaerobic hood using 100 mL each of SRC and CDC added to 100 g of hay- or wood-rich organic mixture in 1 L bottles filled with deoxygenated synthetic tailings pond water without selenium (Table 1). Inoculum pH was adjusted to 8.

### Packing and operation of bioreactors

Organic material was pre-soaked overnight in Postgate-B, adjusted to pH 8 and deoxygenated by sparging with N<sub>2</sub>. Crushed (+20 and –10 mesh) limestone (4%<sub>w</sub>) was added to facilitate pH control. Inoculum (500 mL) was added to the organic material inside an anaerobic hood before loading

**Table 1** | Composition of influent feed

Chemical compound	Postgate-B medium (g/L)	Synthetic tailings pond water (g/L)
Total SO <sub>4</sub> <sup>2-</sup>	1.650	0.6
CaSO <sub>4</sub> · 2H <sub>2</sub> O	1.26	0.483
Na <sub>2</sub> SO <sub>4</sub>	–	0.253
MgSO <sub>4</sub>	0.977	0.094
FeSO <sub>4</sub> · 7H <sub>2</sub> O	0.5	0.25
KH <sub>2</sub> PO <sub>4</sub>	0.5	0.0475
NH <sub>4</sub> Cl	1	0.026
Sodium lactate	3.5	–
Selenium (as sodium selenate) (mg/L)	–	0.015
pH	7.5–8	7.5–8

the mixtures into the reactor columns. An additional 1.5–2 L of deoxygenated Postgate-B was added to each column.

All bioreactors were left in batch mode for 40 days and sulphate concentration was monitored from the middle sampling port. When sulphate dropped to less than half of the initial concentration, continuous flow of Postgate-B was initiated for 45 days (hydraulic retention time in each bioreactor was 15 days) after which it was replaced by synthetic tailings pond water. A previous study indicated that this start-up procedure was needed for effective performance of the bioreactors (Mirjafari *et al.* 2011). All feed solutions were deoxygenated by sparging with N<sub>2</sub> and fed from tightly closed plastic bags to minimize oxygen infiltration.

### Initial characterization of the organic materials

For total dissolved organic carbon (DOC), 200 mL of dH<sub>2</sub>O was added to 2.5 g-dw of finely ground solids and shaken overnight at 200 rpm (20 °C). The mixture was filtered (0.45 µm) and total organic carbon (TOC) measured using a Shimadzu TOC analyzer (Shimadzu Corp., Kyoto, Japan; TOC-V<sub>CPH</sub>). Total dissolved carbohydrates were measured with the phenol-sulphuric acid Dubois assay (Dubois *et al.* 1956) and organic acids using a Waters (Milford, MA, USA) high pressure liquid chromatography system with an IC-Pak ion exclusion column and UV detection at 214 nm. Determination of the crude fibre components (recalcitrant materials such as hemicellulose, cellulose and lignin) was done using a sequential wash with neutral detergent (Vansoest *et al.* 1991): 3.6 g NaOH, 27 g sodium lauryl sulphate, 13.15 g EDTA, 6.13 g sodium borate decahydrate, 4.105 g

disodium hydrogen phosphate and 9 mL tri-ethylene-glycol in 900 mL de-ionized water (Hall 2000). All the chemicals were American Chemical Society grade from Sigma-Aldrich (St Louis, MO, USA). The residue of the neutral detergent wash was used to measure holocellulose, α-cellulose, hemicellulose and lignin following the procedures suggested by Wieder & Starr (1998).

### Chemical analysis of the bioreactor effluents

Concentrations of dissolved organic compounds in the effluent were measured regularly to assess the availability of carbon sources. These were DOC, total dissolved carbohydrates, proteins and organic acids, as previously described. Environmental conditions pH, oxidation-reduction potential (Pt ORP probe referenced to Ag/AgCl) and conductivity were measured immediately after sampling with a pH/ORP/Cond 7200 pH meter and probes (WTW, Weilham, Germany). Dissolved oxygen (DO) was measured with a Symphony SP50D DO meter (VWR, Radnor, PA, USA). Sulphate was measured using the turbidimetric barium sulphate American Water Works Association method 4500 – SO<sub>4</sub><sup>2-</sup> and sulphide was measured with the methylene blue American Water Works Association 4500-S<sup>2-</sup> method (Eaton *et al.* 2005). Total selenium concentrations were determined using high-resolution inductively coupled plasma mass spectroscopy (ICP-MS) by an analytical laboratory (ASL, Burnaby, BC, Canada).

## RESULTS AND DISCUSSION

### Start-up and operation of wood-rich bioreactors

Organic material characteristics correlated with effective sulphate-reduction in other studies include DOC and the C:N ratio (Cocos *et al.* 2002; Zagury *et al.* 2006; Schmidtova & Baldwin 2011). Some mine-influenced water contains nitrate (Banks *et al.* 1997) and N might not be limiting even if the organic material has a very high C:N ratio. Dissolved organics are washed out during continuous flow and DOC also might not be a good predictor of reactor performance. Nevertheless, several characteristics of the hay used for these bioreactors indicated that it was a suitable substrate. Hay contained a larger fraction of water soluble organic carbon and a more favourable C:N ratio than woodchips (Table 2). The vast majority of the hay was organic matter (OM) with little ash from inorganic compounds. The higher resin acid content of the woodchips was expected to contribute to their slower biodegradation (Liss *et al.* 1997).

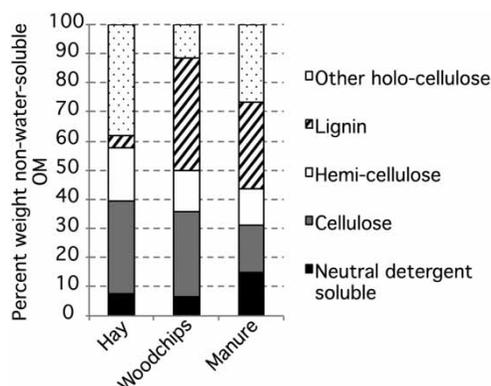
**Table 2** | Characteristics of the hay, woodchips and cow manure used for the BCRs (percent dry weight of organic material prior to water wash)

Characteristic	Hay	Wood	Manure
TOC	43.1	48.9	44.9
DOC	7.46	0.57	2.51
TKN	1.34	0.214	0.795
C/N (TOC/TKN) (ratio)	32.16	228.5	56.48
Total resin acids (mg/kg)	<0.6	8920	508
OM (% <sub>dw</sub> non-water-soluble solids)	98.2 ± 1.2	89.3 ± 1.7	71.1 ± 1.5

TKN: total Kjeldahl nitrogen.

To further explore the degradability of the hay, woodchips and manure, composition of the water-non-soluble OM fraction (Figure 1) was measured. Woodchips were composed of mainly lignin, cellulose and hemicellulose, whereas a significant portion of the hay comprised unidentified neutral detergent non-soluble material. Lignin, which is resistant to decomposition (Zagury *et al.* 2006), composed more than one-third by weight of the woodchips compared to less than 5%<sub>dw</sub> OM of the hay. These tests confirmed that hay contained more labile compounds than wood.

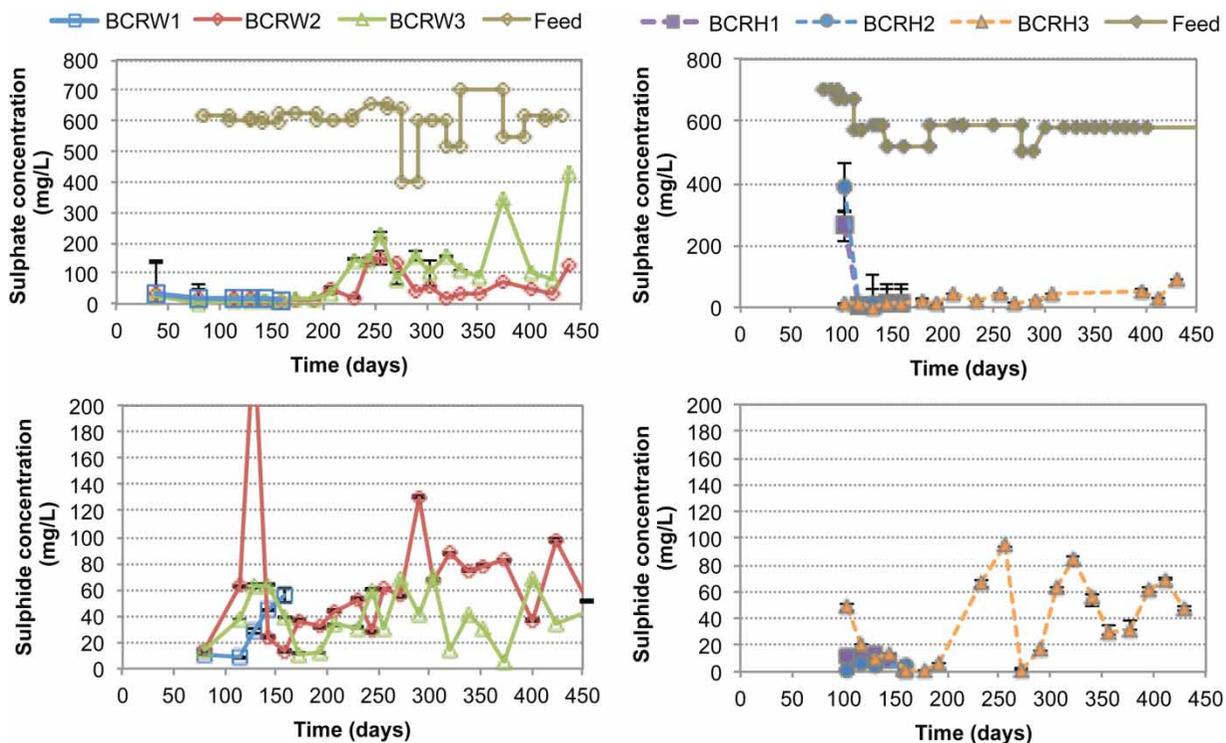
During the batch acclimation period, pH decreased below 6.5 (Figure S1, available online at <http://www.iwaponline.com/wst/072/276.pdf>), the lower limit for effective sulphate-reduction (Postgate 1984). Most likely, this was due to leaching of acidic compounds, such as phenolic and resin acids, from the organic materials, particularly wood (Venner *et al.* 2009), and release of organic acids due to fermentation and acetogenesis. Regular pH adjustment to 7.5–8.0 was required for up to 114 days in some cases. Thereafter, NaOH addition was needed for pH adjustment only sporadically. In all wood-rich bioreactors, the ORP was between –250 and –380 mV (Figure S1), which is within the range reported for other mixed organic bioreactors (for example, Song *et al.* 2012) and

**Figure 1** | Relative composition of the OM portion of the non-water-soluble material.

favorable for sulphate-reduction (Stumm & Morgan 1996). DO was always below 1 mg/L, except for a spike on Day 374 (Figure S1).

All three wood-rich bioreactors removed sulphate to well below the 100 mg/L targeted limit over the first 200 days after start up (Figure 2). One bioreactor (BCRW1) was sacrificed after 159 days so as to capture the microbial community and organic material characteristics during the successful operating phase (data not shown). In the duplicate long-term bioreactors, effluent sulphate concentrations increased above 100 mg/L after 230 days of operation and then fluctuated cyclically. Performance variability increased in BCRW3; however, in BCRW2, sulphate decreased again to below 100 mg/L (Figure 2(a)). Sulphide concentrations in the wood-rich bioreactors were initially low at 14 mg/L (Figure 2) and increased to above 50 mg/L over the first approximately 100 days, but then decreased and fluctuated for the rest of the operation. Comparing the changes in sulphate and sulphide concentrations revealed that these two variables were alternating. Iron, as an Fe salt, was added to the influent so as to control sulphide concentration through precipitation of FeS. Sulphide is toxic to microorganisms (O'Flaherty *et al.* 1998), and also may oxidize to reform sulphate (Mirjafari *et al.* 2011). For influents lacking sufficient metals to scavenge sulphide, the addition of Fe is recommended. Selenium was removed from the influent concentration of 15 µg/L to less than 0.2 µg/L in the effluent for all times and all bioreactors.

Prior to start-up of continuous flow of synthetic tailings pond water, the DOC was very high (Figure S2, available online at <http://www.iwaponline.com/wst/072/276.pdf>), but then diminished within approximately three pore volumes after commencement of continuous flow. There was evidence of replenishment of dissolved organics as the DOC increased and decreased cyclically over the long term (inset in Figure S2). To further explore the nature of dissolved organic compounds present, total dissolved carbohydrates and organic acids were monitored in the effluent leaving the bioreactors (Figure S3 (available online at <http://www.iwaponline.com/wst/072/276.pdf>) and Figure 3). Dissolved carbohydrates were detected with a cyclical trend similar to that observed for the DOC, indicating that they were being produced and consumed throughout the bioreactor operation. Organic acids, malic acid, lactate, formate, propionate and acetate were present at the beginning, either from the original inoculum and organic materials or due to fermentation reactions during the acclimation period (Figure 3). An increase in acetate concentration after start-up of the synthetic tailings pond water indicated that acetogenic bacteria were active. In all three wood-rich bioreactors, excess acetate was undetectable after 150 days.



**Figure 2** | Sulphate (top panels) and sulphide (bottom panels) concentrations versus time for the wood-rich (left-hand-side panels) and hay-rich (right-hand-side panels) bioreactors. Error bars represent the standard deviation of three replicate analyses.

### Start-up and operation of hay-rich bioreactors

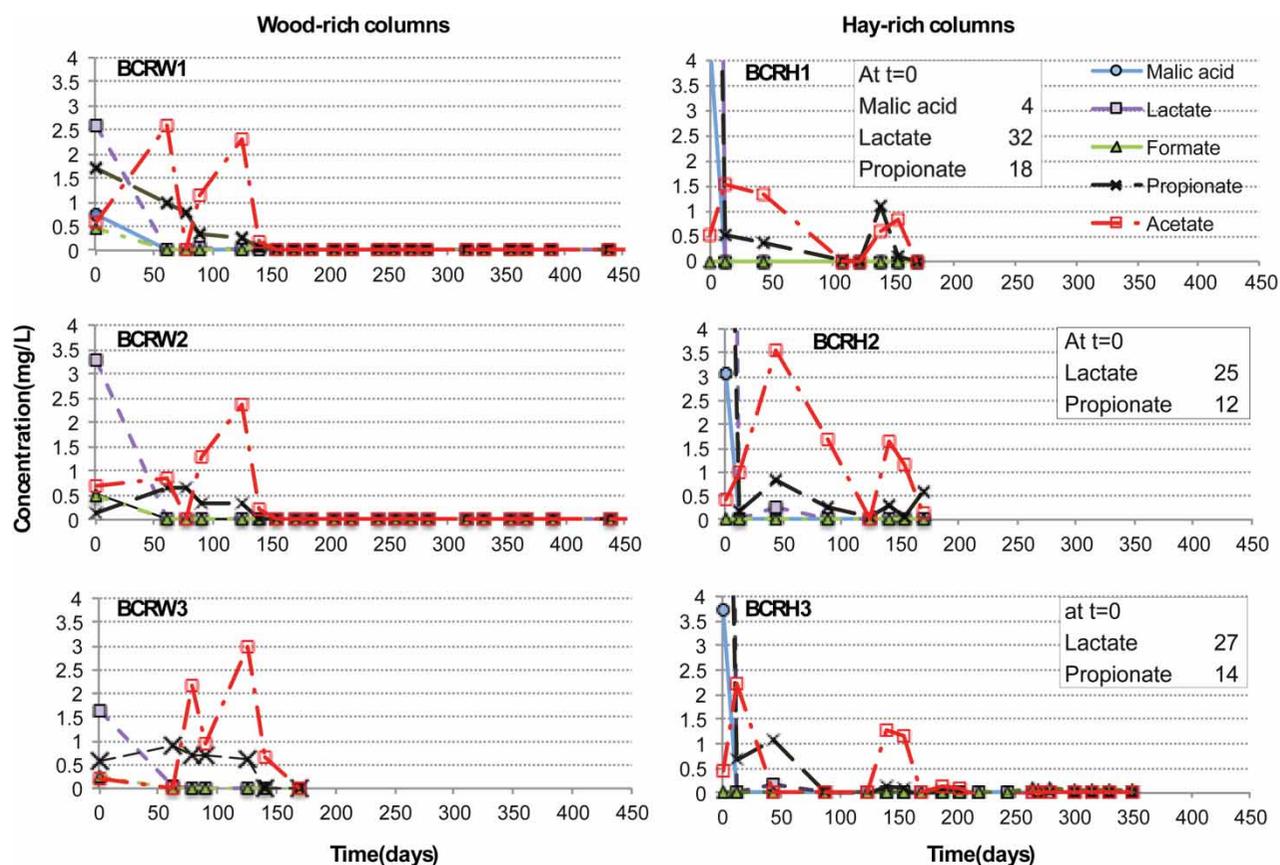
In contrast to the wood-rich bioreactors, the hay-rich bioreactors took longer to reach optimal pH and ORP conditions (Figure S1B). For example, in BCRH3, it took 193 days before the pH could be sustained above 6.5. The ORP in the hay-rich bioreactors was initially higher than that observed in the wood-rich bioreactors, but it did drop eventually to the same levels as seen in the wood-rich bioreactors. Two duplicate hay-rich bioreactors were operated for the short duration only. In the long-term hay-rich bioreactor (BCRH3), ORP decreased below  $-300$  mV after 144 days (Figure S1F). DO in all three bioreactors was below 1 mg/L, except for a spike on Day 130 (Figure S1H).

All hay-rich bioreactors achieved sulphate-reduction to below 100 mg/L, even the one long-term bioreactor (BCRH3) (Figure 2(C)). Unlike the wood-rich bioreactors, there were no measurable fluctuations in sulphate concentration in the effluent. However, sulphide concentrations fluctuated between concentrations as low as 1 mg/L and as high as 95 mg/L (Figure 2). Similarly to the wood-rich bioreactors, selenium removal was achieved ( $15 \mu\text{g/L}$  was reduced to  $0.1 \mu\text{g/L}$  in the effluent for all reactors and time points). These results demonstrate that it is possible

to remove sulphate and selenium to below the required concentrations using bioreactors with mixtures of woodchips, hay and manure. This approach could be one alternative to active treatment processes that require prohibitively costly reagents. Supplementing woodchips with more labile organic material such as hay improved the reliability of sulphate removal over the long term.

There was much more DOC in the hay-rich bioreactors initially (4,000 to 5,700 mg/L) and DOC replenishment was more pronounced than in the wood-rich bioreactors (Figure S2). The greater availability of dissolved organic components in the hay-rich bioreactors was confirmed in the profiles of dissolved carbohydrates (Figure S3). Excess acetate and propionate production occurred in the hay-rich bioreactors, even as late as 200 days after setting up the columns (Figure 3). Interestingly, there was a consistency to the appearance of the second acetate peak in all replicate columns. Like the wood-rich bioreactors, the hay-rich bioreactors were organic carbon limited over the long term, but no performance decline was observed for the long-term hay-rich bioreactor.

Other researchers observed a performance decline after a similar time period to the one seen for the wood-rich bioreactors: 240 days (Pulles & Heath 2009); 270 days (Neculita & Zagury 2008). Higher sulphate-reduction rates



**Figure 3** | Plot of organic acid concentrations versus time for the wood-rich (left-hand-side panels) and hay-rich (right-hand-side panels) bioreactors. Initial concentrations, where they were greater than the y-axis range, are given in the insets.

than those measured in this study (41 mg/(L day)) reported in the literature might be over-estimates since they were measured early on before washout of DOC, and in some cases the sulphate-reduction rate dropped by more than half after only a month of operation (Hulshof *et al.* 2003).

## CONCLUSIONS

It was possible to remove sulphate and selenium to below the required concentrations with a bioreactor using complex organics. Presence of more labile organic material in the original mixture gave the hay-rich bioreactors an advantage over the long term and no 'crash phase' was observed over the duration of operation.

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