



Novel Two-stage Biochemical Process for Hybrid Passive/Active Treatment of Mine-influenced Water

Lauren Lundquist¹ · Susan A. Baldwin¹

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Abstract

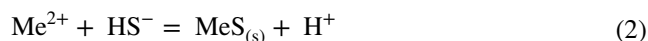
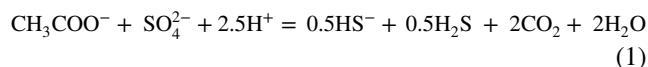
Current passive treatment systems for metal removal from sulphate-rich mine-influenced water, such as biochemical reactors and permeable reactive barriers, are based on a one-time application of a solid waste organic matrix. These are prone to declining efficiency and reliability as the carbon source is depleted over time. Also, in many of these systems, metal sulphide precipitates are retained in situ, creating a legacy liability on the mine site. A novel hybrid passive/active treatment system was designed to overcome these shortcomings by supplying a liquid nutrient source derived in a separate upstream step through fermentation of waste organic material, and by carrying out sulphidogenesis and chemical metal precipitation in two separate packed-bed column reactors, in which sulphide is fed back from the bioreactor into the upstream metal sulphide precipitation reactor. A separate metal precipitation module will enable eventual removal of the metals from the mine site. Liquid fermentation products from a woodchip/hay-grass mixture were compared with molasses as nutrient sources. The systems were tested for a duration of 96 days, demonstrating that the tested liquid carbon sources reduced sulphate at maximum rates of 41.2 and 28.7 mg-SO₄²⁻L⁻¹ d⁻¹, respectively, and removed 85% and 90% Zn, respectively, in the chemical precipitation step, making it possible to remove these from the mine site for recycling.

Keywords Zinc · Sulphate · Sulphate reducing bacteria · Metals · Circular economy

Introduction

Mining contributes to the supply of essential metals needed for the low carbon economy, such as Zn, Ni, Cu, Co, Li, Mn, Fe, and rare earths. Dwindling high concentration ore bodies and the challenging regulatory environment for establishing new mines threaten the supply of these metals (de Koning et al. 2018). Currently, water from mine workings, waste storage facilities, and mineral processing, whether neutral pH or acidic, is viewed as a liability and a challenge to treat. These mine-influenced waters (MIWs) present an opportunity for concomitant metal recovery along with treatment with novel treatment processes.

Industry standard high density sludge precipitation plants use neutralizing agents to remove metals from MIW, but the residues produced are unstable and need to be disposed of in an environmentally acceptable manner (Zinck and Griffith 2013). Passive and semi-passive treatment systems (Skousen et al. 2017), such as permeable reactive barriers (PRBs) and biochemical reactors (BCRs), use complex organic matter, often forestry or agricultural wastes, as slowly decomposing material to supply low molecular weight carbon compounds to microorganisms, such as sulphate-reducing bacteria (SRB). These bacteria reduce the sulphate present in many MIWs to sulphide (Eq. 1) that then reacts chemically with dissolved metal ions (Me²⁺) to precipitate them as sparingly soluble metal sulphides (Eq. 2) (Jeen et al. 2014; Johnson and Hallberg 2005; Mattes et al. 2011; Nielsen et al. 2018; Shabalala 2013).



✉ Susan A. Baldwin
 sue.baldwin@ubc.ca

¹ Department of Chemical and Biological Engineering,
 University of British Columbia, 2360 East Mall, Vancouver,
 BC V6T 1Z3, Canada

These are less costly to build and operate than high density sludge treatment plants but have several downsides. Usually, PRBs and BCRs are installed in the ground and the precipitated metals accumulate within the complex organic matter matrix. Over time, this becomes a contaminated site, and the accumulated metals present a risk and liability. Also, the performance of PRBs and BCRs declines as the easily degradable compounds that are broken down by hydrolysing and fermenting bacteria into low molecular weight compounds are exhausted (Mirjafari and Baldwin 2016; Neculita et al. 2007). Some systems fail as early as a few hundred days after start-up (Logan et al. 2005; Mirjafari and Baldwin 2016). When this happens, it is difficult to replace the spent material with fresh organics. Additionally, due to the heterogeneous nature of the complex organic matter, hydraulic failures can occur, causing short circuiting (Ness et al. 2014). To overcome limited longevity and reliability, and to reduce metal accumulation on site, we propose a new hybrid passive/active treatment system.

The system is decoupled into three steps: (1) waste organic matter decomposition, (2) biological sulphidogenesis, and (3) metal precipitation. Fuelling of biological sulphate reduction by fermented complex organic matter (e.g. silage) leachate has achieved high sulphate reduction rates in previous studies compared with use of the organic matter directly, and enabled use of a high kinetic rate bioreactor operating on liquid carbon feed only (Li and Baldwin 2011). In addition, use of aqueous-phase carbon sources in passive MIW treatment systems improves the kinetics of sulphide production and avoids plugging of the bioreactor (Sobolewski 2010). Performing complex organic matter fermentation in a separate step allows for replenishment of raw organic matter when needed to sustain the nutrient supply, and additionally provides a liquid flow stream of a controlled amount of dissolved organic carbon and other nutrients to support SRB in the biological reactor step. The biological reactor is downstream of the metal precipitation reactor that receives MIW directly from the source so as to recover metals prior to sulphate reduction. The metal precipitation reactor receives a return flow of biogenic sulphide from the SRB bioreactor in order to remove metals as metal sulphides. Also, removing metals before the sulphidogenesis step prevents metal inhibition of SRB growth. This process can be implemented as a modular system, with the metal precipitation module easily removed for recovery of the metal values at a location away from the mine site. To achieve such a process, hybrid passive/active components are needed, such as packed bed reactors for metal precipitation and biosulphide production and a simple anaerobic fermenter. Waste organic materials can still be used to keep reagent costs low, and SRB are already present in most MIW from sulphidic mine wastes

(Nielsen et al. 2018). Such hybrid approaches are expected to become more popular as mining companies look for ways to recover metals from their waste streams and meet water quality guidelines (Nancucheo et al. 2012).

As a proof of concept for this process, liquid ferments were produced from several waste organic materials. These were tested for their ability to support biological sulphate reduction and sulphide production, and to determine dosing levels of the solid organic wastes needed to extract the amounts of dissolved organic carbon required per sulphate to be reduced. Supernatants from these ferments containing biogenic sulphide were tested for chemical precipitation of metals from a typical MIW in batch tests. Based on the successful results, continuous flow packed bed columns were set up at a mine site to treat the MIW and test the removal of metals in a metal precipitation column separate from the sulphidogenesis bioreactor.

Methods

Site Description

The field site used for testing the novel hybrid passive/active system is a closed mine site situated on private land in southeastern British Columbia (BC), where reclamation efforts are ongoing. The site currently collects water from both underground and above ground sources and directs them to a water treatment facility for metal removal prior to discharge to the environment. The MIW from one of the underground sources was investigated in this study as it exceeds the British Columbia Water Quality Guidelines for Aquatic Life (BC WQGs; The Government of British Columbia 2021) for Cd, Co, Fe, Pb, Mn, and Zn (Table 1).

Batch Screening Tests

Spruce-pine-fir (SPF) woodchips, a 50:50 hay-grass mixture, and pulp mill secondary wastewater treatment biosolids, respectively, were selected as sources for fermentation broths expected to contain carbon compounds suitable for supporting SRB. To produce the fermentation broths, each solid organic material was mixed with unfiltered river water from Spanish Banks Creek in Vancouver, British Columbia, Canada, and incubated at 25 °C for 5 days. Unsterilized materials and river water were used so that the native microorganisms in the organic material and in the Spanish Banks Creek water could contribute to fermentation. After 5 days, the fermentation broth solution from each organic material was obtained by filtering (0.45 µm). Each fermentation solution was added in the appropriate amount to a growth medium based on the Postgate G recipe (Postgate 1983)

(1700 mg/L SO_4^{2-}) to achieve a concentration of 4000 mg/L chemical oxygen demand (COD). All growth media were sterilized by autoclaving at 120 °C for 15 min. After autoclaving, the pH of each growth medium was adjusted to 7.0 and oxygen eliminated through sparging with N_2 gas for 20 min. Finally, each growth medium was inoculated with 10%v mine sediment that was previously collected from a site impacted by seepage containing elevated levels of metals, especially Zn. Triplicate cultures for each fermentation solution growth medium were incubated at 28 °C for 16 days. Filtered (0.45 μm) liquid samples were taken every 3 days.

To test metal precipitation with biogenic sulphide, 20 mL filtered (0.45 μm) supernatant from the fermentation solution SRB cultures was added to 100 mL of MIW (Table 1) in separate containers. The biogenic sulphide was added at a 1:1 mass ratio to the amount of Zn in the MIW. Dissolved metal samples were taken at time points of 30 m, 1 h, 5 h, and 24 h.

Column Reactor Experimental Set-Up

The novel hybrid passive/active system consisted of two up-flow, packed-bed columns in series with a feedback loop for biogenic sulphide from the second column into the first column (Fig. 1). The first column was targeted for metal precipitation and had an empty bed volume of 450 mL and a hydraulic retention time of 11 h, whereas the second column, the biogenic sulphide reactor, had an empty bed volume of 2.5 L and a hydraulic retention time of 4 d. The organic matter ferment was prepared separately following procedures similar to those used in the batch tests.

Three separate two-stage column systems were set up to compare two different carbon sources. Carbon source 1 was a woodchip/hay-grass mix fermentation solution, carbon

source 2 was molasses (an industry standard), and the third system was an abiotic control with no added carbon source. First, the second stage (biological) column was filled with 250 mL of mine sediment, as an inoculum, and 2,250 mL of a mixture of the carbon source fermentation solution and MIW. The amount of carbon source solution added to the MIW targeted a COD: SO_4^{2-} molar ratio of 2 to match the ratio used in the previous batch experiments. The columns were then left to sit in batch mode until biological activity was noted. All three hybrid systems were run in continuous mode for 96 days to determine the effectiveness and reliability of the design.

Analytical Methods

For characterization, the solid organic materials were analysed by the ALS analytical laboratory in Burnaby, British Columbia. Total organic carbon (TOC) was calculated by the difference between total carbon and total inorganic carbon, method 21.2 in (Skjemstad and Baldock 2007). The metal content of the organic materials were measured by collision/reaction cell inductively coupled plasma mass spectrometry (CRC ICPMS), using U.S. Environmental Protection Agency (USA EPA) methods 200.2 & 6020A. Total nitrogen (TN) was measured by combustion (Swift 1996). The pH of the materials was measured by an extraction procedure, which involved mixing the dried (at ≈ 60 °C) and sieved (no. 10/2 mm) sample with deionized water at 1:2 mass ratio of sediment to water, and subsequently measured using a standard pH probe (WTW SenTix@41 pH/T probe, Xylem Analytics, Yellow Springs OH, USA).

Total sulphide was measured using a colorimetric method adapted from APHA Method 4500-S2 (Eaton et al. 2005). Sulphate concentrations in the batch cultures were measured using turbidimetric method APHA 4500-SO4 (Eaton et al.

Table 1 Source water quality in metals and treatment objectives

Metal	Total concentration ($\mu\text{g/L}$)	Dissolved concentration ($\mu\text{g/L}$)	BC Water Quality Guidelines based on water hardness of 142 mg/L
Cadmium (Cd)	24.1–28.4	22.2–26.0	Chronic = 0.27 $\mu\text{g/L}$ dissolved Acute = 0.84 $\mu\text{g/L}$ dissolved
Cobalt (Co)	17.2–17.7	16.3–17.0	Chronic = 4 $\mu\text{g/L}$ total Acute = 110 $\mu\text{g/L}$ total
Iron (Fe)	94–181	< 50	Total Fe = 1000 $\mu\text{g/L}$ Dissolved Fe = 350 $\mu\text{g/L}$
Lead (Pb)	117–161	72.1–86.7	Chronic = 8.3 $\mu\text{g/L}$ total Acute = 127 $\mu\text{g/L}$ total
Manganese (Mn)	2130–2170	2020–2120	Chronic = 1200 $\mu\text{g/L}$ total Acute = 2100 $\mu\text{g/L}$ total
Zinc (Zn)	22,700–22,800	21,000–22,600	Chronic = 292 $\mu\text{g/L}$ total Acute = 1287 $\mu\text{g/L}$ total

2005). Sulphate in the column studies was measured by ion chromatography (USA EPA method 300.1) with conductivity and/or UV detection. Total metal concentrations in water were measured by CRC ICPMS (USA EPA method 200.2/6020A), in which samples are digested with nitric acid before analysis. Dissolved metals were measured by CRC ICPMS (APHA method 3030B/6020A; Eaton et al. 2005), in which samples are filtered through 0.45 µm and preserved with nitric acid. Chemical oxygen demand was measured colourimetrically using procedures adapted from APHA method 5220 (Eaton et al. 2005).

DNA Extraction and Sequencing

Samples for taxonomic analysis were collected from the batch and continuous flow bioreactors by filtering the mixed liquor suspended solids through 0.45 µm filters, which were then processed for DNA extraction using the Qiagen Power-Soil Pro kit (Qiagen Inc., Toronto) following the manufacturer's instructions. Sequencing of the 16S rRNA V4 variable region, amplified using 515f and 806r primers (Caporaso et al. 2012), was performed on an Illumina MiSeq with v3 chemistry in the Biofactorial Laboratory at the University of British Columbia. Paired end reads were processed with the Qiime2 suite of tools using the DADA2 pipeline (Callahan et al. 2015) to obtain amplicon sequence variants (ASVs; Bolyen et al. 2019). The SILVA release 138 SSU database (Quast et al. 2013) was used to assign taxonomy to the representative sequences for the dominant (those representing > 1% of the total population) ASVs.

Results and Discussion

Characterization of Organic Materials

After collection, the organic materials were analysed for bulk properties such as TOC, TN, pH, and trace metals (Table 2). These were all compared to a sample of the mine sediment as a baseline to determine the deficiencies intrinsic to the environment on the mine site. Trace metals were also analysed to determine if the organic materials met organic matter recycling regulation (OMRR) and soil amendment code of practice (SACoP) land application guidelines (SYLVIS Environmental 2008). This would ensure that any material used at the mine site would not in turn be detrimental to the environment.

It is important to balance the availabilities of the C:N ratio of lignocellulosic organic material to decomposing microorganisms (Jílková et al. 2020). Despite this, C:N ratio has been a poor predictor of complex organic matter suitable for the support of SRB in passive treatment systems (Schmidtova and Baldwin 2011; Zagury et al. 2006).

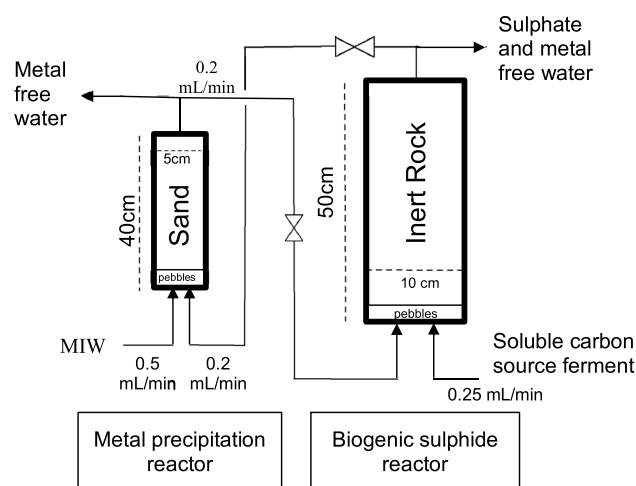


Fig. 1 Schematic of the column experimental apparatus set-up

Not surprisingly, SPF woodchips had the highest C:N ratio, suggesting that this material might be N deficient. The hay-grass and biosolids contained relatively more TN than the woodchips. The pH of the woodchips was less than optimal (circumneutral, 5.5–7.4) for growth of SRB (Postgate 1983), while the hay-grass mixture pH was slightly higher (Table 2). Therefore, the characteristics of a 50/50 mixture of SPF woodchips and hay-grass together would have both high available carbon and nitrogen content, and a pH closer to neutral. Other studies of passive treatment systems for sulphate reduction have found that a mixture of organic materials was better for supporting sulphate reduction than using a single type of lignocellulose material (Schmidtova and Baldwin 2011; Zagury et al. 2006). Consequently, this mixture was chosen for fermentation into a soluble leachate product for the following tests. The pulp mill secondary wastewater treatment biosolids also had desirable properties (C, N content, pH, Table 2) and have been used successfully previously for MIW treatment (Baldwin et al. 2015, 2016). After performing a 5-day fermentation to decompose longer chain, high molecular weight carbon compounds into lower molecular weight, soluble molecules that SRB can use, a solid to liquid mass ratio of 1:6 50/50 SPF woodchip/hay-grass mixture in water provided 7,000 mg/L of soluble chemical oxygen demand (sCOD) with a pH of 6. A 1:1 solid to liquid mass ratio of biosolids in water yielded 4000 mg/L sCOD with a higher pH of 8. These concentrations exceed what is needed for complete reduction of the 1700 mg/L sulphate used in the batch bioreactors, based on the recommended requirement of 0.8 to 1.2 gCOD gSO₄⁻² (Kosińska and Miśkiewicz 1999). Furthermore, the pH remained stable in the circumneutral range of 6–8 during the 5-day fermentation, which is within the range for optimum SRB growth.

Batch Tests Results for Biogenic Sulphide Production and Metal Precipitation

Sulphate removal was observed in both fermentation leachates (Fig. 2a). In the bioreactors containing the 50:50 woodchip/hay-grass mix leachate (Fig. 2a), the SO_4^{2-} concentration decreased from 1971 to 677 mg/L (66% removal) after 14 days, whereafter it then plateaued. Less overall SO_4^{2-} removal was achieved in the bioreactors containing biosolid leachate, 1460 mg/L–966 mg/L (34% removal), with no further SO_4^{2-} removal occurring after 9 days. Compared to the lactate standard, a lag period exhibited in the complex organic matter leachates suggested that a shift on the order of 7 days in microbial community composition was required for each ferment culture before noticeable SO_4^{2-} reduction started.

All SRB cultures produced sulphide at concentrations expected from the 1:1 molar stoichiometric conversion of SO_4^{2-} (96 g/mole MW) to S^{2-} (32 g/mole MW) (Fig. 2b). When an aliquot of the biogenic sulphide supernatant was added to synthetic MIW based on a ratio of 1 mol S^{2-} per 1 mol of metal cations, the metals Cd, Pb, and Zn were removed by > 90% extent within the first time point of 30 min for all ferment leachates (Table 3). This was expected since the solubility product constants (K_{spa}) for these metal sulphides are less than 10^{-2} .

Fe was removed only by the lactate biogenic S^{2-} , whereas, in contrast, Fe concentrations increased when the supernatants from the complex organic matter ferment leachate cultures were added to the metal solution. It is possible that complexation of Fe with dissolved organic carbon compounds in the leachates retained it in solution. The poor Mn removal were not unexpected; previous reports that have demonstrated requirements of high pH (8–10) for rapid formation of MnS (Logan et al. 2005). The 5% increase in Mn observed when the biosolids leachate biogenic S^{2-} was used might have originated from the pulp & paper mill residues having elevated levels of Ca, Mg, and Mn due to the thermochemical processes used in this industry (Asemaninejad et al. 2018). Cobalt was removed only when biogenic

S^{2-} from biosolids leachate was used and increased in the case of lactate and the woodchip/hay-grass mix. The high standard deviation values for Fe and Co removal using any of the biogenic sulphides suggests that there is high variability within the natural environment for speciation of these metals.

Column Test Results Using Actual MIW

Sulphate Removal and Sulphide Production

The sulphate concentration in the MIW fed into the columns over the first 60 days was 175 mg/L. The concentration was less at 125 mg/L in the second batch of MIW used after 60 days until the end of the experiment. Both biological columns exhibited an acclimation period of ≈ 43 days, during which sulphate removal did not exceed 44% for the woodchip/hay-grass leachate column and was no more than 17% for the molasses column (Fig. 3a). The higher removals in the woodchip/hay-grass leachate column were due to SRB in the mine sediment inoculum being able to readily utilize the low molecular weight fermentation by-products, such as organic acids, as carbon sources, as was observed in the batch reactor experiments. Molasses consists of carbohydrates, such as glucose and fructose, which might require a longer adaptation phase for the microbial community to metabolise and ferment into low molecular weight carbon compounds preferred by SRB. It is not unusual for a passive treatment system to require a long acclimation period due to the slow growth of SRB, as well as the time required for decomposition and fermentation of complex organic matter (Mirjafari and Baldwin 2011; Nielsen et al. 2019). By day 60, both the woodchip/hay-grass leachate and molasses columns reached their peak sulphate reduction rates (SRR) of $41.2 \text{ mg-SO}_4^{2-} \text{ L}^{-1} \text{ day}^{-1}$ and $28.7 \text{ mg-SO}_4^{2-} \text{ L}^{-1} \text{ day}^{-1}$, respectively. After day 60, these maximum SRRs were maintained for both biological columns until the end of the experiment. The SO_4^{2-} removal percentage from day 60 onward, once peak SRR was reached, increased to 85% and 73% for woodchip/hay-grass leachate and molasses, respectively (Table 4). Operational parameters such as HRT and COD:SO4 ratio remained constant at 4 days and 2:1 molar, respectively, and the influent and effluent pH, and temperature were all relatively constant at 7–8, and $\approx 20^\circ \text{C}$.

The trends in sulphide production mirrored those observed for sulphate reduction (Fig. 3b). During the acclimation phase, sulphide concentrations were low. They increased during the ramp-up phase, reaching steady state concentrations between 25 and 37 mg S^{2-}/L . The percent yield of sulphide from sulphate reduction for the woodchip/hay-grass leachate column was less than expected during the acclimation phase at 40%, but then increased throughout the ramp-up phase to achieve 89% by the time steady-state was

Table 2 Organic material characteristics

Organic material	C:N Ratio	TOC (%w)	TN (%w)	pH	Trace metals*
SPF wood-chips	250	49	0.2	4.3	Yes
Hay-Grass (50/50)	15	42	3	5.6	Yes
Biosolids	15	43	3	7.5	Yes
Mine sediment	36	36	1	7	No

*Concentrations do not exceed guidelines

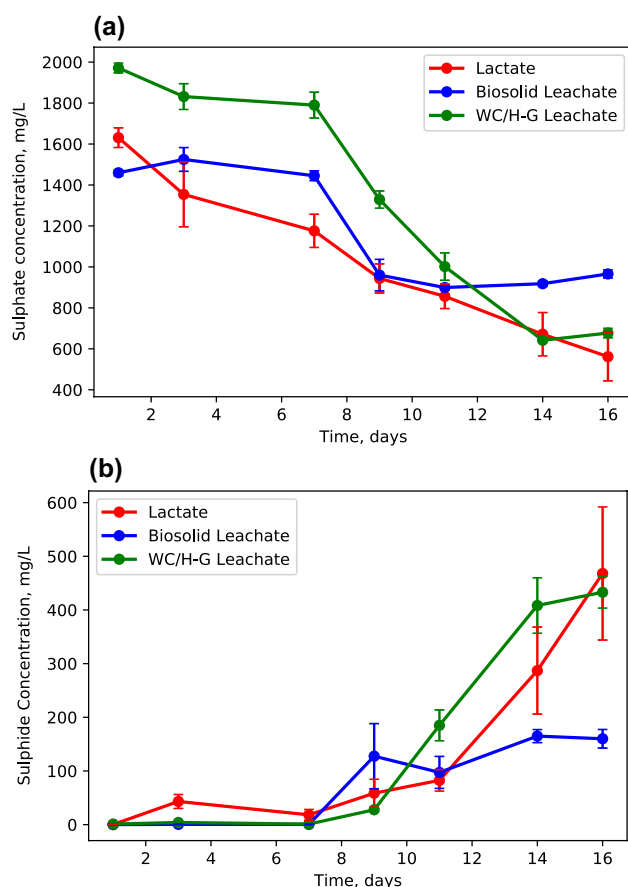


Fig. 2 Sulphate (a) and sulphide (b) concentrations (mg/L) versus time (days) in the batch SRB cultures with three different carbon sources, lactate, the laboratory standard (Lactate, red), 5-day ferment leachate from a 50:50 SPF wood chip/hay-grass mix (WC/H-G Leachate, green) and a 5-day ferment leachate from pulp mill wastewater treatment secondary biosolids (Biosolid Leachate, blue). Error bars represent the standard deviation from the average value for the three duplicate cultures

reached. The molasses column followed a similar trend; the percent yield was initially low at 25% and increased throughout the ramp-up phase to 94%. The percent yields of sulphide expected from the amount of sulphate reduction in the columns were overall less than observed in the batch studies. One likely reason for this is due to precipitation of sulphide as metal sulphide solids within the biological columns as metal removal was observed in these columns as well as in the metal precipitation columns.

The percent yield is important because it allows estimation of the amount of biogenic sulphide that has to be fed back into the first-stage column to achieve the desired metal sulphide precipitation in that column. Initially, the amount of sulphide fed into the first stage metal precipitation column was less than the theoretical and design requirement of 0.5 g S²⁻/g Zn (Fig. 4, acclimation period). After the ramp-up period, during which sulphate reduction in the biological

Table 3 Percentage removal of metals through precipitation with biogenic sulphide. (Initial concentrations are given in Table 1.)

Metal	Biogenic sulphide carbon source		
	Lactate	Woodchip/hay-grass leachate	Biosolids leachate
Cu	94 ± 4%	90 ± 2%	90 ± 0.3%
Pb	98 ± 4%	100 ± 4%	100 ± 2%
Zn	95 ± 2%	99 ± 0.1%	99 ± 0.4%
Fe	78 ± 48%	– 10 ± 28%	– 5 ± 48%
Mn	5 ± 1%	12 ± 1%	– 5 ± 2%
Co	– 521 ± 108%	– 71 ± 50%	50 ± 73%

column improved, the sulphide entering the metal precipitation column reached and was maintained at just above the amount required to precipitate all of the Zn in the MIW entering the column (Fig. 4).

Metal Removal

The negative control column, which received no carbon source, removed ≈ 50% Zn at start-up (Fig. 5a). Thereafter, the amount of Zn removed decreased until the steady-state period during which only a small amount of Zn continued to be removed. The explanation for initial Zn removal is that adsorption of Zn occurred to surfaces in the 1st stage column until the capacity for sorption was reached (Delolme et al. 2004). Removal of Zn in the columns receiving biogenic S²⁻ and not in the control column during the steady-state operational phase suggests that biogenic S²⁻ was required for Zn removal, supporting the hypothesis for its removal as sulphide precipitates. In the woodchip/hay-grass leachate and molasses fed systems, Zn was initially removed at ≈ 50% during the acclimation phase, and as the biogenic sulphide production from the biological columns increased, the Zn removal in the metal precipitation columns increased to 85%. At the end of the study, the British Columbia Water Quality Guideline for acute toxicity was met by the molasses-fed system but not by the woodchip/hay-grass leachate fed system (Fig. 5a). Average removal extents over the steady state period for all of the metals are given in Table 5.

Of the metals present in the MIW, the Cd, Pb, and Zn were removed in the biological columns as well as in the metal precipitation columns (Table 5). Cadmium and Zn were removed to > 85% in both the woodchip/hay-grass leachate and molasses fed systems. Lead was removed to levels below acute guidelines in all systems, even in the negative control. This is the only metal that was removed by the sand filter alone without any biogenic sulphide being present. Removal of Co, Mn, and Fe in the systems was more variable. Manganese was removed up to 12% in the metal precipitation columns, whereas greater removal were achieved

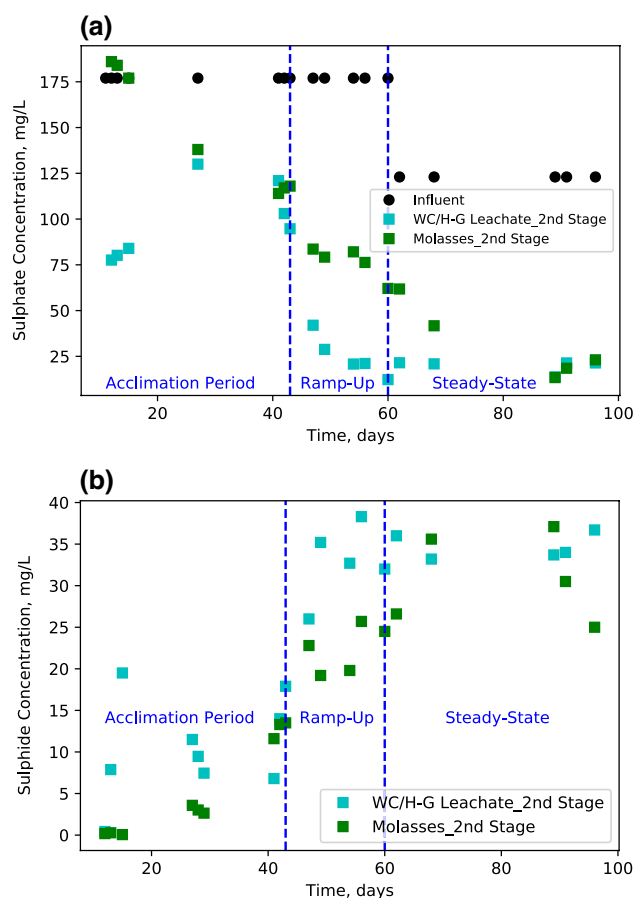


Fig. 3 Sulphate (a) and sulphide (b) concentrations in the biological column (2nd Stage) influent (black circles) and effluents (light blue and green squares) versus time (days). WC/H-G stands for woodchip/hay-grass

in the biological columns (20–33%). This is due to the pH increase, from 7 in the influent to 8 in the effluent observed in the biological columns, which would have allowed MnS formation.

Cobalt was not removed to below chronic guideline levels in the metal precipitation column but eventually reached it after the MIW passed through the biological columns. This suggests that to meet stringent guidelines for some metals, two treatment stages might be required.

The treatment performance for iron removal was highly variable and Fe was released at concentrations up to 3000% higher than those in the influent (Fig. 5b). It was discovered that iron is a component of the sand used in the 1st stage metal precipitation column and this was likely the source of the released Fe. The sand that we used was what was already available on the mine site, and unfortunately, we did not know that it contained Fe. This release was only realized when the systems fed carbon sources released iron, suggesting that the reducing conditions inside the metal precipitation column caused the iron to be released from the sand media. Biological iron reduction occurs at a different redox (ORP = − 80 mv) than sulphate reduction; therefore, the conditions in the metal precipitation column would have been suitable for bio-reduction of iron in the sand media (Madigan et al. 2015, pg. 83). Anaerobic biological treatment of MIW using SRB is known to perform poorly for iron removal; therefore, in some MIW treatment systems, oxidation of Fe(II) to Fe(III) is carried out prior to biosulphide production to remove Fe as insoluble iron hydroxide precipitates (Clyde et al. 2016). Iron oxidation can be carried out biologically using iron-oxidizing bacteria (Hou et al. 2020). In the column bioreactors, Fe reduction and leaching from the sand media appeared to decrease over time (Fig. 5b).

Microbial Population Compositions in the Batch and Continuous Flow Column Tests

In both the batch and column bioreactors, Firmicutes, Bacteroidota, Desulfobacterota, and Proteobacteria were the phyla with the highest relative abundances in the 16S rRNA amplicon sequence libraries (Fig. 6). In the batch bioreactors, Firmicutes were the most relatively abundant, especially in the mine sediment, decreasing in fraction in the batch cultures from lactate > woodchips/hay-grass > biosolids. Within Firmicutes, the dominant classes were (in the order of biosolids, woodchips/hay-grass leachates, lactate, and sediment, respectively) Clostridia (67%, 59%, 24%, and 77%), Negativicutes (22%, 34%, 63%, and 20%) and Desulfotobiaria (2.9%, 2.4%, 0, and 1.5%). Taxa represented within the Clostridia were those with members capable of anaerobic organic matter degradation, notably Lachnospiraceae,

Table 4 Summary of sulphate removal extents and rates

Day range	Phase description	Woodchip/hay-grass leachate column		Molasses column	
		SO ₄ ^{2−} removal (%)	SRR (mgSO ₄ ^{2−} L ^{−1} day ^{−1})	SO ₄ ^{2−} removal (%)	SRR (mgSO ₄ ^{2−} L ^{−1} day ^{−1})
0–43	Acclimation	44%	19.4	17%	6.1
43–60	Ramp-Up to Peak SRR	84%	33.9	55%	22.3
60–96	Steady-State	85%	28.4	73%	23.8

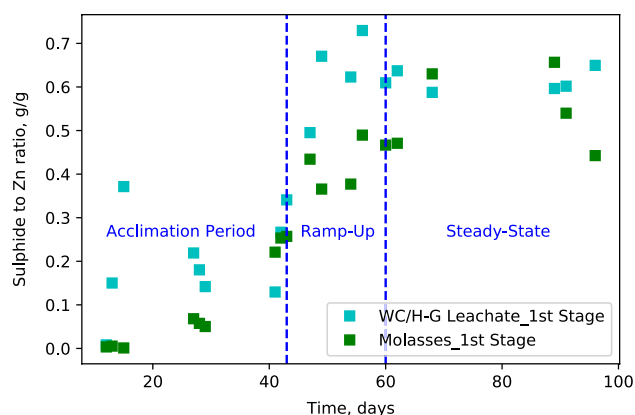


Fig. 4 Ratio (g g^{-1}) of biogenic sulphide to dissolved Zn entering the metal precipitation columns over the duration of the experiment. WC/H-G stands for woodchip/hay-grass

Clostridiaceae, Ruminococcaceae, and Oscillospiraceae (Figs. S-1, S-2; Lombard et al. 2014), some of which also produce fermentation products such as short chain fatty acids (e.g. acetate, butyrate, propionate) and alcohols (e.g. ethanol and propanol). The Negativicutes were dominated by two genera, *Anaeromusa*-*Anaeroarcus* (an amino-acid fermenting clade; Baena et al. 1999) and *Pelosinus* (Figs. S-3, S-4). The Firmicutes in the batch bioreactors were presumably decomposing and fermenting the soluble plant-derived carbohydrates and proteins in the leachates to produce the low molecular weight carbon sources that SRB can readily use. The Firmicutes phylum was also represented by taxa known to play roles in Fe and S cycling. The other dominant Negativicutes genus, *Pelosinus*, are iron-reducing bacteria known to use lactate as their electron acceptor (Brown et al. 2012). The Desulfitobacteriia class was dominated by *Desulfosporosinus* species that are SRB known to thrive in metal-contaminated environments (Burkhardt et al. 2011) (Fig. 7).

Species within the Bacterioidota phylum were also dominant in the batch bioreactors (Figs. S-5, S-6). Members of this phylum in the batch bioreactor are known to degrade complex polysaccharides into smaller fatty acid molecules (Thomas et al. 2011). Like the Firmicutes, these contribute to the supply of carbon sources for SRB from the plant-derived dissolved organic matter in the leachates.

The phylum Proteobacteria was also highly represented in the batch bioreactors even though they were only a small fraction of the microbial population present in the sediment used for inoculation. Most of the lactate culture Proteobacteria were known cultured species of *Pseudomonas* and *Aeromonas* (Figs. S-7, S-8). *Pseudomonas* are common, metabolically versatile heterotrophic bacteria that mostly grow under aerobic conditions but can use nitrogen compounds as electron acceptors, enabling them to grow anaerobically. *P. putida* and *P. stutzeri* are two species that are important, for

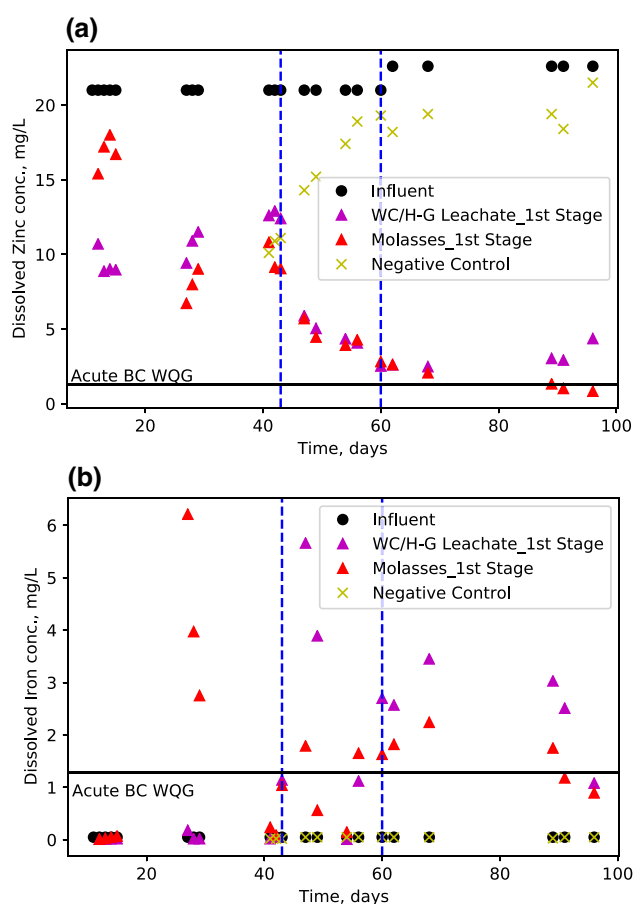


Fig. 5 Dissolved zinc (a) and iron (b) concentrations (mg/L) in the influent (black circles) and effluents (purple and red triangles, and yellow Xs) from the metal precipitation columns (1st Stage) over time (days). WC/H-G stands for woodchip/hay-grass

example, for bioremediation of environments contaminated with hydrocarbons or selenium (Wu et al. 2011). The *Pseudomonas* species in the batch reactors were using fermentation by-products for their electron donors. The *Aeromonas* in the batch bioreactors are facultative anaerobes reducing nitrate coupled to metabolism of glucose and polysaccharides to produce fatty acids. *Tolumonas* and *Delftia* species were dominant Proteobacteria in the biosolids and woodchip/hay-grass leachate cultures, as well as *Pseudomonas* and *Aeromonas* species. *Tolumonas* are closely related to *Aeromonas*. *Delftia* are Proteobacteria that grow on fatty acids as their preferred carbon sources, and some species are capable of metal reduction (Das et al. 2015). The woodchips/hay-grass leachate culture had the most ASVs that could not be classified at the genus level. However many of these ASVs were classified at the family level and were in the same families as those in the other leachates. A notable exception was the woodchips/hay-grass culture, which supported a wider variety of Proteobacteria families than were observed in the other leachate microbial populations. This

Table 5 Average percentage removal of metals during the steady-state phase

Metal	Woodchip/hay-grass leachate		Molasses		Negative control
	Metal precip. column	Biogenic sulphide reactor	Metal precip. column	Biogenic sulphide reactor	Metal precip. column
Cd (Dissolved)	100%	100%	100%	100%	-27%
Pb (Total)	100%	89%	100%	99%	99%
Zn (Total)	85%	90%	90%	94%	9%
Co (Total)	64%	90%	59%	85%	2%
Mn (Total)	0%	19%	12%	33%	0%
Fe (Total)	-3040%	-28%	-2384%	-31%	31%

might be due to a wider range of fermentation by-products produced from the many forms of soluble, plant-derived organic compounds in this leachate. Overall, most Proteobacteria in the batch bioreactors were using the fermentation by-products produced by decomposition of plant-based polysaccharides.

Firmicutes *Desulfosporosinus* SRB were present in the batch bioreactors with biosolids and woodchips/hay-grass leachates but were not present in the lactate culture. Other types of SRB are classified in the phylum Desulfobacterota

(Waite et al. 2020) by the SILVA Align taxonomic assignment tool (Quast et al. 2013). The taxa in this phylum are known to share the trait of reducing sulphate to sulfide. The Desulfobacterota were represented in the batch bioreactors by 11%, 8.8%, 23%, and 5.6% of the 16S rRNA amplicon reads in the sequence libraries obtained from the biosolids and woodchips/hay-grass leachates, lactate and sediment, respectively. The lactate culture had the highest relative abundance of SRB, which is not surprising since lactate is a preferred carbon source for SRB and is often

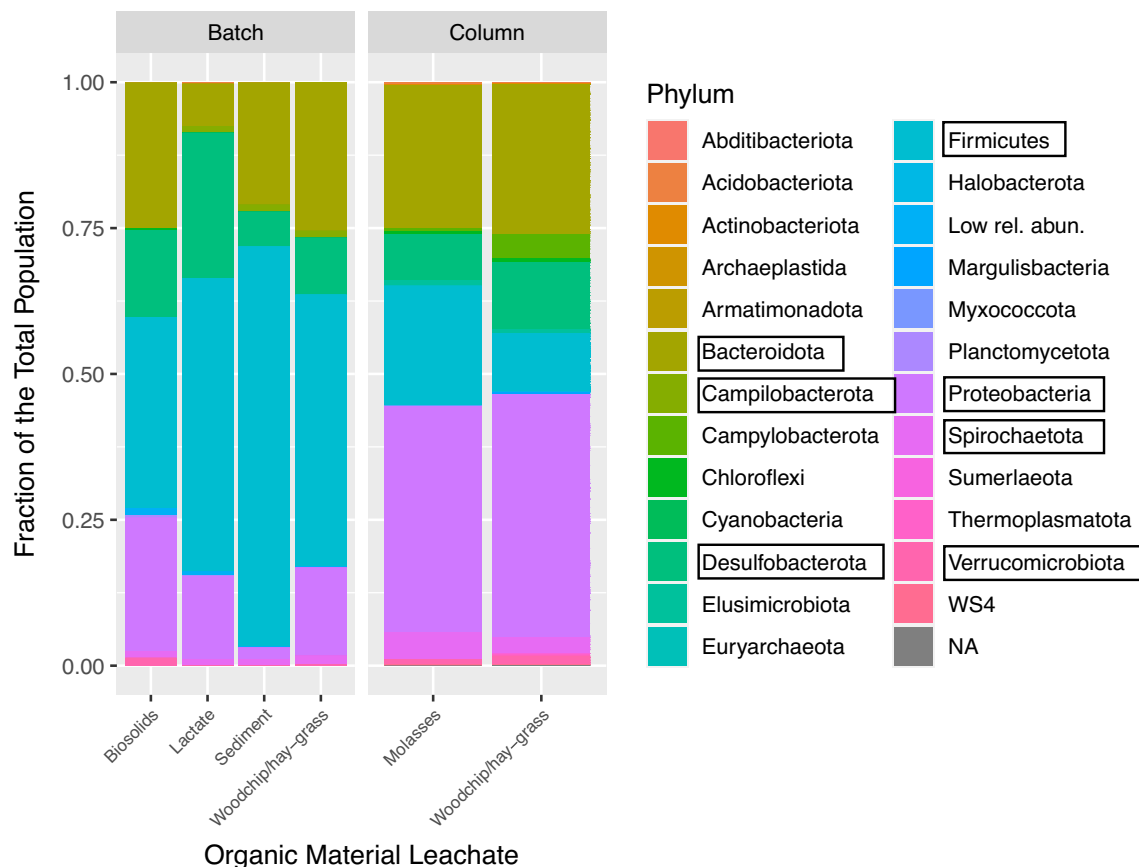


Fig. 6 Relative abundance of dominant phyla in the batch and column bioreactors versus the type of organic material used. The highest relatively abundant phyla are highlighted in boxes on the key

used in growth media for enriching for SRB (e.g. Postgate B medium). Overall, six different species of SRB were growing on the leachates. The leachates and lactate had more diverse SRB species than the mine sediment, which was dominated by *Desulfovibrio*. All these species of SRB are known to be adapted to metal-rich environments. This indicates that all of the leachates were capable of supporting SRB and a community of synergistic microorganisms decomposing plant-derived polysaccharides and amino acids into low molecular weight compounds suitable as carbon sources for SRB.

In the column bioreactors, the most highly represented phylum was Proteobacteria. Most Proteobacteria were assigned to known genera, unlike the batch bioreactor Proteobacteria, many of which were unassigned (Fig. S-9). The dominant Proteobacteria species were *Pseudomonas* sp., as in the batch reactors, but also *Ferribacterium* sp. were present (Fig. S-9). There is only one known *Ferribacterium* species that is an obligate anaerobe reducing Fe(III) coupled with acetate oxidation (Cummings et al. 1999). This species may have been responsible for the Fe released from the column bioreactors observed during the continuous flow operation as Fe(II) is more soluble than Fe(III).

Most Bacteroidota in the column bioreactors were classified into the same genera as in the batch bioreactors, with the molasses columns hosting more diverse Bacteroidota (Fig. S-10), indicating that these organisms contribute to breaking down complex polysaccharides into lower molecular weight compounds available for growth of SRB.

Firmicutes in the column bioreactors were remarkably diverse, with 35 different genera represented (Fig. S-11). Most of these were in the Clostridia class that comprised 63% and 50% of the Firmicutes in the woodchip/hay-grass and molasses columns, respectively. The Negativicutes were relatively less abundant in the column bioreactors (2.8% and 4.0% of the Firmicutes in the woodchip/hay-grass and molasses columns, respectively) than in the batch bioreactors. Sulphate-reducing bacteria, Desulfotomaculalia, were similarly present, with more (22.5%) within the Firmicutes of the woodchip/hay-grass columns than in the molasses columns (12%). The column bioreactors supported an additional class of Firmicutes SRB, Desulfotomaculalia (3.5% and 9.2% of Firmicutes in the woodchip/hay-grass and molasses columns, respectively). There were also Bacilli present in the column bioreactors. Only a few of the column Firmicutes genera were also present in the batch bioreactors, indicating a much different and more diverse community of organic matter degraders in the column bioreactors due to the feed to these bioreactors coming from the environment. This indicates that many species were present in the column bioreactors to metabolise the plant-derived soluble

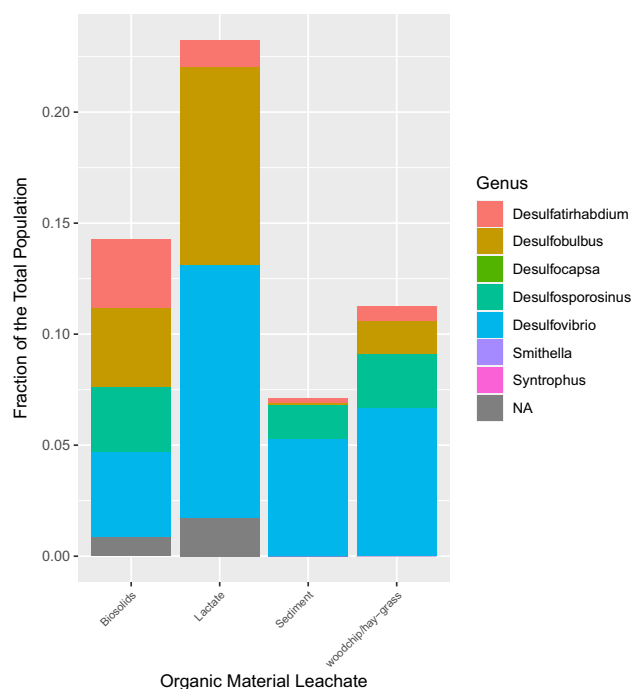


Fig. 7 Relative abundance (fraction of the total bacterial population) of sulphate-reducing bacteria genera in the batch bioreactors. NA indicates unassigned taxa

compounds into organic acids and other small molecules for the SRB and other bacteria, such as the Proteobacteria, to use for their growth. The Firmicutes contained four different SRB genera that contributed to 2.5% of the total bacterial population in the hay columns and 3.6% of the total bacterial population in the molasses columns.

Sulphate-reducing bacteria comprised less of the total population in the column bioreactors than in the batch bioreactors, with the woodchip/hay-grass leachate supporting proportionally more SRB than the molasses-fed column bioreactors (Fig. 8). Genera within the phylum Desulfobacterota, such as *Geobacter sulfurreducens*, were present, which is known to also reduce sulphate (Caccavo et al. 1994). A wider variety of SRB genera were present in the column reactors than the batch ones. The four main SRB genera in the batch bioreactors were also among the main SRB in the column bioreactors. The additional sulphate-reducing genus present in the column bioreactors, *Desulfofarcimen*, have members that are acetate-oxidizing species (Watanabe et al. 2018). Not all SRB are capable of completely oxidizing organic acids and some produce acetate as a by-product. Complete oxidation of organic acids and acetate is important in bioreactors to prevent acetate accumulation and lowering of the pH as well as product inhibition. Thus, acetate-utilizing SRB and microorganisms such as Proteobacteria play a key role.

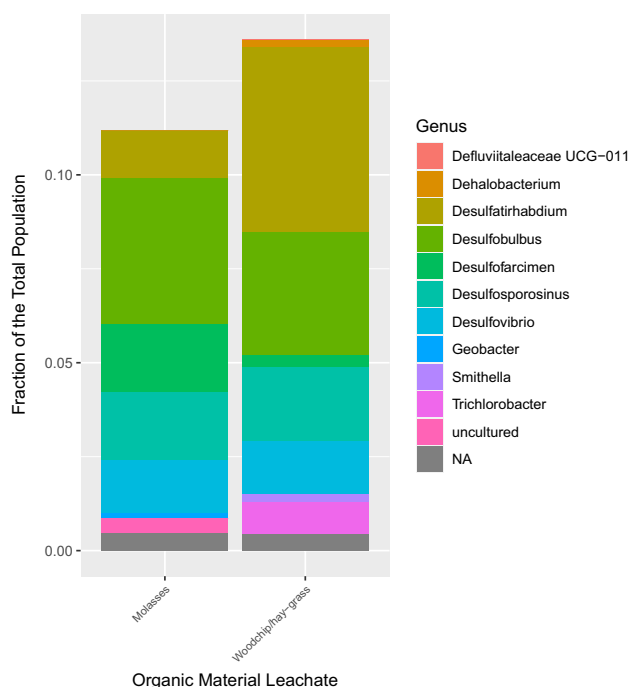


Fig. 8 Relative abundance (fraction of the total bacterial population) of sulphate-reducing bacteria genera in the column bioreactors. NA indicates unassigned taxa

Implications for Practice

The proposed process is referred to as a hybrid passive/active system since additional components will be required than in a typical PBR or BCR, contributing to the capital and potentially, operating costs. The fermentation leachate can be produced in a tank or containment vessel to which fresh organic material can be added when needed. In the study described in this manuscript, this was performed statically; agitation was not required. An extra filtration step will be needed to recover the liquid fermentation leachate solution. Spent solid organic material can be composted and used for reclamation purposes on the mine site. For metal precipitation with biosulphide, pumps and control valves will be needed. The hybrid system retains some passive components in that waste organic material is used as the nutrient source and natural biogeochemical processes are leveraged for metal removal and sulphate reduction.

Conclusions

A novel hybrid passive/active biological treatment system fed with a liquid nutrient solution effectively removed metals from mine-impacted water using chemical precipitation decoupled from biogenic sulphide production. Five-day

fermentation leachate solutions from a woodchip/hay-grass mixture supported sulphate reduction and biogenic S^{2-} production at expected stoichiometric ratios, and at rates comparable to the laboratory standard, lactate, and molasses, a carbon source currently used in industrial practice. Biogenic sulphide fed back into the metal precipitation column from the downstream biological column allows for a modular design enabling metal removal from the mine site, and potential recovery of the metals for use. Microbial community analysis confirmed the presence of taxonomic groups within the phyla Firmicutes, Bacteroidota, and Proteobacteria involved in the complex degradation and fermentation of organic compound to produce the low molecular weight fermentation products known to be preferred carbon sources for SRB. The suitability of the woodchip/hay-grass fermentation leachate as a nutrient source for this process was supported by the diverse SRB represented by the eight different genera observed in the biological column.

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