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ORIGINAL PAPER

Coupled physicochemical and bacterial reduction mechanisms for passive remediation of sulfate- and metal-rich acid mine drainage

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Abstract Treatment of acid mine drainage (AMD) highly rich in sulfate and multiple metal elements has been investigated in a continuous flow column experiment using organic and inorganic reactive media. Treatment substrates that composed of spent mushroom compost (SMC), limestone, activated sludge and woodchips were incorporated into bacterial sulfate reduction (BSR) treatment for AMD. SMC greatly assisted the removals of sulfate and metals and acted as essential carbon source for sulfate-reducing bacteria (SRB). Alkalinity produced by dissolution of limestone and metabolism of SRB has provided acidity neutralization capacity for AMD where pH was maintained at neutral state, thus aiding the removal of sulfate. Fe, Pb, Cu, Zn and Al were effectively removed (87–100%); however, Mn was not successfully removed despite initial Mn reduction during early phase due to interference with Fe. The first half of the treatment was an essential phase for removal of most metals where contaminants were primarily removed by the BSR in addition to carbonate dissolution function. The importance of BSR in the presence of organic materials was also supported by metal fraction analysis that primary metal accumulation occurs mainly through metal adsorption onto the organic matter, e.g., as sulfides and onto Fe/Mn oxides surfaces.

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Introduction

Acid mine drainage (AMD) has been known as a serious environmental issue; however, the cost for rehabilitating the mine-impacted area has become an impediment to developing an appropriate remediation technology. AMD remains a toxic legacy of excess contaminants to the accessible waterways affecting the quality of water environments. In Malaysia, several cases of AMD occurrence have been reported as a result of metal mining activities. For instance, some former mining areas have experienced serious environmental deterioration and it appears to be a significant pollution at the post-mining phase (Abdullah et al. 2008; Jopony and Tongkul 2009). In terms of environmental degradation, the Mamut former copper mining area has been reported to cause severe water pollution in a nearby river several kilometers away from the source. The mining activities came to an end after 24 years of operation, and it was known as a residual pollution in the nearby waterways and was the worst AMD scenario in this country that has given catastrophic impact to the environment (Abdullah et al. 2008). Irrespective of abandoned or active mines, some trace elements were found exceeding the permissible limits of the National Water Quality Standards as the mine discharges enter the watercourses (Alshaebi et al. 2009; Yaacob et al. 2009; Kusin et al. 2016a). This can be a problem especially when the river is intended for use as raw water resource for potable water consumption (Kusin et al. 2016b).

Passive AMD treatment was adopted in this study because the treatment is relatively low in cost, the materials are easily available, and it does not require frequent maintenance. A sulfate-reducing bioreactor has the potential of removing metals and sulfates and neutralizing acidic water, which is essential for the remediation of AMD (Neculita et al. 2007). This study applies the concept of passive bioremediation of AMD using sulfate-reducing bacteria (SRB) as the key treatment mechanism. The SRB-mediated sulfate reduction mechanism functions are given in Eqs. 1 and 2 (Dvorak et al. 1992):

$$2CH_2O + SO_4^{2-} = H_2S + 2HCO_3^- \quad (1)$$

$$H_2S + M^{2+} = MS(s) + 2H^+ \quad (2)$$

Sulfate-reducing bioreactor provides an environment in which organic carbon is oxidized to HCO_3^- and SO_4^{2-} which is the terminal electron acceptor reduced to H_2S as shown in Eq. 1. SRB utilizes sulfate as an electron acceptor to reduce sulfates to sulfides, which then react with dissolved metals to form solid metal sulfides (Cheong et al. 2010; Vasquez et al. 2016). The bacterial sulfate reduction (BSR) utilizes organic molecules as electron donors (Ayala-Parra et al. 2016). BSR will vigorously occur as long as high pH can be maintained for the SRB to perform key reduction mechanism. A suitable pH range and sufficient source of carbon, nutrient and a solid matrix on which the SRB can sustain their growth are required for effective BSR (Cheong et al. 2010).

More recent alternatives of carbon source for SRB have been the use of organic wastes such as animal manure, woodchips, sawdust, spent mushroom compost, maize straw, risk and coconut husks and yard waste (Cheong et al. 2010; Mayes et al. 2011; Wakeman et al. 2010; Márquez-Reyes et al. 2013; Kim et al. 2014; Kijjanapanich et al. 2014; Muhammad et al. 2015, 2017; Zhang et al. 2016). The carbon source obtained from the carbonaceous material is present as labile cellulose-rich materials for later breakdown to sustain the bacterial activity. The degradation products of complex organic materials can be used as carbon and electron sources for SRB in many sulfate-reducing bioreactor applications (Wakeman et al. 2010; Kim et al. 2014).

Sulfate-reducing bioreactors have been used for the treatment of AMD where heavy metals and sulfate are the primary contaminants of concern (e.g., Behum et al. 2011; Mayes et al. 2011; Song et al. 2012; Bai et al. 2013; Kijjanapanich et al. 2014; Shi et al. 2015; Zhang et al. 2016). High concentrations of heavy metals such as Fe, Cu, Cd and Zn have been found removed at $> 99.9\%$, and sulfate removal of up to 88% and slightly alkaline pH of between 7.3 and 7.8 have been observed in a bioreactor filled with immobilized SRB beads using maize straw as the carbon source supplemented with sodium lactate (Zhang et al. 2016). Sulfate removal of more than 61% and Cu and Fe removals of 99 and 86%, respectively, with pH of 6.2 have been found from an AMD treatment by SRB using iron in bench-scale runs (Bai et al. 2013). An effective BSR treatment has also been observed in a full-scale, on-site remediation of AMD whereby removals of Fe, Al, Ni and Zn were found between 90 and 99% with significant removal of sulfate (Behum et al. 2011). Performance of a sulfate-reducing bioreactor can be enhanced by optimizing the hydraulic retention time of the system such as those observed from the work of Vasquez et al. (2016) that pH and alkalinity were efficiently increased, sulfate was removed at $> 60\%$, and metals (Fe and Zn) were reduced between 70 and 100%.

In this study, performance of a sulfate-reducing bioreactor was sufficiently evaluated as to reflect treatment concept using a reducing and alkalinity-producing system (RAPS). Fundamentally, the RAPS incorporates the use of calcite dissolution for neutralizing acidity and raising the pH and the use of SRB in the compost bed layer that provides suitable environment to facilitate metal removal. Specifically, this study aims to evaluate treatment performance of a continuous flow sulfate-reducing bioreactor for alkalinity generation, sulfate and heavy metals reduction (Mn, Fe, Cu, Pb, Zn and Al) so as to provide a treatment option for mine-impacted water. To date, none of such treatment application has ever been developed in this country. It has been known that passive remediation of AMD highly rich in sulfate and containing multiple metal elements may often be difficult. Therefore, this study will emphasize on the importance of both physicochemical treatment and bacterial contaminant reduction mechanisms in the remediation of such AMD. The study was carried out between December 2015 and June 2016 in the laboratory of the Faculty of Environmental Studies, Universiti Putra Malaysia.

Materials and methods

Preparation of synthetic AMD

Synthetic mine water was used in the experiments because it was not possible to obtain actual mine water from the mining site. The compositions of the synthetic mine water were made up according to the average concentration of the constituents in a highly polluted former copper mining pond in Mamut, Sabah. The synthetic AMD was prepared using analytical laboratory-grade salts added with distilled water to ensure consistency of the heavy metals concentrations. The compositions of the synthetic AMD used in the column experiment were as follows: Al (44 mg/L,

$Al_2(SO_4)_3 \cdot 18H_2O$), Mn (20 mg/L, $MnSO_4 \cdot H_2O$), Fe (5.8 mg/L, $FeSO_4 \cdot 7H_2O$), Cu (4.6 mg/L, $CuSO_4 \cdot 5H_2O$), Pb (0.5 mg/L, $Pb(NO_3)_2$), Zn (5.9 mg/L, $ZnSO_4 \cdot 7H_2O$), Mg (39 mg/L, $MgSO_4 \cdot 7H_2O$), Ca (27 mg/L, $Ca(CH_3OO)_2 \cdot H_2O$), K (5.8 mg/L, K_2HPO_4), SO_4 (1600 mg/L, $Na_2SO_4 \cdot 10H_2O$) and Cl (170 mg/L, NaCl). Hydrochloric acid was added into the solution to reduce the pH into acidic condition to pH 3.1.

Continuous upward flow column test

Column substrates

Selected media used in column experiment were determined from a series of batch test conducted earlier, i.e., mixed substrates composed of limestone (LS, 40% v/v), spent mushroom compost (SMC, 30%), activated sludge (AS, 20%) and woodchips (WC, 10%). SMC was used as an electron donor (organic carbon source) to feed the SRB, AS as the source of SRB, limestone as an alkalinity-generating agent and WC to aid in permeability in the treatment reactor (Cheong et al. 2010; Mayes et al. 2011). The LS and WC were sourced from a local factory, AS was obtained from a sewage treatment plant, and SMC was obtained from a mushroom farm. The activated sludge was filtered through a 125-mm sieve to separate between the solids and water, and the SMC used was of *Pleurotus ostreatus* species, which is known as the oyster mushroom.

Column experimental setup and operation

A series of experimental AMD treatment using two bioreactor columns was conducted (i.e., up-flow anaerobic packed-bed bioreactor) (Fig. 1). Each column has internal diameter of 64 mm and height of 500 mm, namely column 1 (C1) and column 2 (C2). 1300 cm^3 of mixed substrates were inserted into the column before feeding with synthetic AMD. A Longer Pump 100 series peristaltic pump was set up to give each column a flow rate of 1 mL/min and hydraulic retention time of 24 h. An upward flow bioreactor was applied in the experiment to prevent channeling and system blocking. The continuous flow column experiment was conducted throughout 30 days with an intermediate aeration to aid carbon dioxide degassing. The effluent from each column reactor was sampled according to the specified time intervals.

Sample analyses

Measurements of water sample from the column reactors for pH, redox potential (Eh), total dissolved solids (TDS), electrical conductivity (EC) and temperature were taken using a Myron 6P Ultrameter. Sample alkalinity was examined through the titration of sample against 0.16 N sulfuric acid with phenolphthalein indicator powder and bromcresol green-methyl indicator using a HACH Alkalinity Test Kit AL-AP. Sulfate was determined using a HACH spectrometer D2800 with SulfaVer4 powder pillow reagent according to USEPA Method 8051 of the standard methods for the examination of water and wastewater. Chemical oxygen demand (COD) was determined using HACH COD HR TNT 822 based on USEPA Reactor Digestion Method 8000. Heavy metals (Fe, Mn, Al, Cu, Pb, Zn) were analyzed using a PerkinElmer ICP-OES Optima 8300, while the major cations (Ca, Mg, Na, K) were determined using flame atomic absorption spectrometry (FAAS) PerkinElmer 3300. The samples were filtered using 0.2-μm nylon membrane filters before acidified with 1% v/v nitric acid (HNO_3) for analysis using the ICP-OES or the FAAS.

Column substrates characterization

Physicochemical characteristics

The physicochemical parameters analyzed for the column substrates (drained substrates) were pH, EC, Eh, loss on ignition (LOI) and cation exchange capacity (CEC). pH, EC and Eh were tested by mixing 10-g air-dried substrates (< 2 mm) with 20 mL distilled water with solid-to-liquid ratio of 1:2 into 50-mL centrifuge tubes. The tubes were shaken in an orbital shaker for about 30 min. The samples were left for 1 h and were then measured by specific instruments. LOI was measured to determine the organic matter content in the mixed substrates whereby the LOI was calculated as the difference of the initial and final weight of samples after heating, divided by the weight of sample used. CEC was calculated to indicate the amount of negative charges on the surface of sediment particles (e.g., clay or organic matter) available for cation exchangeable in the reactive media and was determined using cations displacement method. From the CEC value, percent base saturation (PBS) was determined to estimate the relative availability of each of those cations. PBS (%) was calculated by dividing the milliequivalents of each element by the CEC value.

Sequential extraction

The substrates used in column reactors were analyzed for sequential extraction based on Tessier et al. (1979) method and modified from procedure used by Jena et al. (2013). There are five fractions to indicate heavy metals immobilization according to where they have been bound in the reactive media; fraction 1 is for soluble and exchangeable metals, fraction 2 is for metals that bound to carbonates,

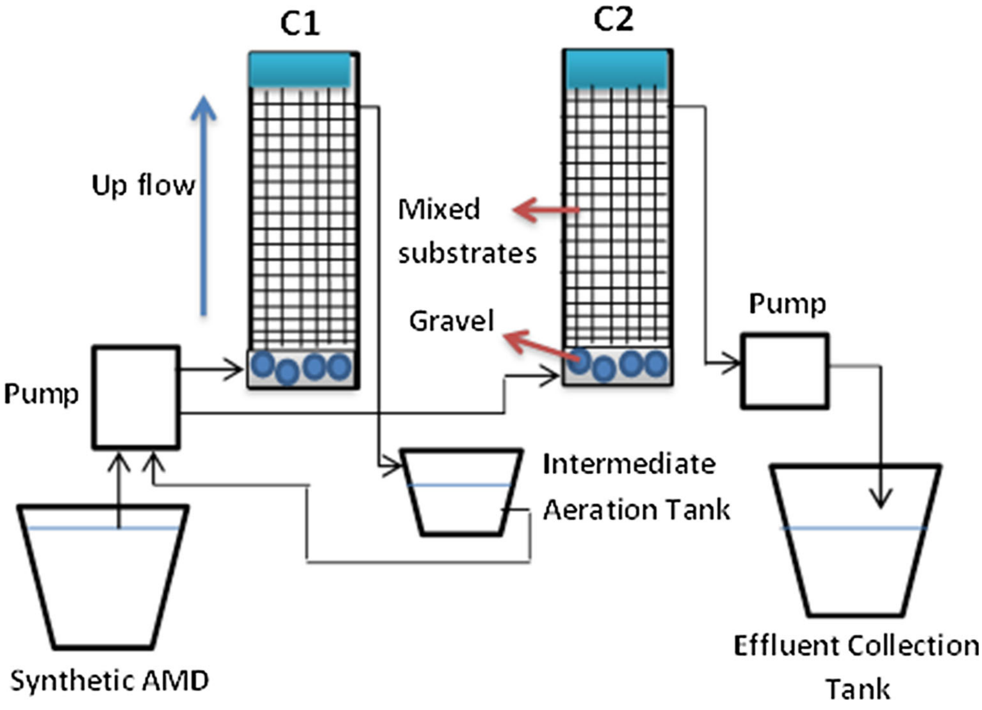

Fig. 1 Continuous upward flow column experimental setup (laboratory-scale experiment)

fraction 3 is for metals that bound to Fe and Mn oxides, fraction 4 is for metals that bound to organic matter, and fraction 5 is for metals that bound to soil matrix which is residual phase. Between all the consecutive extractions, the extractants were filtered through a 0.45-µm membrane and the residues from each fraction were preserved for use in next fraction.

Bacterial identification

Sulfide–indole–motility (SIM) medium from Oxoid was used for microorganism differentiation on the basis of hydrogen sulfide (H_2S) production, indole production and motility for activated sludge sample and the column substrates. Positive results from black precipitate as ferric ammonium citrate and sodium thiosulfate discover the production of H_2S. Sulfate-reducing bacteria—BART or known as SRB-BART—was used for visual determination of SRB. It is a biodetector aiding in identifying the presence and activity of various bacteria. The presence of SRB forms either a black slime in the base of the tube or an irregular black ring around the ball. The samples were also analyzed of their bacterial species in the substrates using bacterial species barcoding analysis. The samples were cultivated and isolated on iron sulfide agar manufactured by Oxoid. After the single colony of each sample has been isolated into a slant agar, the species barcoding results were gained by NCBI 16S ribosomal RNA sequences analysis.

Results and discussion

Column test

Physicochemical parameters

Physicochemical parameters tested in the column experiment included pH, alkalinity, EC, Eh, TDS, COD and selected major ions such as Ca and SO_4 (Fig. 2). pH of the initially acidic water has been increased and maintained in circum-neutral condition over the course of the experiment. While AMD effluent in column 1 (C1) showed fluctuating pH throughout the experiment, column 2 (C2) had greater pH increase than C1. This is because C1 has been fed with untreated AMD, while C2 received influent water that has been treated in C1. Therefore, the acidity has been initially reduced in C1 and C2 was capable in maintaining the pH within neutral state until the end of experiment. This was likely due to alkalinity generation through the dissolution of limestone that was also supported during sulfate reduction that has provided acidity neutralization capacity for the AMD as per following equation (Sawyer et al., 2003):

$$SO_4^{2-} + OM \rightarrow HS^- + HCO_3^- \quad (3)$$

While alkalinity was greatly produced throughout the experiment, there was a gradual decrease in alkalinity for both columns within the first 15 days. However, alkalinity started to increase until the end of experiment. As with the pH, the alkalinity of effluent water was significantly higher

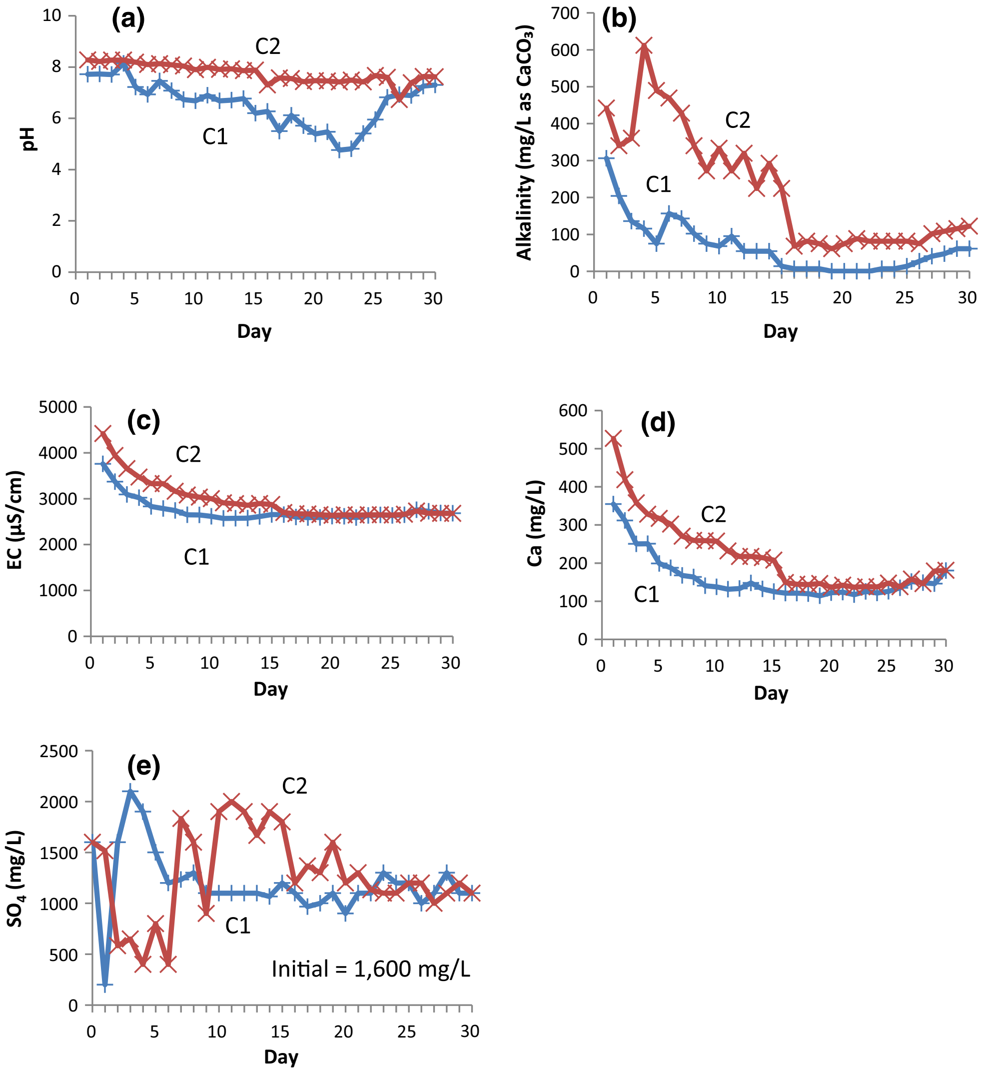

Fig. 2 Physicochemical parameters **a** pH, **b** alkalinity, **c** electrical conductivity and selected major ions, **d** Ca and **e** sulfate in column effluents during continuous flow experiment

(Mann–Whitney $W = 125$, $p < 0.001$) in C2 compared to C1. The drop in alkalinity might be attributed to limestone passivation due to precipitation of sulfate that formed throughout the experiment. The precipitation of sulfate has restricted the dissolution of calcite and production of alkalinity, which could occur a few hours after exposure to AMD (Lefticariu et al. 2015; Genty et al. 2011). The alkalinity and Ca showed similar patterns where they decreased within the first 15 days and both gradually increased from day 15 until the end of experiment. This suggests that bacterial sulfate reduction producing bicarbonate alkalinity vigorously occurred during the first half of the experiment in addition to the dissolution of calcite. However, after 15 days bacterial sulfate reduction has started to slow down while alkalinity generation was dominated by calcite dissolution. The dissolution of calcite neutralizes proton acidity, and bicarbonate alkalinity was generated when calcite reacted with water.

EC for both columns was reduced throughout the experiment. This indicates that the total dissolved solids, the solids which are commonly mineral salts that dissolved in the water, have been reduced. C2 showed significant EC decrease ($W = 228$; $p < 0.001$) compared to C1. The Eh was found to be fluctuating throughout the experiment. While a more reduced condition prevails within the first 15 days, the Eh showed a more oxidized phase after day 15. This might be due to the presence of organic matter and variety of organic compounds in the reactor systems.

Sulfate removal

Sulfate showed a small reduction in the effluents from both columns at the end of experiment ($\sim$ 35%) despite some significant reductions during early experiment especially in C2. This suggests that the treatment media in C2 could reduce more sulfates at the early of experiment since a more reducing condition was maintained in the treatment reactors. Similarly, small reduction in sulfate has been anticipated and observed in other AMD treatment using sulfate-reducing bioreactor (e.g., Ayala-Parra et al. 2016), but removal may be enhanced with addition of external carbon source (Mayes et al. 2011). Sulfate reduction in AMD treatment using SRB depends on SRB capability to degrade sulfates into H_2S, which easily attach to metals and produce soluble precipitates (Lefticariu et al. 2015). The presence of SRB was observed by the formation of black precipitates vertically slant in both column reactors throughout the experiment. The odor produced in both effluents indicates the presence of H_2S in the system (Mayes et al. 2011). Continuing reduction of sulfate maintained after 30 days suggests that the organic carbon source was not exhausted. Sulfate removal can be related with the removal of COD in the bioreactor. The effluent COD/SO_4 ratio was 0.058 for C1 and 0.064 for C2. The low COD/SO_4 ratio indicates that high accumulation of sulfides had occurred in the system. Notwithstanding this, there are two possible factors that may cause slow degradation of sulfates in such reactors: (1) The COD was inadequate in the effluent to allow the reduction of sulfate, and (2) major production of sulfides affects the SRB in terms of toxicity by dissolved sulfide and associated H_2S concentration.

Metal removal

Removal of heavy metals (Mn, Fe, Cu, Pb, Zn and Al) in the bioreactor has been investigated for 30 days (Fig. 3). For Mn, it was initially reduced at lower concentrations for a few days (i.e., 5 days in C1 and 15 days in C2) before suddenly followed by a rapid increase to above initial Mn concentration of 20 mg/L. The concentrations were even higher after day 28; therefore, both column reactors were not effective in removing Mn at day 30. According to Mayes et al. (2011), Mn was not expected to be removed in large quantities in such column reactors. In their study, removal of Mn was only noted at about 48% when external carbon source such as methanol was added to the bioreactor. In this study, it has been observed that the role of SRB that has become less significant after 15 days, as the metabolism of SRB kept reducing until the end of experiment. It has also been suggested that Mn removal was generally less effective than other metals in a study using sulfate-reducing bioreactor for AMD treatment containing high concentration of Cu, Fe, Mn, Zn and SO_4, in which Mn was only found removed at about 50% (Bai et al. 2013). In this study, removal of Mn was not sufficiently supported by the bacterial Mn reduction given very low pH condition to enable Mn oxidation to occur. Mn is a difficult metal to be removed from AMD due to complex interactions between Mn and other metals, which may affect the solubility of Mn (Zahar et al. 2015). It requires abiotic oxidation of high pH, greater than 8 that converts from Mn^{2+} to non-soluble Mn^{4+} and difficult to form sulfide minerals (Johnson and Hallberg 2005). The pH of the solution in C1 was in neutral state where the Mn removal mostly occurred due to precipitation as carbonates and also associated with adsorption onto Fe/Mn oxides and exchangeable fraction (see "Speciation of heavy metals" section). Even though the pH of the solution in C2 was mostly maintained at pH 8, the condition was still insufficient to enable effective oxidation of Mn. In fact, removal of metals by the precipitation with sulfide is to a great extent depending on the solubility products of the metal sulfide precipitates. It has been reported that the solubility product of manganese sulfide ($Ksp = 2.5 \times 10^{-13}$) was to some extent higher than those of soluble metal concentration (Bai et al. 2013). Therefore, Mn was present almost entirely in dissolved form throughout the treatment.

In contrast to Mn, Fe concentration was reduced to 0.62 mg/L in C1 compared to initial concentration of 5.8 mg/L (89% Fe removal) at the end of experiment, while Fe in C2 was reduced to 0.73 mg/L (87% removal). Both the column reactors successfully removed Fe given the pH values of within circum-neutral range which is favorable for the oxidation of Fe. Additionally, ferrous iron (Fe^{2+}) will oxidize to ferric iron (Fe^{3+}) in the presence of oxidized Mn in which Mn acts as an electron acceptor based on the following equation:

$$Mn^{4+} + 2Fe^{2+} = Mn^{2+} + 2Fe^{3+} \qquad (4)$$

Mn will solubilize again into the solution if any Mn complexes be in contact with the solution. Therefore, the interaction between Fe and Mn suggests that Fe should be removed first to allow Mn removal from the solution. Based on Eh–pH diagram for Mn calculated by the Geochemist's Workbench, when pH is around 7 and Eh is between 0 and 0.5 V, Mn is still present in $MnSO_4$ aqueous state which is in soluble phase, while Fe is in reduced condition as Fe^{2+} in the solution. This shows that the presence of Fe will affect the precipitation of Mn at near-neutral pH (Balintova et al. 2012; Goldani et al. 2013). It is also known that the removal of Fe may to a great extent be assisted by bacterial sulfate reduction through the formation of metal sulfide (Song et al. 2012; Kijjanapanich et al. 2014). Therefore, the roles of SRB and carbonate dissolution that occurred throughout the experiment were both essential for the reduction in Fe.

Meanwhile, Cu and Pb concentrations were found below detection limit (< LOD) for both column effluents in most

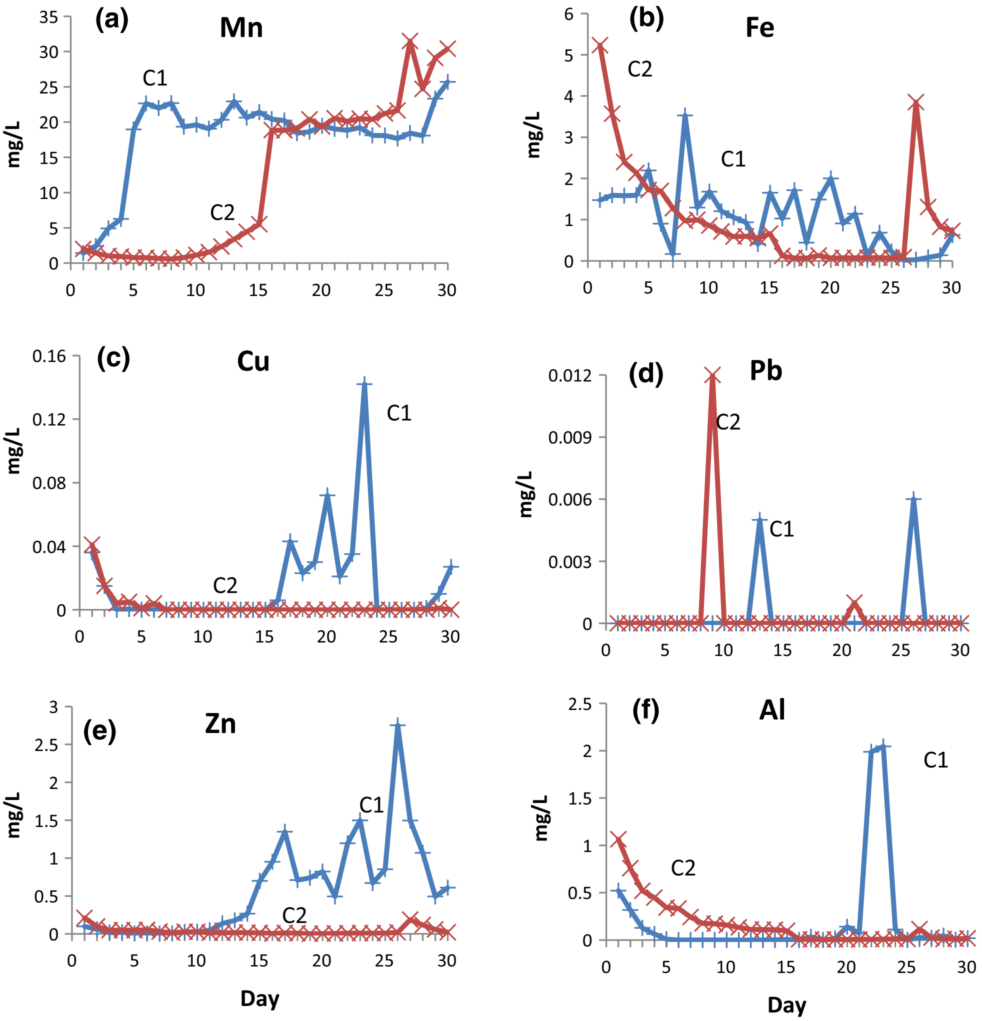

Fig. 3 Metal concentrations of **a** Mn **b** Fe, **c** Cu, **d** Pb, **e** Zn and **f** Al in column effluents of the continuous flow reactors. Irregular change in Mn concentration was noted in (**a**) due to treatment complexity in the presence of multiple metal elements

of the time during the experiment. Cu reached < LOD as early as day 3 for C1 and day 7 for C2. Despite this, Cu in C1 was found increased after day 15 for 10 days before reducing back to < LOD. This was likely associated with the role of SRB as noted earlier. Cu showed great reduction in C1 (99% removal) from the 4.6 mg/L influent to 0.027 mg/L at day 30, while C2 showed the reduction of 99.97% at day 29 with 0.001 mg/L. The efficient removal of Cu is also associated with the low solubility product of copper sulfide ($K\mathrm{sp} = 6.3 \times 10^{-36}$) that favors its removal from the water column.

The concentration of Pb diminished from 0.5 mg/L to 0.006 mg/L at day 26 (99% removal) for C1 and 0.004 mg/L at day 21 (99% removal) for C2 as Pb has reached < LOD after day 21. The high removal efficiency of Pb was due to the adsorption of the metal onto the substrates. This condition is associated with two occurrences: (1) The reduction in positive charges is likely to lead into the accumulation on the surface of the adsorbent, promoting the metal ions onto the active sites of the substrates; and (2) the reduction in positive charges also leads in an increasing of coulombic attraction of the sorbing metal ions (Zvinowanda et al. 2009). Notwithstanding this, metal adsorption has been a common mechanism for removal of various metal ions from aqueous solution such as for removal of Co(II), Cd(II), U(IV) and for organic pollutant removal (e.g., Zhang et al. 2014; Chen et al. 2015; Liu et al. 2016; Duan et al. 2016).

Zn removal in C1 was 89% which was 0.61 mg/L from initially 5.9 mg/L, while C2 showed the greatest reduction of 0.021 mg/L (99% removal). Apparently, the removal of Zn occurred as early as day 1 for both columns. This was also observed by Mandadi (2012) that Zn is removed to a maximum level after 24 h, and it was proved by the kinetic studies. Based on Eh–pH diagram for Zn, when pH of both C1 and C2 was between 7.3 and 7.62 and Eh between 0.10 and 0.15 V, Zn removal is favorable. Additionally, as with the removal of Cu, a high level of Zn removal was established due to the low solubility product of zinc sulfide

($Ksp = 2.93 \times 10^{-25}$) with independent of solution pH value (Bai et al. 2013).

Al was reduced in both columns with 99.96% from initial concentration of 44 mg/L. Al in both column reactors clearly showed that it followed first-order kinetics where higher initial concentration leads to higher removal rate in a system. The successive removal of Al might be because of the Al^{3+} ions were in state where it turned into aluminate ions $Al(OH)_4^-$ at pH 6 and above according to Eh–pH diagram for Al. At pH 5–6, Al may precipitate as basaluminite ($Al_4(SO_4)(OH)_{10} \cdot 5H_2O$) such as for those pH found on day 19–25 in C1 (Liu et al. 2008). Despite a spike in Al concentration between day 20 and 25, Al was successfully removed from both columns at day 30.

Generally, the removal efficiency of the heavy metals in the bioreactor can be ranked in the order of Al > Pb > Cu > Zn > Fe > Mn. For all metals (Fe, Cu, Pb, Zn and Al) except for Mn, the final concentrations fell below the recommended acceptable value of Malaysian Ministry of Health (MOH) for raw water resource (Table 1). In addition, these metals were removed to within Class II of National Water Quality Standard (NQWS), which is suitable for use as raw water resource such as for raw water supply (although conventional treatment is still required) and for recreational uses.

Substrate characterization

Substrate surface morphological structure

The SEM morphological structure of mixed substrates used in the columns experiment is shown in Fig. 4a. Based on EDX analysis in Fig. 4b, the mixed substrates were composed of carbon (52.77%), oxygen (36.04%), magnesium (0.83%), aluminum (0.99%), potassium (0.83%), calcium (6.79%) and iron (0.64%). This supports the presence of some metals and cations being attached onto the substrates after the treatment. Various elements were found which were to complement treatment requirement using organic and inorganic compounds.

Physicochemical parameters

The physicochemical parameters of the mixed substrates which are the media before the treatment (MS), and the substrates used for column experiment in C1 and C2 (drained column substrates) that were separated for analysis after the treatment are presented in Table 2. The pH of the substrate in MS and C1 was in neutral state, while the substrate in C2 has slightly acidic pH. The Eh in all substrates was in oxidizing state. The conductivity of MS was the lowest among all, whereas the substrates in C1 and C2 have higher values of conductivity due to ion adsorption by the media from the synthetic AMD after the treatment. The LOI values indicated that there were sufficient amount of organic matter in the substrates to aid removal of contaminants in the synthetic AMD. The treatment media have been analyzed for their cation exchange capacity as shown

Table 1 Compliance of results with regulatory requirements

Element	Concentration (mg/L)			
	MOH$^+$ standard	NQWS*	Initial	Final (after 30 days)
Cu	1.0	0.02 (Class II)	4.6	0.001
Fe	1.0	1.0 (Class II)	5.8	0.73
Mn	0.2	0.1 (Class II)	20	33
Pb	0.1	0.05 (Class II)	0.5	< LOD
Zn	5.0	5.0 (Class II)	5.9	0.021
Al	–	0.06 (Class III)	44	< LOD

$^+$MOH (Ministry of Health)—raw untreated water

*NQWS (National Water Quality Standards) classification

Class I conservation of natural environment; water supply I—practically no treatment necessary; fishery I—very sensitive aquatic species

Class IIA water supply II—conventional treatment required; fishery II—sensitive aquatic species

Class IIB recreational use with body contact

Class III water supply III—extensive treatment required; fishery III—common of economic value and tolerant species; livestock drinking

Class IV irrigation

Class V none of the above

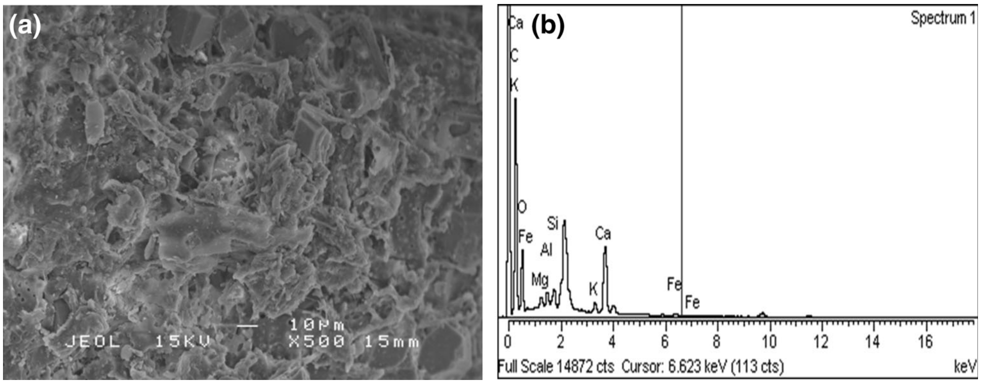

Fig. 4 **a** SEM morphological structure of mixed substrates and **b** EDX analysis for elemental composition in the mixed substrates of column test

Table 2 Physicochemical parameters of the media before and after treatment

Parameter	Unit	MS	C1	C2
pH	–	7.82	6.49	5.97
Eh	mg/L	120.00	133.00	162.00
Cond.	µS	978.00	1291.00	1511.00
LOI	%	87.32	89.94	91.98

in Table 3. Obviously, after the treatment, the substrates in C1 and C2 were found to contain lesser amount of cations and hence lower CEC values. This suggests that there was decreasing organic matter content in the substrates of C1 and C2 whereby the organic matter acts as negatively charged particles that hold the cations. The percent base saturation that indicates the relative availability of the cations in the substrates is shown in Fig. 5. Notably, Ca and Mg were more dominant cations during the removal processes in C1 and C2.

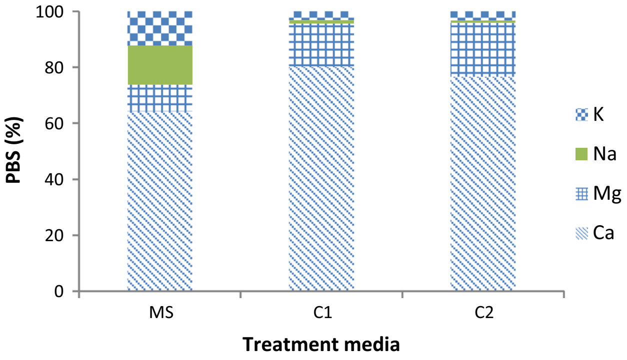

Fig. 5 Percent base saturation of the treatment media used

Speciation of heavy metals

As noted earlier, the accumulation of heavy metals during the continuous flow AMD treatment occurred when the contaminants were transferred from the water column into the treatment substrates. The fraction distribution of heavy metals in the precipitates (Fig. 6) has been determined by means of sequential extraction as to understand the preferential phase for the accumulation of the heavy metals (Wali et al. 2014). It was found that, Mn accumulation phase in MS followed the order of Fe/Mn oxides > organic > carbonate > exchangeable > residual. The Mn concentration in Fe/Mn oxides fraction has a mean of 147.43 mg/kg. The Mn accumulation in C1 and C2 was mostly associated with exchangeable fraction (mean of 139.42 and 316.58 mg/kg, respectively), followed by Fe/Mn oxides, carbonate and organic residual fractions. The preference of Mn removal onto the exchangeable fraction was likely associated with the preferential cation exchange providing some surfaces for Mn adsorption. Fe was mostly bound to organic matter fraction (51%, mean of 1880 mg/kg) in MS. Fe in C1 and C2 was mostly associated with Fe/Mn oxides fraction (67% in C1 with 1734 mg/kg and 55% in C2 with 2276 mg/kg). This has been anticipated because Fe oxides surfaces act as surface catalyst for the adsorption of more ferrous or ferric iron.

Table 3 Cation exchange capacity of the media before and after treatment

Cation/Sample	MS	C1	C2
Ca	5.07	1.09	1.05
Mg	0.75	0.21	0.26
Na	1.11	0.02	0.01
K	0.96	0.04	0.04
CEC	7.89	1.36	1.37

The units of all cations and CEC are in meq/100 g

The concentration of Cu for all treatment substrates was predominantly associated with organic matter fraction which was 73% in MS, 93% in C1 and 88% in C2. The Cu association with organic matter fraction was likely because organic fraction is often considered the fraction where

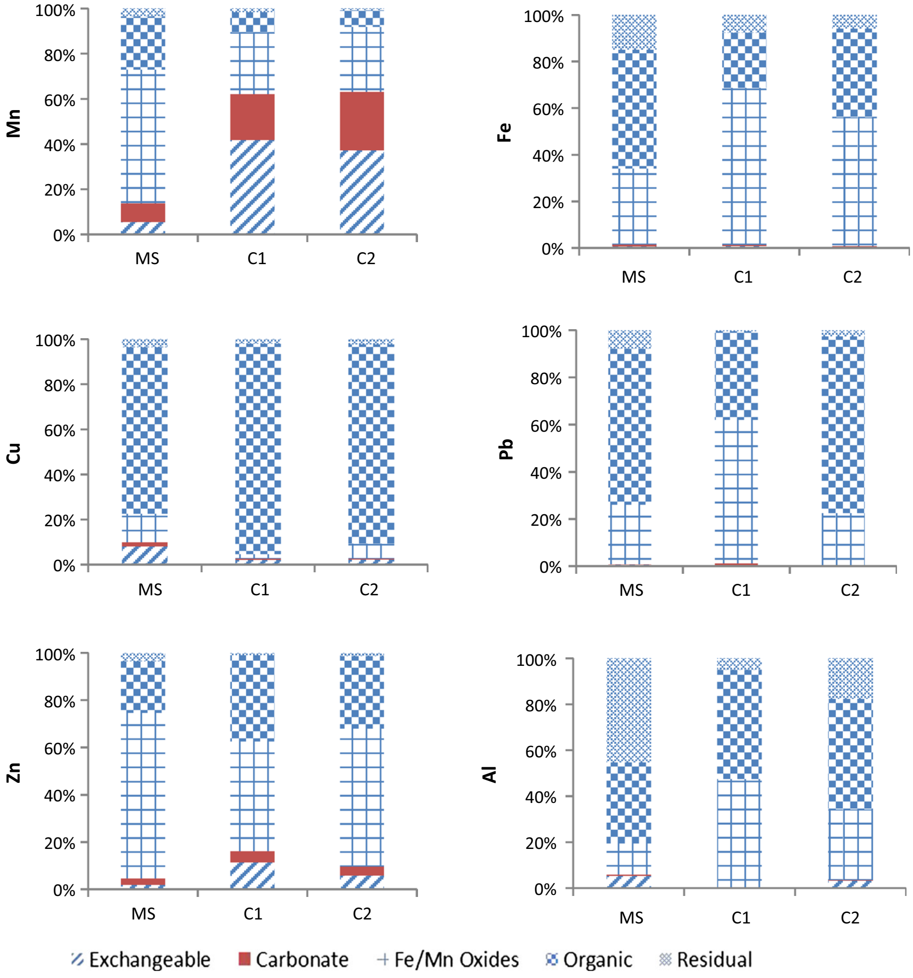

Fig. 6 Heavy metals speciation in treatment substrates used before (MS) and after treatment (C1 and C2) based on sequential extraction

sulfides are recovered (ChaguÉ-Goff, 2005). Likewise, Pb mostly bound to the organic matter fraction in MS and C2 with mean concentrations of 2.2 mg/kg (66%) and 10.96 mg/kg (75%), respectively. However, Pb was mainly associated with Fe/Mn oxides in C1 (60%, mean of 132 mg/kg). Most of Zn was associated with Fe/Mn oxides for all treatment, i.e., 96 mg/kg in MS (69%), 638 mg/kg in C1 (46%) and 228 mg/kg in C2 (58%). This agrees with the findings of Mayes et al. (2011) that Fe/Mn oxides was the dominant sink for Zn, while some portion was associated with the organic matter. Al has mostly bound to residual fraction (45%) and organic fraction in MS. In C1 and C2, Al was mostly bound to organic matter fraction (48%) and some portion associated with Fe/Mn oxides. Notably, organic matter and Fe/Mn oxides were the dominant fractions for metal sink in the AMD treatment, while carbonate fraction appears to be less significant sink for the contaminants. This suggests that the role of organic carbon source was a more dominant mechanism, e.g., removal of metals as sulfides compared to carbonate dissolution, while significant removals were also assisted by the adsorption onto Fe/Mn oxides surfaces.

Bacterial sulfate reduction

The production of hydrogen sulfide H_2S that indicates the activity of SRB in the substrates has been determined by the formation of black precipitates on the SIM agar. The activated sludge (AS) which was used as the source of SRB demonstrated black precipitates covering all agar surfaces, indicating the presence of microbe that reduces sulfur-containing compounds to sulfides during metabolism processes. The agar plates of C1 showed less black precipitates than C2. Meanwhile, from the BART test results, all three samples of AS, C1 and C2 were found to have dense population of anaerobic bacteria that were dominated by *Desulfovibrio desulfuricans*. This was proved by the reaction that can be seen on day 1 that the black slimes appeared in the bottom of the sample tubes. The results showed that the bacterial population was estimated to about 6.8×10^7 cfu/mL based on the slime population. Similar findings have also been found that synergistic interaction between SRB (*Desulfovibrio desulfuricans*) and coexisting fermentative bacteria could be the key factor for the utilization of organic substrate as carbon and nutrient sources for BSR (Zhang et al. 2016). The species barcoding analysis indicated that the samples contained *Bacillus cereus* strain which is from gram-positive bacteria. Activated sludge typically has mixed bacterial populations from different domestic sources. Thus, the AS that was used as the source of SRB might have both gram positive and gram negative in it. According to Fig. 7, the bacteria from C2 column were found in coccus-shaped and a small population of rod-shaped bacteria.

Conclusion

The findings demonstrated that the sulfate-reducing bioreactor that composed of spent mushroom compost, limestone, activated sludge and woodchips were effective for AMD treatment of sulfate- and metal-rich mine water. The spent mushroom compost has greatly assisted the removals of sulfate and metals in the treatment. Alkalinity was greatly produced by the dissolution of limestone and metabolism of SRB, thus maintaining pH at neutral state. Although Fe, Pb, Cu, Zn and Al were effectively removed (87–100%), Mn was not successfully removed despite initial Mn reduction during early phase of the experiment. The first half of the treatment was an essential phase for removal of most metals where contaminants were primarily removed by bacterial sulfate reduction in reducing condition and carbonate dissolution function. The metals in the substrates were mostly bound to organic matter fraction, while some metals have greater affinity to Fe/Mn oxides fraction. Therefore, this study has highlighted the importance of both physicochemical and bacterial treatment functions in the remediation of AMD highly rich in sulfate and heavy metals.

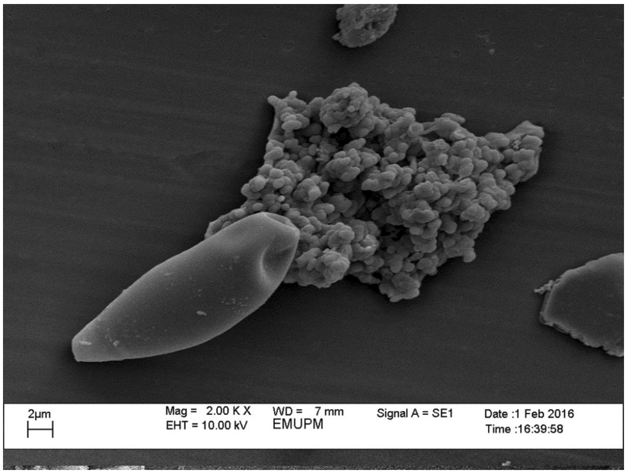

Fig. 7 SEM morphology of media in C2 after treatment

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