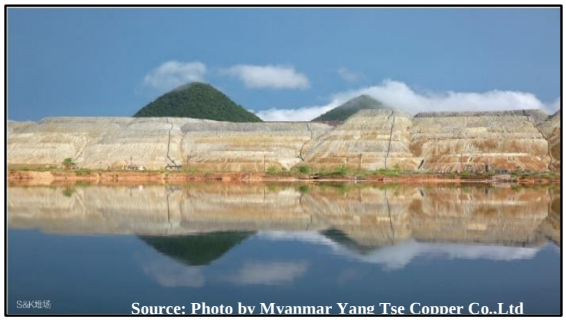
Leaflet No.

The Republic of the Union of Myanmar
Ministry of Natural Resources and Environmental Conservation
Department of Mines

Competitive Growth of Iron-Reducing Bacteria(IRBs)/ Sulfate-Reducing Bacteria (SRBs) with Bioleaching Acidophiles for Bioremediation under Anaerobic Conditions



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February, 2023

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Abstract

The Monywa copper mine in Myanmar, a high-sulfidation deposit rich in pyrite, faces significant environmental challenges due to acid mine drainage (AMD) generated by bioleaching microbes that oxidize sulfide minerals. AMD releases sulfuric acid and heavy metals, contaminating ecosystems for centuries. Traditional remediation methods are costly and unsustainable. This study explores the use of sulfate-reducing bacteria (SRBs) and iron-reducing bacteria (IRBs) for in situ AMD control by competing with local iron-oxidizing bacteria (IOBs) and sulfur-oxidizing bacteria (SOBs) in acidic pyritic heap bioleaching residues. The research successfully cultured acidic SRBs and IRBs at pH 2.0–3.0, demonstrating their potential to inhibit AMD through biogenic mineralization. Key findings revealed that organic matter and elevated pH (achieved via lime addition) are critical for SRB and IRB growth, enabling them to outcompete IOBs and SOBs. Under these conditions, pH increased from acidic to nearly neutral, Eh decreased, and metals precipitated with microbially generated sulfides, significantly reducing copper in pore solutions. Column leaching experiments under aerobic conditions further confirmed that SRBs and IRBs can dominate over bioleaching microbes, transforming bio-oxidation into biogenic mineralization. Organic matter input, particularly when combined with lime, promoted SRB and IRB growth (e.g., *Clostridiacea*e and *Peptococcaceae*) while inhibiting IOBs and SOBs. This shift resulted in the complete inhibition of sulfide mineral oxidation, effectively controlling AMD at its source. The study highlights the potential of SRBs and IRBs for sustainable, in situ remediation of pyritic waste rocks, offering a cost-effective alternative to conventional methods. By leveraging microbial competition and environmental modifications, this approach provides a promising strategy for AMD source control and long-term environmental protection in mining regions.

Keywords: Acid mine drainage, Bioremediation, Source control, Iron-and sulfate- reducing bacteria, Microbial Competition, In Situ Remediaiton

ကြေးနီသတ္တုတွင်းဝန်းကျင်ရှိ အကျိုးပြုအဏုဇီဝပိုးမွှားနှင့် Heap Bioleaching အကြွင်းအကျန်များမှ Acid Mine Drainage (AMD) ဖြစ်နိုင်ချေကို အကဲဖြတ်လေ့လာခြင်းနှင့် အရင်းအမြစ်ထိန်းချုပ်မှုအားဖြင့် AMD လျော့ပါး သက်သာအောင် ကုစားရေးတွင် အဏုဇီဝပိုးမွှားတို့ကို အသုံးပြု၍ ၎င်းယှဉ်ပြိုင်မှုတို့ကို လေ့လာခြင်း

စာတမ်းအကျဉ်း

စစ်ကိုင်းတိုင်းဒေသကြီး မုံရွာခရိုင်၊ ဆားလင်းကြီးမြို့နယ်အနီးရှိ ကြေးနီသတ္တုတွင်းသည် Heap bio leaching နည်းစဉ်ဖြင့် ကြေးနီသတ္တုထုတ်လုပ်မှုလုပ်ငန်း ဆောင်ရွက်နေခြင်း ဖြစ်ပါသည်။ Leaching ပြုလုပ်ပြီး ကြွင်းကျန်သောအရာများမှာ Heap bio leaching Residues စွန့်ပစ်မြေစာနှင့် ကျောက်များဖြစ်ပါသည်။ ဤသုတေသနသည် Heap bio leaching Residues မှ မြေနမူနာများ ရယူ၍ ဝိသေသလက္ခဏာရပ်များအပေါ် အကဲဖြတ်ခြင်း၊ မူရင်းမျိုးရင်းဖြစ်သော အဏုဇီဝပိုးများအားလေ့လာခြင်းနှင့် ဆာလ်ဖိုက်ဓာတ်ပြယ်စေရန် အကျိုးပြု အဏုဇီဝပိုးများ ထည့်သွင်းလေ့လာခြင်း စသော အဏုဇီဝဗေဒလုပ်ငန်းစဉ်များအား အဓိကထားလုပ်ဆောင်ခြင်း ဖြစ်ပါသည်။ Pyrite ကြွယ်ဝကာ ဆာလဖာအများအပြား ပါဝင်သည့် သတ္တုသိုက်ဖြစ်ပြီး Sulfide minerals များကို ဓာတ်တိုးစေသော Bioleaching microbes များကြောင့် ဖြစ်ပေါ်လာသော Acid mine drainage (AMD) မိုင်းအက်စစ် ယိုစိမ့်မှုဖြစ်ပေါ်စေကာ ကြီးမားသော ပတ်ဝန်းကျင်ဆိုင်ရာစိန်ခေါ်မှုများကို ရင်ဆိုင်ရနိုင်ပါသည်။ Pyrite နှင့် Chalcopyrite ကဲ့သို့သော သတ္တု ဆာလ်ဖိုက်များ ပါဝင်သည့် စွန့်ပစ် ကျောက်အများအပြားသည် သဘာဝအားဖြင့် လေနှင့် ရေတို့ ထိတွေ့ ဓာတ်ပြုပြီး အက်စစ် ဓာတ်ရှိသော အက်ဆစ်ပျော်ရည်အဖြစ် ပြောင်းလဲသွားပါသည်။ AMD သည် ဆာလဖျူရစ် အက်ဆစ်နှင့် Heavy metals များကို ထုတ်လွှတ်ကာ ပတ်ဝန်းကျင် ဂေဟစနစ်များကို ရာစုနှစ် များစွာ ညစ်ညမ်းစေနိုင်ပါသည်။ ရှေးရိုးရာနည်းလမ်းများမှာ ကုန်ကျ စရိတ်များပြီး ရေရှည် တည်တံ့နိုင်မှုမရှိပါ။ လေ့လာမှုတွင် Sulfate–reducing bacteria (SRBs) နှင့် Iron–reducing bacteria (IRBs) များကို အသုံးပြု၍ In situ AMD ထိန်းချုပ်မှုကို လေ့လာခဲ့ပြီး အက်ဆစ် ဓာတ်ပါသော Pyritic heap bioleaching residues များတွင် ဒေသခံ Iron–oxidizing bacteria (IOBs) နှင့် Sulfur–oxidizing bacteria (SOBs) တို့ကို ယှဉ်ပြိုင်နိုင်စေရန် ရည်ရွယ် ခဲ့ခြင်းဖြစ်ပါသည်။ ဓာတ်ခွဲစမ်းသပ်သည့် နမူနာများမှ NAG test, Paste pH test, Paste EC test, XRF, XRD, ICP–OES ကဲ့သို့ အမျိုးမျိုးသော စမ်းသပ်မှုများနှင့် ခွဲခြမ်းစိတ်ဖြာမှုများ ပြုလုပ် ခဲ့ပါသည်။ Column leaching စမ်းသပ်မှုအရ ကျောက်နမူနာများတွင် Metal ions များ ဖြစ်သည့် Fe, Cu, and SO_4^{2-} များ ပျော်ဝင်နေပြီး pH တန်ဖိုး 3 ထက် လျော့နည်းနေသည်ကို တွေ့ရှိရပါသည်။ ထို့ကြောင့် AMD ဖြစ်စေသည့် sulfides ဓာတ်တိုးမှုကိုထိန်းချုပ်ခြင်း နှင့် သတ္တုများပျော်ဝင်မှုအား လျှော့ချခြင်းကဲ့သို့သော သင့်လျော်သော အစီအမံများ ပြုလုပ်ရန် အလွန် အရေးကြီးပါသည်။ သုတေသနသည် pH 2.0–3.0 တွင် အက်ဆစ်ဓာတ် ပါသော SRBs နှင့် IRBs များကို အောင်မြင်စွာမွေးမြူနိုင်ခဲ့ပြီး၊ ၎င်းတို့သည် biogenic mineralization

မှတစ်ဆင့် AMD ကို တားဆီးနိုင်စွမ်းရှိပါသည်။ တွေ့ရှိချက်များအရ အော်ဂဲနစ်ပစ္စည်းများနှင့် ထုံး အသုံးပြုခြင်းဖြင့် pH ကို မြှင့်တင်ခြင်းသည် SRB နှင့် IRB များ၏ ကြီးထွားမှုအတွက် အရေးကြီးပြီး၊ ၎င်းတို့သည် IOBs နှင့် SOBs များကို ယှဉ်ပြိုင်နိုင်စေခဲ့ပါသည်။ ဤအခြေအနေများတွင် pH သည် အက်ဆစ်ဓာတ်မှ Neutral အထိ တိုးစေခြင်း၊ Eh လျော့ကျခြင်း၊ သတ္တုများသည် microbial –generated sulfides များနှင့် အတူ အနည်ထိုင်စေခြင်းနှင့် Pore solution များတွင် ကြေးနီ ပါဝင်မှုပမာဏ သိသိသာသာ လျော့ကျသွားခြင်းတို့ ဖြစ်စေခဲ့ပါသည်။ စမ်းသပ်မှုများမှ SRBs နှင့် IRBs များသည် Bioleaching microbes များထက် သာလွန်နိုင်ပြီး၊ Bio–oxidation ကို Biogenic mineralization အဖြစ်သို့ ပြောင်းလဲနိုင်ကြောင်း အတည်ပြုခဲ့ပါသည်။ SRB နှင့် IRB များ (ဥပမာ၊ *Clostridiaceae* နှင့် *Peptococcaceae*) ၏ ကြီးထွားမှုကို မြှင့်တင်ပေးပြီး၊ IOBs နှင့် SOBs များကို တားဆီးနိုင်ပါသည်။ ထိုပြောင်းလဲမှုသည် sulfide mineral oxidation ကို လုံးဝ တားဆီးနိုင်ခဲ့ပြီး၊ AMD ကို အရင်းအမြစ်အဆင့်တွင် ထိရောက်စွာ ထိန်းချုပ်နိုင်ခဲ့ပါသည်။ ဤ လေ့လာမှုအရ SRBs နှင့် IRBs များ၏ စွမ်းဆောင်ရည်ကို မီးမောင်းထိုးပြခဲ့ပြီး Pyritic waste rocks များအတွက် ရေရှည်တည်တံ့သော In situ remediation နည်းလမ်းတစ်ခုအဖြစ် ကုန်ကျ စရိတ် သက်သာစေနိုင်ပါသည်။ Bioremediation နှင့် Microbial competition ဇီဝနည်းဖြင့် ပြုပြင်ကုစားသည့်နည်းလမ်းများအသုံးပြုခြင်းဖြင့် AMD အရင်းအမြစ်ထိန်းချုပ်မှုနှင့် သတ္တုတွင်းဒေသများတွင် ရေရှည်ပတ်ဝန်းကျင်ကာကွယ်မှုအတွက် အထောက်အကူဖြစ်စေသော သုတေသန တစ်ခုအဖြစ် စမ်းသပ် လေ့လာနိုင်ခဲ့ပါသည်။

အဓိကစကားလုံးများ : မိုင်းအက်စစ်ယိုစိမ့်မှု၊ ဇီဝနည်းဖြင့်ပြုပြင်ကုစားခြင်း၊ သတ္တုဆာဖိုဒ်၊ သံဓာတ်နှင့် ဆာလဖိတ်ဓာတ်လျော့ချပေးသော ဘက်တီးရီးယားများ

Competitive Growth of Iron-Reducing Bacteria(IRBs)/ Sulfate-Reducing Bacteria (SRBs) with Bioleaching Acidophiles for Bioremediation under Anaerobic Conditions

1. Introduction

Mining operations generate significant amounts of waste, primarily consisting of sub-economic waste rock and tailings. These waste materials often contain sulfide minerals, with pyrite being the most common. When these sulfides are exposed to air and, in particular, when autotrophic iron- and sulfur-oxidizing microorganisms naturally colonize them, the oxidation of sulfide minerals is accelerated. This process leads to the production of large quantities of acid mine drainage (AMD). (Johnson, D.B.; Hallberg et al., 2005; Neculita et al., 2008; Schippers et al., 2009). Mining activities often generate acidic drainage, which is commonly linked to elevated levels of heavy metals and metalloids. This type of drainage is regarded as one of the most significant contributors to heavy metal pollution in the environment. (Akcil et al., 2006)

It was usually employed to treat an AMD solution by growing SRBs, and the AMD solution is treated using a microbial process that reduces metals and sulfates. This process results in the production of alkalinity and the reduction of metal accessibility through the precipitation of sulfide minerals (Rodríguez-Galán M et.,al 2019, Dong Y-R et al., 2019). These processes are exploited in ex-situ treatment of AMD after it comes out from rocks (Zhang et., al, 2016, Becerra et.,al 2010).

However, a few researchers have used the SRBs to directly address the acid-generating waste rocks in situ, inhibit sulfide minerals' oxidation, and induce the simultaneous precipitation of metal with sulfide. This approach restricts the release of acid mine drainage (AMD) and facilitates the subsequent restoration of the waste rocks. It was investigated that the predominance of oxidation processes resulted in the acidification of the waste rock(Benner et al., 2000) rather than the reducing processes in the tailing ponds. Tailings were usually fine and low in oxygen concentration inside the dam; the IRBs and SRBs can co-exist with IOBs and SOBs(Bruce Wielinga, 1999), indicating the presence of biogeochemical iron and sulfur cycle in acidic natural conditions(Schippers, 2009). Although not dominant by the reducing microbes, this fact still gives us confidence for the in situ competition over IOBs and SOBs by SRBs and IRBs by creating suitable conditions for them. We investigated the competition between reducing microbes and oxidizing microbes to illustrate specific microbes' community and diversity under specific conditions and gave implications to bio-remediation.

Case Studies on the Bioremediation of Acid Mine Drainage Using Sulfate-Reducing Bacteria (SRBs): The application of sulfate-reducing bacteria (SRBs) in bioremediation processes has been extensively studied . Appendix Table 5 summarizes various case studies, highlighting the use of different bioreactors, site-specific conditions, and targeted metals for remediation. These studies focus on mitigating acid mine drainage (AMD) generated from mining activities, providing insights into effective remediation strategies.

2. Objectives

The objective is to understand how these microbes compete with the local autotrophic bioleaching microbes under acidic conditions. In addition, the succession of the SRBs community during the remediation process with altered environmental conditions were investigated, and the main influencing factors were figured out. The findings gave implications for in situ controlling of acid mine drainage from acid-generating rocks in their natural environment. The contents of this research include:

(1) Cultivation of iron- and sulfate-reducing microbes in acid mine drainage

(2) Influence of different organic matter on chemical and biological pyrite oxidation

(3) Competitive growth of sulfate-reducing bacteria with bioleaching acidophiles for bioremediation under anaerobic condition

3. Materials and Methods

3.1. Site description, sample collection, and characterization

The iron- and sulfate-reducing bacteria solutions were incubated with the AMD solutions from Monywa heap leaching residue (Figure.1 and 2 (a,b)). The two samples collected are AMD solution samples near a raffinate (from the SX plant) pond and heap-leached dumps residues (from 3 different Cells). Local mine-impacted anaerobic soil (pH about 4.0) was also collected near the acid mine drainage.

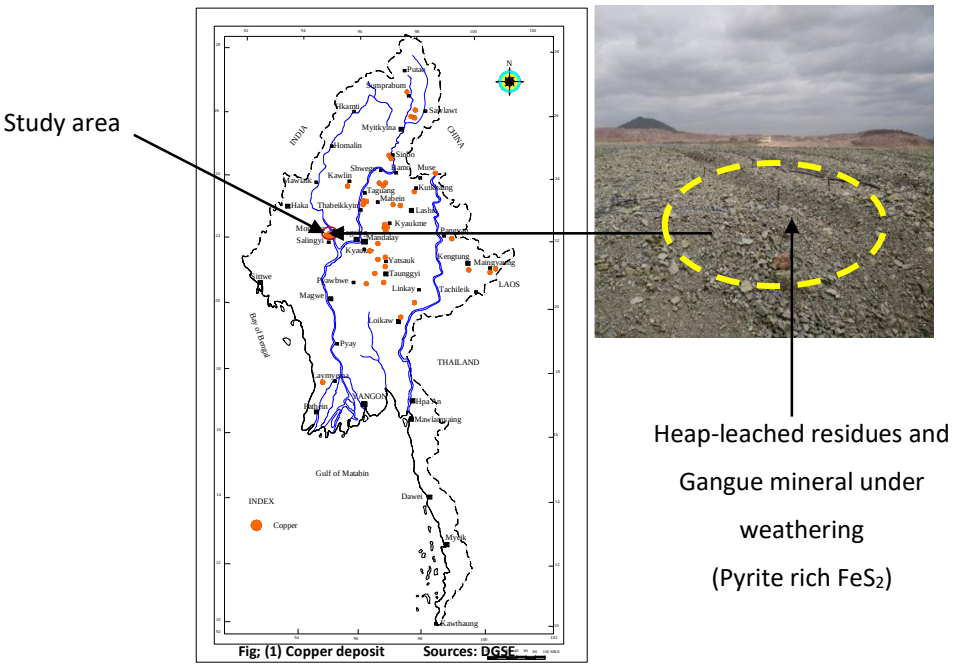

Figure 1. Site location view of heap bio-leached residues at Monywa copper mine site

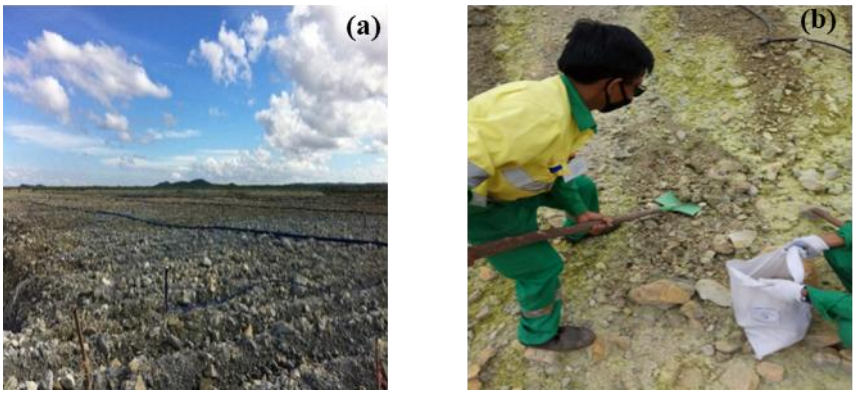

Figure 2. Heap bioleaching process and its residues: (a) view of heap bioleaching cell at irrigation and (b) Sample collected from heap bioleached residues

The study was conducted by sampling bio-leached wastes from 3 heap leaching cells at the Monywa copper mine in Myanmar in 2018. The heap bioleaching facility is located in a region characterized by a tropical environment, where the average annual temperature ranges from 20 to 40 degrees Celsius. Before the heap bioleaching, the composition of the copper ore

consisted of approximately 9% pyrite, around 0.8% copper sulfides (mostly chalcocite), 50% silica, and various other gangue minerals, with a minimal presence of alkaline gangues. The heap leaching process involved stacking up the run-of-mine, and the oxidation of pyrite contributed to an excess of acid and iron in the solution used for leaching. Following heap bioleaching, the remaining material remains uncovered and exposed to weathering, producing acid mine drainage. The leach AMD was collected from the bottom of the cells. Cation ions were analyzed using atomic absorption spectrometry (AAS) with the ZEEnit@700P Analytik Jena. Anions were quantified using ion chromatography (IC) with the ICS600 instrument from PerkinElmer, located in Waltham, MA, USA. Free acid was determined through titration, while the ionic strength was assessed by precipitating all elements and measuring the total weight; results are presented in (Table 1).

The leaching cells have an average height of 30-40 meters and contain a total of 150 million tons of residue that formed accumulated over about 20 years. The element content was analyzed using ICP-MS, and the net acid-producing potential was determined. Hydrogen peroxide (H_2O_2) is used through oxidation, and sodium hydroxide (NaOH) is consumed. The average concentration of total sulfur is 6.42%, while the concentration of sulfide sulfur is 3.81% (mainly pyrite, with a small amount of unleached chalcocite). This can produce an average of 77.8 kg of sulfuric acid per ton (Table 2). The average concentration of copper in the residue is around 0.07%.

Table 1. Element assay of the acid mine drainage from leached cells

Fe	**Cu**	**Ca**	**Mg**	**K**	**Na**
mg/L	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**
11520	1644	630	1002	7.8	790
Al	**As**	**Cd**	**Zn**	**Pb**	**Mn**
mg/L	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**
1697	16.9	2.6	85.1	<0.1	271
Hg	**Cr**	**Co**	**Se**	**Si**	**Ag**
mg/L	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**
<0.1	0.8	8.6	<0.1	133	<0.1
pH	**Eh**	**SO_4^{2-}**	**Cl^-**	**Free acid**	**Total ionic strength**
	mV, vs SHE	**mg/L**	**mg/L**	**g/L**	**%**
1.10	850	73800	2018	9.21	8.08

Table 2. Characteristics of the heap bioleaching residue

Sample	**Cu %**	**Fe %**	**Ca %**	**Mg %**	**Al %**	**Na %**	**K %**	**Total sulfur %**	**Reduce sulfur %**	**Net acid production potential kg/t**
Cell 1	0.064	5.70	0.10	0.12	8.45	0.28	2.66	5.60	3.57	72.9
Cell 2	0.078	6.86	0.09	0.08	9.27	0.26	2.93	9.02	5.71	116.5
Cell 3	0.074	4.00	0.12	0.12	9.51	0.28	2.61	4.63	2.16	44.1
Average	0.070	5.52	0.10	0.11	9.08	0.27	2.73	6.42	3.81	77.8

(Adapted from Aungkyawphyo et al., 2020-modified)

Microbial communities in Monywa Mine, as the previous study(Jia et al., 2016) reported in Figure.3, that community possessed a significant amount (about 89%) of microbes able to ferrous oxidation, whereas a small number (about 9%) included those that were able to sulfur-oxidizing microbes. Minimal amounts (less than 3%) of other heterotrophic bacteria, such as *Alicyclobacillus* and *Acidiphilium*, were identified. The presence of thermophilic acidophilic microbes, namely sulfur-oxidizing microbes such as *F.cupricumulans*, *A. caldus*, *Thermogymnomonas acidicola*, *S. acidophilus*, and *S. thermosulfidooxidans*, was identified.

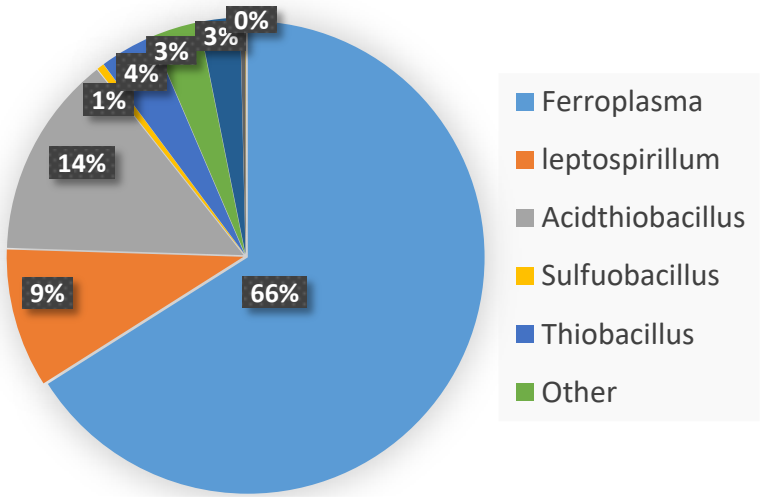

Figure3. Microbial community in the acid mine drainage

3.2. Anaerobic bacterial culture

The anaerobic soil was stirred, and the resulting solution was collected as filtrate. The pH of AMD solutions is adjusted with NaOH to achieve a varied pH at levels of 2.0, 3.0, 4.0, and 5.0, as illustrated in (Figure .4). After that, different organic matters, such as 2 g/L yeast powder, 2 g/L sodium lactate, and 2 g of pure pyrite, were added to each 100 ml AMD solution with varying pH levels. The anaerobic bottles were stored in the anaerobic culture box at a temperature of 35 °C (Figure .4). After the inoculation, microbes in the solution and the final precipitates in bottles were collected for further assay. The Fe^{2+}concentration in the bottles was tested every week during the culture. The microbes in the solutions were collected during the 180 days of incubation.

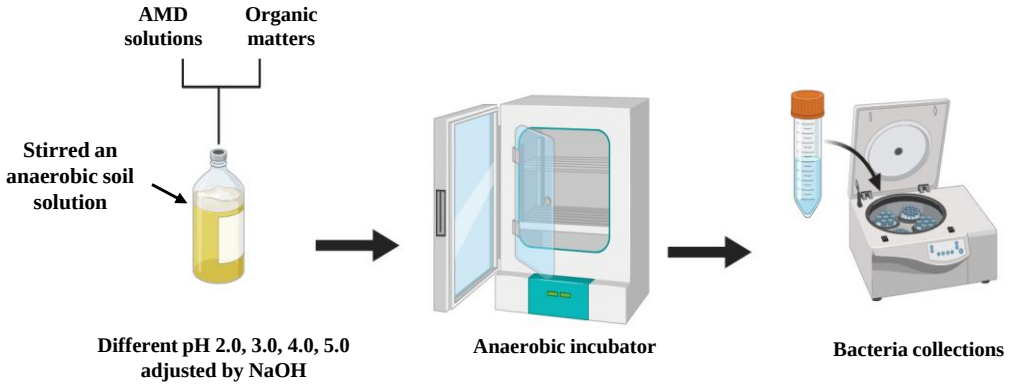

Figure 4. Anaerobic culture of the microbes in AMD at pH 2.0, 3.0, 4.0, and 5.0 in the anaerobic bottles

3.3. Physiochemical assay of the solution

The pH 2.0 and 3.0 microbial population was examined by amplifying the 16S rDNA and assessed using MiSeq high-throughput sequencing techniques. In addition, the two microbial consortiums that were cultivated at pH 2.0 and 3.0 were used as the inoculation source of IRBs and SRBs in the anaerobic column tests. The pH value was determined using a pH meter (Mettler Toledo, seven Go). At the same time, the redox potential of the solution was measured using the Ag/AgCl reference electrode immediately after collection (Mettler Toledo). The enumeration of microbes in the fluid was performed using a hemocytometer (0.1 mm, 1/400 mm2) under a microscope (CX31, Olympus, Japan) fitted with a phase contrast condenser and a Charge Coupled Device (NC7030, Oplenic, China). A 100× plan achromat objective was utilized to verify the cell count. After cultivation, the solutions were centrifuged. Then, the microbes were used for DNA extraction. Iron concentration was measured by UV-8000 spectroscopy devices (Ultra-violence visible light UV analysis), and ferrous iron was tested by titration.

3.4. Anaerobic column test

The heap leach residue was crushed by a jaw crusher to a size of about P_{80} = 20mm for column leaching tests. One of them was also pulverized for chemical assay, and four samples were used for column tests. Four acid-proof 316 stainless-steel columns (100 cm high and 10 cm inner diameter with a rubber liner) with a water jacket were used in the column experiment. Each column was charged with the same 6 kg non-sterile (the ore was agglomerated with 4 wt% sterilized water) and then operated at 35 °C maintained by a water bath. The columns include 6 kg ore were set as column 1 as the control (Control), column 2 added 0.65% lime (Lime), and column 3 added organic matter (yeast powder and sodium lactate, each 1%) (OM), column 4 add with lime (0.65%) and organic matters (yeast powder and sodium lactate, each 1%) (Lime + OM). SRBs and IRBs from the pH 2.0 and pH 3.0 anaerobic bottles in Chapter 2 were added to each column of 10 ml. A nylon net syringe tube with a connecting hole thin tube for the purpose of solution sample collecting was hanging in the middle of the column. Water was added to each column and kept at a water level above the ore of 3 cm.

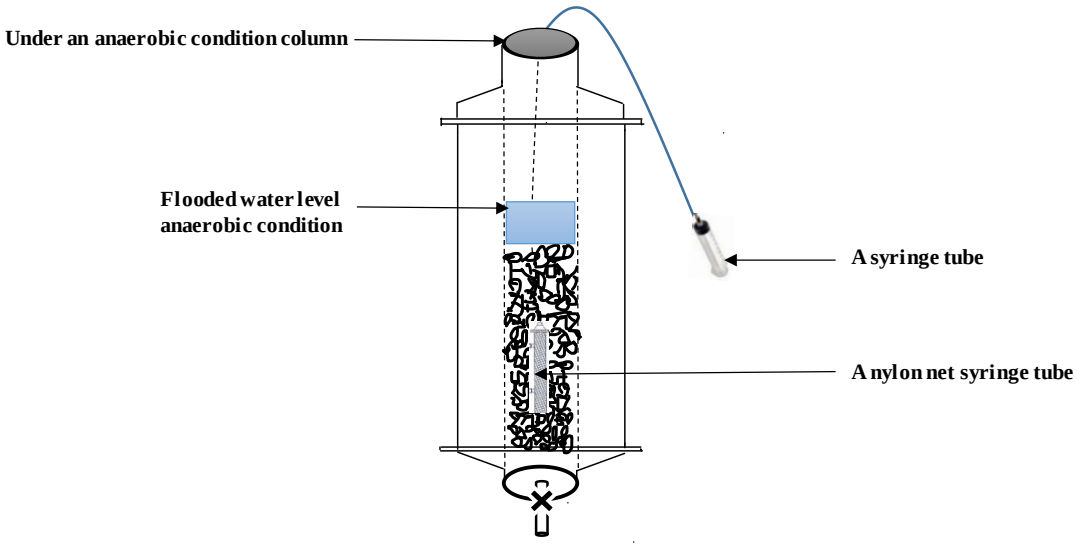

Figure 5. Schematic diagram of the cultivation experiment under anaerobic conditions

Note: Each column was charged with the same 6 kg non-sterile, flooded with water, and then operated at a set temperature of 35 °C, maintained by a water bath. Four treatments are:
C1-Control: no addition;
C2-Lime 0.65% lime;

C3-OM: chicken manure 2%, sodium lactate 1%;
C4-Lime +OM: 0.65% lime, chicken OM manure 2%, sodium lactate 1%

The solution of 10 ml in the columns was collected by syringe and immediately measured; the sample for chemical analysis such as pH, Eh, TFe, Fe^{2+}, Cu, TOC, and cell number weekly, and microbial community test was performed at 30 days, 90 days and 150 days. For the microbial community collection, four representative liquid samples (100 ml) were sampled and then immediately collected with 0.22 μm filter paper for DNA extraction and barcoded pyrosequencing. After 180 days of incubation, the columns were drained at the end of the test, and solution samples were collected into anaerobic bottles with rubber seals for chemical analysis. Additionally, a 3 kg residue sample was dried at 50°C for three days and then assayed for element contents. The other 3 kg residue was frozen at -80 °C for DNA extraction and microbial community analysis.

3.5. Physiochemical analysis of sample

The solution samples were periodically collected using a syringe-driven filter (100 mesh) buried inside the column. The pH value, redox potential, and microbial count were measured. After cell counting, the monthly washed solutions (100 mL) were filtered through a Millipore filter (0.22 μm). The microbes present on the filter paper were then used for DNA extraction, following the method described in (Jia Y et al., 2015). SO_4^{2-} was measured by UV spectroscopy devices using $BaSO_4$ to determine sulfate concentration in the samples (Anan P et al., 2013). The copper and total iron concentration was determined using ICP-OES (Optima 5300DV, PerkinElmer). Analysis of TOC was conducted using a spectrophotometer (TOC-L, CPH, Shimadu). A Mineral Liberation Analyzer determined the proportions of sulfide minerals. The residue samples were dried in an oven at 50°C and pulverized into a fine powder to analyze copper, iron, total sulfur, and reduced sulfur. The copper and total iron concentration was determined using ICP-OES (Optima 5300DV, PerkinElmer).

3.6 Microbial communities analysis

The manufacturer's methodology was followed to identify the presence of microbial communities in the anaerobic bottles. It was done by extracting the DNA using a Fast DNA Spin kit (Bio 101, MP Biomedicals, Santa Ana, CA, USA). The F515 (50-GTGYCAGCM GCCGCGG TAA) and R806 (50-GGACTACNVGGGTWTCTAAT) primers were employed to amplify the V4 hypervariable region of the bacterial and archaeal 16S rDNA genes(Bates et al., 2011).

The PCR reaction was conducted (Table .3). According to the manufacturer's instructions, the amplifications were taken out, the DNA samples were separated on 2% agarose gels and then purified using the Axyprep DNA Gel Extraction Kit from Axygen Biosciences, located in Union City, CA, USA.. Using an Illumina MiSeq platform, purified amplicons were sequenced following industry standards. Data have undergone examination and quality filtration with the aid of Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al.,2010). The UPARSE software (version 7.1, available at http://drive5.com/uparse/) was utilized to cluster operational units (OTU) based on a sequence similarity threshold of 97%. Additionally, UCHIME was employed to examine and create chimeric sequences. The sequences were compared to the NCBI using BLAST (Jia Y et al., 2016).

Table 3. PCR procedure of the 16s rDNA amplification

Move	Temperature (°C)	Time (seconds)	Periodic number
Initial denaturation	95	180	1
Denaturation	98	20	30
Annealing	55	15	
Extension	72	15	
Final extension	72	60	1

4. Results and Discussions

4.1 Microbial communities in different columns

The study demonstrates that microbial community and microbial numbers during the incubation were tested in the pore solution at day 30, 90, and 150 see Figure .6, Figure 7, Figure 8. and Table 4.) was also tested in residue at the end of the tests (180 days).

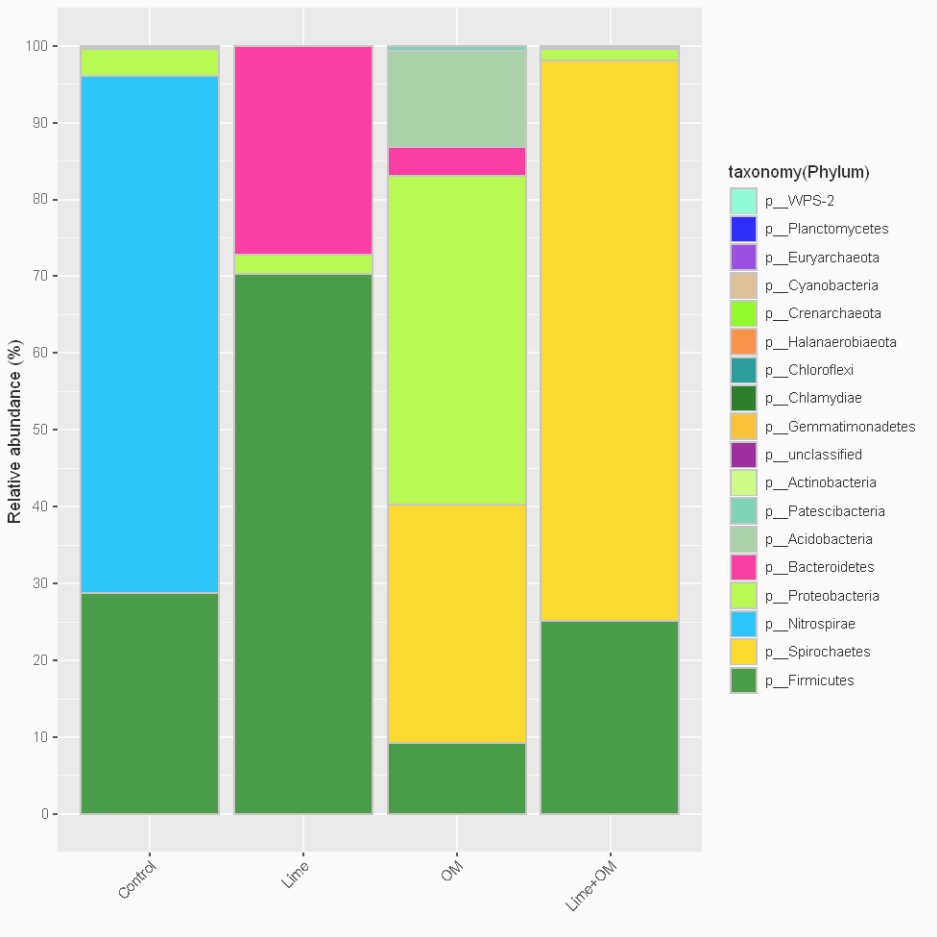

Table 6. Phylum of the microbial community in the solution phase of the four columns at day 150 (Aungkyawphyo et al., 2020)

Organic matter and lime (to elevate pH) were added to the incubation columns to support the SRBs and iron-reducing microbes to compete with the iron- and sulfur-oxidation microbes.

It is shown that organic matter significantly promotes the growth of the SRBs. The autotrophic bioleaching microbes were no longer detected, and adding lime inhibited the autotrophic acidophiles see (Figure 6 and Figure 7).

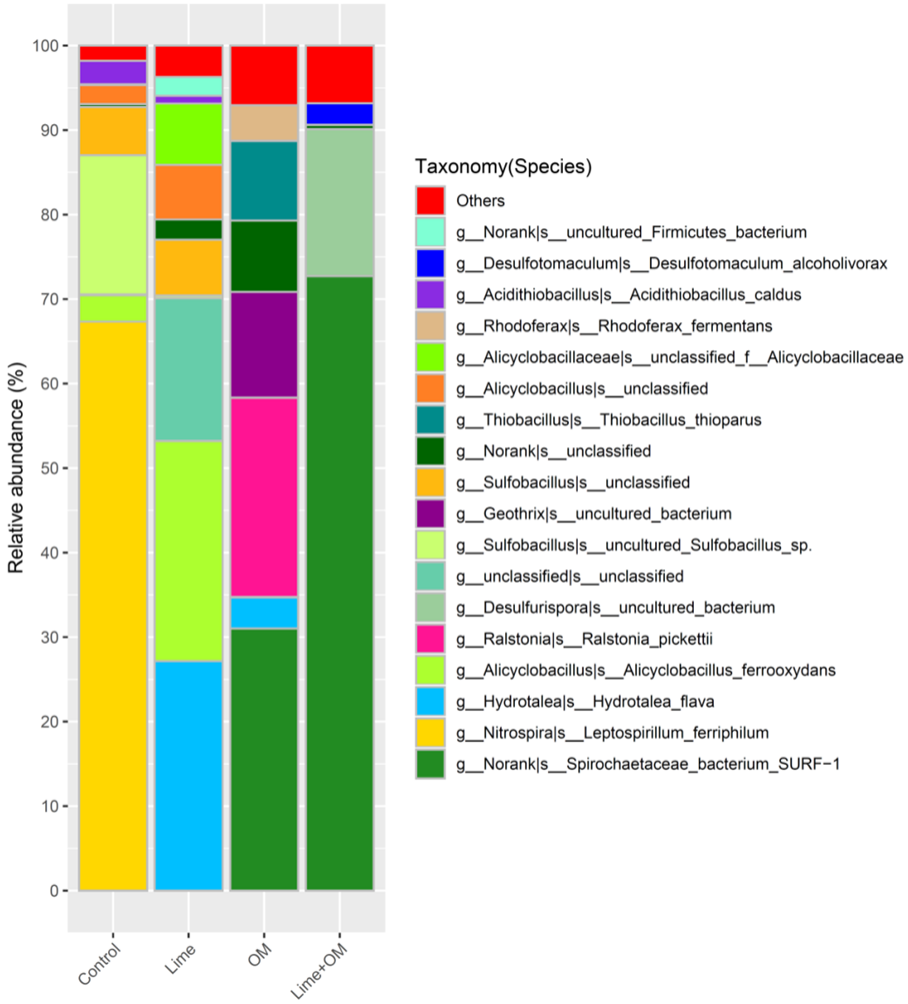

Figure 7. Species of the microbial community in the solution phase of the four columns at day 150

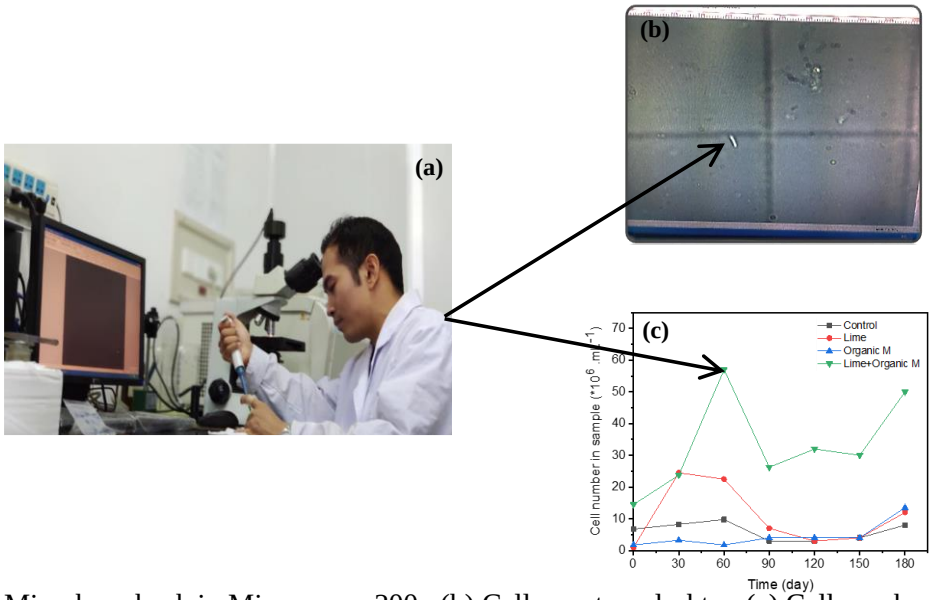

Figure 8. (a) Microbes check in Microscope 200x (b) Cell count on desktop (c) Cell number in the solution phase of the four columns

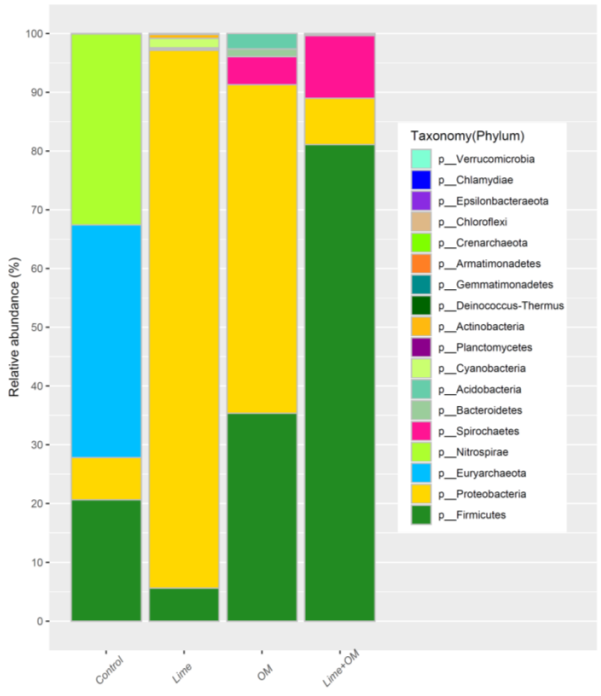

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Figure 9. Phylum of the microbes in the residue of the four columns at the end of the inoculation of 180 days

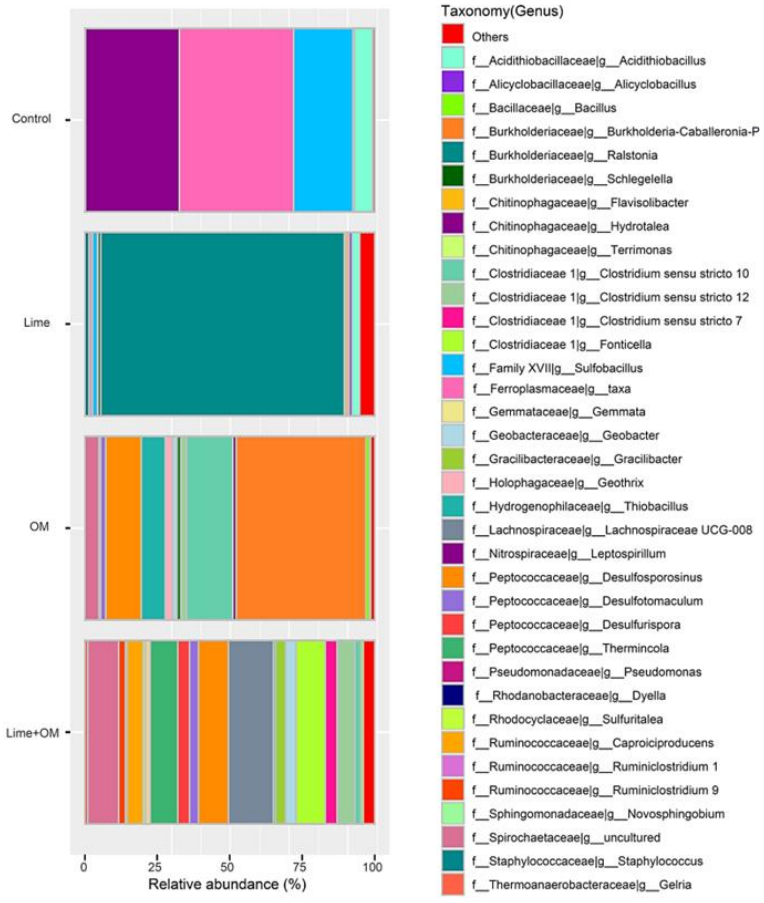

Figure 10. Genus of the microbial community in the residue of the four columns at the end of the incubation in 180 days

Table 4. Dominant species in the solution of organic addition columns (OM, Lime + OM) during the incubation in the column (abundance >5%) (Aungkyawphyo et al., 2020-modified)

Sample Time (Day)	**Cl-3 (OM)**	**Cl-4 (Lime+OM)**
30	*Alicyclobacillus ferrooxydans: 32.7%* *Desulfosporosinus acidiphilus: 23.0%* *Clostridium sensu stricto 10: 17.1%* *Hydrotalea flava: 11.6%* *Penicillium Janthinellum*: 7.9%	*Clostridium sensu stricto 10: 66.8%* *Desulfosporosinus acidiphilus:27.2%*
90	*Hydrotalea flava: 21.3%* *Geothrix: 13.5%* *Ralstonia pickettii: 6.2%* *Desulfotomaculum: 5.5%* *Unclassified Anaerovorax: 9.4%* *Pseudolabrys: 8.3%* *Desulfotomaculum: 6.6%*	*Spirochaetaceae bacterium SURF-1: 70.5%* *Desulfovibrio: 8.5%*
150	*Spirochaetaceae bacterium SURF-1: 31.0%* *Ralstonia pickettii: 23.6%* *Geothrix: 12.5%* *Norank Family XVIII: 8.4%* *Thiobacillus_thioparus: 9.4%*	*Spirochaetaceae bacterium SURF-1:72.7%* *Desulfurispora: 17.4%*

For the microbial community in the pore solution, microbes in the control treatment were dominant by phylum Firmicutes and Nitrospirae, which are mainly the species of *Leptospirillum ferriphilum, Sulfobacillus*, some *Acidithiobacillus caldus*, and some *Alicyclobacillus ferrooxydans*. In addition, in the lime addition treatment, pH was elevated from 2.0 to about 3.9; the solution's microbial community changed to the dominant of the phylum of Firmicutes and Bacteroidetes, with a high percentage species of *Hydrotalea flava* and *Alicyclobacillus*, and also the growth of *Clostridiales*, with a small portion of *Sulfobacillus* and *Acidithiobacillus caldus*; however, *Leptospirillum* was not detected anymore. This suggested that *Leptospirillum* was not accustomed to the higher pH (at the end of the test, pH of 3.9), and the higher pH resulted in the growth of a small amount of *Clostridiales*. Additionally, the treatment with organic matter showed a microbial community in the pore solution of *Spirochaetaceae* bacterium SURF-1 (31.0%), *Ralstonia pickettii* (23.6%), *Geothrix* (12.5%), *Norank Family* XVIII (8.4%), and *Thiobacillus thioparus* (9.4%), while in the treatment with organic matter and lime together suggested the species of *Spirochaetaceae bacterium SURF-1* (72.7%) and *Desulfurispora* (17.4%) in the incubation solutions. Adding lime and organic matter together promotes the solution cell number (Figure . 8). The species during the incubation at 30, 90, and 150 days suggested the dominance of SRBs of Spirochaetaceae bacterium SURF-1 in the solution (Table 4). It is reasonable for the growth of Spirochaetaceae bacterium SURF 1 since there is abundant sulfate in the residue, along with the organic matter input, the gradually increased pH, and the more anaerobic condition.

microbes in the residue of the treatment Lime + OM promote the different SRBs and iron-reducing groups (most in the phylum of Firmicutes) in the Control column with Firmicutes, Nitrospirae and Euryarchaeota and single lime or organic matter addition column with the predominance of *Proteobacteria* (Figure 9). Microbial community diversity in species in the residue after 180 days is much higher than that in the pore solution (Fiure 10), suggesting that most of the microbes like to live in the residue rather than in the pore solution, while

Spirochaetaceae bacterium SURF-1 was more likely to exist in the solution, and in the residue, it was only in a proportion of about 10% (Figure 10) in the treatment of Lime + OM. In the residue of the control treatment, *Acidithiobacillus, Alicyobacillus, Sulfobacillus,* and *Ferroplasma* were the main genus. The microbial community in the treatment with lime was high in the genus of *Ralstonia* and in the OM treatment, *Burkholderia, Clostridium, Thiobacillus,* and *Desulfosporosinus.* The most abundant and diversity of microbes was seen in the Lime + OM treatment, with a high diversity of SRBs groups (such as in *Clostridiaceae, Peptococcaceae*) and also in iron-reducing microbes such as *Geobacter, Geothrix, Therminocola*. The bacterial population also included other organisms, such as fermenting bacteria and methanogens, some of which help to hydrolyze and ferment complex carbons to readily available substrates for the SRBs(Figure 10). It showed a higher diversity index of Shannon and Simpson in the Lime + OM treatment (Shannon index of 4.36 in column 4 with Lime + OM, while columns 1–3 of 2.18, 1.61, and 2.94, respectively; Simpson index of 0.92 in column 4 with Lime + OM, while columns 1–3 of 0.70, 0.30, and 0.75 respectively).

Reduced sulfur species produced by sulfate-reducing bacteria (SRBs) support sulfur-oxidizing microbes, creating a cycle of iron and sulfur oxidation and reduction (Benner et al., 2000). However, low organic matter in tailing dams or bioleaching residues limits SRB activity, necessitating organic matter addition to enhance their growth (Jia et al., 2019). Ferric iron acts as an oxidant in acidic conditions, while autotrophic acidophiles facilitate sulfide mineral dissolution. Creating anoxic conditions inhibits iron- and sulfur-oxidizing bacteria but promotes SRBs and iron-reducing bacteria, effectively preventing acid rock drainage (ARD) (Kuyucak et al., 2002). Flooding heap bioleaching residues to establish anaerobic conditions is crucial, as acidophilic bacteria are inhibited by organic substances, while SRBs thrive on complex organics like yeast extract and sodium lactate (Zhang et al., 2017; Choudhary et al., 2011). Organic matter oxidation coupled with ferric and sulfate reduction generates alkalinity, favoring SRB growth and iron-reducing microbes in sulfide waste rock incubation (Vardanyan et al., 2019).

It can be summarized that, under the oxygen deficiency condition in the acidic sulfide waste rocks, species favor or can grow in an anaerobic environment, such as *Acidiphillum*, *Ferroplasma,* and *Alicyclobacillus* will grow and compete with the aerobic *Acidithiobacillus* and *Leptospirillum* (Jia Y et al., 2019). As most anaerobic microbes rely on organic substances, the organic substance input will further promote this anaerobic genus while reducing Fe^{3+} to Fe^{2+} (such as *Ferroplasma and Acidophilium*), which will facilitate the pH increase. If the pH increases above 3, some acid-tolerant SRBs groups, such as in the family *Clostridiaceae,* then *Peptococcaceae* will grow, and Fe^{3+} and SO_4^{2-} reducing happened in higher reaction rate and increase the pH continuously.

4.2 Physiochemical properties in different treatments

The physicochemical characteristics were significantly different between treatments (Figure 11). It showed that the organic matter addition (OM, Lime+OM) finally resulted in the pH increase to nearly neutral, while the lime addition alone did not finally increase the pH (Figure 11-a), suggesting the importance of the organic matter, as also shown in the microbial community while the SRBs was significantly promoted. During the incubation, the Fe^{2+} in the organic matter addition treatments (OM, Lime+OM) showed an increased Fe^{2+} at about 40 and 70 days, respectively (Figure 11-b); this time is also correlated to the time of the pH increase, suggesting the competitive growth of the SRBs and iron-reducing microbes at that time. Then, the Fe^{2+} concentration decreased, suggesting the co-precipitate with the S^{2-}. The control treatment also had a higher Fe^{2+} concentration in the beginning because the pH was lower, and the pyrite dissolved after the start of the incubation. The decrease of the redox potential in the organic matter addition treatments (OM, Lime+OM) also occurred at the same time as the start

of iron and sulfur reduction (Figure 11-c). With the reduction of Fe^{3+} to Fe^{2+} and SO_4^{2-} to S^{2-}, the Eh decreased significantly, while the control and lime addition treatment is relatively stable, suggesting the lesser reducing reaction.

It was reported that below the Eh of about 450 mV (vs. Ag/AgCl), pyrite oxidation was negligible even though it was entirely covered with bioleaching microbes on the surface (Liu C et al., 2017). So, in all four treatments, the pyrite oxidation is very slow as their Eh were all lower than 450 mV; thus, the pH in the column was not decreased, showing the effectiveness of the anaerobic condition. Organic matter oxidation further consumes the dissolved oxygen, which further inhibits the autotrophic microbes. The dissolved organic carbon in the solution decreased during inoculation (Figure 11-d) and may be used by heterotroph microbes for growth. The overall process results in improved pore water quality due to the precipitation of metals as sulfides with the H_2S generated in an organic substrate and neutralization of the acidity due to the bicarbonate released during sulfate reduction.

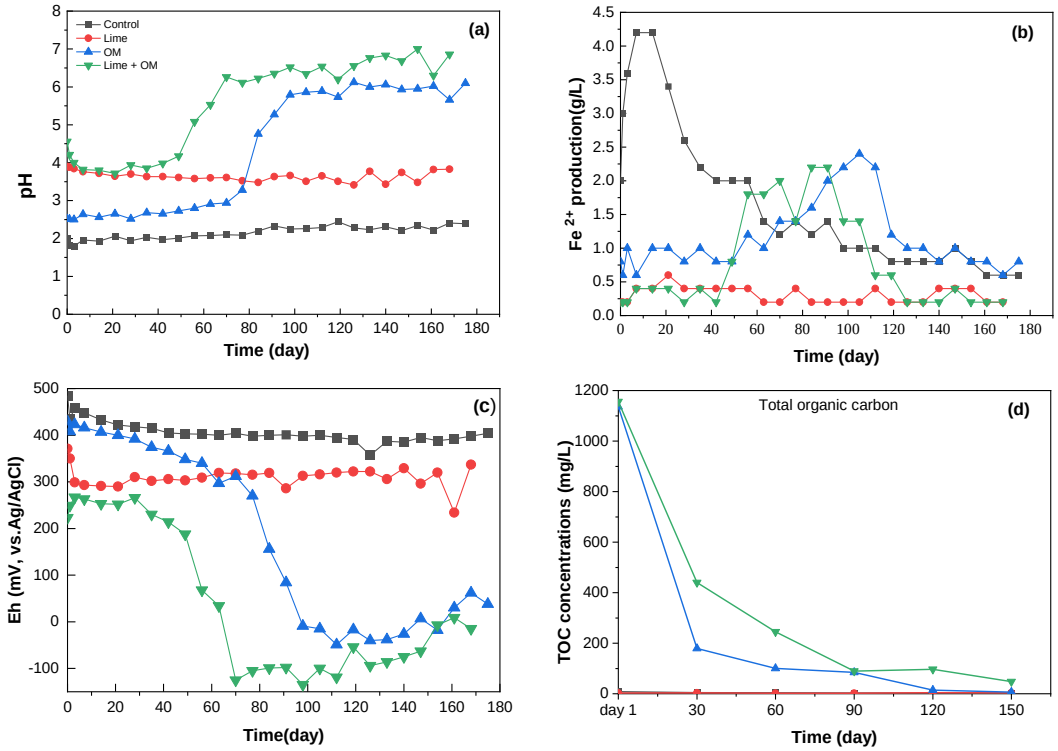

Figure 11. pH value (a), Fe^{2+} concentration(b), Eh (c), and total organic carbon (d) in the solutions from the four columns during the experiment (Control, Lime, OM, and Lime + OM), respectively (Aungkyawphyo et al., 2020)

As pH increases, aqueous metal species tend to precipitate as hydroxide, oxyhydroxide, or hydroxy sulfate minerals. Also, dissolved metals may adsorb onto the surfaces of these secondary minerals and other surfaces present in the environment and increase hydrolysis of metal ions at circum-neutral pH (Nordstrom DK et al., 1986). With the co-precipitation of the Cu together with the microbial-produced sulfide, the final Cu concentration in the solution of treatment with lime and organic matter together is below the detection limitation (<1 mg/L), while in the treatments of lime addition and organic matter are 56 mg/L and 26 mg/L, respectively. They are much lower than the control treatment of 400 mg/L. As previously reported, this process is also particularly effective for removing heavy metals such as cadmium, copper, lead, mercury, zinc, and iron to low concentrations (Kuyucak et al., 2002). Researchers have tried to use SRBs to remediate AMD in the last several years, but most were the ex-situ treatment after the AMD had already come out from the sulfide waste rocks (Zhang et al., 2016; Jamil I et al., 2013; Becerra C A et al., 2010).

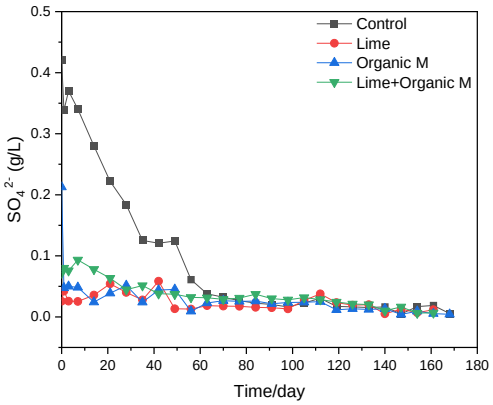

Figure 12. Sulfate SO_4^{2-} concentrations in the solutions from the four columns during the experiment (Control, Lime, OM, and Lime + OM), respectively

Source control to inhibit sulfide mineral oxidation in waste ore was studied, showing pH elevation due to the activity of iron-reducing microbes and sulfate-reducing bacteria (SRBs). Inoculation with local anaerobic SRBs and organic matter addition increased pH to neutral levels. Iron-reducing microbes, active even at very low pH, likely initiate the process, followed by sulfate reduction (Wendt-Potthoff et al., 2002). Microbial alkalinity generation occurs first through iron reduction and later through both iron and sulfate reduction. Bioremediation of acidic sulfide waste rocks initially relies on acidophilic iron reduction to raise pH to ~3.0, followed by SRBs and iron-reducing bacteria growth to precipitate metals and sulfate, further increasing pH. Even small populations of these microbes consume oxygen, reduce Fe^{3+} and SO_4^{2-}, lower Eh, and inhibit pyrite oxidation. Additionally, if these microbes become dominant, they will finally reverse the reactions, causing acid mine drainage, producing alkalinity, attenuating the movement of metals by the precipitation of sulfide minerals, and, finally, bio-remediate the waste rocks (Figure 12.). For the rehabilitation of acidic waste rocks, one layer of lime and organic matter will be helpful to inhibit the microbial-assisted sulfide mineral oxidation in the oxidation front of the waste rocks and, therefore, provide a protection layer for upper plant growth. For the remediation of acidic lakes in mining, SRBs-combined organic matter input will help neutralize and precipitate heavy metals in the lakes.

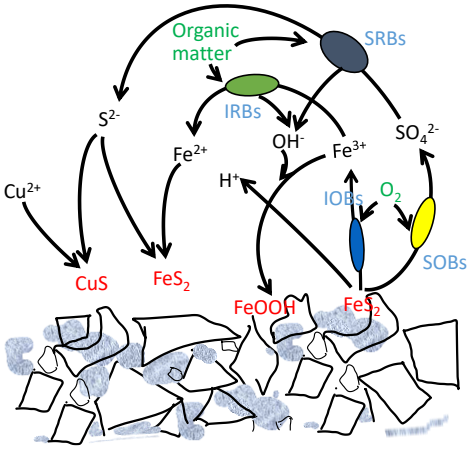

Figure 13. Scheme of the microbial-induced transformation of the minerals for bioremediation of pyritic waste rocks , (Aungkyawphyo et al., 2020)

Note: Sulfide minerals, such as pyrite, are oxidized by IOBs and SOBs to Fe^{3+} and SO_4^{2-} under aerobic conditions and form acid mine drainage. After the creation of the anaerobic condition and input of organic matter, the acidic IRB reduces Fe^{3+} to Fe^{2+} and elevate the

pH, and then the less acid-tolerant SRBs and IRBs grow; thus the pH increases gradually, and, finally, the metal ions precipitate together with the microbial generated sulfide and with the elevated pH. SOB: sulfur-oxidizing microbes, IOBs: iron-oxidizing microbes, IRB: iron-reducing microbes, SRBs: sulfur-reducing microbes.

5. Conclusion

The acidic IRBs and SRBs consortiums were applied to the heap bioleaching residue from a copper mine to investigate the competitive growth of IRBs and SRBs with the local autotrophic acidophiles under anaerobic conditions. It is proved that there is a growth of the anaerobic heterotrophic microbes under anaerobic acidic conditions, mainly the iron-reducing microbes, such as *Acidiphillum*, *Ferroplasma,* and *Alicyclobacillus*, especially when the pH value is lower (pH < 3), and then this gradually turns to the growth of SRBs and IRBs together if the pH increases. If there is input of organic matter, the SRBs and iron-reducing microbes will be significantly promoted, such as in the family *Clostridiaceae*, *Peptococcaceae*, and *Spirochaetaceae*. They will result in the co-precipitation of the metals with biogenic sulfide and also elevate pH, finally resulting in heavy metal precipitation in situ of the waste rocks.

Oxygen depletion is essential for the growth of iron-reducing bacteria (IRBs) and sulfate-reducing bacteria (SRBs). By adding organic matter and creating anaerobic conditions in heap bioleaching residues, pH can increase from acidic to neutral, promoting IRB and SRB activity. Naturalized residues enhance microbial growth, enabling in situ remediation of acid waste rocks. This process inhibits pyrite oxidation, reverses sulfide mineral oxidation, and facilitates metal-sulfide co-precipitation. SRB cultivation is effective both ex situ for acid mine drainage (AMD) treatment and in situ for preventing acid generation and forming protective barriers. This approach supports waste rock rehabilitation, neutralizes AMD lakes, and aids in heavy metal precipitation.

6. Acknowledgements

We are grateful to the Institute of Process Engineering, Beijing, China PRC (Belt & Road Program, University of Chinese Academy of Sciences -UCAS) for their financial support. We acknowledge the kind and strong support from the Director General and staff members of the Department of Mines, Ministry of Natural Resources and Environmental Conservation, Myanmar, for this research. The authors would like to sincerely thank to Myanmar Wanbao Mining Copper Ltd. for granting permission to conduct the fieldwork and sampling for the current study.

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8. Appendix

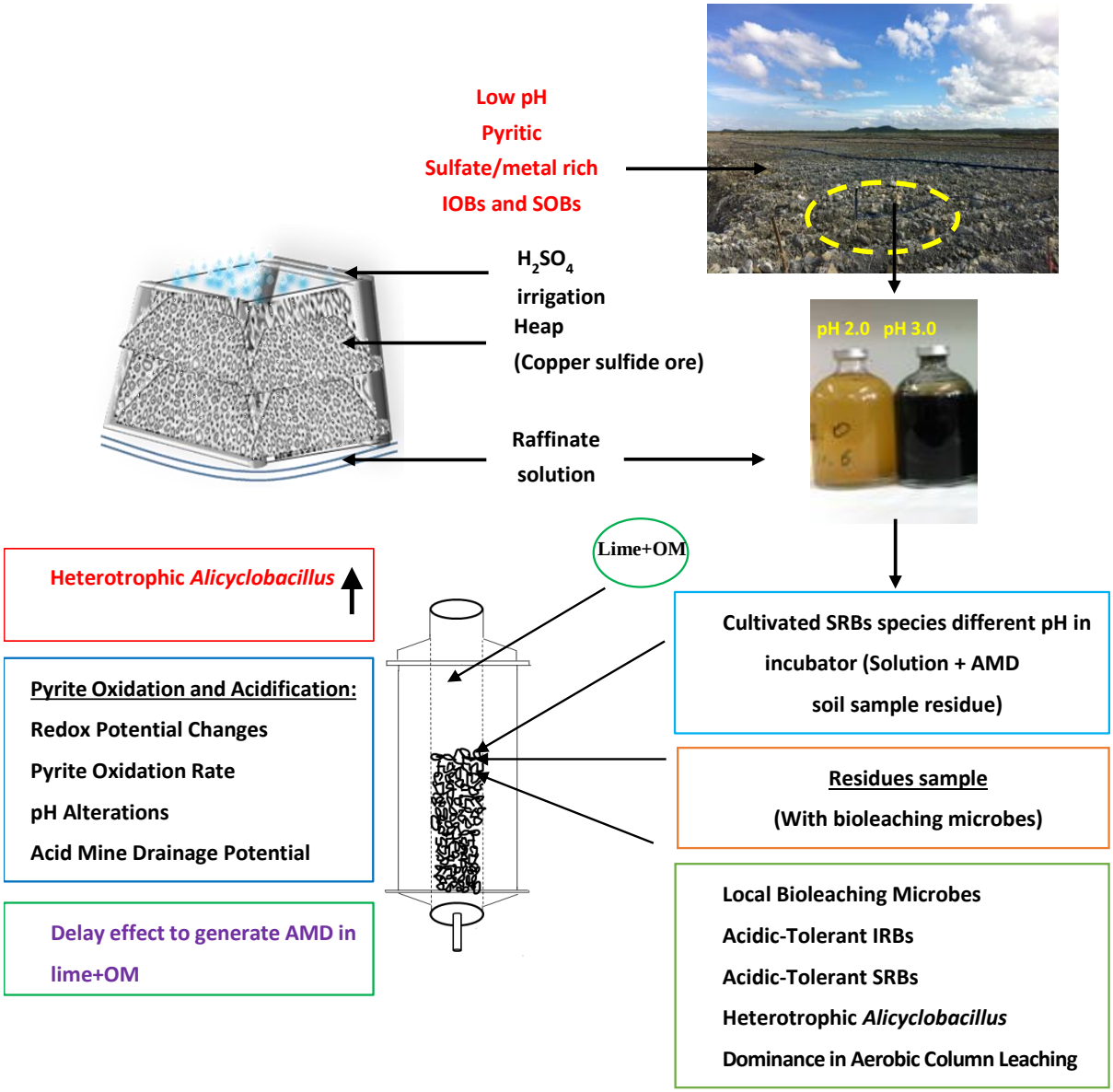

Figure 14. Illustration of the overall experimental steps and schematic diagram.

(Microbial-chances and acidifying pyritic waste rocks)

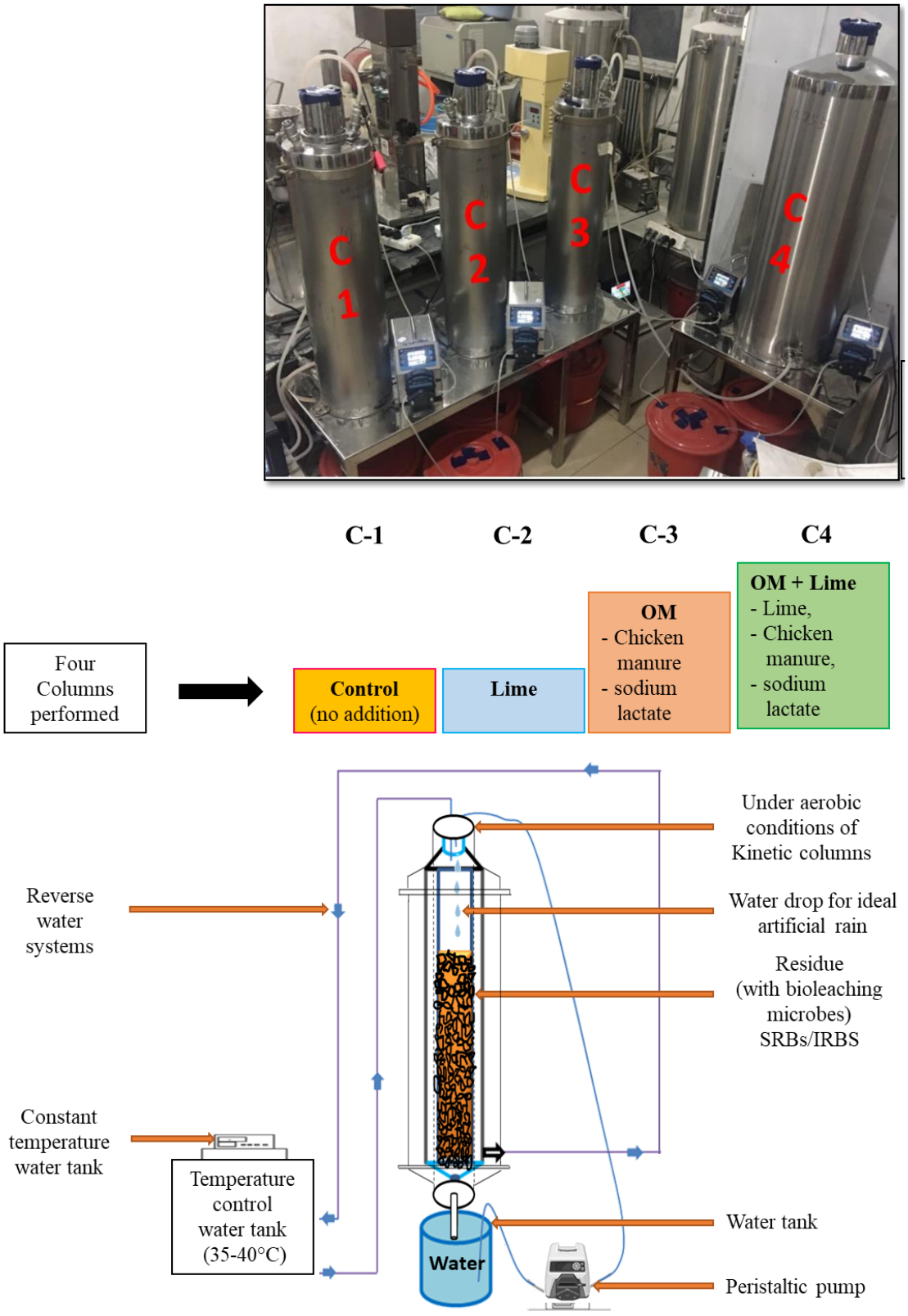

Figure 15. Experimental performed for water flooded anaerobic incubation under different treatments (Control, Lime, OM, Lime + OM) (Aungkyawphyo et al., 2020- modified)

Table 5. Case studies of bioremediation of acid mine drainage using SRBs

Technology	**Location**	**Site**	**Metals removed**	**Reference**
1. Compost bioreactor	Yellow Creek, Pennsylvania	Abandoned underground coal mine	Fe, Al	(Doshi et al., 2006)
2. Compost bioreactor	Carnon Valley, Cornwall, UK	Wheal Jane tin mine	Zn, Fe	(Hallberg and Johnson et al., 2002; Whitehead, Hall et al., 2005)
3. Compost bioreactor	Reynolds County, Missouri	Doe Run West Fork Mine (active underground Pb–Zn mine)	Pb, Zn	(Doshi et al., 2006)
4. In situ bioreactor	Elliston, Montana	Lilly/Orphan Boy Mine	Al, As, Cd, Cu, Zn	(Doshi et al., 2006)
5. Compost bioreactor	Ellison, Montana	Surething abandoned mine	Al, As, Cd, Cu, Fe, Pb, Mn, Zn	(Bless, Joyce et al., 2006)
6. Permeable reactive barrier	Sudbery, Ontario, Canada	Nickel Rim abandoned Ni–Cu mine	Al, Cu, Ni, Zn	(Benner, Blowes et al., 2002)
7. Permeable reactive barrier	Shoshone County, Idaho	Success Mine and Mill	Cd, Pb, Zn	(Conca and Wright et al., 2006)
8. In−lake treatment	Black Hills, South Dakota	Ancher Hill Pit Lake	Al, As, Cd, Cu, Fe, Se, Zn	(Doshi et al., 2006)

(Adapted from Barton et al., 2009)