



**Universität für Bodenkultur Wien**  
University of Natural Resources  
and Life Sciences, Vienna

Doctoral Dissertation

# **Urban mining – Potential bioleaching applications for metal recovery from low grade artificial ores**

submitted by

**DI Klemens Kremser, BSc**

in partial fulfilment of the requirements for the academic degree

**Doktor der Bodenkultur (Dr.nat.techn.)**

**Supervisor:**

**Univ. Prof. Dr. Georg M. Gubits**

University of Natural Resources and Life Science Vienna

Department of Agrobiotechnology, IFA Tulln

Institute of Environmental Biotechnology

Konrad-Lorenz-Straße 20

3430 Tulln

Vienna  
2021



## Table of Contents

<b>I. Preamble</b>	6
<b>II. Abstract</b>	8
<b>III. Kurzfassung</b>	11
<b>1. Aim of the thesis</b>	14
<b>2. Introduction</b>	15
<b>2.1. Artificial ores for secondary metal recycling and recovery</b>	15
2.1.1. Ashes and slags from different industries	17
2.1.2. Shredder residues	20
2.1.3. Multilayer packaging waste	21
2.1.4. Other potential waste streams	22
<b>2.2. Bioleaching organisms and mechanisms</b>	22
2.2.1. Bacterial bioleaching	22
2.2.2. Fungal bioleaching	25
2.2.3. Direct and indirect bioleaching	26
2.2.4. Factors influencing the bioleaching performance	27
<b>2.3. Bioleaching processes</b>	29
2.3.1. Irrigation-type bioleaching	30
2.3.2. Stirred-tank bioleaching	31
<b>2.4. Potential ways of metal recovery</b>	32
2.4.1. Biosorption	32
2.4.2. Bioelectrochemical systems	34
2.4.3. Electrowinning and Precipitation	35
<b>2.5. References</b>	36
<b>3. Leachability of metals from waste incineration residues by iron-and sulfur oxidizing bacteria</b>	46
<b>3.1. Abstract</b>	47
<b>3.2. Introduction</b>	48
<b>3.3. Material and methods</b>	50
3.3.1. Waste incineration ashes and slags	50
3.3.2. Bacteria and growth conditions	52
3.3.3. Adaptation of <i>A. ferrooxidans</i> to slags and ashes	52
3.3.4. Bioleaching batch tests	53
3.3.5. Sample characterization	54
3.3.6. Calculation of the leaching efficiency	55
3.3.7. Statistical evaluation	55

<b>3.4. Results and discussion</b>	56
3.4.1. Characterization of MWIP ashes and slags	56
3.4.2. Bioleaching efficiency – Effect of adaptation and sulfur addition	58
3.4.3. Bioleaching efficiency	62
3.4.3.1. <i>Leptospirillum ferrooxidans</i>	62
3.4.3.2. <i>Acidithiobacillus ferrooxidans</i>	63
3.4.3.3. <i>Acidithiobacillus thiooxidans</i>	64
3.4.3.4. Susceptibility of incineration residues for sulfur- and iron oxidizing bacteria	64
<b>3.5. Conclusion</b>	68
<b>3.6. Acknowledgements</b>	69
<b>3.7. References</b>	69
<b>3.8. Supplementary information</b>	75
<b>4. Biogenic sulfuric acid production for pH control in heap bioleaching systems</b>	79
4.1. Abstract	80
4.2. Introduction	81
4.3. Material and methods	83
4.3.1. Waste incineration slag	84
4.3.2. Bacteria and growth media	84
4.3.3. Biological sulfuric acid production batch experiments	84
4.3.4. Sulfuric acid reactor and heap-bioleaching system	85
4.3.5. Calculation of total acidity and leaching efficiency	87
4.3.6. Nucleic acid extraction and quantification	88
4.4. Results and discussion	90
4.4.1. Biogenic sulfuric acid production	90
4.4.2. Population dynamics during biogenic sulfuric acid production	92
4.4.3. Biogenic acid production in CSTR	94
4.4.4. Heap bioleaching using biogenic sulfuric acid for pH control	96
4.4.5. Economical assessment	98
4.5. Conclusion	100
4.6. Acknowledgements	101
4.7. References	101
<b>5. Stirred-tank and heap-bioleaching of shredder-light-fractions (SLF) by acidophilic bacteria</b>	105
5.1. Abstract	106
5.2. Introduction	107
5.3. Materials and methods	110

5.3.1.	Shredder-light-fraction (SLF) samples.....	110
5.3.2.	Bacteria and growth media.....	111
5.3.3.	Bioleaching batch tests.....	111
5.3.4.	Tank and heap-bioleaching experiments.....	112
5.3.5.	Calculation of the leaching efficiency .....	114
<b>5.4.</b>	<b>Results and discussion .....</b>	<b>115</b>
5.4.1.	Characterization of the original shredder-light-fraction.....	115
5.4.2.	Bioleaching efficiencies by pure bacterial cultures.....	116
5.4.3.	Leaching efficiency of a bioleaching co-culture .....	118
5.4.4.	Influence of substrate concentration.....	120
5.4.5.	CSTR and heap-bioleaching.....	122
5.4.6.	CSTR and heap-bioleaching efficiency .....	125
<b>5.5.</b>	<b>Conclusion .....</b>	<b>127</b>
<b>5.6.</b>	<b>Acknowledgements .....</b>	<b>128</b>
<b>5.7.</b>	<b>References .....</b>	<b>128</b>
<b>6.</b>	<b>A new bioleaching strategy for the selective recovery of aluminum from multi-layer beverage cans.....</b>	<b>132</b>
<b>6.1.</b>	<b>Abstract.....</b>	<b>133</b>
<b>6.2.</b>	<b>Introduction.....</b>	<b>134</b>
<b>6.3.</b>	<b>Material and methods .....</b>	<b>137</b>
6.3.1.	Aluminum can.....	137
6.3.2.	Acid dissolution of aluminum cans.....	138
6.3.3.	Bacteria and growth media.....	138
6.3.4.	Bioleaching batch-tests.....	138
6.3.5.	Iron-determination.....	139
6.3.6.	Sulfate measurement .....	139
6.3.7.	Aluminum determination .....	140
6.3.8.	Surface characterization.....	140
6.3.9.	Aluminum recovery .....	141
<b>6.4.</b>	<b>Results and discussion .....</b>	<b>141</b>
6.4.1.	Acid dissolution and characterization of UBC .....	141
6.4.2.	Bioleaching of the aluminum layer .....	143
6.4.2.1.	<i>Acidithiobacillus ferrooxidans</i> .....	143
6.4.2.2.	<i>Acidithiobacillus thiooxidans</i> .....	144
6.4.2.3.	<i>Acidithiobacillus caldus</i> .....	145
6.4.3.	Surface characterization.....	147

6.4.4. Aluminum precipitation and recovery .....	149
<b>6.5. Conclusion .....</b>	<b>150</b>
<b>6.6. Acknowledgements .....</b>	<b>151</b>
<b>6.7. References .....</b>	<b>151</b>
<b>7. General conclusion .....</b>	<b>156</b>
<b>8. Appendix.....</b>	<b>159</b>
8.1. Publications .....	159
8.2. Oral presentations as presenting author .....	160
8.3. Poster presentations as presenting auhtor .....	160
8.4. Acknowledgements .....	161

## **I. Preamble**

This thesis covers the potential use and application of extreme acidophilic bacteria for biological metal solubilization and extraction from different waste streams. Therefore, it is divided into five sections, starting with a general introduction, followed by four scientific publications giving a more detailed insight into the bio-extraction and recovery of valuable metals.

The introduction gives an overview about different waste materials, which can be classified as artificial ores for urban metal mining due to their incorporated metal concentrations. Furthermore, a more detailed description of the different bioleaching mechanisms and bacteria is given and factors influencing the bioleaching efficiency are highlighted. Finally, different bioleaching processes are mentioned and potential ways for the recovery of the dissolved metals are described.

The first publication outlines the potential of iron- and sulfur-oxidizing bacteria in the bio-extraction of metals from waste incineration residues such as ashes and slags. It focusses on factors, influencing the bioleaching efficiency, especially bacterial adaptation, pH-value, and nutrient supply. Herein, ferrous iron and sulfur supply were found to be key factors for efficient metal extraction.

In the second publication, two sulfur-oxidizing bacteria were investigated for the efficient and biological generation of sulfuric acid. A combined culture of both was found to be most effective in sulfuric acid production in batch and stirred tank experiments. The generated acid was used for pH-regulation of a heap-bioleaching system, in which an iron oxidizing bacterium was used to extract metals from waste incineration slag. The calculated value of liberated metals was found to be much higher compared to the potential sulfuric acid costs.

Another waste material for biological metal extraction was investigated in the third publication. Herein, shredder light fractions, generated during the treatment and shredding of end-of-life vehicles were analysed and tested for bioleaching applications. The iron- and sulfur-oxidizing bacterium *Acidithiobacillus ferrooxidans* was applied in stirred-tank and heap-bioleaching operations to extract valuable metals from the shredder fraction.

In the fourth paper, a potential new bioleaching application was investigated. Three different acidophilic bacteria were tested according to their efficiency to selectively remove aluminium from multi-layer packaging materials. In this study, beverage cans which include an epoxy layer on the inside of the can to prevent direct contact of food and aluminium were investigated as model material. Two out of three bacteria were found to be effective in the selective removal of aluminium, without affecting the polymer structure.

At the end, a general conclusion summarizes the findings and perspectives of this thesis, followed by a list of publications, oral presentations and grants in the appendix section.



## II. Abstract

Incineration of hazardous and residual waste presents one of the most frequently used waste treatment technique worldwide. In fact, the reduction of waste volume and the generation of energy are beneficial and contribute to a waste-to-energy principle. Nevertheless, almost one third of residuals such as different ashes and slags remain after incineration. These residuals can pose an environmental risk on the one hand but contain economically interesting metal concentrations on the other hand. The application of extreme acidophilic bacteria for the bio-extraction of these metals can provide an efficient and environmentally friendly alternative to their disposal. In this thesis, high metal extraction efficiencies were achieved with the iron- and sulfur-oxidizing bacterium *Acidithiobacillus ferrooxidans*. Additionally, parameters such as pH-value of the medium and nutrient supply with ferrous iron and sulfur were investigated on their effect on the bioleaching efficiency. Herein, it was found that the combination of low pH, together with ferrous iron and elemental sulfur addition were key factors for efficient bio-extraction of the incorporated metals.

Almost all bioleaching applications have in common that they require external acid addition for pH-adjustment of cultivation media or during bioleaching experiments. Most frequently, sulfuric acid is used in different molarities. Sulfuric acid represents the world's largest bulk chemical produced and finds numerous applications. Nevertheless, the production is energy intensive and the transportation of huge amounts of concentrated acid pose a potential risk. In order to develop an independent, effective and environmentally friendly bioleaching process, in this thesis two sulfur-oxidizing bacteria were tested for their application in biogenic sulfuric acid production. Therefore, different setups with pure and combined cultures of both bacteria were tested at different temperature and with different concentrations of elemental sulfur.

The combined culture reached a maximum acidity of around 1.4 M in a 1.5 L stirred tank reactor. After successful biogenic acid production, the generated acid was used for pH-regulation of a heap-bioleaching system in which the iron- and sulfur-oxidizing bacterium *A. ferrooxidans* was tested in the bio-extraction of metals out of waste incineration slag. A pH-value of 2 could be successfully maintained over a period of three weeks resulting in the liberation of valuable metals with a total potential value higher than the costs for purchasing the required amount of sulfuric acid. This study revealed a potential new application of bioleaching, combining the two most effective mechanisms of iron- and sulfur-oxidation.

Similar to waste incineration residuals, other waste streams need to be considered as artificial ores for potential secondary metal recycling. In this sense shredder light fractions, evolving during the treatment and size reduction of end-of-life vehicles, were investigated in another part of this thesis. Containing high concentrations of copper, nickel and zinc, the biological leaching of these residues was tested in stirred-tank and heap-bioleaching systems with two different iron-oxidizing bacteria. In the stirred-tank bioleaching, increasing concentrations of shredder fractions from 10 – 80 g L<sup>-1</sup> were successfully treated, and metals were bio-extracted by up to 100%. Furthermore, heap-bioleaching was proven to be an effective and cheap alternative to the more expensive operation of a stirred tank reactor. By preventing these residues from incineration, metals could be efficiently extracted which is contributing to the idea of a circular economy.

Applying bioleaching for the recycling and selective recovery of aluminium from multilayer materials presents a completely new application for acidophilic bacteria. Used beverage cans represent one of the biggest resources for secondary aluminium recycling with over 220 billion cans consumed worldwide per year. Around 70% of the

consumed cans are collected and used in secondary aluminium production, which needs only 5% the amount of energy compared to primary aluminium production. Nevertheless, most of the consumers do not know that there is an additional plastic epoxy layer on the inside of the aluminium can, to prevent direct contact of food and aluminium. To test our hypothesis that bioleaching can be used for the selective dissolution of the aluminium layer, three acidophilic bacteria were tested according to their leaching efficiency. It was found that two out of three bacteria were able to effectively dissolve the aluminium layer, without affecting the polymer structure. This mild treatment method therefore prevents the epoxy layer from incineration, increasing the recyclability of both, aluminium, and polymer. Furthermore, bioleaching might find application in the recycling of other, more complex multilayer materials such as liquid packaging boards.

### III. Kurzfassung

Die Verbrennung von Gefahrgut und Restmüll ist eine der weltweit meistverbreiteten Techniken zur Müllbehandlung. Durch die Müllverbrennung kann das Abfallvolumen reduziert und elektrische Energie durch die Verbrennung erzeugt werden welches dem Prinzip von Abfall-zu-Energie entspricht. Dennoch bleiben nach der Verbrennung rund ein Drittel Reststoffe wie Aschen und Schlacken zurück. Diese Reststoffe stellen einerseits eine Gefahr für die Umwelt dar, enthalten jedoch andererseits ökonomisch interessante Konzentrationen an Metallen. Die Anwendung von acidophilen Bakterien zur biologischen Extraktion dieser Metalle kann eine umweltfreundliche und effektive Alternative darstellen - verglichen zur Entsorgung dieser Reststoffe. Durch die Anwendung dieser Methode konnten mit dem Eisen- und Schwefel-oxidierenden Bakterium *Acidithiobacillus ferrooxidans* hohe Effizienzen in der Extraktion unterschiedlicher Metalle erzielt werden. Zusätzlich wurden Parameter wie pH-Wert, und Nährstoffzufuhr mit Eisen und Schwefel auf deren Effekt bezüglich der Laugungseffizienz getestet. Dabei stellte sich heraus, dass die Kombination von einem niedrigen pH-Wert in Verbindung mit Eisen- und Schwefelzugabe zur höchsten Effizienz bei der Extraktion führte.

Bei fast allen Anwendungen der biologischen Laugung ist es notwendig den pH-Wert von Medien einzustellen oder diesen während dem Laugungsprozess mit Säure zu regulieren. Schwefelsäure findet dabei die meiste Anwendung und zählt daher zu einer der meistproduzierten Chemikalien weltweit. Die Produktion ist jedoch mit einem hohen Energieaufwand verbunden und der Transport von großen Mengen konzentrierter Säure stellt durchaus auch ein Risiko dar. Um einen unabhängigen, effektiven und umweltfreundlichen Prozess zur biologischen Säureproduktion zu entwickeln, wurden zwei Schwefel-oxidierende Bakterien auf deren Anwendbarkeit

getestet. Dazu wurden Rein- und Mischkulturen der beiden Bakterien bei unterschiedlichen Temperaturen und mit unterschiedlichen Schwefelkonzentrationen getestet. Mit einer Mischkultur konnte biologisch Schwefelsäure mit einer Konzentration von bis zu 1,4 M in einem 1,5 L Rührreaktor produziert werden. Diese Säure wurde danach zur Regelung des pH-Wertes in einem Haldenlaugungssystem eingesetzt in welchem das Eisen-oxidierende Bakterium *A. ferrooxidans* zur biologischen Extraktion von Metallen aus Müllverbrennungsschlacke verwendet wurde. Dabei konnte erfolgreich ein pH-Wert von 2 über drei Wochen gehalten und Metalle mit hoher Effizienz extrahiert werden. Der Wert der extrahierten Metalle übersteigt dabei beträchtlich jene Kosten die notwendig wären, um die entsprechende Menge an Schwefelsäure zu erwerben. Diese Studie hat eine neue Anwendungsmöglichkeit der biologischen Laugung gezeigt, indem die zwei effektivsten Mechanismen der Eisen- und Schwefeloxidation kombiniert wurden.

Zusätzlich zu Reststoffen der Müllverbrennung gibt es weitere Abfallströme die als Metallquellen erschlossen werden können. Shredder-leicht-Fractionen entstehen bei der Behandlung und Zerkleinerung von Altfahrzeugen und enthalten hohe Konzentrationen an Metallen wie Zink, Nickel und Kupfer. Um die Metalle biologisch zu extrahieren, wurden zwei Eisen-oxidierende Bakterien in der Rührreaktor- und Haldenlaugung dieser Stoffe getestet. Im Rührreaktor wurden Konzentrationen von 10 – 80 g L<sup>-1</sup> Shredder-Fraktion erfolgreich gelaugt und die darin enthaltenen Metalle mit einer Effektivität von bis zu 100% extrahiert. Des Weiteren konnte die Haldenlaugung als effektive und kostengünstigere Methode im Vergleich zur Rührreaktorlaugung etabliert werden. Durch die Rettung dieser Reststoffe vor einer möglichen Verbrennung konnten die darin enthaltenen Metalle erfolgreich extrahiert werden was die Idee der Kreislaufwirtschaft stärkt.

Die Anwendung der biologischen Laugung im Recycling und selektiven Rückgewinnen von Aluminium aus Mehrschicht-Materialien stellt eine komplett neue Herangehensweise dar. Gebrauchte Aluminium-Getränkedosen stellen eine der größten Ressourcen zur Sekundärproduktion von Aluminium dar. Von den jährlich rund 220 Milliarden konsumierten Dosen werden rund 70% weltweit gesammelt und recycelt. Bei der Sekundärproduktion von Aluminium wird allerdings nur 5% der Menge an Energie benötigt als für die vergleichbare Menge an Rohaluminium. Viele der Konsumenten wissen jedoch nicht, dass sich im Inneren der Dose eine Plastikschiicht aus Epoxidharz befindet, welche den direkten Kontakt von Lebensmitteln und Aluminium verhindert. Um unsere Hypothese zu testen - dass die biologische Laugung verwendet werden kann, um die Aluminiumschicht von der Plastikschiicht zu trennen - wurden drei acidophile Bakterien anhand ihrer Laugungseffizienz getestet. Dabei stellte sich heraus, dass zwei von drei Bakterien in der Lage waren das Aluminium selektiv zu lösen, ohne die Struktur des Plastiks zu beschädigen. Durch diese milde Behandlungsmethode kann verhindert werden, dass die Plastikschiicht verbrannt wird, wodurch die Recyclingfähigkeit beider Materialien erhöht werden kann. Des Weiteren kann diese Methode auch Anwendung im Recycling von noch komplexeren Mehrschicht-Materialien wie Getränkekratons finden.

## **1. Aim of the thesis**

The aim of this thesis was to investigate the potential of bioleaching in the extraction of valuable metals from different waste streams. Therefore, various approaches regarding the characterization of different waste materials, the applicability of different iron- and sulfur-oxidizing bacteria and the potential of novel and innovative bioleaching applications were investigated. Having an increased demand in metal resources as well as decreasing metal concentrations of natural ores in mind, it is important to consider different waste streams as artificial ores for urban metal mining. Herein, bioleaching presents an effective and environmentally friendly alternative compared to waste disposal or other hydro- or pyrometallurgical recycling methods. By the exploitation of potential new bioleaching applications like the biogenic sulfuric acid production or the recycling of multilayer packaging waste, already well-known recycling techniques might be improved or replaced at certain time. In this way, bioleaching can help to reduce the carbon footprint and strengthen the concept of a circular economy and the closure of a material cycle.

This work was supported by the European fund for regional development, under the program Interreg V-A Austria-Czech Republic, project ATCZ183, IRAS (Innovative Recycling technology for Ashes and Slags).

## 2. Introduction

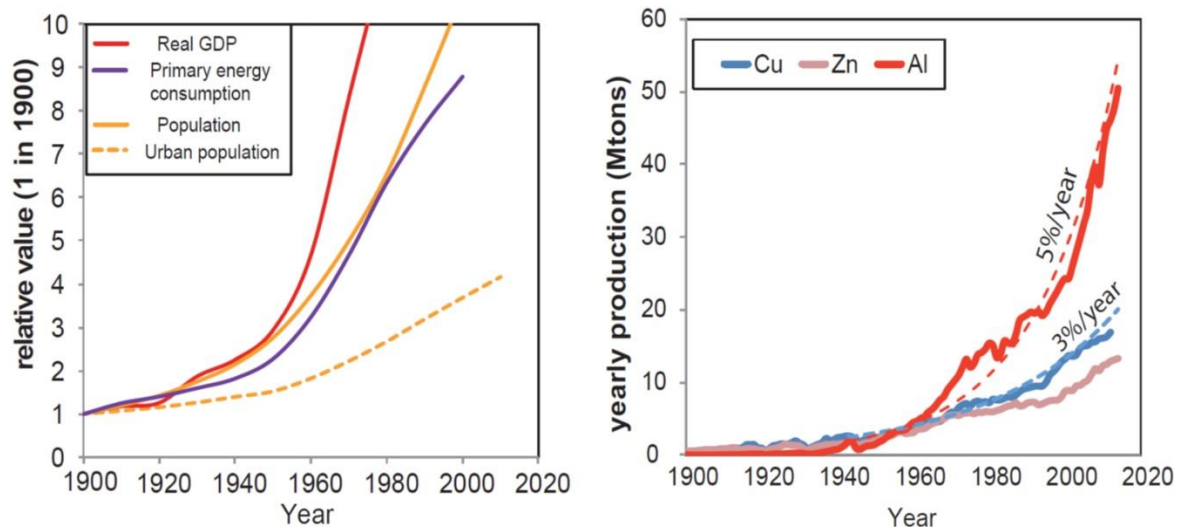
Bioleaching, as part of the biohydrometallurgy, describes the solubilisation of metals from insoluble ores or waste streams by naturally occurring microorganisms such as bacteria or fungi (Mishra and Rhee, 2010). In this sense, different artificial ores might find application to provide high metal concentrations for the recovery and secondary recycling.

### 2.1. Artificial ores for secondary metal recycling and recovery

Globally, the consumption of mineral resources is increasing which is related to the energy consumption, gross domestic production (GDP) and population growth, going hand in hand with the rapid industrialization in the 20<sup>th</sup> century (Steffen et al., 2015). In figure 1, a clear similarity between the before mentioned parameters and the yearly production rate of metals such as Al (5%/year), Cu (3%/year) and Zn can be seen, estimating an exponential growth and further increase in production rate and demand in the 21<sup>st</sup> century (Vidal et al., 2017). Already by now, humanity is using an estimated value of about 70 Gt of mineral resources per year (Wiedmann et al., 2015). With increasingly high demands of metal resources, the ultimately mineable resources will deplete, resulting in or will result in a maximum production maxima for certain metals



followed by a collapse due to depleting mineral stocks (Ali et al., 2017; Kerr, 2014; Mohr et al., 2018).



**Figure 1:** Relationship between population growth and other factors with metal production rates (Vidal et al., 2017). Left: GDP, energy consumption and world population. Right: Yearly production rates of copper, zinc and aluminium.

Having the depleting resources in mind, additional concerns regarding the environmental impacts of mineral mining become more severe. Failures in the construction or operation of dams which are built to collect mineral tailings can lead to leakage and breakage of the dam, causing environmental catastrophes. For example, the failure of a mineral tailing dam in Brazil in November 2015, resulted in the death of 19 people and contamination of 650 km of rivers with toxic and heavy metals (Hatje et al., 2017). Generally, Brazil is one of the major countries in mineral mining, causing environmental pollution by a series of accidents (Silva et al., 2021). Especially in lower income countries, mineral mining is one of the biggest industrial sectors, having severe environmental and social impacts (Kitula, 2006).

In 2017, the European Union (EU) declared a list of critical and economically interesting metals (Tab. 1), which highlights the future demand of these raw materials (Commission, 2017). The end-of-life recycling rates of most of the mentioned metals

are low, showing the urgent need to exploit new secondary resources for metal recycling. With this in mind, different potential resources for secondary metal recycling will be mentioned in the following sections.

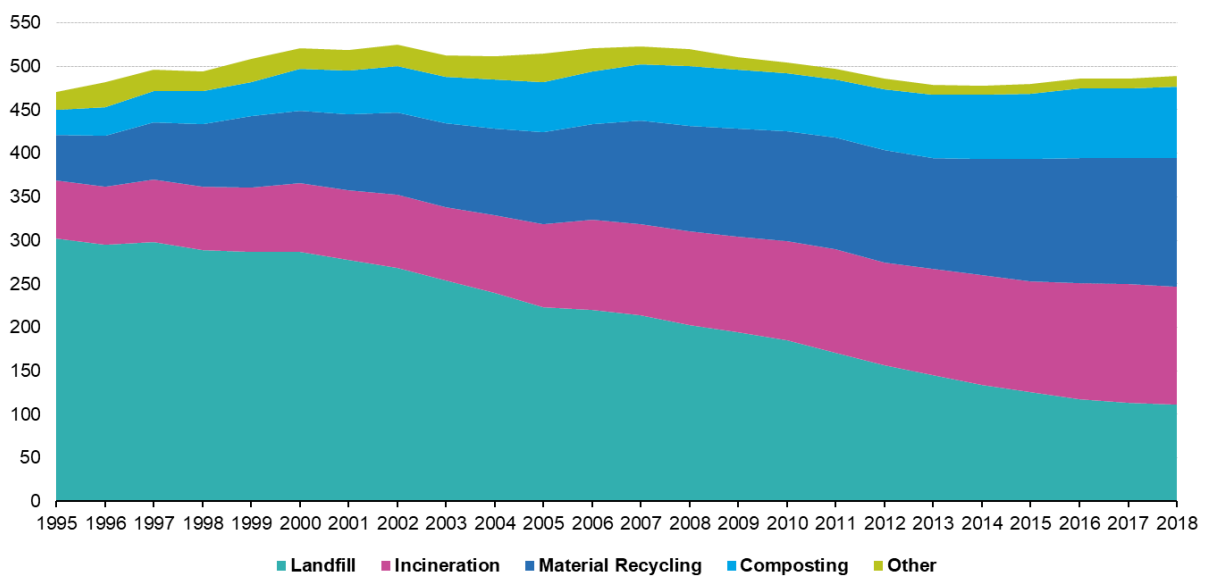
**Table 1:** Critical raw materials and raw materials with a high relative economic importance listed by the European Union (Commission, 2017).

<b>critical raw materials and materials with high economic importance on EU level</b>		
<b>Critical raw materials</b>	Antimony	Indium
	Beryllium	Magnesium
	Cobalt	Niobium
		Platin group
	Fluorspar	metals
	Gallium	Rear earths
	Germanium	Tantalum
	Graphite	Tungsten
<b>Raw materials with high relative economic importance</b>	Aluminium	Molybdenum
	Bauxite	Nickel
	Chromium	Rhenium
	Iron	Tellurium
	Magnesite	Vanadium
	Manganese	Zinc

#### 2.1.1. Ashes and slags from different industries

Waste incineration is one of the most common treatment techniques for municipal solid waste (MSW) worldwide and in the EU, tackling the waste to energy principle. On a global scale, the number of generated waste will increase to 2.2 billion tons per year until 2025 (Hoorweg and Bhada-Tata, 2012). In the EU, incineration accounts for the second most frequently applied treatment technique for MSW reaching around 28% compared to recycling (30%), landfilling (23%) and composting (17%) (Eurostat Waste Statistics, 2017). While landfilling is decreasing year per year, the percentage of incineration is steadily increasing (Fig. 2).

**Municipal waste treatment, EU-28, 1995-2018**  
(kg per capita)



**Figure 2:** Overview of different waste treatment techniques applied in the European Union from 1995-2018 (Eurostat Waste Statistics, 2017).

Around 25% of the initial waste volume remain after incineration as different types of ashes and slags. Within this residuals high and economically interesting concentrations of valuable metals are incorporated, making them a potential secondary resource for urban mining. Comparing different incineration plants in Finland, Germany and Austria, the amount of metals is quite different (Tab. 2). Metals with higher economic impact such as Cu, Zn, Al, Cr, Ni, Fe and Mn arise in concentrations of 0.9 – 9, 3.2 – 61.8, 32.4 – 99.4, 0.1 – 1.5, 0.1 – 0.4, 11.3 – 136.7 and 0.7 – 2.8 g kg<sup>-1</sup>. Estimated values of this residuals with actual marked values of the incorporated metals account for 131 – 403 \$USD per ton of residual, depending on the type of ash and slag. Especially in the case of copper, natural ore grades are declining showing concentrations of around 0.5% (Northey et al., 2014). On the other hand, incineration fly-ash for example contains around 0.8% of copper, exceeding the concentrations of natural ores by 0.3%. This, and the fact of easy and increasing availability of incineration residues makes them interesting artificial ores for urban metal mining.

**Table 2:** Comparison of the metal content and potential value of the incorporated metals in different residuals after waste incineration. The potential value was calculated in \$ USD per t of residue according the metal market prices.

Element	Marked value \$ USD/kg	Fly-ash <sup>1</sup>		Filter-ash <sup>2</sup>		Kettle-ash <sup>2</sup>		Bottom-ash <sup>2</sup>		Bottom-ash <sup>3</sup>		PIR <sup>4</sup>	
		g/kg	value \$ USD/t	g/kg	value \$ USD/t	g/kg	value \$ USD/t	g/kg	value \$ USD/t	g/kg	value \$ USD/t	g/kg	value \$ USD/t
Co	45.2 <sup>a</sup>	0.01	0.54	0.06	2.76	0.06	2.71	0.15	6.78	n.d.	n.d.	n.d.	n.d.
Al	2.5 <sup>a</sup>	32.4	81.0	45.2	113	n.d.	n.d.	42	105	38.0	95.0	99.4	248.5
Cr	8.4 <sup>b</sup>	1.5	12.6	0.37	3.11	1.1	9.24	0.74	6.22	0.16	1.34	0.74	6.22
Fe	0.5 <sup>a</sup>	11.3	5.65	29.8	14.9	39.0	19.5	75.8	37.9	89.0	44.5	136.7	68.35
Mn	2.5 <sup>b</sup>	0.7	1.75	1.2	3.0	2.8	7.0	1.8	4.5	n.d.	n.d.	2.7	6.75
Ni	18 <sup>a</sup>	0.09	1.62	0.2	3.6	0.3	5.4	0.4	7.2	0.11	1.98	0.42	7.56
Zn	2.9 <sup>a</sup>	14.4	41.76	61.8	179.22	24.3	70.47	6.0	17.4	3.2	9.28	5.0	14.5
Cu	10 <sup>a</sup>	0.9	9.0	8.9	89.0	2.3	23.0	5.8	58.0	1.9	19.0	5.1	51.0
Cd	2.4 <sup>c</sup>	n.d.		0.67	1.61	0.17	0.41	0.01	0.02	n.d.	n.d.	0.002	0.005
Total [\$ USD]			153.92		392.19		137.73		131.2		171.10		402.88

<sup>1</sup>(Funari et al., 2017), <sup>2</sup>(Kremser et al., 2021b), <sup>3</sup>(Mäkinen et al., 2019), <sup>4</sup>(Abramov et al., 2018)

<sup>a</sup>London Metal exchange (5.5.2021), <sup>b</sup>SMM, <sup>c</sup>U.S. Geological Survey, Mineral Commodity Summaries, January 2020

PIR....Processed incineration residues

### 2.1.2. Shredder residues

A lot of recycling procedures require an initial shredding step in order to prepare the materials for further processing. Especially bigger waste materials such as end-of-life vehicles (ELV's) and waste electronic and electric equipment (WEEE) need to undergo shredding procedures to reduce the particle size. During shredding, a small fraction (SLF) containing high amounts of metals arises, accounting for around 25% of the treated material. Depending on the origin, different concentrations of valuable metals can be part of this fraction (Tab. 3). SLF generated during the shredding of ELV's contain up to 59.4, 29.3, 13.1, 9.9, 4.9, 4.1 and 2.8 g kg<sup>-1</sup> of Fe, Al, Zn, Cu, Ba, Ti and Mn, respectively (Cheminfo Services, 2014; Kremser et al., 2020; Margarido and Nogueira, 2011). On the other hand, fractions arising during the treatment of WEEE contain precious metals like Cu, Zn, Ag, Au, Pd and Nd in concentrations of 33.5, 8.2, 0.3, 0.009, 0.02 and 0.09 g kg<sup>-1</sup> (Marra et al., 2018). Independent from the type of SLF, the concentration of certain metals exceeds the ones in natural ores, showing the high potential of this waste materials.

**Table 3:** Metal concentrations of different shredder residues

Element	SLF <sup>1</sup>	SLF <sup>2</sup> [g kg <sup>-1</sup> ]	WEEE dust <sup>3</sup>
Fe	95.4	22	16
Al	29.3	13	31.8
Zn	13.1	12	8.2
Cu	9.9	30	33.5
Ba	4.9	n.d.	n.d.
Ti	4.1	n.d.	n.d.
Ni	0.51	0.33	1.3
Mo	0.15	n.d.	n.d.
Cr	0.5	0.3	n.d.
Ag	n.d.	n.d.	0.3
Au	n.d.	n.d.	0.009
Pd	n.d.	n.d.	0.02
Nd	n.d.	n.d.	0.09

<sup>1</sup>(Kremser et al., 2020), <sup>2</sup>(Cheminfo Services, 2014),

<sup>3</sup>(Marra et al., 2018)

### 2.1.3. Multilayer packaging waste

Aluminium is listed by the EU as raw material with a high relative economic importance. Indeed, Aluminium is one of the most frequently used metals in construction, automotive and packaging sector accounting for an annual, global production of 65 million tons in 2021 (Statista, 2021). Primary aluminium production faces disadvantages like a high energy demand, the generation of around 120 million tons of highly alkaline red-mud per year, the release of large amounts of CO<sub>2</sub> and the emission of perfluorocarbons (Das et al., 2010; Milačič et al., 2012). On the other hand, recycling of aluminium requires 95% less energy and avoids greenhouse gas emissions, making aluminium one of the most frequently recycled metals with high recycling rates of up to 76% globally (International Aluminum Institute, 2021, 2009).

In packaging, aluminium is introduced to provide structural integrity, light- and oxygen-barriers to protect the packaged goods. Aluminium beverage cans are produced in huge amounts all over the world reaching an expected marked value of 61.4 billion USD \$ by 2025 (Deshwal and Panjagari, 2020). To avoid direct contact of food and aluminium, different polymer coatings are applied on the internal surface of the beverage can, making them a multilayer packaging material. While the recycling rate of aluminium cans with 70% (International Aluminum Institute, 2021) is relatively high, other multilayer materials are more complex to recycle.

Liquid beverage packaging (i.e. milk and juice cartons) represent the most complex multilayer packaging materials consisting of different layers of polymer, paperboard and aluminium (Gesellschaft für Verpackungsmarktforschung GmbH, 2016). State-of-the-art recycling of these materials can only recycle the paperboard, leaving the plastic-aluminium reject behind for incineration (Zawadiak, 2017). Currently, new methods are investigated to increase the recycling rates for aluminium and plastic from the reject

material, including bioleaching and other innovative technologies (Georgiopoulou et al., 2021; Kaiser, 2020).

#### 2.1.4. Other potential waste streams

Beside incineration ashes and slags, other industries produce similar residues which do not find any application by now. In steel and copper industry, reasonable amounts of slag are generated for example steel slag containing metals such as Cu, Al, Fe, Mg, Cr and Ni in economically interesting concentrations (Gomes et al., 2018; Hocheng et al., 2014). Other ashes such as coal fly ash from coal incineration contain critical and economically important raw materials like Mo, Mg, V and Fe in different concentrations (Ertit Taştan, 2017; Fan et al., 2019). Furthermore, different sludges from wastewater treatment plants can be taken into account as artificial metal ores, revealing different concentrations of Cu, Zn, Cr, Ni within this residues (Gu et al., 2018a; Pathak et al., 2009).

## 2.2. Bioleaching organisms and mechanisms

Bioleaching is part of the biohydrometallurgy, describing the solubilisation of metals from ores or waste streams by microorganisms such as bacteria or fungi (Mishra and Rhee, 2010). Via the so-called bio-oxidation, metals present in the form of sulphides or oxides undergo solubilisation into metal cations (Rohwerder et al., 2003). In the following, typical bacteria (chemolithotrophs) and fungi (organotrophs) frequently applied in bioleaching will be described and the solubilization pathways and leaching mechanisms of metal extraction will be discussed in more detail.

#### 2.2.1. Bacterial bioleaching

Lacking the need of an organic carbon source as energy source, chemolithotrophs are named according their ability to utilise inorganic compounds such as ferrous iron ( $\text{Fe}^{2+}$ )

and reduced inorganic sulfur compounds (RISC's) (Quatrini and Johnson, 2018). While a classification according their preferred growth temperature (i.e. mesophilic, moderately thermophilic and thermophilic) is favourable, all of them have in common that their pH-optimum and tolerance is around pH 2 and below.

Among bioleaching bacteria, the ferrous iron and sulfur-oxidizing *Acidithiobacillus ferrooxidans* represents one of the most frequently applied and intensively investigated organisms in bioleaching. Growing at a temperature- and pH-optimum of around 30 °C and 1.5 – 2.0, respectively, *A. ferrooxidans* can oxidize  $\text{Fe}^{2+}$  and RISC's for energy generation (Valdés et al., 2008). This process can be divided into uphill and downhill pathways using different enzymes and proteins involved in the oxidation of  $\text{Fe}^{2+}$  and fixation of  $\text{CO}_2$  (Zhan et al., 2019). Other mesophiles are only capable of oxidizing either  $\text{Fe}^{2+}$  or RISC's. *Leptospirillum ferrooxidans* for example, grows by oxidation of ferrous iron only, without being able to oxidize RISC's (Hallmann et al., 1992). On the other hand, the RISC's oxidizing *Acidithiobacillus thiooxidans* lacks on the ferrous iron oxidizing pathway. *A. thiooxidans* is known for the effective metabolization of RISC's at a temperature- and pH-optimum of 28 – 30 °C and 2.0 related to the expression of enzymes involved in sulfur metabolization, allowing growth even at pH=0.5 (Wang et al., 2019). Another sulfur-oxidizing bacterium, frequently applied in bioleaching applications, is the moderately thermophilic *Acidithiobacillus caldus*. At a temperature- and pH-optimum of 40 – 45 °C and 2.0 – 2.5, *A. caldus* can metabolize RISC's via the upregulation of genes, responsible for the expression of proteins and enzymes important for sulfur-metabolization at pH-values bellow 2 (Mangold et al., 2013, 2011). Further moderately thermophilic organisms include eubacteria, in example *Acidimicrobium* sp., *Ferromicrobium* sp., and *Sulfobacillus* sp. (Srichandan et al., 2019). Thermophilic bacteria, growing at temperatures between 60 and 80 °C,



oxidizing  $\text{Fe}^{2+}$  and RISC's are dominantly of *Sulfolobus sp.* (i.e. *Sulfolobus metallicus*, *Sulfolobus acidocaldarius* and *Sulfolobus solataricus*) and have been investigated in several bioleaching studies (Roshani et al., 2017). A list of commonly applied and investigated bacteria within the *Acidithiobacillus sp.* can be seen in table 4.

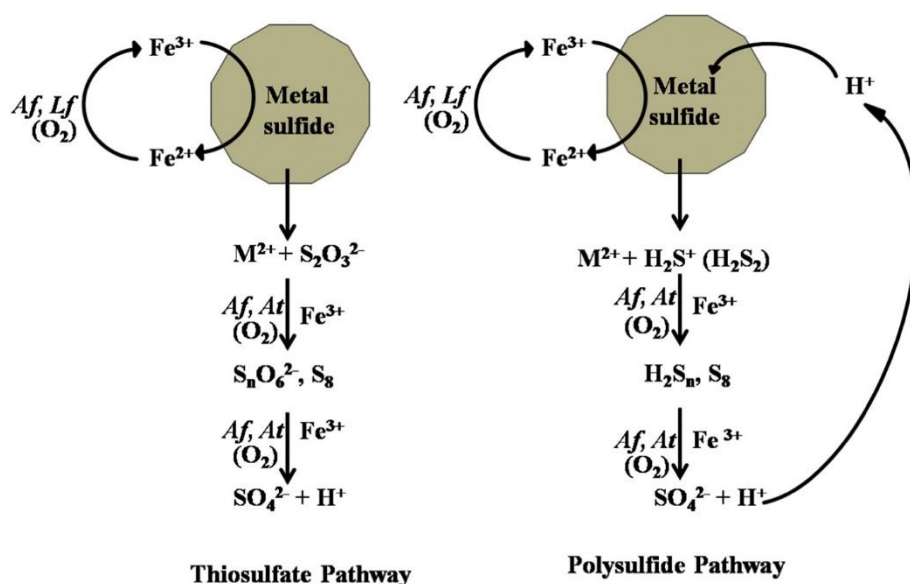
**Table 4:** Comparison of four different, frequently applied and well investigated bioleaching organisms within the *Acidithiobacillus sp.* modified from (Wang et al., 2019). The most important parameters such as optimum growth temperature and pH-value are compared to other parameters such as nutrient requirements, gram stain and motility.

	<i>A. ferrooxidans</i>	<i>A. ferridurans</i>	<i>A. thiooxidans</i>	<i>A. caldus</i>
Gram strain	-	-	-	-
Motility	+/-	+	+	+
Growth pH (optimum)	1.3-4.5 (2.0-2.5)	1.4-3.0 (2.1)	0.5-5.5 (2.0-3.0)	1.0-3.5 (2.0-2.5)
Growth T/°C (optimum)	10-37 (30-35)	10-37 (29)	10-37 (28-30)	32-52 (40-45)
Oxidation of RISC's	+	+	+	+
Oxidation of $\text{Fe}^{2+}$	+	+	-	-
Growth on sulfide minerals	+	+	-	-
Growth on hydrogen	+	+	-	+
Anaerobic growth with $\text{Fe}^{2+}$	+	+	-	-
N <sub>2</sub> fixation	+	NR	-	-

+, positive; -, negative; NR, not reported

All of the before mentioned organisms share two common pathways for metal solubilization from metal sulphides; the thiosulfate and the polysulfide pathway (Rohwerder et al., 2003). In thiosulfate pathway, acid non-soluble metal sulphides are oxidized via electron extraction by ferric iron ( $\text{Fe}^{3+}$ ). In this,  $\text{Fe}^{3+}$  is reduced to  $\text{Fe}^{2+}$  which can then be continuously regenerated by iron-oxidizing microbes. Metal and sulfur compounds are released as thiosulfate and metal cations, respectively. Thiosulfate is afterwards oxidized to tetrathionate and further to sulfuric acid ( $\text{H}_2\text{SO}_4$ ). The elemental sulfur produced within this pathway can be oxidized by RISC's oxidizing

bacteria to additionally form sulfuric acid and lower the pH. A schematic presentation of the thiosulfate and polysulfide pathway can be seen in figure 3.



**Figure 3:** Schematic presentation of the thiosulfate (left) and polysulfide pathway (right) performed by the bioleaching bacteria to solubilize metal sulfides and metal oxides (Rohwerder et al., 2003 and Srichandan et al., 2019).

Acid soluble metal sulphides are dissolved by electron extraction comprising a combined action of  $\text{Fe}^{3+}$  and hydrogen ions ( $\text{H}^+$ ) in the polysulfide pathway. In here,  $\text{H}_2\text{S}$  is released, being further oxidized to elemental sulfur. Sulfur is afterwards further oxidized by sulfur oxidizing bacteria to form sulfuric acid. The acid soluble metals can be dissolved by the generated sulfuric acid. This is also the case for metal oxides which are first converted to soluble metal sulphates via the generated sulfuric acid.

### 2.2.2. Fungal bioleaching

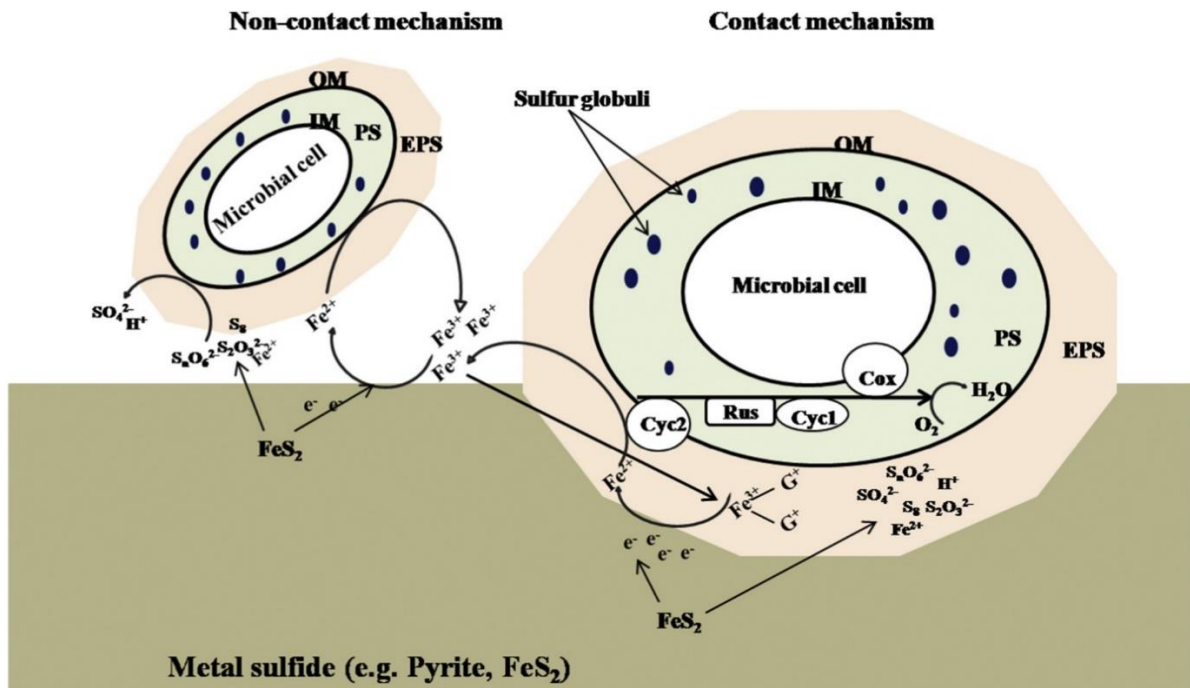
Organotrophic fungi such as *Aspergillus niger*, *Aspergillus flavus* and *Penicillium chrysogenum* produce organic metabolites (i.e. gluconic acid, citric acid, oxalic acid, etc.) which can find application in bioleaching (Ilyas et al., 2013). Fungi used in bioleaching grow at temperatures of 25 – 35 °C and at a pH of 3.0 – 7.0. Metal solubilization is performed by the generated organic acids via acidolysis, complexolysis, redoxolysis or bioaccumulation (Asghari et al., 2013). In acidolysis,

generated protons attack the ore surface, weakening bonds and remove metal ions whereas in complexolysis metal ions are stabilized by forming complexes with organic acids or amino acids. Redoxolysis results in the oxidation or reduction of metals, increasing the metal mobility, whereas in bioaccumulation soluble metals are transported into the cell and accumulate as solid particles.

### 2.2.3. Direct and indirect bioleaching

Knowing the different pathways of metal sulphide solubilization, bioleaching organisms can be used in two mechanisms namely direct and indirect bioleaching. In direct bioleaching, bacteria oxidize  $\text{Fe}^{2+}$  and RISC's via contact or non-contact mechanism in which the generated  $\text{Fe}^{3+}$  solubilizes metals according the before mentioned thiosulfate or polysulfide pathway (Fig. 4). In the contact mechanism, bacteria attach to the surface via their extracellular polymeric substances (EPS) in which  $\text{Fe}^{3+}$  is complexed with glucouronic acid.  $\text{Fe}^{3+}$  is reduced to  $\text{Fe}^{2+}$  which may afterwards be again regenerated by different enzymes (Huang and Li, 2014). In the non-contact mechanism, planktonic cells oxidize  $\text{Fe}^{2+}$  and RISC's without contact to the mineral surface leading to the formation of  $\text{Fe}^{3+}$  and sulfuric acid. Overall, the direct bioleaching

mechanism requires active action of the bacteria on the desired material used for the treatment.



**Figure 4:** Schematic presentation and details of the contact and non-contact mechanism performed by the bioleaching bacteria in direct and indirect bioleaching (Srichandan et al., 2019). EPS, OM, PS and IM represent extracellular polymeric substances, outer membrane, periplasmic space and outer membrane. Enzymes involved in the metabolism of ferrous iron are mentioned as Cyc2, Rus, Cyc1 and Cox.

The major difference of indirect bioleaching is that a direct contact or action of the bacteria is not required and intended. Herein, bacterial metabolites such as organic and inorganic acids (i.e  $\text{HCl}$ ,  $\text{H}_2\text{SO}_4$ , oxalic acid, gluconic acid, etc.), ferric iron and biosurfactants are the desired actors of interest. In the so-called spent medium leaching pure and mixed acidophilic bacteria or fungi are cultivated until a certain concentration of biosurfactants, acidity or ferric iron is reached (Boxall et al., 2018; Faraji et al., 2018). This leaching reagent is afterwards used to leach out metals from different wastes or ores.

#### 2.2.4. Factors influencing the bioleaching performance

One of the crucial parameters effecting the bioleaching efficiency of different bacteria and substrates is the pH-value. It has impact on the growth and activity of the

microorganisms used, as well as on the solubilization and stability of metals in solution (Asghari and Mousavi, 2014). To keep a low pH, optimal for bacterial growth (1.8 – 2.5), different strategies have been applied. Addition of elemental sulfur for example results in the generation of sulfuric acid by sulfur-oxidizing bacteria (Wang et al., 2019). Furthermore, the addition of ferrous iron ( $\text{Fe}^{2+}$ ) on the one hand prevents excessive pH shift due to a buffering effect on elemental sulfur and on the other hand  $\text{Fe}^{2+}$  and elemental sulfur are bio oxidized leading to bioaugmentation (Asghari and Mousavi, 2014; Fonti et al., 2016). Additionally, external pH-regulation with different acids to keep a constant pH-value is frequently applied.

Another factor influencing the biological leaching is the redox potential of the lixiviant. During bio-oxidation of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  the redox potential increases, which was reported to be beneficial for metal leaching from ores or solid waste (Abramov et al., 2018; Kremser et al., 2021). Maintaining a high redox potential by the addition of ferrous iron containing substrates and optimal bacterial growth, represents key factors for successful bioleaching operations.

Pulp density or concentration of solids, and the particle size of the treated materials represent further important parameters effecting the leaching efficiency. An increase in pulp density has negative effects on the leaching efficiency due to higher toxicity of heavy metals, adsorption of metal ions and insufficient oxygen supply by inadequate mixing (Gu et al., 2018). In general, 1 - 2% (w/v) of solids concentration was proven to provide optimum conditions for metal bio-extraction from different waste materials (Chi et al., 2011; Fan et al., 2019; Horeh et al., 2016; Tong et al., 2020). On the other hand, the particle size plays another important role in effective bioleaching operations. In general, the smaller the grain size of the particles, the faster bacterial extraction of metals occurs due to an increased surface to volume ratio (Yin and Chen, 2021).

Furthermore, in the case of waste incineration residues, the finer fraction after sieving was identified to have more extractable metals (Abramov et al., 2018), making size reduction an critical parameter for successful bioleaching applications.

Sufficient nutrient supply represents another important factor in the biological treatment of waste residues. Elemental sulfur is added to provide an energy source for RISCs oxidizing microorganisms and to acidify the leaching medium due to resulting sulfuric acid generation. This leads to a higher metal ion mobility, increased extraction efficiencies and favours bacterial growth. Another important nutrient in bioleaching is ferrous iron. Most likely, it is provided as  $\text{FeSO}_4$  as part of the leaching medium. Optimum  $\text{Fe}^{2+}$  concentrations were found to be in the range of 3 – 9 g L<sup>-1</sup>, varying between the different waste materials treated (Hubau et al., 2018; Li et al., 2017; Xiang et al., 2010). Insufficient oxygen supply due to inappropriate aeration or stirring can lead to a reduction of oxidation rates for  $\text{S}^0$  and  $\text{Fe}^{2+}$ . In general, an dissolved oxygen (DO) concentration between 1.5 – 4.0 g L<sup>-1</sup> was reported to be the optimum for microbial activities (De Kock et al., 2004). Furthermore,  $\text{CO}_2$  supply seems to be of importance as needed for the growth of autotrophic bacteria. The optimal gas concentration of  $\text{CO}_2$  for ferrous iron oxidation was found to be in the range of 7 – 17% (v/v) (De Kock et al., 2004).

### **2.3. Bioleaching processes**

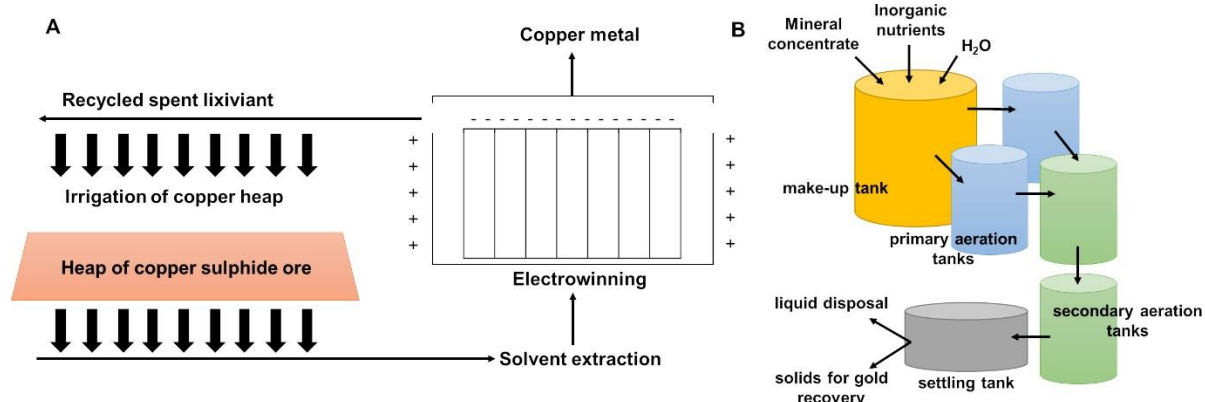
Laboratory scale investigations of bioleaching applications involving different microorganisms and substrates, are most likely performed in small scale experiments in shaking flasks or similar laboratory equipment. Nevertheless, up-scaling is a crucial step to make bioleaching applicable for large-scale applications in metal mining from minerals, ores and waste (Petersen, 2010). In the following, the most prominent

examples for big-scale, bioleaching operations will be described and compared according their applicability on different metal containing materials.

### 2.3.1. Irrigation-type bioleaching

Low grade ores or dumps containing copper and other metals are often too less concentrated to be recovered profitably by commercial techniques. In irrigation-type processes, the lixiviant containing microorganisms and their metabolites, is percolated through crushed ores or other waste materials which were stacked into heaps, columns or dumps (Rawlings, 2002). In heap bioleaching, pre-treatment especially to reduce the particle size, is necessary to increase the efficiency of bio extraction (Yin and Chen, 2021). By crushing the material, particle sizes less than 250 mm are reached. The material is afterwards piled up and the lixiviant is pumped over it from the top of the heap. The solution flows through the pile by gravity and is collected via a drainage system at the bottom of the heap (Fig. 5A) (Rawlings, 2002). When a certain concentration of metal in the solution is reached, the lixiviant is used in further recovery processes to extract the pure metal. In contrast to heap-bioleaching, dump-bioleaching is operated without applying the lixiviant from the top and pump it in a circular way over the dump. In dump-bioleaching, up to 350 m high piles are constructed in which the microorganisms can grow and convert insoluble metal sulphides to soluble metal sulphates by the addition of nutrient rich, mining waste water. Nevertheless, the bio

extraction via dump-bioleaching can take several years, making it a no longer frequently used process.



**Figure 5:** Schematic process overview of a heap-bioleaching system (A) in which the piled-up heap is sprinkled with the lixiviant from the top. When a certain concentration of metal in the solution is reached, metal recovery is performed for example via electrowinning. In B, a standard stirred-tank bioleaching for big scale application is shown. Most frequently the tanks are connected in series and the ore substrate is leached until the desired metal (in this case gold) is available in a sufficient concentration. This figure was modified from Rawlings, 2002

Especially in copper leaching, bio extraction using acidophilic microorganisms is a frequently applied technique for big-scale copper mining. Still in 2010, at least 18 copper heap-bioleaching plants were operated around the world extracting tons of copper with efficiencies of up to 80% showing the potential of this technique also for other low grade metal ores (Brierley and Brierley, 2013; Gentina and Acevedo, 2016; Ruan et al., 2011; Yin and Chen, 2021). The number of commercial heap-bioleaching plants even increased to around 40 worldwide, treating different metal containing resources (Li et al., 2021).

### 2.3.2. Stirred-tank bioleaching

Due to higher construction and operation costs, stirred-tank bioleaching is mainly applied for high-value ores and concentrates. In most of the cases, bioreactors are connected in a series of tanks, flowing from one to the other until the bio-oxidation of the substrates is sufficiently complete (Fig. 5B). These tanks are sufficiently aerated, continuously stirred and supplemented with nutrient solutions to ensure bacterial



growth and effective bio-oxidation. Most of the stirred-tank operations are used for the pre-treatment of gold bearings, to reduce the pyrite/arsenopyrite content which helps to extract the finely distributed incorporated gold. One of the biggest plants operated in this field is most probably located in Ghana consisting of 24 tanks and can process up to 1000 tons of gold concentrate per day (Rawlings, 2002). Furthermore, other metals such as Ni and Co are interesting for stirred-tank leaching applications and are currently under investigation for commercial applications (Cameron et al., 2021).

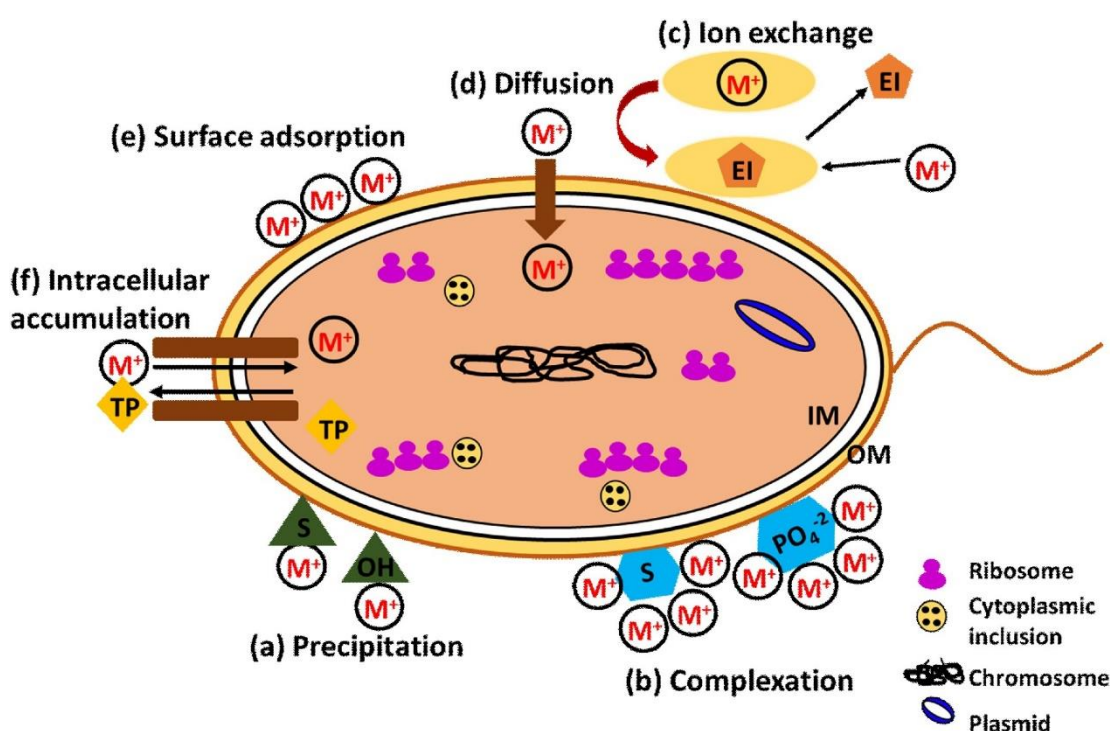
## **2.4. Potential ways of metal recovery**

Successful biological extraction of metals from different waste streams is the first step towards an efficient and environmentally friendly recovery technique. Nevertheless, recovery of the dissolved metals from the saturated metal solution to bring them back into a re-usable and re-processable form, is a crucial step to build up an economically feasible process. Talking about an industrial scale, different biological techniques such as biosorption or the use of bioelectrochemical systems, as well as non-biological techniques like selective precipitation or electrowinning can provide possible solutions.

### **2.4.1. Biosorption**

Biosorption is a well investigated and industrially applied process for heavy metals binding to the cell wall of or the active uptake by different microorganisms. The process of biosorption can be divided into metabolism dependent or independent mechanism (Robalds et al., 2016). While some of the processes related to the metabolism dependent mechanisms refer to the action of living biomass, most of the mechanisms involved in biosorption are carried out in an independent way by dead biomass. Herein, biosorption can be divided into physisorption, chemisorption, ion exchange and microprecipitation, in which chemisorption is further divided into complexation and covalent binding and further into chelation and coordination (de Freitas et al., 2019;

Robalds et al., 2016). In more detail, the processes of metal biosorption involves precipitation (interaction between cell surface and metal), complexation (interaction of metal ions with surface active groups), ion exchange (exchange with counter ions present on the cell wall), diffusion, surface adsorption (metal cations bind to the cellular surface) and intracellular accumulation (transport of metal ions across the membrane) (Fig. 6) (Priyadarshane and Das, 2021).



**Figure 6:** Schematic representation of the processes involved in biosorption of heavy metals (Priyadarshane and Das, 2021). OM-Outer Membrane, IM-Inner Membrane, EI-Exchangeable Ions, TP-Transport Proteins, M<sup>+</sup>-Metal cations

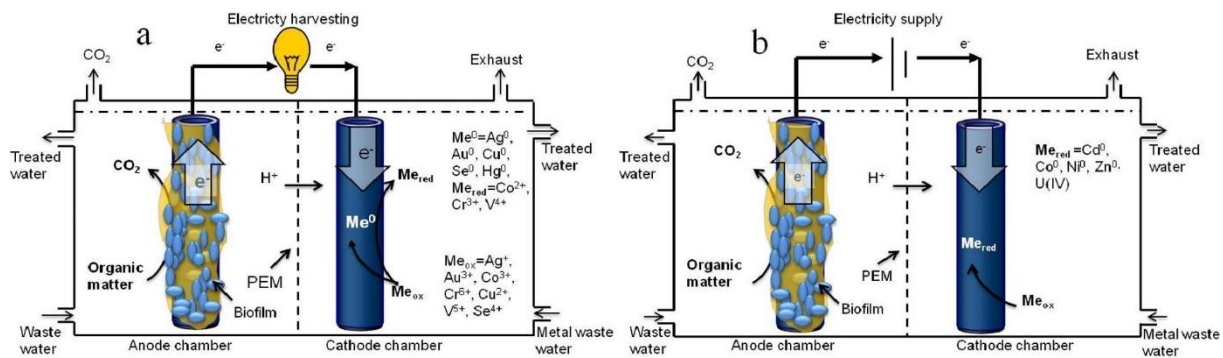
Factors influencing the biosorption of heavy metals include pH, temperature, type of metal and ionic strength. Nevertheless, the pH value seems to play a key role in biosorption, affecting the metal solution chemistry and the activity of surface functional groups. In recent years, continuous biosorption processes have been brought to an industrial scale, using adsorption and desorption processes in packed columns. Herein, microorganisms such as different bacteria, fungi and yeast have been successfully applied to remove heavy metals like Zn, Cu, Ni, Cd, Cr and Pb from

different waste waters, leaching solutions and contaminated soils (de Freitas et al., 2019; Priyadarshane and Das, 2021).

#### 2.4.2. Bioelectrochemical systems

Being classified as an emerging technology, bioelectrochemical systems (BES) gained more importance over the last ten years due to their possible application in harnessing energy, generating value added bio-products and metal recovery (Elmekawy et al., 2014; Huang et al., 2018). Talking about the application of BES, microbial fuel cells (MFCs) and microbial electrolysis cells (MECs) have emerged, in which microorganisms grow in biofilms on anode material, converting biodegradable organic matter (i.e. from waste water) into CO<sub>2</sub>, electrons and protons (Nanchaiah et al., 2015). While CO<sub>2</sub> and protons are released into the solution, electrons are passed to the anode and are further transported to the cathode via an external circuit. The principle of metal removal via BES is that biological growth and conversions in the anode biofilm related to direct contact, electron shuttle or conductive pili mediation in the microorganisms deliver electrons to the cathode, where a reductive precipitation of metals is driven by using them as terminal electron acceptors (TEAs) (Fig. 7a). In this way, MFCs are used for selective precipitation of metals with a higher redox potential than the anodic potential in BES such as Ag(I), Au(III), Cr(VI), Co(III) and Cu(II) (Rene et al., 2017; Wang and He, 2020). MECs are used for the reduction of metals with lower potential than the anode. Therefore, an external power supply is needed to direct the electrons from the anode to the cathode (Fig. 7b). In this way, metals such as Ni(II), Cd(II) and Zn(II) were successfully recovered (Luo et al., 2014). Furthermore, the metal recovery performance and efficiency is also dependent and strongly influenced by the

microbial community in the biofilm and the type of anode/cathode material (Spiess et al., 2021).



**Figure 7:** Overview of bioelectrochemical systems comprising microbial fuel cells (MFCs) (a) and microbial electrolysis cells (MECs) with external power supply (b) (Nanchaiah et al., 2015). In both BES, microorganisms grow in biofilms on the anode and metabolize organic matter from waste water to form CO<sub>2</sub>, protons and electrons. In MFCs, electrons are transported to the cathode via an external circuit and metals with a higher redox potential than the anode potential are precipitated. In MECs, an external power supply is needed to shuttle the electrons to the cathode.

#### 2.4.3. Electrowinning and Precipitation

Electrowinning is another recovery process for electrochemical processing or utilization of metals from aqueous solutions, reversing the process of metal dissolution (Free, 2007; Moats and Free, 2007). The principle of electrowinning is similar to the before mentioned MEC having electrons transported from the anode (i.e. titanium coated with different metal oxides) to the cathode (very often stainless steel) by applying an external current. In this way, metals in solution like copper can be plated and recovered on the cathode. Using electrowinning, copper could be successfully recovered after bioleaching of printed circuit boards (PCBs) or copper smelt fly ash (Baniasadi et al., 2021; Lei et al., 2021).

Selective precipitation of dissolved metals from leaching solutions as metal oxides, hydroxides, sulphides, carbonates and phosphates, might present another potential way for metal recovery (Blais et al., 2008). By adding alkaline reagents, such as sodium hydroxide (NaOH) metals such as copper, iron, zinc and aluminium can be separated

after precipitation as metal-hydroxides according the solution pH (Blais et al., 2008). By adjusting the pH with NaOH, different researchers showed the possibility to selectively recover iron and aluminium from acid mine drainage and bioleaching lixiviants (Kremser et al., 2021; Wei et al., 2005).

## 2.5. References

- Abramov, S., He, J., Wimmer, D., Lemloh, M.L., Muehe, E.M., Gann, B., Roehm, E., Kirchhof, R., Babechuk, M.G., Schoenberg, R., Thorwarth, H., Helle, T., Kappler, A., 2018. Heavy metal mobility and valuable contents of processed municipal solid waste incineration residues from Southwestern Germany. *Waste Manag.* 79, 735–743. <https://doi.org/10.1016/j.wasman.2018.08.010>
- Ali, S.H., Giurco, D., Arndt, N., Nickless, E., Brown, G., Demetriades, A., Durrheim, R., Enriquez, M.A., Kinnaird, J., Littleboy, A., Meinert, L.D., Oberhänsli, R., Salem, J., Schodde, R., Schneider, G., Vidal, O., Yakovleva, N., 2017. Mineral supply for sustainable development requires resource governance. *Nature* 543, 367–372. <https://doi.org/10.1038/nature21359>
- Asghari, I., Mousavi, S.M., 2014. Effects of key parameters in recycling of metals from petroleum refinery waste catalysts in bioleaching process. *Rev. Environ. Sci. Biotechnol.* 13, 139–161. <https://doi.org/10.1007/s11157-013-9329-8>
- Asghari, I., Mousavi, S.M., Amiri, F., Tavassoli, S., 2013. Bioleaching of spent refinery catalysts: A review. *J. Ind. Eng. Chem.* 19, 1069–1081. <https://doi.org/10.1016/j.jiec.2012.12.005>
- Baniasadi, M., Graves, J.E., Ray, D.A., De Silva, A.L., Renshaw, D., Farnaud, S., 2021. Closed-Loop Recycling of Copper from Waste Printed Circuit Boards Using Bioleaching and Electrowinning Processes. *Waste and Biomass Valorization* 12, 3125–3136. <https://doi.org/10.1007/s12649-020-01128-9>
- Blais, J.F., Djedidi, Z., Cheikh, R.B., Tyagi, R.D., Mercier, G., 2008. Metals precipitation from effluents: Review. *Pract. Period. Hazardous, Toxic, Radioact. Waste Manag.* 12, 135–149. [https://doi.org/10.1061/\(ASCE\)1090-025X\(2008\)12:3\(135\)](https://doi.org/10.1061/(ASCE)1090-025X(2008)12:3(135))

- Boxall, N.J., Cheng, K.Y., Bruckard, W., Kaksonen, A.H., 2018. Application of indirect non-contact bioleaching for extracting metals from waste lithium-ion batteries. *J. Hazard. Mater.* 360, 504–511. <https://doi.org/10.1016/j.jhazmat.2018.08.024>
- Brierley, C.L., Brierley, J.A., 2013. Progress in bioleaching: part B: applications of microbial processes by the minerals industries. *Appl. Microbiol. Biotechnol.* 97, 7543–7552. <https://doi.org/10.1007/s00253-013-5095-3>
- Cameron, R.A., Lastra, R., Thibault, Y., Morin, L., Gould, W.D., 2021. Stirred-tank bioleaching of nickel and cobalt from pyrrhotite-rich tailings from Sudbury, Ontario. *Hydrometallurgy* 105592. <https://doi.org/10.1016/j.hydromet.2021.105592>
- Cheminfo Services, 2014. Background Study on the Content of Shredder Residue.
- Chi, T.D., Lee, J.C., Pandey, B.D., Yoo, K., Jeong, J., 2011. Bioleaching of gold and copper from waste mobile phone PCBs by using a cyanogenic bacterium. *Miner. Eng.* 24, 1219–1222. <https://doi.org/10.1016/j.mineng.2011.05.009>
- Commission, E., 2017. COMMUNICATION FROM THE COMMISSION TO THE EUROPEAN PARLIAMENT, THE COUNCIL, THE EUROPEAN ECONOMIC AND SOCIAL COMMITTEE AND THE COMMITTEE OF THE REGIONS on the 2017 list of Critical Raw Materials for the EU.
- Das, S.K., Green, J.A.S., Kaufman, G., 2010. Aluminum recycling: Economic and environmental benefits. *Light Met. Age* 68, 42–46.
- de Freitas, G.R., da Silva, M.G.C., Vieira, M.G.A., 2019. Biosorption technology for removal of toxic metals: a review of commercial biosorbents and patents. *Environ. Sci. Pollut. Res.* 26, 19097–19118. <https://doi.org/10.1007/s11356-019-05330-8>
- De Kock, S.H., Barnard, P., Du Plessis, C.A., 2004. Oxygen and carbon dioxide kinetic challenges for thermophilic mineral bioleaching processes. *Biochem. Soc. Trans.* 32, 273–275. <https://doi.org/10.1042/BST0320273>
- Deshwal, G.K., Panjagari, N.R., 2020. Review on metal packaging: materials, forms, food applications, safety and recyclability. *J. Food Sci. Technol.* 57, 2377–2392. <https://doi.org/10.1007/s13197-019-04172-z>

- Elmekawy, A., Hegab, H.M., Pant, D., 2014. The near-future integration of microbial desalination cells with reverse osmosis technology. *Energy Environ. Sci.* 7, 3921–3933. <https://doi.org/10.1039/c4ee02208d>
- Ertit Taştan, B., 2017. Clean up fly ash from coal burning plants by new isolated fungi *Fusarium oxysporum* and *Penicillium glabrum*. *J. Environ. Manage.* 200, 46–52. <https://doi.org/10.1016/j.jenvman.2017.05.062>
- Eurostat Waste Statistics, 2017. Municipal waste statistics 1–6. <https://doi.org/10.2785/408702>
- Fan, X. lu, Lv, S. qing, Xia, J. lan, Nie, Z. yuan, Zhang, D. rui, Pan, X., Liu, L. zhu, Wen, W., Zheng, L., Zhao, Y. dong, 2019. Extraction of Al and Ce from coal fly ash by biogenic Fe<sup>3+</sup> and H<sub>2</sub>SO<sub>4</sub>. *Chem. Eng. J.* 370, 1407–1424. <https://doi.org/10.1016/j.cej.2019.04.014>
- Faraji, F., Golmohammadzadeh, R., Rashchi, F., Alimardani, N., 2018. Fungal bioleaching of WPCBs using *Aspergillus niger*: Observation, optimization and kinetics. *J. Environ. Manage.* 217, 775–787. <https://doi.org/10.1016/j.jenvman.2018.04.043>
- Fonti, V., Dell'Anno, A., Beolchini, F., 2016. Does bioleaching represent a biotechnological strategy for remediation of contaminated sediments? *Sci. Total Environ.* 563–564, 302–319. <https://doi.org/10.1016/j.scitotenv.2016.04.094>
- Free, M.L., 2007. The fundamentals of electrometallurgy in aqueous media. *Jom* 59, 28–33. <https://doi.org/10.1007/s11837-007-0062-z>
- Funari, V., Mäkinen, J., Salminen, J., Braga, R., Dinelli, E., Revitzer, H., 2017. Metal removal from Municipal Solid Waste Incineration fly ash: A comparison between chemical leaching and bioleaching. *Waste Manag.* 60, 397–406. <https://doi.org/10.1016/j.wasman.2016.07.025>
- Gentina, J.C., Acevedo, F., 2016. Copper bioleaching in Chile. *Minerals* 6. <https://doi.org/10.3390/min6010023>
- Georgiopoulou, I., Pappa, G.D., Vouyiouka, S.N., Magoulas, K., 2021. Recycling of post-consumer multilayer Tetra Pak® packaging with the Selective Dissolution-Precipitation process. *Resour. Conserv. Recycl.* 165, 105268. <https://doi.org/10.1016/j.resconrec.2020.105268>

- Gesellschaft für Verpackungsmarktforschung GmbH, 2016. Flexible Plastic Packaging Market in Germany and in Europe. Trends and Perspectives until 2021 by Product Categories.
- Gomes, H.I., Funari, V., Mayes, W.M., Rogerson, M., Prior, T.J., 2018. Recovery of Al, Cr and V from steel slag by bioleaching: Batch and column experiments. *J. Environ. Manage.* 222, 30–36. <https://doi.org/10.1016/j.jenvman.2018.05.056>
- Gu, T., Rastegar, S.O., Mousavi, S.M., Li, M., Zhou, M., 2018a. Advances in bioleaching for recovery of metals and bioremediation of fuel ash and sewage sludge. *Bioresour. Technol.* 261, 428–440. <https://doi.org/10.1016/j.biortech.2018.04.033>
- Gu, T., Rastegar, S.O., Mousavi, S.M., Li, M., Zhou, M., 2018b. Advances in bioleaching for recovery of metals and bioremediation of fuel ash and sewage sludge. *Bioresour. Technol.* 261, 428–440. <https://doi.org/10.1016/j.biortech.2018.04.033>
- Hallmann, R., Friedrich, A., Koops, H., Pommerening-Röser, A., Rohde, K., Zenneck, C., Sand, W., 1992. Physiological characteristics of *thiobacillus ferrooxidans* and *leptospirillum ferrooxidans* and physicochemical factors influence microbial metal leaching. *Geomicrobiol. J.* 10, 193–206. <https://doi.org/10.1080/01490459209377920>
- Hatje, V., Pedreira, R.M.A., De Rezende, C.E., Schettini, C.A.F., De Souza, G.C., Marin, D.C., Hackspacher, P.C., 2017. The environmental impacts of one of the largest tailing dam failures worldwide. *Sci. Rep.* 7, 1–13. <https://doi.org/10.1038/s41598-017-11143-x>
- Hocheng, H., Su, C., Jadhav, U.U., 2014. Bioleaching of metals from steel slag by *Acidithiobacillus thiooxidans* culture supernatant. *Chemosphere* 117, 652–657. <https://doi.org/10.1016/j.chemosphere.2014.09.089>
- Hoornweg, D., Bhada-Tata, P., 2012. What a Waste : A Global Review of Solid Waste Management. *Urban Dev. Ser. Knowl. Pap.* 29–43. <https://doi.org/10.1201/9781315593173-4>
- Horeh, N.B., Mousavi, S.M., Shojaosadati, S.A., 2016. Bioleaching of valuable metals from spent lithium-ion mobile phone batteries using *Aspergillus Niger*. *J. Power*



- Sources. <https://doi.org/10.1016/j.jpowsour.2016.04.104>
- Huang, L., Zhou, Q., Quan, X., 2018. Recovery of metals from wastes using bioelectrochemical systems, *Bioelectrochemistry Stimulated Environmental Remediation: From Bioelectrorespiration to Bioelectrodegradation*. [https://doi.org/10.1007/978-981-10-8542-0\\_6](https://doi.org/10.1007/978-981-10-8542-0_6)
- Huang, T., Li, D., 2014. Presentation on mechanisms and applications of chalcopyrite and pyrite bioleaching in biohydrometallurgy - A presentation. *Biotechnol. Reports* 4, 107–119. <https://doi.org/10.1016/j.btre.2014.09.003>
- Hubau, A., Minier, M., Chagnes, A., Jouliau, C., Perez, C., Guezennec, A.G., 2018. Continuous production of a biogenic ferric iron lixiviant for the bioleaching of printed circuit boards (PCBs). *Hydrometallurgy* 180, 180–191. <https://doi.org/10.1016/j.hydromet.2018.07.001>
- Ilyas, S., Chi, R.A., Lee, J.C., 2013. Fungal bioleaching of metals from mine tailing. *Miner. Process. Extr. Metall. Rev.* 34, 185–194. <https://doi.org/10.1080/08827508.2011.623751>
- International Aluminum Institute, 2021. Aluminium Recycling Factsheet.
- International Aluminum Institute, 2009. Global Aluminium Recycling : A Cornerstone of Sustainable Development. *Int. Alum. Inst.* 1–36.
- Kaiser, K.M.A., 2020. Recycling of multilayer packaging using a reversible cross-linking adhesive. *J. Appl. Polym. Sci.* 137, 1–12. <https://doi.org/10.1002/app.49230>
- Kerr, R.A., 2014. The coming copper peak. *Science* (80-. ). 343, 722–724. <https://doi.org/10.1126/science.343.6172.722>
- Kitula, A.G.N., 2006. The environmental and socio-economic impacts of mining on local livelihoods in Tanzania: A case study of Geita District. *J. Clean. Prod.* 14, 405–414. <https://doi.org/10.1016/j.jclepro.2004.01.012>
- Kremser, K., Gerl, P., Pellis, A., Guebitz, G.M., 2021a. A new bioleaching strategy for the selective recovery of aluminum from multi-layer beverage cans. *Waste Manag.* 120, 16–24. <https://doi.org/10.1016/j.wasman.2020.11.012>
- Kremser, K., Thallner, S., Schoen, H., Weiss, S., Hemmelmaier, C., Schnitzhofer, W.,

- Aldrian, A., Guebitz, G.M., 2020. Stirred-tank and heap-bioleaching of shredder-light-fractions (SLF) by acidophilic bacteria. *Hydrometallurgy* 193, 105315. <https://doi.org/10.1016/j.hydromet.2020.105315>
- Kremser, K., Thallner, S., Strbik, D., Spiess, S., Kucera, J., Vaculovic, T., Vsiansky, D., Haberbauer, M., Mandl, M., Guebitz, G.M., 2021b. Leachability of metals from waste incineration residues by iron- and sulfur-oxidizing bacteria. *J. Environ. Manage.* 280, 111734. <https://doi.org/10.1016/j.jenvman.2020.111734>
- Lei, T., Shu, J., Deng, Y., Hu, L., Chen, S., Chen, M., Huang, W., 2021. Enhanced recovery of copper from reclaimed copper smelting fly ash via leaching and electrowinning processes. *Sep. Purif. Technol.* 273, 118943. <https://doi.org/10.1016/j.seppur.2021.118943>
- Li, H., Ye, M., Zheng, L., Xu, Y., Sun, S., Du, Q., Zhong, Y., Ye, S., Zhang, D., 2017. Optimization of kinetics and operating parameters for the bioleaching of heavy metals from sewage sludge, using co-inoculation of two *Acidithiobacillus* species. *Water Sci. Technol.* 2017, 390–403. <https://doi.org/10.2166/wst.2018.167>
- Li, J., Yang, H., Tong, L., Sand, W., 2021. Some Aspects of Industrial Heap Bioleaching Technology: From Basics to Practice. *Miner. Process. Extr. Metall. Rev.* 00, 1–19. <https://doi.org/10.1080/08827508.2021.1893720>
- Luo, H., Liu, G., Zhang, R., Bai, Y., Fu, S., Hou, Y., 2014. Heavy metal recovery combined with H<sub>2</sub> production from artificial acid mine drainage using the microbial electrolysis cell. *J. Hazard. Mater.* 270, 153–159. <https://doi.org/10.1016/j.jhazmat.2014.01.050>
- Mäkinen, J., Salo, M., Soini, J., Kinnunen, P., 2019. Laboratory Scale Investigations on Heap (Bio)leaching of Municipal Solid Waste Incineration Bottom Ash. *Minerals* 9, 290. <https://doi.org/10.3390/min9050290>
- Mangold, S., Rao Jonna, V., Dopson, M., 2013. Response of *Acidithiobacillus caldus* toward suboptimal pH conditions. *Extremophiles* 17, 689–696. <https://doi.org/10.1007/s00792-013-0553-5>
- Mangold, S., Valdés, J., Holmes, D.S., Dopson, M., 2011. Sulfur metabolism in the extreme acidophile *Acidithiobacillus caldus*. *Front. Microbiol.* 2, 1–18.

<https://doi.org/10.3389/fmicb.2011.00017>

Margarido, F., Nogueira, C.A., 2011. Characterisation of automotive shredder residue 1–5.

Marra, A., Cesaro, A., Rene, E.R., Belgiorno, V., Lens, P.N.L., 2018. Bioleaching of metals from WEEE shredding dust. *J. Environ. Manage.* 210, 180–190.  
<https://doi.org/10.1016/j.jenvman.2017.12.066>

Milačič, R., Zuliani, T., Ščančar, J., 2012. Environmental impact of toxic elements in red mud studied by fractionation and speciation procedures. *Sci. Total Environ.* 426, 359–365. <https://doi.org/10.1016/j.scitotenv.2012.03.080>

Mishra, D., Rhee, Y.-H., 2010. Current research trends of microbiological leaching for metal recovery from industrial wastes. *Curr Res Technol Educ Top. Appl Microbiol Microb Biotechnol* 2, 1289–1292.

Moats, M., Free, M., 2007. A bright future for copper electrowinning. *Jom* 59, 34–36.  
<https://doi.org/10.1007/s11837-007-0128-y>

Mohr, S., Giurco, D., Retamal, M., Mason, L., Mudd, G., 2018. Global projection of lead-zinc supply from known resources. *Resources* 7.  
<https://doi.org/10.3390/resources7010017>

Nancharaiah, Y. V., Venkata Mohan, S., Lens, P.N.L., 2015. Metals removal and recovery in bioelectrochemical systems: A review. *Bioresour. Technol.* 195, 102–114. <https://doi.org/10.1016/j.biortech.2015.06.058>

Northey, S., Mohr, S., Mudd, G.M., Weng, Z., Giurco, D., 2014. Modelling future copper ore grade decline based on a detailed assessment of copper resources and mining. *Resour. Conserv. Recycl.* 83, 190–201.  
<https://doi.org/10.1016/j.resconrec.2013.10.005>

Pathak, A., Dastidar, M.G., Sreekrishnan, T.R., 2009. Bioleaching of heavy metals from sewage sludge: A review. *J. Environ. Manage.* 90, 2343–2353.  
<https://doi.org/10.1016/j.jenvman.2008.11.005>

Petersen, J., 2010. Modelling of bioleach processes: Connection between science and engineering. *Hydrometallurgy* 104, 404–409.  
<https://doi.org/10.1016/j.hydromet.2010.02.023>

- Priyadarshane, M., Das, S., 2021. Biosorption and removal of toxic heavy metals by metal tolerating bacteria for bioremediation of metal contamination: A comprehensive review. *J. Environ. Chem. Eng.* 9, 104686.  
<https://doi.org/10.1016/j.jece.2020.104686>
- Quatrini, R., Johnson, D.B., 2018. Microbiomes in extremely acidic environments: functionalities and interactions that allow survival and growth of prokaryotes at low pH. *Curr. Opin. Microbiol.* 43, 139–147.  
<https://doi.org/10.1016/j.mib.2018.01.011>
- Rawlings, D.E., 2002. Heavy metal mining using microbes. *Annu. Rev. Microbiol.* 56, 65–91. <https://doi.org/10.1146/annurev.micro.56.012302.161052>
- Rene, E.R., Sahinkaya, E., Lewis, A., Lens, P.N.L., 2017. Sustainable heavy metal remediation Volume 2: Case studies. <https://doi.org/10.1007/978-3-319-58622-9>
- Robalds, A., Naja, G.M., Klavins, M., 2016. Highlighting inconsistencies regarding metal biosorption. *J. Hazard. Mater.* 304, 553–556.  
<https://doi.org/10.1016/j.jhazmat.2015.10.042>
- Rohwerder, T., Gehrke, T., Kinzler, K., Sand, W., 2003. Bioleaching review part A: *Appl. Microbiol. Biotechnol.* 63, 239–248. <https://doi.org/10.1007/s00253-003-1448-7>
- Roshani, M., Shojaosadati, S.A., Safdari, S.J., Vasheghani-Farahani, E., Mirjalili, K., Manafi, Z., 2017. Bioleaching of molybdenum by two new thermophilic strains isolated and characterized. *Iran. J. Chem. Chem. Eng.* 36, 183–194.
- Ruan, R., Liu, X., Zou, G., Chen, J., Wen, J., Wang, D., 2011. Industrial practice of a distinct bioleaching system operated at low pH, high ferric concentration, elevated temperature and low redox potential for secondary copper sulfide. *Hydrometallurgy* 108, 130–135. <https://doi.org/10.1016/j.hydromet.2011.03.008>
- Silva, L.B., Comini, I.B., Alves, E.B.B.M., da Rocha, S.J.S.S., Jacovine, L.A.G., 2021. Compensating the negative environmental impacts of mining with financial mechanisms in Brazil. *Land use policy* 104.  
<https://doi.org/10.1016/j.landusepol.2021.105351>
- Spiess, S., Kucera, J., Seelajaroen, H., Sasiain, A., Thallner, S., Kremser, K., Novak, D., Guebitz, G.M., Haberbauer, M., 2021. Impact of carbon felt electrode

- pretreatment on anodic biofilm composition in microbial electrolysis cells. *Biosensors* 11. <https://doi.org/10.3390/bios11060170>
- Srichandan, H., Kumar, R., Kumar, P., 2019. Hydrometallurgy Bioleaching approach for extraction of metal values from secondary solid wastes : A critical review. *Hydrometallurgy* 189, 105122. <https://doi.org/10.1016/j.hydromet.2019.105122>
- Statista, 2021. Global aluminium consumption from 2016 to 2023 [WWW Document]. URL <https://www.statista.com/statistics/863681/global-aluminum-consumption/>
- Steffen, W., Broadgate, W., Deutsch, L., Gaffney, O., Ludwig, C., 2015. The trajectory of the anthropocene: The great acceleration. *Anthr. Rev.* 2, 81–98. <https://doi.org/10.1177/2053019614564785>
- Tong, L., Zhao, Q., Kamali, A.R., Sand, W., Yang, H., 2020. Effect of graphite on copper bioleaching from waste printed circuit boards. *Minerals* 10, 1–10. <https://doi.org/10.3390/min10010079>
- Valdés, J., Pedroso, I., Quatrini, R., Dodson, R.J., Tettelin, H., Blake, R., Eisen, J.A., Holmes, D.S., 2008. *Acidithiobacillus ferrooxidans* metabolism: From genome sequence to industrial applications. *BMC Genomics* 9, 1–24. <https://doi.org/10.1186/1471-2164-9-597>
- Vidal, O., Rostom, F., François, C., Giraud, G., 2017. Global trends in metal consumption and supply: The raw material-energy nexus. *Elements* 13, 319–324. <https://doi.org/10.2138/gselements.13.5.319>
- Wang, R., Lin, Jian Qiang, Liu, X.M., Pang, X., Zhang, C.J., Yang, C.L., Gao, X.Y., Lin, C.M., Li, Y.Q., Li, Y., Lin, Jian Qun, Chen, L.X., 2019. Sulfur oxidation in the acidophilic autotrophic *Acidithiobacillus* spp. *Front. Microbiol.* 10, 1–20. <https://doi.org/10.3389/fmicb.2018.03290>
- Wang, Z., He, Z., 2020. Frontier review on metal removal in bioelectrochemical systems: mechanisms, performance, and perspectives. *J. Hazard. Mater. Lett.* 1, 100002. <https://doi.org/10.1016/j.hazl.2020.100002>
- Wei, X., Viadero, R.C., Buzby, K.M., 2005. Recovery of iron and aluminum from acid mine drainage by selective precipitation. *Environ. Eng. Sci.* 22, 745–755. <https://doi.org/10.1089/ees.2005.22.745>

- Wiedmann, T.O., Schandl, H., Lenzen, M., Moran, D., Suh, S., West, J., Kanemoto, K., 2015. The material footprint of nations. *Proc. Natl. Acad. Sci. U. S. A.* 112, 6271–6276. <https://doi.org/10.1073/pnas.1220362110>
- Xiang, Y., Wu, P., Zhu, N., Zhang, T., Liu, W., Wu, J., Li, P., 2010. Bioleaching of copper from waste printed circuit boards by bacterial consortium enriched from acid mine drainage. *J. Hazard. Mater.* 184, 812–818. <https://doi.org/10.1016/j.jhazmat.2010.08.113>
- Yin, W., Chen, K., 2021. Effect of the particle size and microstructure characteristics of the sample from HPGR on column bioleaching of agglomerated copper ore. *Hydrometallurgy* 200, 105563. <https://doi.org/10.1016/j.hydromet.2021.105563>
- Zawadiak, J., 2017. Tetra Pak Recycling – Current Trends and New Developments. *Am. J. Chem. Eng.* 5, 37. <https://doi.org/10.11648/j.ajche.20170503.12>
- Zhan, Y., Yang, M., Zhang, S., Zhao, D., Duan, J., Wang, W., Yan, L., 2019. Iron and sulfur oxidation pathways of *Acidithiobacillus ferrooxidans*. *World J. Microbiol. Biotechnol.* 35, 1–12. <https://doi.org/10.1007/s11274-019-2632-y>

### 3. Leachability of metals from waste incineration residues by iron-and sulfur oxidizing bacteria

Published in: Journal of Environmental Management, 15 February 2021, Volume 280  
<https://doi.org/10.1016/j.jenvman.2020.111734>

**Klemens Kremser<sup>a</sup>, Sophie Thallner<sup>b</sup>, Dorina Strbik<sup>a</sup>, Sabine Spiess<sup>b</sup>, Jiri Kucera<sup>c</sup>, Tomas Vaculovic<sup>d</sup>, Dalibor Vsiansky<sup>e</sup>, Marianne Haberbauer<sup>b</sup>, Martin Mandl<sup>c</sup>, Georg M. Guebitz<sup>a\*</sup>**

<sup>a</sup>University of Natural Resources and Life Sciences Vienna BOKU, Dept. of Agrobiotechnology, IFA-Tulln, Inst. of Environmental Biotechnology, Konrad-Lorenz-Straße 20, 3430 Tulln and der Donau, Austria

<sup>b</sup>K1-MET GmbH, Stahlstraße 14, 4020 Linz, Austria

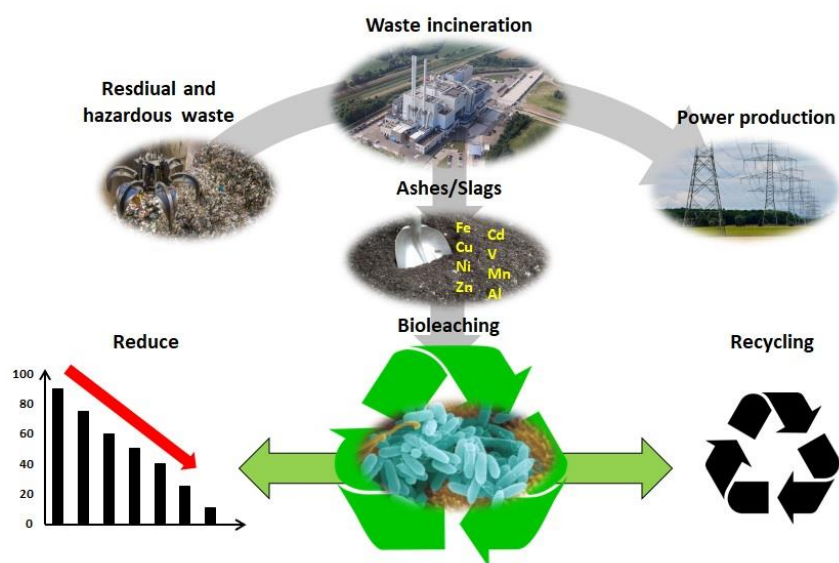
<sup>c</sup>Department of Biochemistry, Faculty of Science, Masaryk University, Kotlarska 2, 61137 Brno, Czech Republic

<sup>d</sup>Department of Chemistry, Faculty of Science, Masaryk University, Kotlarska 2, 61137 Brno, Czech Republic

<sup>e</sup>Department of Geological Sciences, Faculty of Science, Masaryk University, Brno, Czech Republic

**Keywords:** Waste incineration residues, artificial metal ore, bioleaching, metal recovery, iron- and sulfur-oxidizing acidophiles

## Graphical abstract



### 3.1. Abstract

Hazardous waste disposal via incineration generates a substantial amount of ashes and slags which pose an environmental risk due to their toxicity. Currently, these residues are deposited in landfills with loss of potentially recyclable raw material. In this study, the use of acidophilic bioleaching bacteria (*Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*, and *Leptospirillum ferrooxidans*) as an environmentally friendly, efficient strategy for the recovery of valuable metals from incineration residues was investigated. Zinc, Cobalt, Copper, and Manganese from three different incineration residues were bio-extracted up to 100% using *A. ferrooxidans* under ferrous iron oxidation. The other metals showed lower leaching efficiencies based on the type of culture used. Sulfur-oxidizing cultures *A. ferrooxidans* and *A. thiooxidans*, containing sulfur as the sole substrate, expressed a significantly lower leaching efficiency (up to 50%). According to ICP-MS, ashes and slags contained Fe, Zn, Cu, Mn, Cr, Cd, and Ni in economically attractive concentrations between 0.2 – 75 mg g<sup>-1</sup>. Compared to conventional hydrometallurgical and pyrometallurgical processes, our biological approach provides many advantages such as: the use of a limited amount



of used strong acids ( $\text{H}_2\text{SO}_4$  or  $\text{HCl}$ ), recycling operations at lower temperatures ( $\sim 30^\circ\text{C}$ ) and no emission of toxic gases during combustion (i.e., dioxins and furans).

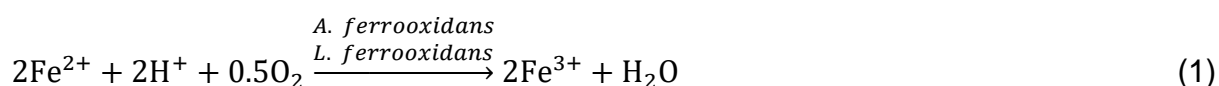
Keywords: Waste incineration residues, artificial metal ore, bioleaching, metal recovery, iron- and sulfur-oxidizing acidophiles

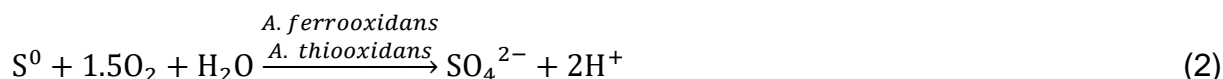
### **3.2. Introduction**

Incineration is one of the most widely applied processes worldwide to reduce the volume and mass of municipal solid waste (MSW). In 2012, the global generation of MSW reached approximately 1.3 billion tons per year. With an increasing world population, the generated waste will further increase, reaching a level of around 2.2 billion tons per year in 2025 (Hoorweg and Bhada-Tata, 2012). Besides material recycling (75%), incineration is still one of the most common waste treatment strategies in the EU, accounting for approximately 70%, followed by landfilling (57%) and composting (43%) (Eurostat Waste Statistics, 2017). In this study, ashes and slags from a state-of-the-art municipal waste incineration plant (MWIP) in Austria (EVN, Dürnröhr) were investigated for microbial metal recovery. Although the waste incineration tackles the principle of waste to energy and reduces the waste volume up to 80% (Wei et al., 2011), there are still residuals like ashes and slags accounting for approximately 80 – 90% of the total residual mass (EVN Abfallverwertung, 2017; Zhu et al., 2018). These residues contain economically attractive concentrations of valuable metals such as Cu, Zn, Cd, Ni, Mn, and others (Table 2). However, due to their heavy metal content, ashes and slags are declared as hazardous waste (European Parliament and Council, 2008), ending most frequently up on landfills and underground disposal sites. Different attempts have been made to prevent these resources from being landfilled by using them, for example, as additives for various construction materials (Blasenbauer et al., 2020; Cristelo et al., 2020; Joseph et al., 2018).

Nevertheless, using ashes and slags in construction still means a loss of valuable resources. Krebs et al. considered these residues as “artificial ores” for the approach of microbial recovery (Krebs et al., 1997).

In this study, the potential of chemo lithotrophic acidophiles to recover metals from waste incineration slags and ashes was investigated. Solubilization of metals by microbial action called “bioleaching” or “biomining” is an already well-established process and finds various applications in mineral industries (Banerjee et al., 2017; Schippers et al., 2013; Sethurajan et al., 2018; Werner et al., 2018). Furthermore, different studies have been made on the applicability of bioleaching to other processes like remediation of contaminated soils (Akinci and Guven, 2011), metal recovery from sewage sludge (Pathak et al., 2009) and other industrial residues (Mishra and Rhee, 2010, 2014; Solisio et al., 2002). The commonly used bioleaching bacteria are extremely acidophilic and mesophilic microorganisms (pH < 3), known to obtain energy by the oxidation of Fe(II), elemental sulfur and reduced inorganic sulfur compounds (RISCs) (Quatrini and Johnson, 2018; Rohwerder et al., 2003). Although sulfates are end products of bacterial oxidation, the combination of the degree of acidity and redox conditions represents a key factor influencing the reaction course and the mechanism of bacterial leaching of sulfide minerals. Thus, the major role of bioleaching microorganisms is the regeneration of Fe<sup>3+</sup> (Eq. 1) and the production of sulfuric acid according to equation 2 or directly from (iron) sulfides (Borilova et al., 2018). Bacterial leaching of metal oxides from solid materials (i.e., ashes and slags) is mainly facilitated by biogenic production of inorganic and organic acids and the secretion of complexing agents (Vestola et al., 2010).





For the first time, three different incineration residues, arising in higher quantities during incineration such as slag, kettle-ash, and filter-ash, were assessed for bioleaching by pure cultures of iron- and sulfur-oxidizing bacteria in this study. The bioleaching efficiency was examined using pure cultures of three bacterial species: iron-oxidizing *Leptospirillum ferrooxidans*, sulfur-oxidizing *Acidithiobacillus thiooxidans*, and iron- and sulfur-oxidizing *Acidithiobacillus ferrooxidans*. The influence of various factors comprising heavy metal content, media composition, sulfur-addition, and cellular adaptation, was evaluated and the positive effect of iron-oxidizing bacteria on metal recovery was demonstrated.

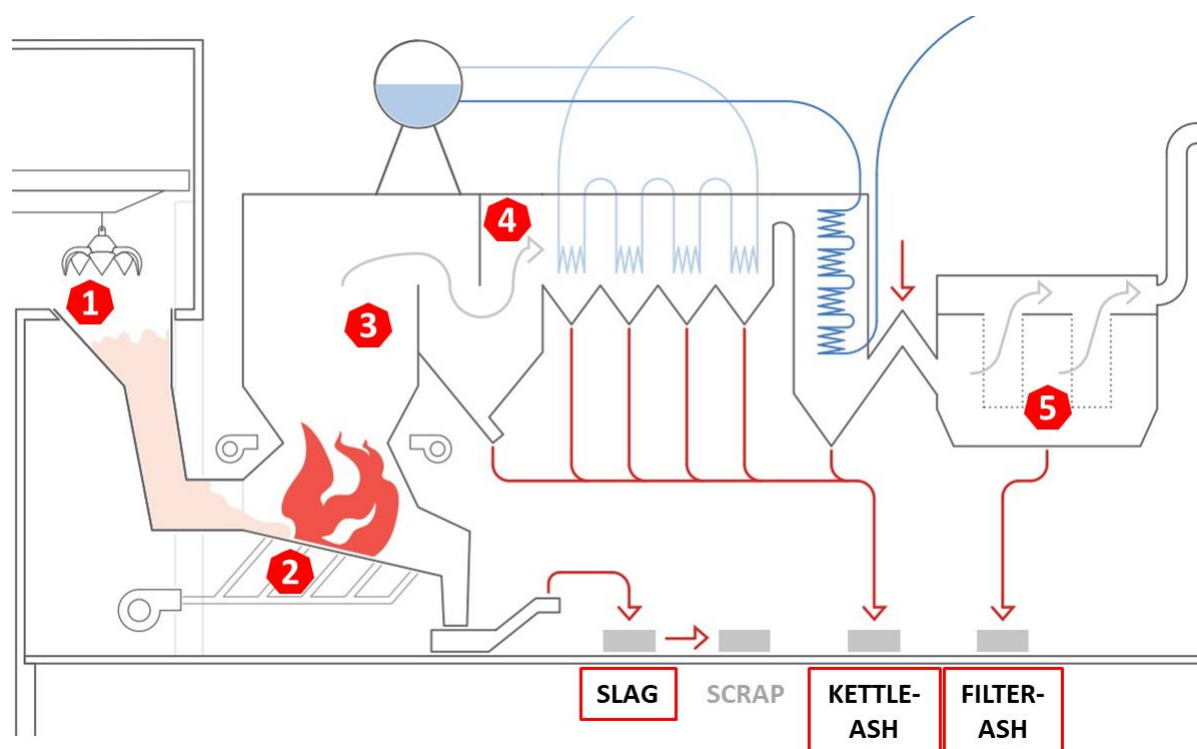
### 3.3. Material and methods

All chemicals were of analytical grade unless otherwise specified and purchased from Sigma-Aldrich (Sigma-Aldrich, Vienna, Austria). Deionized water was used for preparation of cultivation media and stock solutions. Cell number determination was done by counting bacterial cells in a Neubauer improved hemocytometer with 0.01 mm depth (BRAND GmbH, Wertheim, Germany) under an Olympus BX43 optical microscope (Olympus, Hamburg, Germany). For pH measurements, a Mettler Toledo S220 pH meter with a combined glass electrode was used.

#### 3.3.1. Waste incineration ashes and slags

Three different ashes and slags were obtained from a local municipal waste incineration plant (MWIP) operated by the energy producer EVN (EVN Waste Processing, Dürnrohr, Austria). The investigated site shows a capacity of around 500,000 t waste per year. After incineration of residual and hazardous waste, about 1/4 of the initial volume is remaining as so-called kettle-slag. Additionally, around

20 kg t<sup>-1</sup> of ash (kettle-ash) are separated from the off-gas after incineration in the kettle. The third primary residue is filter-ash (around 30 kg t<sup>-1</sup>) arising by a final filtration *via* fabric filter elements (Figure 1) In total, around 300 kg residues per ton of incinerated waste arise, accounting for a total annual residual output of around 150,000 t (EVN Abfallverwertung, 2017). Prior to experiments, all ashes and slags were sieved to reach a particle size of less than 2.8 mm. Especially in case of slag, it was previously reported, that the concentration of extractable heavy metals is increasing within a particle size <2 mm (Abramov et al., 2018). All samples were washed three times in double-distilled water (10% w/v) and filtrated through a Whatman® cellulose filter paper with a pore size of 11 µm to remove water-soluble salts (Wang et al., 2009). Prior to use, ashes and slags were dried at 60 °C for 48 h to remove the remaining water.



**Figure 1:** Process scheme of the municipal waste incineration plant located in Dürnrohr, Austria. The waste is loaded into the incinerator (1) and incinerated in the kettle (2). The exhaust air is passing by a gravity separation step (3 and 4), followed by fabric membrane filtration (5). The main residual fraction (SLAG) is separated after incineration in the kettle, followed by the first filtration step (KETTLE-ASH) and the filtration through fabric filter elements (FILTER-ASH). The exhaust air is finally cleaned through a liquid washing procedure and ammonia (not shown) (EVN Abfallverwertung, 2017).

### 3.3.2. Bacteria and growth conditions

Following bacteria were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) and used in this work; *Acidithiobacillus ferrooxidans* DSM 583, *Leptospirillum ferrooxidans* DSM 2705, and *Acidithiobacillus thiooxidans* DSM 504. Ferrous iron-oxidizing *L. ferrooxidans* was cultivated in DSMZ medium 882 containing 20 g l<sup>-1</sup> of ferrous sulfate heptahydrate with pH adjusted to 1.8 with sulfuric acid. Ferrous iron- and sulfur-oxidizing *A. ferrooxidans* was cultivated either in DSMZ medium 70 containing 33.3 g l<sup>-1</sup> of ferrous sulfate heptahydrate with pH adjusted to 1.4 or in a basal salts medium (Wakeman et al., 2008) containing 10 g l<sup>-1</sup> of elemental sulfur with an initial pH 4.5. Sulfur-oxidizing *A. thiooxidans* was cultured in DSMZ medium 35 containing 10 g l<sup>-1</sup> of elemental sulfur with an initial pH 4.5. Bacteria were cultured at 30 °C in 250-ml Erlenmeyer flasks containing 90 ml of selected media and 10 ml of inoculum with shaking at 130 rpm until cell density of 1 × 10<sup>8</sup> cells ml<sup>-1</sup> was reached.

### 3.3.3. Adaptation of *A. ferrooxidans* to slags and ashes

Ferrous iron- and sulfur-oxidizing *A. ferrooxidans* was adapted to increasing concentration of slag, kettle-ash, and filter-ash in a two-step process prior to the batch tests. First, 10 ml of ferrous iron-grown *A. ferrooxidans* culture was inoculated into 90 ml of the DSMZ 70 medium containing additionally 10 g l<sup>-1</sup> of elemental sulfur and 1 g of ash/slag (1% w/v) in 250-ml Erlenmeyer flasks and incubated at 30 °C with shaking at 130 rpm. After two weeks of incubation, 10 ml culture from the first adaptation step was used to inoculate 90 ml of fresh DSMZ 70 medium containing additionally 10 g l<sup>-1</sup> of elemental sulfur and 2 g of ash/slag (2% w/v) in 250-ml Erlenmeyer flasks. After further two weeks of adaptation, culture from the second adaptation step was used as an inoculum for batch tests.

### 3.3.4. Bioleaching batch tests

Batch tests with selected acidophiles were carried out in biological duplicates as previously described (Kremser et al., 2020a) with some adaptations according initial media pH and substrate addition. Tests were performed in a total of five variants (Table 1 and Figure S1). The overall pulp density in all experiments was 10 g l<sup>-1</sup>. In order to determine the difference between microbial and chemical leaching, one abiotic control (BLANK) containing only medium, was performed in parallel for each mode. Furthermore, one chemical control containing 0.05 M sulfuric acid only (same sulfuric acid concentration as in the used medium) was performed for each incineration residue. All 100-ml batch tests (bacteria and abiotic controls) were allowed to leach in 250-ml Erlenmeyer flasks at 30 °C with shaking at 130 rpm for two weeks. The pH values were measured at beginning and end of each incubation. Aliquots for elemental analysis were collected at the end of the experiment. Prior to elemental analysis and pH measurement, leachates were filtrated through a CHROMAFIL® Xtra Nylon filter (Macherey-Nagel GmbH) with a pore size of 0.45 µm to remove solid particles.

**Table 5.** Setup of batch experiments with different iron- and sulfur-oxidizing bacteria

Organism	Initial pH	FeSO <sub>4</sub>	S <sup>0</sup>	Inoculum [% v/v]	Pulp density [g l <sup>-1</sup> ]
<i>L. ferrooxidans</i>	1.8	+	-	10	10
<i>A. ferrooxidans</i>	1.4	+	-	10	10
<i>A. ferrooxidans</i>	1.4	+	+	10	10
<i>A. ferrooxidans</i>	4.5	-	+	10	10
<i>A. thiooxidans</i>	4.5	-	+	10	10

\*A more detailed description of bath experiments is shown in the supplementary material (Fig. S1)

### 3.3.5. Sample characterization

For ICP-MS analysis, samples of ashes and sludges were dried, grinded, and homogenized prior to decomposition. To determine elements in slags and ashes, samples were decomposed by a four-step procedure. 1 g of each sample was leached in a mixture containing nitric acid and hydrogen peroxide and evaporated to dryness. Next, samples were leached in hydrofluoric acid and evaporated to dryness, followed by leaching in perchloric acid and evaporation to dryness. Finally, samples were leached in hydrogen chloride and evaporation to dryness. Decomposed samples were afterwards analyzed using inductively coupled plasma mass spectrometry (ICP-MS). To minimize the matrix effect and to get the best LOD, all samples were diluted with MiliQ water by factor 100, and a solution of Sc ( $400 \mu\text{g l}^{-1}$ ) was used as internal standard before analysis. Equally, microbial leachates were diluted with MiliQ water by factor 100 before ICP-MS analysis. Sc, as internal standard ( $400 \mu\text{g l}^{-1}$ ), was used to suppress the possible matrix effect. For quantification, a set of calibration solutions was prepared.

X-ray diffraction analysis was used to determine mineral phases of the used incineration residues. Samples were pulverized in a Retsch PM 100 planetary ball mill with agate grinding jar and balls and homogenized with 10 wt.% of zincite (ZnO). Zincite was used as an internal standard for the quantification of the amorphous phase. Powder X-ray diffraction (XRD) analysis was conducted using a Panalytical X'Pert PRO MPD diffractometer with reflection geometry equipped with a cobalt tube ( $\lambda\text{K}\alpha = 0.17903 \text{ nm}$ ), Fe filter, and 1-D RMTS (X'Celerator) detector. Step size:  $0.017^\circ 2\theta$ , time per step: 320 s, angular range:  $4 - 100^\circ 2\theta$ , total scan duration: 247 min. Acquired data were processed using Panalytical HighScore 4 plus and Bruker AXS Topas 4 software. Quantitative phase analysis was done by the Rietveld method.

To determine the particle size distribution in the slag, a separation using a stacked sieve tower was performed in order to fractionate the material into 5 fractions (I: >8.0 mm, II: 2.8 – 8.0 mm, III: 1.4 – 2.8 mm, IV: 1.0 – 1.4 mm and V: <1.0 mm).

### 3.3.6. Calculation of the leaching efficiency

Leaching efficiency was calculated by the ratio of the metal concentration in the leachate to the metal content of the untreated ash/slag. To minimize the effect of trace amounts of dissolved metals included in culture media and inoculum, leaching efficiency calculation equation (3) was modified as follows:

$$L_{eff} [\%] = \frac{((c_L - c_M) * V_B) - (c_C * V_C)}{c_F * M_F} * 100 \quad (3)$$

where  $c_L$  is the metal concentration in the leachate,  $c_M$  is the metal concentration in the culture media,  $V_B$  is total batch volume,  $c_C$  is the metal concentration in the inoculum,  $V_C$  is the inoculum volume,  $c_F$  metal concentration in the untreated ash/slag, and  $M_F$  is total mass used for the leaching experiments.

### 3.3.7. Statistical evaluation

Results of the bioleaching efficiency under different experimental conditions were evaluated using a t-test. Differences between corresponding groups were detected based on duplicates. A significance level of 0.05 was used to evaluate significance of differences. To characterize a dominant effect of a defined culture and compare it with another one, the following majority criterion was applied: a significantly higher leaching effect of one bacteria culture is evident if the leaching efficiency for 4 out of 7 metals, or 5 out of 8 metals in the case of all waste materials was higher compared to another culture or blank.



### **3.4. Results and discussion**

#### **3.4.1. Characterization of MWIP ashes and slags**

In a first step, the particle size distribution of the coarse slag was measured resulting in a distribution of 0.42% (Phase I), 26.34% (Phase II), 35.46% (Phase III), 15.07% (Phase IV) and 22.71% (Phase V). As mentioned in section 2.1, more extractable heavy metals are present in slag particles <2 mm. Therefore, all fractions <2.8 mm (73.2% of the total material) were used for metal analysis and bioleaching experiments. The chemical composition of the tested slag and the two ashes was measured via ICP-MS and revealed notable differences in metal content (Table 2). The predominant metal in the slag was Fe with a content of around 76 mg g<sup>-1</sup>. Other precious metals such as Zn, Cu, and Ni were found in contents ranging from 0.4 – 6.0 mg g<sup>-1</sup>. More toxic heavy metals such as Pb, Mn, and Cd were present in lower amounts (0.01 – 1.77 mg g<sup>-1</sup>) compared to both types of ashes. Fe content was decreasing with every gravity separation and membrane filtration step (76 > 40 > 30 mg g<sup>-1</sup>) resulting in the lowest concentration in the final filter-ash. A different trend can be observed on heavy metals such as Cu, Zn, Pb, and Cd. The content of these metals was increasing over the filtration process, reaching its maximum of around 8.9, 61.8, 15.2, and 0.7 mg g<sup>-1</sup> for Cu, Zn, Pb, and Cd, respectively. The content of Fe and Cd in both ashes was in agreement with previous studies on waste incineration fly ashes, whereas concentrations of Cu, Zn, and Pb were significantly higher for the tested filter-ash (Krebs et al., 1999; Wang et al., 2009).

**Table 2.** Metal content in three different MWIP substrate types determined by ICP-MS analysis.

Element	SLAG	KETTLE-ASH	FILTER-ASH
	$c_F \pm SD$ (mg g <sup>-1</sup> )	$c_F \pm SD$ (mg g <sup>-1</sup> )	$c_F \pm SD$ (mg g <sup>-1</sup> )
<b>Fe</b>	75.8 ± 1.3	39.01 ± 0.91	29.77 ± 0.72
<b>V</b>	0.058 ± 0.001	0.064 ± 0.002	0.027 ± 0.001
<b>Cr</b>	0.744 ± 0.015	1.105 ± 0.097	0.365 ± 0.012
<b>Mn</b>	1.766 ± 0.055	2.762 ± 0.077	1.201 ± 0.042
<b>Co</b>	0.148 ± 0.004	0.063 ± 0.002	0.061 ± 0.002
<b>Ni</b>	0.401 ± 0.010	0.291 ± 0.009	0.219 ± 0.005
<b>Cu</b>	5.81 ± 0.11	2.32 ± 0.06	8.89 ± 0.18
<b>Zn</b>	5.98 ± 0.10	24.32 ± 0.54	61.8 ± 1.6
<b>Pb</b>	1.12 ± 0.03	5.72 ± 0.24	15.2 ± 0.4
<b>Sb</b>	0.393 ± 0.007	2.759 ± 0.047	5.296 ± 0.069
<b>Cd</b>	0.007 ± 0.008	0.169 ± 0.005	0.669 ± 0.013

\*Note:  $c_F$  indicates metal concentration; SD means standard deviation (n = 3).

In incineration ashes and slags, metals are mainly present in their oxide forms (Funari et al., 2017; Wang et al., 2009), which was also proved by phase analysis. Mineral phase analysis revealed that minerals like quartz, hematite, calcite, magnetite, anhydrite, akermanite, and gehlenite are the most abandoned amongst all three different residues but the concentration of the different minerals differs within the residues (Table 3 and Figure S2). The results obtained are in arrangement with previous studies on mineral phases in waste incineration residues (Abramov et al., 2018).

**Table 3.** XRD mineral phase analysis of the three waste incineration residues.

Mineral name	Idealized chemical formula	SLAG	KETTLE-ASH	FILTER-ASH
[wt. %]				
<b>Quartz</b>	SiO <sub>2</sub>	8.3	1.5	1.4
Cristobalite	SiO <sub>3</sub>			1
<b>Akermanite</b>	Ca <sub>2</sub> MgSi <sub>2</sub> O <sub>7</sub>	2.3		3.8
<b>Gehlenite</b>	Ca <sub>2</sub> Al(AlSi)O <sub>7</sub>	0.8	3.2	6.2
<b>Magnetite</b>	Fe <sub>3</sub> O <sub>4</sub>	3.7		0.6
Maghemite	γ-Fe <sub>2</sub> O <sub>3</sub>		1.7	0.2
<b>Hematite</b>	α-Fe <sub>2</sub> O <sub>3</sub>	0.7	3.3	2.3
Clinopyroxene	(Ca,Na)(Mg,Fe,Al,Ti)(Si,Al) <sub>2</sub> O <sub>6</sub>	1.5		
Ferrosilicon	FeSi		1.2	
Alkali feldspar	(Na,K)AlSi <sub>3</sub> O <sub>8</sub>	1.2	2.5	
Larnite	β-Ca <sub>2</sub> SiO <sub>4</sub>	7.3		
Sylvite	KCl		1.6	2.3
Halite	NaCl			15.8
<b>Anhydrite</b>	CaSO <sub>4</sub>	0.6	13.5	5.1
Bassanite	2CaSO <sub>4</sub> ·H <sub>2</sub> O		4.9	
Gypsum	CaSO <sub>4</sub> ·2(H <sub>2</sub> O)	1.3		
Ettringite	Ca <sub>6</sub> Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> (OH) <sub>12</sub> ·26(H <sub>2</sub> O)	0.2		
Salammoniac	NH <sub>4</sub> Cl		1.5	1.3
<b>Calcite</b>	CaCO <sub>3</sub>	7.2	5.4	4.2
Amorphous		64.9	59.8	55.8

\*Minerals in higher concentrations are written in bold letters

### 3.4.2. Bioleaching efficiency – Effect of adaptation and sulfur addition

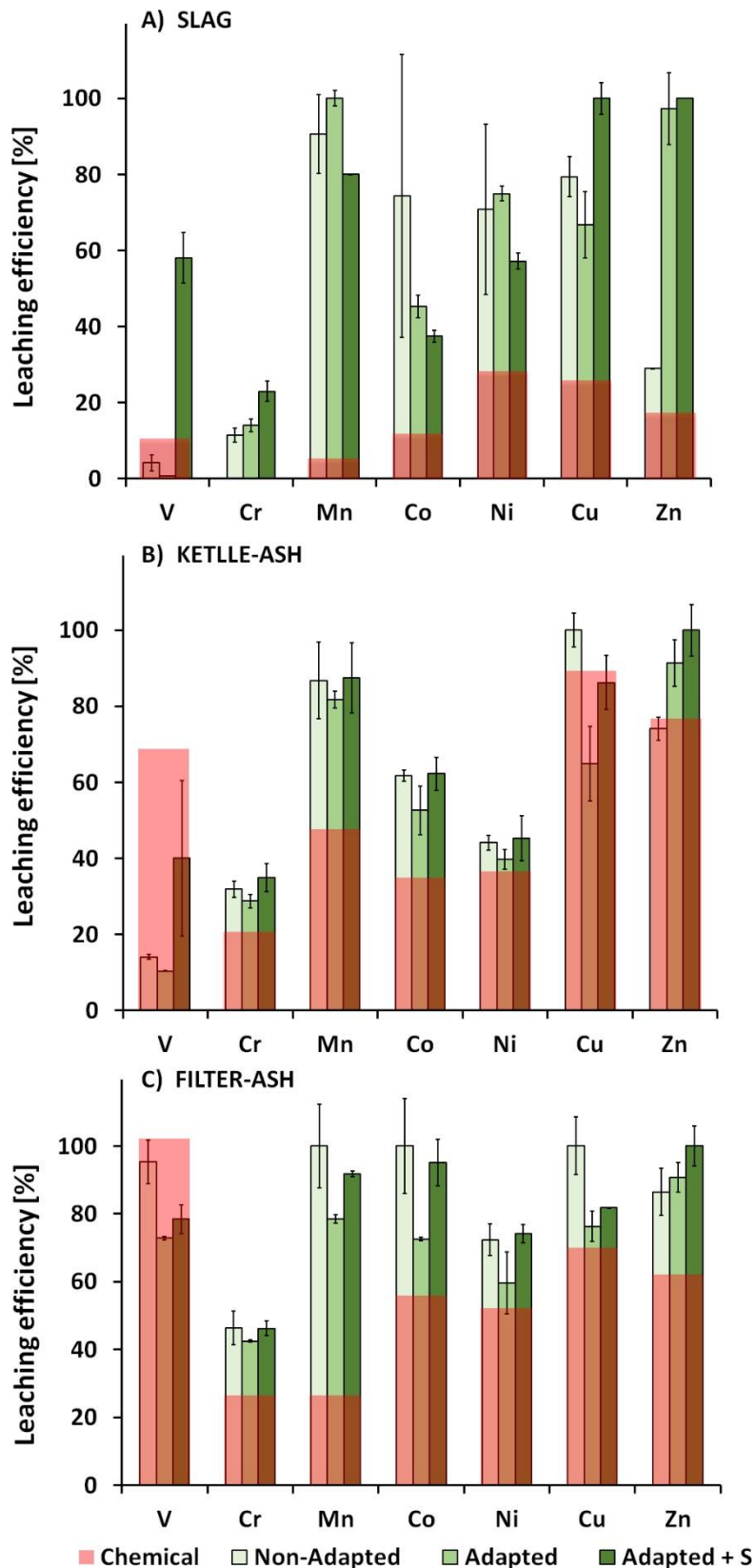
In order to test the effect of adaptation to ashes and slags on the bioleaching efficiency, *A. ferrooxidans* was gradually exposed to increasing MWIP substrate concentration (up to 2% w/v). During batch tests in DSMZ medium 70, the leaching efficiency was compared between the adapted culture, the same adapted culture supplemented with 10 g l<sup>-1</sup> of elemental sulfur, and a non-adapted culture. Bioleaching of the slag indicated a higher efficiency in some cases using the adapted *A. ferrooxidans* cultures supplemented with elemental sulfur, in addition to ferrous iron (Figure 2A). Metals such as V, Cu, and Zn seemed to be leached more efficiently (up to 100%). Within experimental error (using the t-test for the culture pairs), no significant differences between adapted and non-adapted *A. ferrooxidans* cultures were observed. Furthermore, the difference between the adapted *A. ferrooxidans* culture

supplemented with elemental sulfur (in addition to ferrous iron) and any other non-adapted iron-oxidizing cultures was only insignificant ( $P > 0.05$ ). This was detected in all tested waste materials (Fig. 2 A - C) and indicated no effect of both adaptation and elemental sulfur addition.

It is apparent that sulfur-oxidation provided an additional energy source and acidifies the culture, which should be positive for metal extraction. The effect of sulfur addition is evident by comparing the resulting pH of the cultures at the end of the two-week incubation (Table S1). However, all the variants (including the controls) were adjusted at the beginning for acid pH and these acid values were spontaneously kept, although alkalization may be observed in case of the controls (pH 2.2 – 2.8). Under studied conditions, there was no significant difference between iron-oxidizing cultures ( $P > 0.05$ ). In addition to above mentioned majority criterion to evaluate a dominant culture effect, the bacterial adaptation facilitated a more effective bioleaching of Mn and Ni independent of the elemental sulfur supplementation. High standard deviations in the leaching efficiency of some metals most likely result from the inhomogeneity and the relatively low concentration of coarse slag. With an increasing concentration, the negative impact of this effect should decrease. Zn and V could be leached from kettle-ash to a higher efficiency (~100 and 40%, respectively) by the adapted culture supplemented with elemental sulfur. The same trend could be observed for Zn in bioleaching of the filter ash (Figure 2C).

By comparing the leaching efficiencies of the different metals for all three types of MWIP substrates, filter-ash showed the highest recovery rates for all tested metals, followed by kettle-ash and slag. One explanation for the differences in leaching efficiency might be the particle sizes of the materials. Ash particles are tiny (less than 1 mm), resulting in an increased surface area per unit volume for both chemical

and enzymatic reactions. An increased leaching efficiency of the blank for most of the tested metals compared to chemical leaching indicates that leaching in medium at low pH in combination with ferrous-iron leads to a more effective metal extraction.



**Figure 2:** Bioleaching of incineration residues with *A. ferrooxidans* – efficiencies for three types of residues, namely SLAG (A), KETTLE- (B), and FILTER-ASH (C). The amount of chemical leaching related to the sulfuric acid in the medium (red) is shown together with the three different batch tests comprising non-adapted (light-green), adapted (green), and adapted with elemental sulfur addition (dark-green). Error bars indicating the standard deviation of the biological duplicates.

### 3.4.3. Bioleaching efficiency

Metal recovery batch experiments were performed in various media with all MWIP substrate types using the following bacteria *A. ferrooxidans*, *L. ferrooxidans*, and *A. thiooxidans*. pH values for the chemical (medium only) and biological leaching experiments were monitored, and leaching efficiencies were compared based on observed metal concentrations.

#### 3.4.3.1. *Leptospirillum ferrooxidans*

Exclusively ferrous iron-oxidizing bacterium *L. ferrooxidans* was tested in a medium containing ferrous sulfate as the only energy source. The medium was acidified with sulfuric acid to ensure the stability of soluble ferrous iron, as in the case of *A. ferrooxidans* in the above-mentioned iron-containing medium. Therefore, chemical leaching of abiotic controls expectedly increased. *L. ferrooxidans* successfully leached Mn and Cu up to 100% of all three MWIP substrate types (Figure 3A). Leaching efficiencies of Zn, Ni, and Cr were lower compared to *A. ferrooxidans*, and only a little difference between chemical and biological leaching was found. According the significance criteria mentioned in chapter 3.2., leaching efficiency of *L. ferrooxidans* was only insignificantly different compared to the other iron-oxidizing bacterium *A. ferrooxidans*.

The initial low pH of the medium did not result in a significant increase in pH of abiotic controls (~2.6 – 3.4), which facilitated metal solubilization also in absence of bacteria (Figure 5). *L. ferrooxidans* lacks the ability of metabolizing RISCs (Hallmann et al., 1992), leading to an elevated pH value as a result of the oxidation of ferrous iron to ferric iron. Therefore, maximum values after two weeks between 2.1 – 2.3 were reached. Although iron oxidation results in increasing of pH, a subsequent  $\text{Fe}^{3+}$  hydrolysis decreases pH. Thus, some acid solution is kept, in contrast to blank or

chemical leaching, although 2.1 – 2.3 is higher than the pH of cultures with elemental sulfur.

#### 3.4.3.2. *Acidithiobacillus ferrooxidans*

According to before mentioned significance criteria, biological leaching by *A. ferrooxidans* in medium containing both ferrous iron and elemental sulfur was significantly higher compared to both set-ups containing elemental sulfur only. Leaching efficiencies reached up to 100% for Mn, Co, Cu, and Zn, respectively (Figures 3B and S3). Due to the presence of sulfuric acid in the medium (0.05 M H<sub>2</sub>SO<sub>4</sub>), chemical leaching for most of the metals also showed good efficiencies. Most likely, Fe(II) in the medium is chemically oxidized by either atmospheric oxygen or by the incineration residues to Fe(III), which contributes to a higher solubility of other metals in an acidic metal-rich environment. It can also be observed by comparing the pH after two-week leaching (Figure 4). pH of abiotic controls is comparatively low (~ 2.2 – 2.9) but still higher compared to bacterial cultures supplemented with ferrous iron and elemental sulfur (~1.6 – 1.8).

Bioleaching efficiency of *A. ferrooxidans* was further tested using elemental sulfur as only energy source. Under these conditions, bacteria oxidized elemental sulfur to produce sulfuric acid. Biological leaching efficiencies of V, Mn, Cu, and Zn reached up to 50%, whereas chemical leaching was not detectable (Figure S3). Compared to other iron-oxidizing cultures, under studied short-term conditions, *A. ferrooxidans* utilizing elemental sulfur decreased the pH during the two-week incubation to around 1.7. In contrast, controls without bacteria reached maximum values between 6.9 – 8.4 over the same period (Figure S4). Most metals tend to precipitate at neutral and alkaline pH (Blais et al., 2008), making the solubilization more difficult.



#### 3.4.3.3. *Acidithiobacillus thiooxidans*

The predominant sulfur-oxidizing bacterium *A. thiooxidans* is known for its efficient RISCs metabolism to generate sulfuric acid (Wang et al., 2019). Therefore, all three types of MWIP substrates were leached by *A. thiooxidans* in a medium containing elemental sulfur as only energy source. Initial pH of the media in biological and abiotic control batch tests was not adjusted with sulfuric acid. It resulted in an apparent difference between chemical and biological leaching due to the absence of acid in the abiotic control and the ability of *A. thiooxidans* to produce sulfuric acid. Biological leaching efficiencies of V, Mn, Cu, and Zn reached a maximum between 37 and 55%, whereas chemical leaching was not detected (Figure 3C). As expected, the bioleaching efficiency was in the same range as obtained by *A. ferrooxidans* with elemental sulfur as the only energy source. However, according to significance criteria, biological leaching was significantly lower for most of the tested metals compared to all iron-containing set-ups. It confirmed a fundamental role of iron and its contribution to the leaching. A comparison of final pH values demonstrated sufficient elemental sulfur oxidation by *A. thiooxidans* (Figure 4). After two weeks of incubation, *A. thiooxidans* cultures showed the lowest pH of all batch tests (pH ~ 1.5), whereas the pH of the abiotic controls increased up to 7.5.

#### 3.4.3.4. Susceptibility of incineration residues for sulfur- and iron oxidizing bacteria

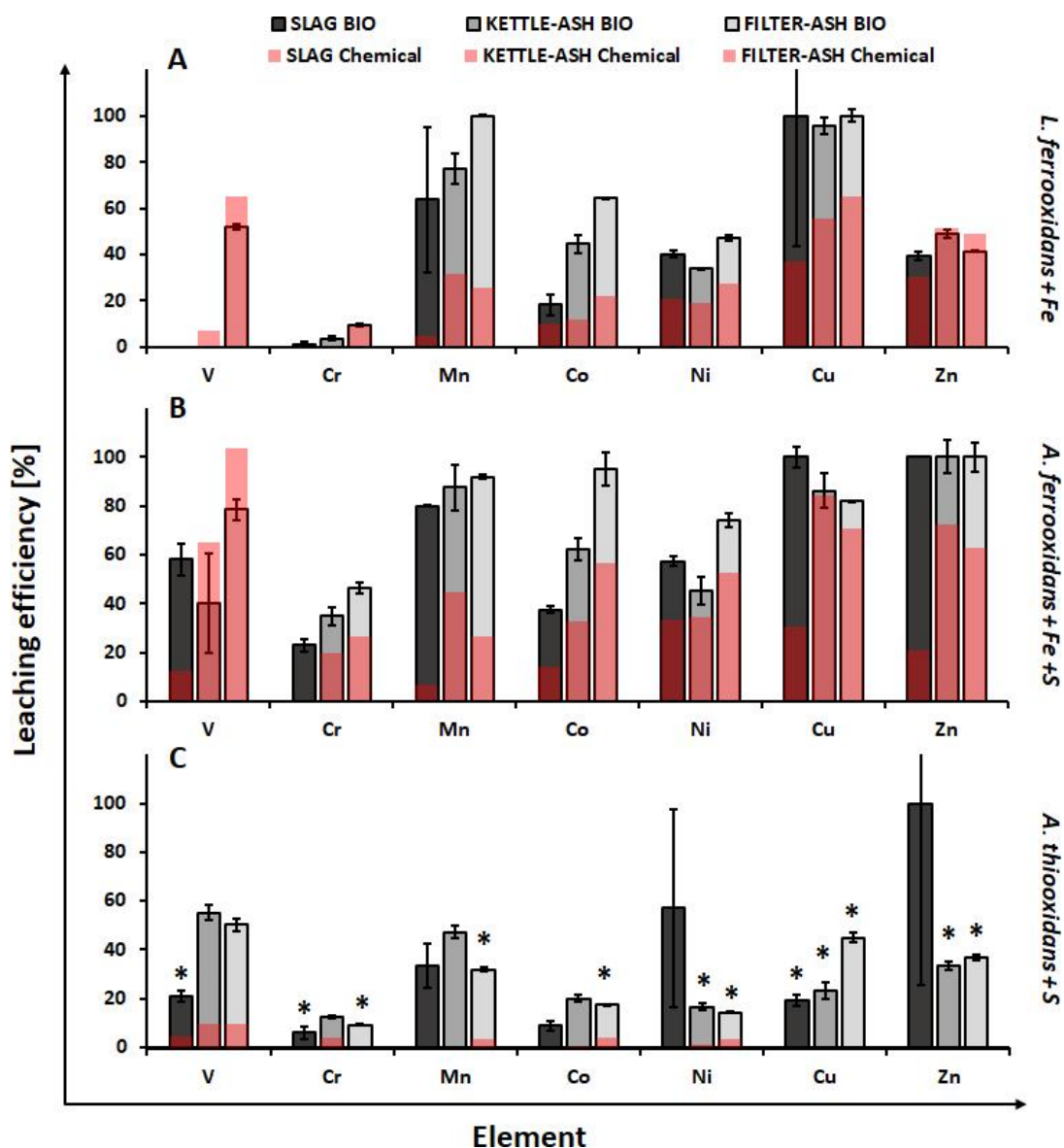
By comparing bioleaching efficiencies of pure bacterial cultures with studies working with mixed acidophilic cultures or fungal bioleaching (Table 4), it is clearly visible that in the case of waste incineration residues (i.e. filter ash), pure cultures of iron-oxidizing bacteria were more efficient. All investigated metals, except Cr, could be leached more effectively by pure *A. ferrooxidans* or *L. ferrooxidans*, compared to *A. thiooxidans*, a mixed acidophilic culture or fungal bioleaching with *A. niger*. A possible explanation for

these findings can be derived from a study by Abramov and co-workers. In their study they showed, that metals incorporated in mineral phases as mentioned in section 3.1 show an increased leachability under oxidizing and reducing conditions compared to acid extraction (Abramov et al., 2018). In case of *A. ferrooxidans* and *L. ferrooxidans*, an effective bio-oxidation of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  is creating such kind of environment which furthermore leads to an increase in redox potential. A high redox potential can further facilitate the metal extraction from solid wastes (i.e. incineration residues), without being significantly affected by the added elemental sulfur (Gu et al., 2018b). In contrast, solely acid generating bacterium *A. thiooxidans* is lacking an iron-oxidizing metabolism, explaining the lower metal extractions yields. Other effects, like a higher heavy metal tolerance (Cabrera et al., 2005; Navarro et al., 2013), resulting in uninhibited metabolism, and faster growth of iron-oxidizing bacteria (Bas et al., 2013) may contribute to higher leaching efficiencies of pure iron-oxidizing bacteria. Additionally, the presence and increased production of biosurfactants by *A. ferrooxidans* in iron-containing medium compared to media containing sulfur only, might contribute positively to metal extraction. Induction of biosurfactant formation in iron-oxidizing cells is considered based on interaction of iron-oxidizing metabolism with the waste materials. The positive effect of biosurfactants in bioleaching processes has been reported previously (Rangarajan and Narayanan, 2018; Sekhon et al., 2012; Shekhar et al., 2015). Our results using the tested waste materials may indicate this effect, which could be related to the metabolism of bacteria that actively oxidize iron. It could be confirmed by further research.

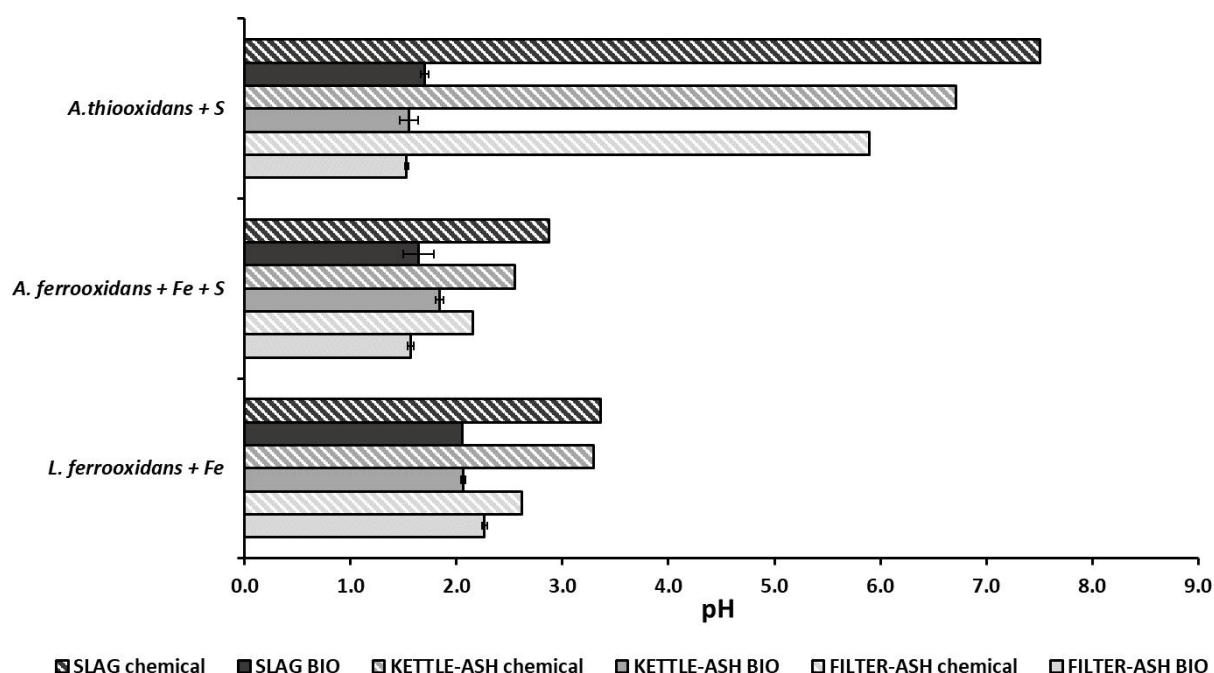
**Table 4.** Comparison of bioleaching efficiencies [%] for certain metals from waste incineration fly ash. Pure bacterial cultures from the present study were compared to results obtained by a mixed acidophilic bacteria culture and bioleaching by the fungus *A. niger*.

Element	Present study			Funari <sup>1</sup>	Wang <sup>2</sup>
	<i>L.f.</i> Leaching efficiency [%] ± SD	<i>A. f.</i> Leaching efficiency [%] ± SD	<i>A.t.</i> Leaching efficiency [%] ± SD	<i>Mixed acidophilic bacteria</i> Leaching efficiency [%] ± SD	<i>A. niger</i>
Cu	100 ± 2.6	81.8 ± 0.1	45 ± 1.8	74 ± 12	56
Zn	41.3 ± 0.2	100 ± 5.9	36.8 ± 1.0	91 ± 0.1	62
Ni	47.1 ± 1.4	74.2 ± 2.7	14.3 ± 0.4	66 ± 11	-
Co	64.4 ± 0.2	95.1 ± 6.9	17.3 ± 0.5	55 ± 17	-
Mn	100 ± 0.4	91.8 ± 0.8	32 ± 1.0	87 ± 16	50
Cr	9.1 ± 0.5	46.3 ± 2.2	9.3 ± 0.4	63 ± 6	20
V	51.7 ± 1.1	78.4 ± 4.3	50.2 ± 2.4	52 ± 6	-

<sup>1</sup>(Funari et al., 2017) <sup>2</sup>(Wang et al., 2009)



**Figure 3:** Efficiency of chemical (red bars) and biological (grey bars) leaching of incineration residues by *L. ferrooxidans* in medium containing ferrous iron (A), *A. ferrooxidans* in medium containing ferrous iron and elemental sulfur (B), and *A. thiooxidans* in medium containing elemental sulfur (C). Three different types of substrates are shown in dark grey (slag), grey (kettle-ash), and light grey (filter-ash). Error bars indicate the standard deviation of the biological duplicate. Stars above the bars indicate a significant decrease ( $P < 0.05$ ) in the metal extraction yield between *A. ferrooxidans* in medium containing ferrous iron plus elemental sulfur (B) and *A. thiooxidans* in media containing elemental sulfur only (C).



**Figure 4:** Final pH of chemical (striped bars) and biological (filled bars) leaching of incineration residues by *A. thiooxidans* in medium containing elemental sulfur, *L. ferrooxidans* in medium containing ferrous iron, and *A. ferrooxidans* in medium containing ferrous iron and elemental sulfur. Three different types of substrates are shown in dark grey (slag), grey (kettle-ash), and light grey (filter-ash). Error bars indicate the standard deviation of the biological duplicate.

### 3.5. Conclusion

This study demonstrates the high potential of bioleaching for metal recovery from waste incineration residues, which usually end up in landfills or find use in construction materials. Different iron- and sulfur-oxidizing acidophiles were compared for leaching efficiency and culture medium composition. Kettle- and filter-ash showed the highest concentrations of valuable metals such as Cu, Cr, Mn, Zn, and Cd. Both types of substrates were leached more efficiently by all tested bacteria compared to the slag. In addition, metal extraction by iron-oxidizing bacteria was significantly higher in media containing iron in addition to sulfur. Bioleaching using only sulfuric acid produced by sulfur-oxidizing strains resulted in an efficiency of about 50% for most of the metals studied. However, the combination of a low pH and the presence of iron in the lixiviant resulted in nearly 100% efficiency for some metals and therefore appears to be optimal for applying biological leaching on waste incineration residues. Furthermore, the acidic

environment, especially for long-term bioleaching period to increase the leaching efficiency, can be easily maintained by adding elemental sulfur to the medium, thereby producing sulfuric acid by strains that oxidize both iron and sulfur such as *A. ferrooxidans*. The application of iron- and sulfur-oxidizing acidophiles could significantly contribute to the recovery of economically attractive metals from the final products of the waste treatment process, as well as their decontamination and subsequent use in construction or environmentally friendly landfill.

### 3.6. Acknowledgements

This work was supported by the EU INTERREG V-A program under grant agreement no. ATCZ183, project IRAS (Innovative Recycling technology for Ashes and Slags). Ash- and slag-samples were kindly provided by the EVN Abfallverwertung NÖ GmbH (Dürnrohr, Austria).

### 3.7. References

- Abramov, S., He, J., Wimmer, D., Lemloh, M.L., Muehe, E.M., Gann, B., Roehm, E., Kirchhof, R., Babechuk, M.G., Schoenberg, R., Thorwarth, H., Helle, T., Kappler, A., 2018. Heavy metal mobility and valuable contents of processed municipal solid waste incineration residues from Southwestern Germany. *Waste Manag.* 79, 735–743. <https://doi.org/10.1016/j.wasman.2018.08.010>
- Akinci, G., Guven, D.E., 2011. Bioleaching of heavy metals contaminated sediment by pure and mixed cultures of *Acidithiobacillus* spp. *Desalination* 268, 221–226. <https://doi.org/10.1016/j.desal.2010.10.032>
- Banerjee, I., Burrell, B., Reed, C., West, A.C., Banta, S., 2017. Metals and minerals as a biotechnology feedstock engineering biomining microbiology for bioenergy applications. *Curr. Opin. Biotechnol.* 45, 144–155. <https://doi.org/10.1016/j.copbio.2017.03.009>
- Bas, A.D., Deveci, H., Yazici, E.Y., 2013. Bioleaching of copper from low grade scrap TV circuit boards using mesophilic bacteria. *Hydrometallurgy* 138, 65–70. <https://doi.org/10.1016/j.hydromet.2013.06.015>
- Blais, J.F., Djedidi, Z., Cheikh, R.B., Tyagi, R.D., Mercier, G., 2008. Metals

- precipitation from effluents: Review. *Pract. Period. Hazardous, Toxic, Radioact. Waste Manag.* 12, 135–149. [https://doi.org/10.1061/\(ASCE\)1090-025X\(2008\)12:3\(135\)](https://doi.org/10.1061/(ASCE)1090-025X(2008)12:3(135))
- Blasenbauer, D., Huber, F., Lederer, J., Quina, M.J., Blanc-Biscarat, D., Bogush, A., Bontempi, E., Blondeau, J., Chimenos, J.M., Dahlbo, H., Fagerqvist, J., Giro-Paloma, J., Hjelm, O., Hyks, J., Keaney, J., Lupsea-Toader, M., O'Caollai, C.J., Orupöld, K., Pająk, T., Simon, F.G., Svecova, L., Šyc, M., Ulvang, R., Vaajasaari, K., Van Caneghem, J., van Zomeren, A., Vasarevičius, S., Wégner, K., Fellner, J., 2020. Legal situation and current practice of waste incineration bottom ash utilisation in Europe. *Waste Manag.* 102, 868–883. <https://doi.org/10.1016/j.wasman.2019.11.031>
- Borilova, S., Mandl, M., Zeman, J., Kucera, J., Pakostova, E., Janiczek, O., Tuovinen, O.H., 2018. Can Sulfate Be the First Dominant Aqueous Sulfur Species Formed in the Oxidation of Pyrite by *Acidithiobacillus ferrooxidans*? *Front. Microbiol.* 9, 1–13. <https://doi.org/10.3389/fmicb.2018.03134>
- Cabrera, G., Gómez, J.M., Cantero, D., 2005. Influence of heavy metals on growth and ferrous sulphate oxidation by *Acidithiobacillus ferrooxidans* in pure and mixed cultures. *Process Biochem.* 40, 2683–2687. <https://doi.org/10.1016/j.procbio.2004.12.005>
- Cristelo, N., Segadães, L., Coelho, J., Chaves, B., Sousa, N.R., de Lurdes Lopes, M., 2020. Recycling municipal solid waste incineration slag and fly ash as precursors in low-range alkaline cements. *Waste Manag.* 104, 60–73. <https://doi.org/10.1016/j.wasman.2020.01.013>
- European Parliament and Council, 2008. Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 on waste and repealing certain directives (Waste framework. LexUriServ. do 3–30. <https://doi.org/2008/98/EC.; 32008L0098>
- Eurostat Waste Statistics, 2017. Municipal waste statistics 1–6. <https://doi.org/10.2785/408702>
- EVN Abfallverwertung, 2017. EVN Abfallverwertung Niederösterreich, MVA Dürnrohr Umwelterklärung 2017.

- Funari, V., Mäkinen, J., Salminen, J., Braga, R., Dinelli, E., Revitzer, H., 2017. Metal removal from Municipal Solid Waste Incineration fly ash: A comparison between chemical leaching and bioleaching. *Waste Manag.* 60, 397–406.  
<https://doi.org/10.1016/j.wasman.2016.07.025>
- Gu, T., Rastegar, S.O., Mousavi, S.M., Li, M., Zhou, M., 2018. Advances in bioleaching for recovery of metals and bioremediation of fuel ash and sewage sludge. *Bioresour. Technol.* 261, 428–440.  
<https://doi.org/10.1016/j.biortech.2018.04.033>
- Hallmann, R., Friedrich, A., Koops, H., Pommerening-Röser, A., Rohde, K., Zenneck, C., Sand, W., 1992. Physiological characteristics of *thiobacillus ferrooxidans* and *leptospirillum ferrooxidans* and physicochemical factors influence microbial metal leaching. *Geomicrobiol. J.* 10, 193–206.  
<https://doi.org/10.1080/01490459209377920>
- Hoornweg, D., Bhada-Tata, P., 2012. What a Waste : A Global Review of Solid Waste Management. *Urban Dev. Ser. Knowl. Pap.* 29–43.  
<https://doi.org/10.1201/9781315593173-4>
- Joseph, A.M., Snellings, R., Van den Heede, P., Matthys, S., De Belie, N., 2018. The use of municipal solidwaste incineration ash in various building materials: A Belgian point of view. *Materials (Basel)*. 11. <https://doi.org/10.3390/ma11010141>
- Krebs, W., Bachofen, R., Brandl, H., 1999. Optimization of metal leaching efficiency of fly ash from municipal solid waste incineration by sulfur oxidizing bacteria. *Process Metall.* 9, 377–386. [https://doi.org/10.1016/S1572-4409\(99\)80038-8](https://doi.org/10.1016/S1572-4409(99)80038-8)
- Krebs, W., Brombacher, C., Bosshard, P.P., Bachofen, R., Brandl, H., 1997. Microbial recovery of metals from solids. *FEMS Microbiol. Rev.* 20, 605–617.  
[https://doi.org/10.1016/S0168-6445\(97\)00037-5](https://doi.org/10.1016/S0168-6445(97)00037-5)
- Kremser, K., Thallner, S., Schoen, H., Weiss, S., Hemmelmair, C., Schnitzhofer, W., Aldrian, A., Guebitz, G., 2020. Stirred-tank and heap-bioleaching of shredder-light-fractions (SLF) by acidophilic bacteria. *Hydrometallurgy*.  
<https://doi.org/https://doi.org/10.1016/j.hydromet.2020.105315>
- Mishra, D., Rhee, Y.-H., 2010. Current research trends of microbiological leaching for metal recovery from industrial wastes. *Curr Res Technol Educ Top. Appl*

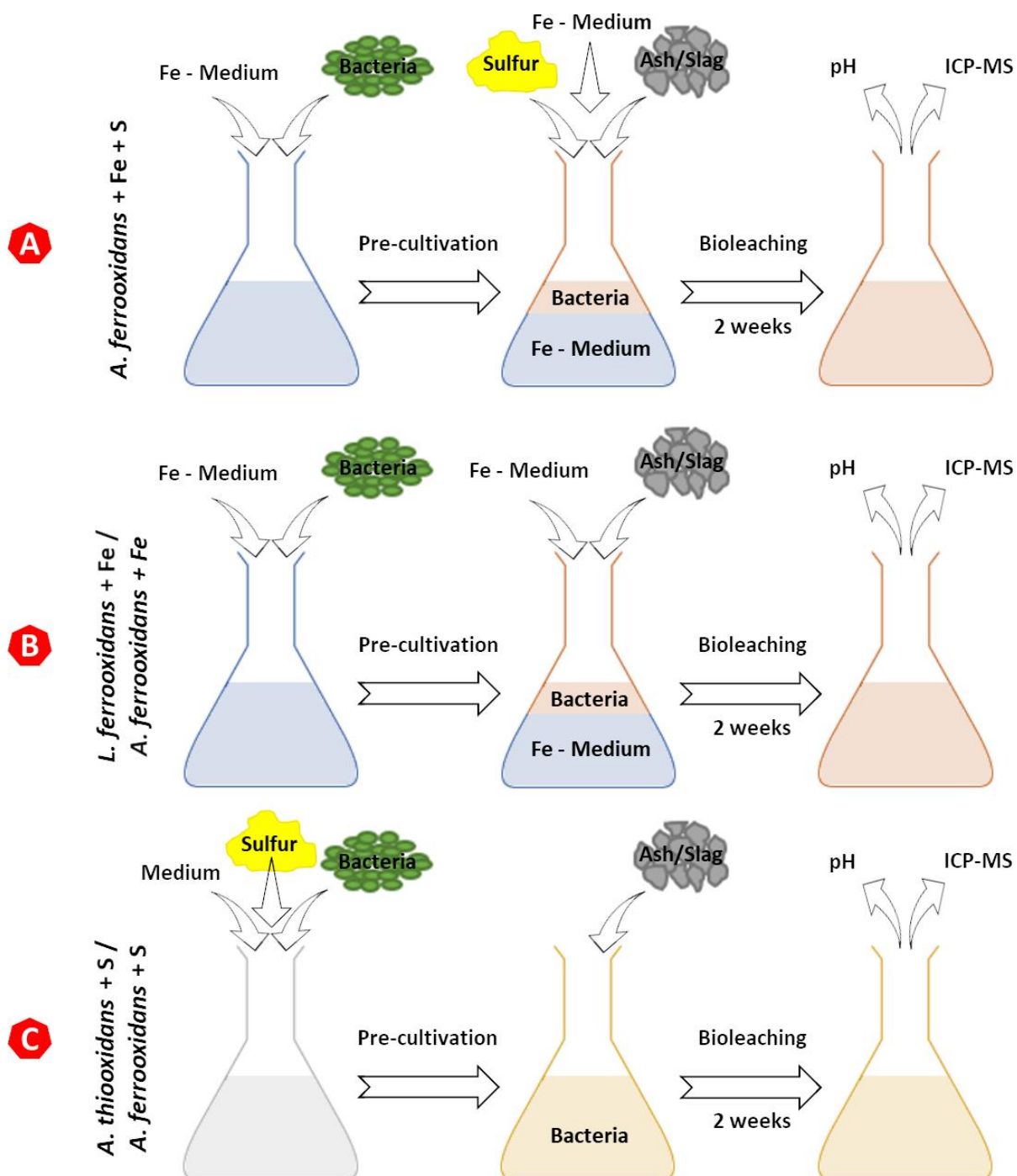


- Microbiol Microb Biotechnol 2, 1289–1292.
- Mishra, D., Rhee, Y.H., 2014. Microbial leaching of metals from solid industrial wastes. *J. Microbiol.* 52, 1–7. <https://doi.org/10.1007/s12275-014-3532-3>
- Navarro, A.C., Von Bernath, D., Jerez, A.C., 2013. Heavy Metal Resistance Strategies of Acidophilic Bacteria and Their Acquisition: Importance for Biomining and Bioremediation. *Biol. Res.* 46, 363–371.
- Pathak, A., Dastidar, M.G., Sreekrishnan, T.R., 2009. Bioleaching of heavy metals from sewage sludge: A review. *J. Environ. Manage.* 90, 2343–2353. <https://doi.org/10.1016/j.jenvman.2008.11.005>
- Quatrini, R., Johnson, D.B., 2018. Microbiomes in extremely acidic environments: functionalities and interactions that allow survival and growth of prokaryotes at low pH. *Curr. Opin. Microbiol.* 43, 139–147. <https://doi.org/10.1016/j.mib.2018.01.011>
- Rangarajan, V., Narayanan, M., 2018. Biosurfactants in Soil Bioremediation. *Adv. Soil Microbiol. Recent Trends Futur. Prospect.* 193–204. [https://doi.org/10.1007/978-981-10-6178-3\\_11](https://doi.org/10.1007/978-981-10-6178-3_11)
- Rohwerder, T., Gehrke, T., Kinzler, K., Sand, W., 2003. Bioleaching review part A: *Appl. Microbiol. Biotechnol.* 63, 239–248. <https://doi.org/10.1007/s00253-003-1448-7>
- Schippers, A., Hedrich, S., Vasters, J., Drobe, M., Sand, W., Willscher, S., 2013. Biomining: Metal Recovery from Ores with Microorganisms. *Geobiotechnology I. Adv. Biochem. Eng.* 123, 127–141. [https://doi.org/https://doi.org/10.1007/10\\_2013\\_216](https://doi.org/https://doi.org/10.1007/10_2013_216)
- Sekhon, K.K., Khannna, S., Cameotra, S.S., 2012. Biosurfactant Production and Potential Correlation with Esterase Activity. *J. Pet. Environ. Biotechnol.* 3. <https://doi.org/10.4172/2157-7463.1000133>
- Sethurajan, M., van Hullebusch, E.D., Nanchaiah, Y. V., 2018. Biotechnology in the management and resource recovery from metal bearing solid wastes: Recent advances. *J. Environ. Manage.* 211, 138–153. <https://doi.org/10.1016/j.jenvman.2018.01.035>

- Shekhar, S., Sundaramanickam, A., Balasubramanian, T., 2015. Biosurfactant producing microbes and their potential applications: A review. *Crit. Rev. Environ. Sci. Technol.* 45, 1522–1554. <https://doi.org/10.1080/10643389.2014.955631>
- Solisio, C., Lodi, A., Veglio', F., 2002. Bioleaching of zinc and aluminium from industrial waste sludges by means of *Thiobacillus ferrooxidans*. *Waste Manag.* 22, 667–675. [https://doi.org/10.1016/S0956-053X\(01\)00052-6](https://doi.org/10.1016/S0956-053X(01)00052-6)
- Vestola, E.A., Kuusenaho, M.K., Närhi, H.M., Tuovinen, O.H., Puhakka, J.A., Plumb, J.J., Kaksonen, A.H., 2010. Acid bioleaching of solid waste materials from copper, steel and recycling industries. *Hydrometallurgy* 103, 74–79. <https://doi.org/10.1016/j.hydromet.2010.02.017>
- Wakeman, K., Auvinen, H., Johnson, D.B., 2008. Microbiological and geochemical dynamics in simulated-heap leaching of a polymetallic sulfide ore. *Biotechnol. Bioeng.* 101, 739–750. <https://doi.org/10.1002/bit.21951>
- Wang, Q., Yang, J., Wang, Q., Wu, T., 2009. Effects of water-washing pretreatment on bioleaching of heavy metals from municipal solid waste incinerator fly ash. *J. Hazard. Mater.* 162, 812–818. <https://doi.org/10.1016/j.jhazmat.2008.05.125>
- Wang, R., Lin, J.Q., Liu, X.M., Pang, X., Zhang, C.J., Yang, C.L., Gao, X.Y., Lin, C.M., Li, Y.Q., Li, Y., Lin, J.Q., Chen, L.X., 2019. Sulfur oxidation in the acidophilic autotrophic *Acidithiobacillus* spp. *Front. Microbiol.* 10, 1–20. <https://doi.org/10.3389/fmicb.2018.03290>
- Wei, Y., Shimaoka, T., Saffarzadeh, A., Takahashi, F., 2011. Mineralogical characterization of municipal solid waste incineration bottom ash with an emphasis on heavy metal-bearing phases. *J. Hazard. Mater.* 187, 534–543. <https://doi.org/10.1016/j.jhazmat.2011.01.070>
- Werner, A., Meschke, K., Bohlke, K., Daus, B., Haseneder, R., Repke, J.-U., 2018. Resource Recovery from Low-Grade Ore Deposits and Mining Residuals by Biohydrometallurgy and Membrane Technology. Potentials and Case Studies. *ChemBioEng Rev.* 5, 6–17. <https://doi.org/10.1002/cben.201700019>
- Zhu, W., Chen, X., Struble, L.J., Yang, E.H., 2018. Characterization of calcium-containing phases in alkali-activated municipal solid waste incineration bottom ash binder through chemical extraction and deconvoluted Fourier transform

infrared spectra. J. Clean. Prod. 192, 782–789.  
<https://doi.org/10.1016/j.jclepro.2018.05.049>

### 3.8. Supplementary information

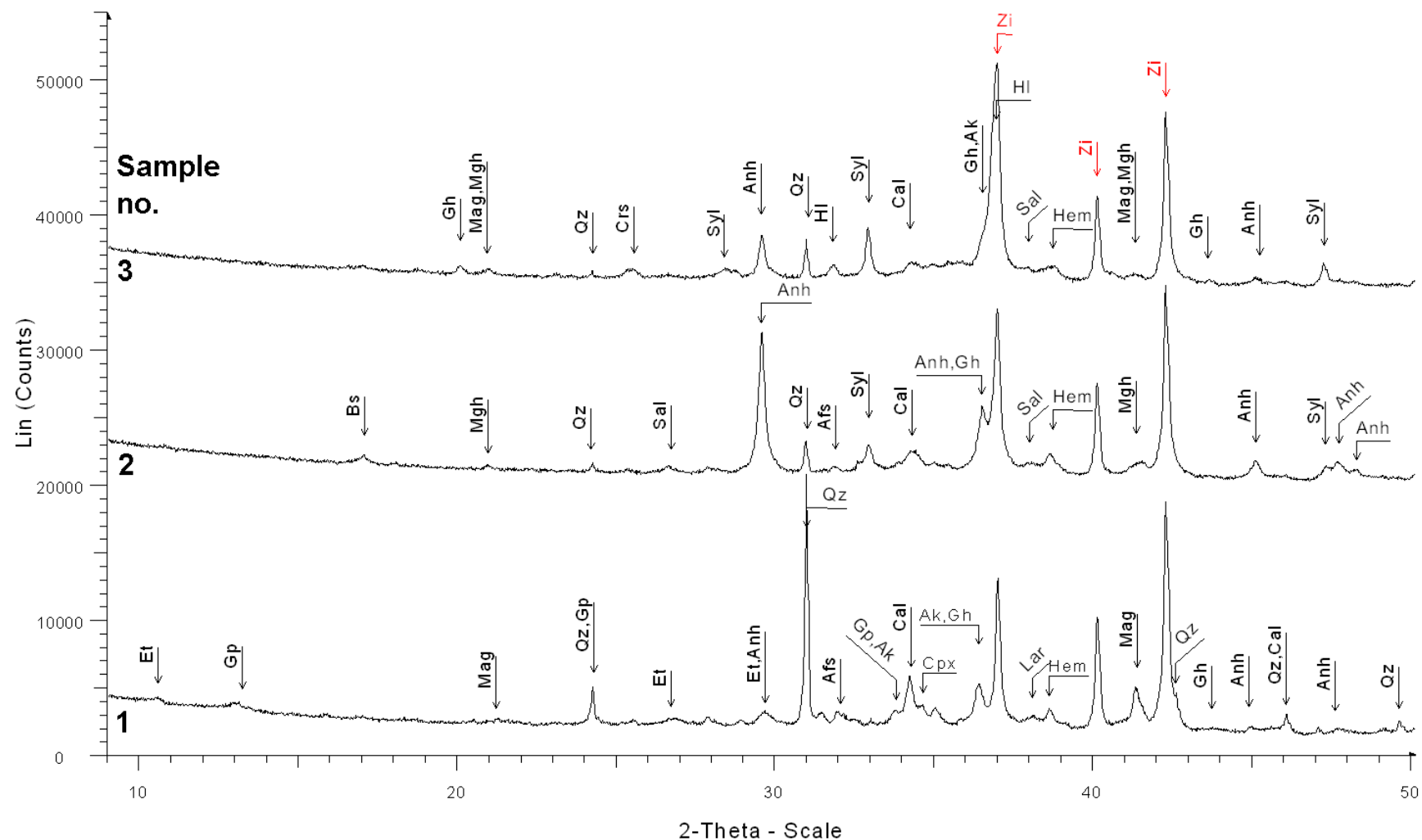


**Figure S1:** Schematic presentation of the bioleaching batch experiments. Bioleaching of incineration residues using *A. ferrooxidans* (A, B, and C), *L. ferrooxidans* (B), and *A. thiooxidans* (C). In mode A, 10 ml of pre-cultivated iron-grown *A. ferrooxidans* culture at a cell density of  $10^8$  cells  $\text{ml}^{-1}$  was inoculated into fresh 90 ml DSMZ medium 70 containing additionally  $10 \text{ g l}^{-1}$  of elemental sulfur and 1 g of dried ash/slag (1% w/v). In mode B, 10 ml of pre-cultivated iron-grown *A. ferrooxidans* or *L. ferrooxidans* culture at a cell density of  $10^8$  cells  $\text{ml}^{-1}$  was inoculated into fresh 90 ml DSMZ medium 70 or 882, respectively, containing additionally 1 g of dried ash/slag (1% w/v). In mode C, 10 ml of pre-cultivated sulfur-grown *A. ferrooxidans* or *A. thiooxidans* culture at a cell density of  $10^8$  cells  $\text{ml}^{-1}$  was inoculated into fresh 90 ml basal salts medium (Wakeman et al., 2008) containing  $10 \text{ g l}^{-1}$  of elemental sulfur. After one week of pre-cultivation, 1 g of dried ash/slag (1% w/v) was aseptically added. A colour changes of the medium from light blue to red represents the oxidation of ferrous to ferric iron (A and B). The oxidation and solubilization of elemental sulfur represent a colour change from transparent to light yellow (C).

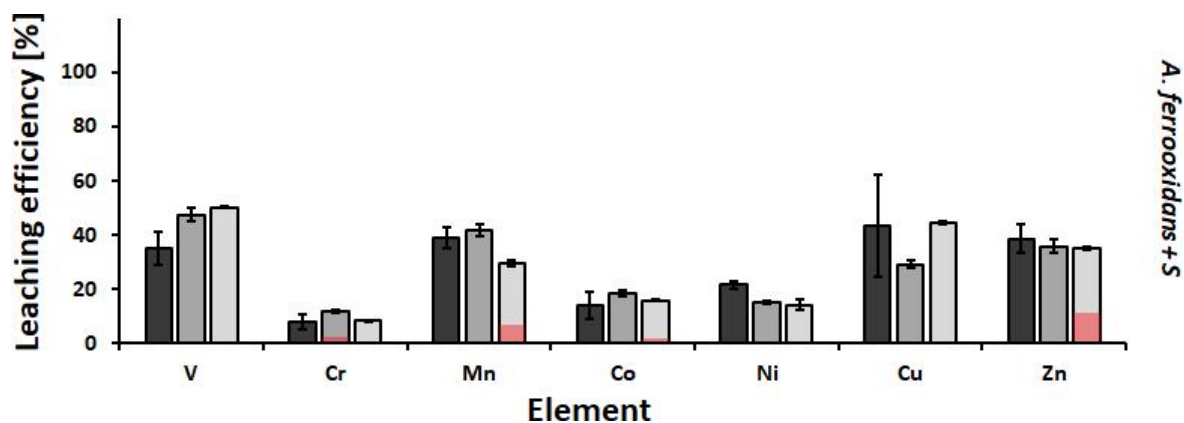
**Table S1:** pH-values of the adaptation experiment with *A. ferrooxidans* at the beginning, and after 14 days of incubation.

Incubation time [d]	SETUP	SLAG	KETTLE-ASH [pH ± SD]	FILTER-ASH
0	0.05 M H <sub>2</sub> SO <sub>4</sub>	1.46	1.66	1.70
	Chemical	1.72	1.99	1.88
	Non-Adapted	1.73 ± 0.01	1.93 ± 0.01	1.79 ± 0.01
	Adapted	1.71 ± 0.01	1.94 ± 0.03	1.82 ± 0.01
	Adapted + S	1.73 ± 0.00	1.87 ± 0.03	1.81 ± 0.01
14	0.05 M H <sub>2</sub> SO <sub>4</sub>	3.80	2.78	1.89
	Chemical	2.87	2.55	2.15
	Non-Adapted	2.02 ± 0.04	1.92 ± 0.01	1.76 ± 0.02
	Adapted	2.11 ± 0.06	2.01 ± 0.00	1.82 ± 0.02
	Adapted + S	1.65 ± 0.15	1.84 ± 0.04	1.57 ± 0.03

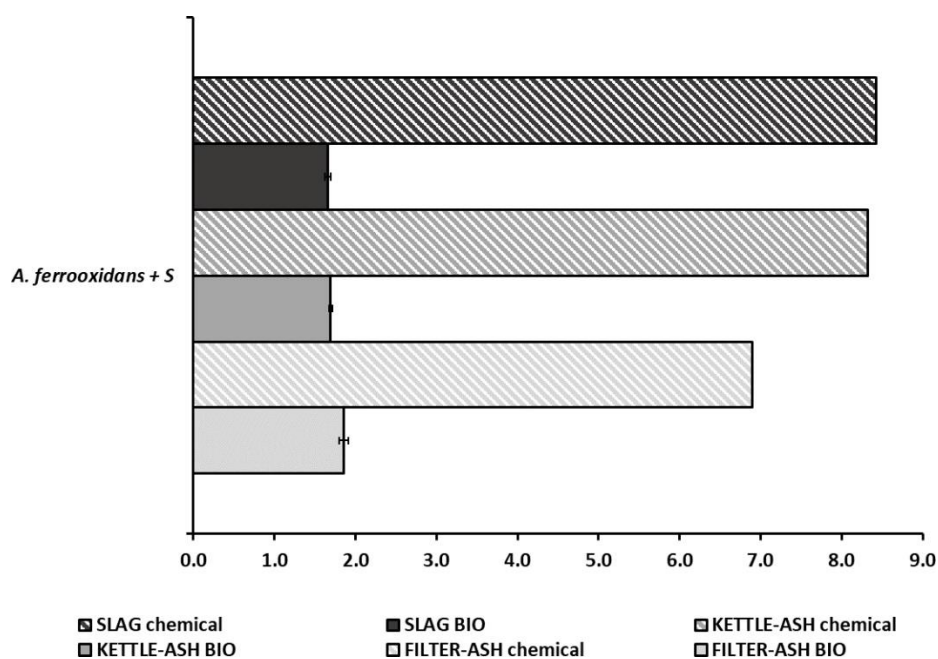
\*Chemical leaching (= culture medium only)



**Figure S2:** 9 – 55 °2 $\theta$  sections of X-ray diffractograms. Afs = alkali feldspar, Ak = akermanite, Anh = anhydrite, Bs = bassanite, Cal = calcite, Cpx = clinopyroxene, Crs = cristobalite, Ett = ettringite, FeSi = ferrosilicon, Gh = gehlenite, Gp = gypsum, Hem = hematite, HI = halite, Lar = larnite, Mag = magnetite, Mgh = maghemite, Qz = quartz, Sal = salammoniac, Syl = sylvite, **Zi = zinc oxide (internal standard)**



**Figure S3:** Efficiency of chemical (red bars) and biological (grey bars) leaching of incineration residues by *A. ferrooxidans* in medium containing only elemental sulfur. Three different types of substrates are shown in dark grey (slag), grey (kettle-ash), and light grey (filter-ash). Error bars indicate the standard deviation of the biological duplicate.



**Figure S4:** Final pH of chemical (striped bars) and biological (filled bars) leaching of incineration residues by *A. ferrooxidans* in medium containing elemental sulfur. Three different types of substrates are shown in dark grey (slag), grey (kettle-ash), and light grey (filter-ash). Error bars indicate the standard deviation of the biological duplicate.

#### **4. Biogenic sulfuric acid production for pH control in heap bioleaching systems**

Submitted to the Journal of Bioresource Technology

**Klemens Kremser<sup>a</sup>, Melanie Maltschnig<sup>a</sup>, Herta Schön<sup>a</sup>, Martin Gajdosik<sup>b</sup>, Tomas Valulovic<sup>c</sup>, Jiri Kucera<sup>b</sup>, Georg M. Guebitz<sup>a\*</sup>**

<sup>a</sup>University of Natural Resources and Life Sciences Vienna BOKU, Dept. of Agrobiotechnology, IFA-Tulln, Inst. of Environmental Biotechnology, Konrad-Lorenz-Straße 20, 3430 Tulln an der Donau, Austria

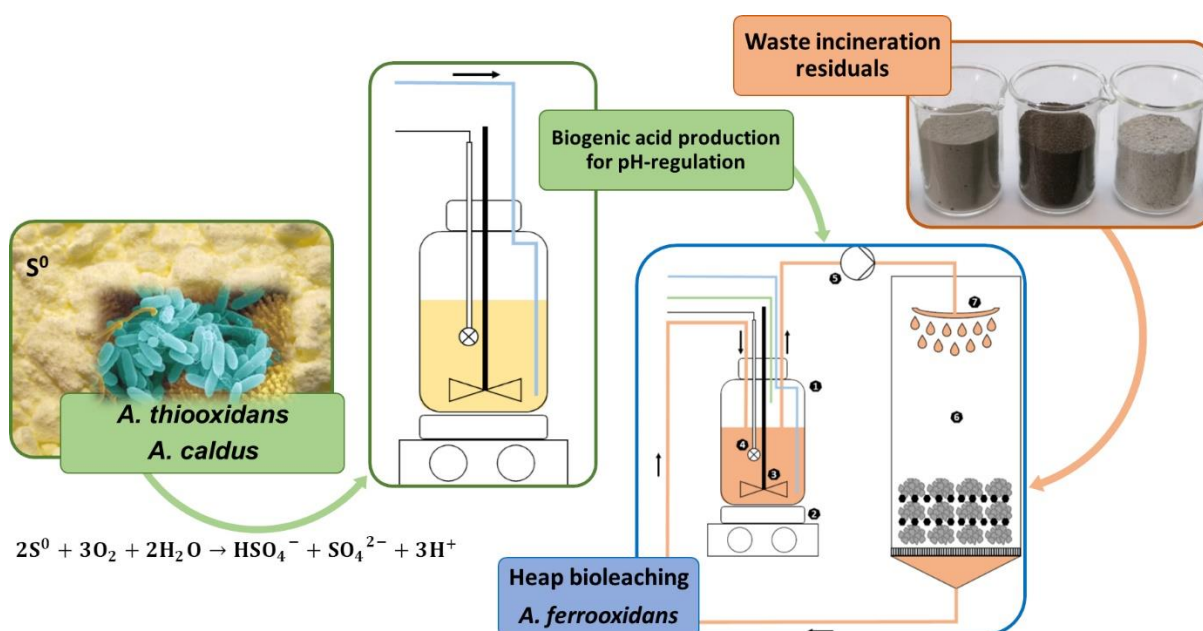
<sup>b</sup>Department of Biochemistry, Faculty of Science, Masaryk University, Kotlarska 2, 61137 Brno, Czech Republic

<sup>c</sup>Department of Chemistry, Faculty of Science, Masaryk University, Kotlarska 2, 61137 Brno, Czech Republic

**Keywords:** Biogenic sulfuric acid; elemental sulfur-oxidation; mixed acidophilic bacteria; heap bioleaching; metal recovery



## Graphical abstract



### 4.1. Abstract

The biological leaching of metals from different waste streams by bacteria is intensively investigated to address metal recycling and circular economy goals. However, usually external addition of sulfuric acid is required to maintain the low pH optimum of the bacteria to ensure efficient leaching. Extremely acidophilic *Acidithiobacillus* spp. producing sulfuric acid and ferric iron have been investigated for several decades in the bioleaching of metal-containing ores. Their application has now been extended to the extraction of metals from artificial ores and other secondary sources. In this study, an optimized process for producing biogenic sulfuric acid from elemental sulfur by two sulfur-oxidizing species, *A. thiooxidans* and *A. caldus* and their combinations, was investigated in batch and stirred tank experiments. Using a combined culture of both species, 1.05 M and 1.4 M biogenic sulfuric acid was produced at 30 °C and 6% elemental sulfur in batch and semi continuous modes, respectively. The acid produced was then used to control the pH in a heap bioleaching system in which iron- and sulfur-oxidizing *A. ferrooxidans* was applied to biologically leach metals from waste

incineration residuals. Metals including Cu, Zn, Ni, Al and Mn were successfully dissolved by up to 99% within three weeks of heap bioleaching. The high potential of extremely acidophilic sulfur-oxidizing bacteria for cheap and efficient production of biogenic sulfuric acid and its use in pH control has been demonstrated.

#### **4.2. Introduction**

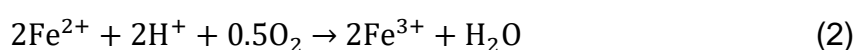
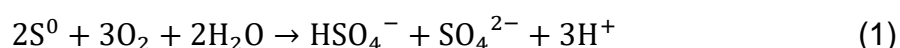
Incineration of municipal solid waste (MSW) and hazardous waste materials is one of the most common waste treatment strategies in the European Union (EU), accounting for approximately 70% after recyclable materials have been removed (Eurostat Waste Statistics, 2017). Although MSW is considered a valuable energy source in terms of waste-to-energy utilization all over the world (Khandelwal et al., 2019; Moya et al., 2017), almost 300 kg of residuals such as ash and slag per ton of waste remain directly after incineration or can be separated from the off-gas. A significant fraction of these residuals presents coarse waste incineration slag (also known as kettle slag or bottom ash), accounting for around 25% of the total residual mass, followed by two types of ash summarized as fly ash (EVN Abfallverwertung, 2017). High concentrations of metals such as Fe, Cr, Mn, Ni, Cu, Zn, Pb, Sn, and Sb remaining in these residuals classify them as a hazardous waste material on the one hand (European Parliament and Council, 2008), but on the other hand make them economically attractive substrates for metal recovery and recycling (Gomes et al., 2020; Krebs et al., 1997). The use of these residuals as additives in construction materials to prevent their disposal has already been investigated but mostly requires chemical or biological pre-treatment to reduce the metal concentrations (Blasenbauer et al., 2020).

The use of iron- and sulfur-oxidizing bacteria in the treatment of these residuals (a process known as bioleaching) has been studied in recent years, revealing its potential for the recovery of valuable metals and reduction in toxicity (Gomes et al., 2020; Gu et

al., 2018; Kremser et al., 2020). Almost all bioleaching processes that operate at extremely low pH (<2) have one thing in common; the need for external addition of sulfuric acid for pH control. Even applications investigating the use of sulfur-oxidizing bacteria, which can oxidize zero-valent sulfur ( $S^0$ ) to sulfuric acid, usually require adjusting the pH of the culture medium. Sulfuric acid reflects the world's largest bulk chemical, with an annual production of approximately 201.6 million tons (Coherent Market Insights, 2021). It is commonly produced by sulfur burning at around 1,100 °C to convert  $S^0$  to sulfur dioxide ( $SO_2$ ), followed by converting  $SO_2$  to sulfur trioxide ( $SO_3$ ) at around 430 °C. In the final step,  $SO_3$  is converted to sulfuric acid ( $H_2SO_4$ ) (King et al., 2013). As this process is burdened with high energy demand, first attempts have already been made to optimize the microbial generation of sulfuric acid using consortia of sulfur-oxidizing acidophilic prokaryotes in laboratory-scale bioreactors at different temperatures (Pakostova and Johnson, 2019). Elemental sulfur is generated in large quantities as a by-product of refining gas, oil, and petroleum (Zhang et al., 2015). Even after its industrial consumption, over 7 million tons remain unused (Steudel, 2003), making it an interesting and cheap substrate for biogenic sulfuric acid production. This study investigated for the first time the combination of external biogenic sulfuric acid generation for pH control in heap bioleaching systems of artificial ores.

In this study, two different sulfur-oxidizing bacteria, namely *Acidithiobacillus thiooxidans* and *Acidithiobacillus caldus* were examined in pure and combined cultures for their potential to generate sulfuric acid effectively. Both bacteria can oxidize  $S^0$  at mesophilic and moderately thermophilic temperatures to produce biogenic  $H_2SO_4$  according to equation 1 (Wang et al., 2019). This simple, environmentally friendly, and inexpensive process was tested in batch experiments, followed by an upscale in a continuous stirred tank reactor (CSTR). The resulting biogenic sulfuric acid was

subsequently used to regulate the pH in a heap reactor system in which the coarse waste incineration slag was leached biologically by the iron- and sulfur-oxidizer *Acidithiobacillus ferrooxidans*. The biological generation of ferric iron ( $\text{Fe}^{3+}$ ) as a strong oxidizing agent by *A. ferrooxidans* (equation 2) in combination with low pH were proven to be critical factors for the efficient leaching of oxide materials, including waste incineration residuals (Abramov et al., 2018; Kremser et al., 2020).



The objective of this study was to investigate the applicability of externally produced biogenic sulfuric acid for pH control in a heap bioleaching system for metal extraction from waste incineration residuals. This study increases the spectrum of bioleaching applications and provides evidence that bioleaching systems can be effectively maintained without the need for chemically produced sulfuric acid for pH regulation.

### 4.3. Material and methods

All chemicals used were of analytical grade and purchased from Sigma-Aldrich (Vienna, Austria) unless otherwise specified. Pulverized elemental sulfur with a purity >99.5% was purchased from Carl-Roth (Karlsruhe, Germany). Deionized water was used for the preparation of cultivation media and stock solutions. Each sample was centrifuged for 1 min at 1,500 g to remove elemental sulfur and slag particles for cell number determination. The optical density (OD) was afterwards measured at 660 nm in a DR3900 spectrophotometer (Hach Lange, Vienna, Austria). For pH measurements, a Mettler Toledo S220 pH meter with a combined glass electrode (Mettler-Toledo GmbH, Vienna, Austria) was used.

#### 4.3.1. Waste incineration slag

Slag samples were provided from a state-of-the-art MSW incineration plant located in Dürnröhr (Austria). Prior to use, samples were washed with deionized water and sieved to a particle size smaller than 2.8 mm, as described previously (Kremser et al., 2020). The samples were then dried at 60 °C for up to 48 h to remove any remaining water. The metal concentration and the composition of the mineral phases have been reported previously (Kremser et al., 2020).

#### 4.3.2. Bacteria and growth media

Sulfur-oxidizing *A. thiooxidans* (DSM 504) and moderately thermophile *A. caldus* (DSM 9466) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The minimal culture medium used for biological sulfuric acid production consisted of 3.00 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.14 g L<sup>-1</sup> CaCl<sub>2</sub> x 2 H<sub>2</sub>O, 0.10 g L<sup>-1</sup> NH<sub>4</sub>Cl, 0.10 g L<sup>-1</sup> MgCl<sub>2</sub> x 6 H<sub>2</sub>O, 10.00 g L<sup>-1</sup> S<sup>0</sup> (pH 4.2). Iron- and sulfur-oxidizer *A. ferrooxidans* (DSM 583), newly classified as *A. ferridurans* (Moya-Beltrán et al., 2021), was used in heap bioleaching experiments and grown in aSRB medium (Ñancucheo et al., 2016) supplemented with 5 g L<sup>-1</sup> FeSO<sub>4</sub> x 7 H<sub>2</sub>O and adjusted to pH 2.0 with sulfuric acid. Bacteria were precultured for two weeks in 250-mL Erlenmeyer flasks with 100 mL minimal culture media with corresponding substrates at 150 rpm and 30 °C (*A. thiooxidans* and *A. ferrooxidans*) or 37 °C (*A. caldus*), respectively.

#### 4.3.3. Biological sulfuric acid production batch experiments

To define the optimum growth conditions and the maximum rate of sulfuric acid generation, pure cultures of both sulfur-oxidizing bacteria and their combinations were tested with different concentrations of S<sup>0</sup>, 1.5, 3.0 and 6.0% (w/v)); and at different

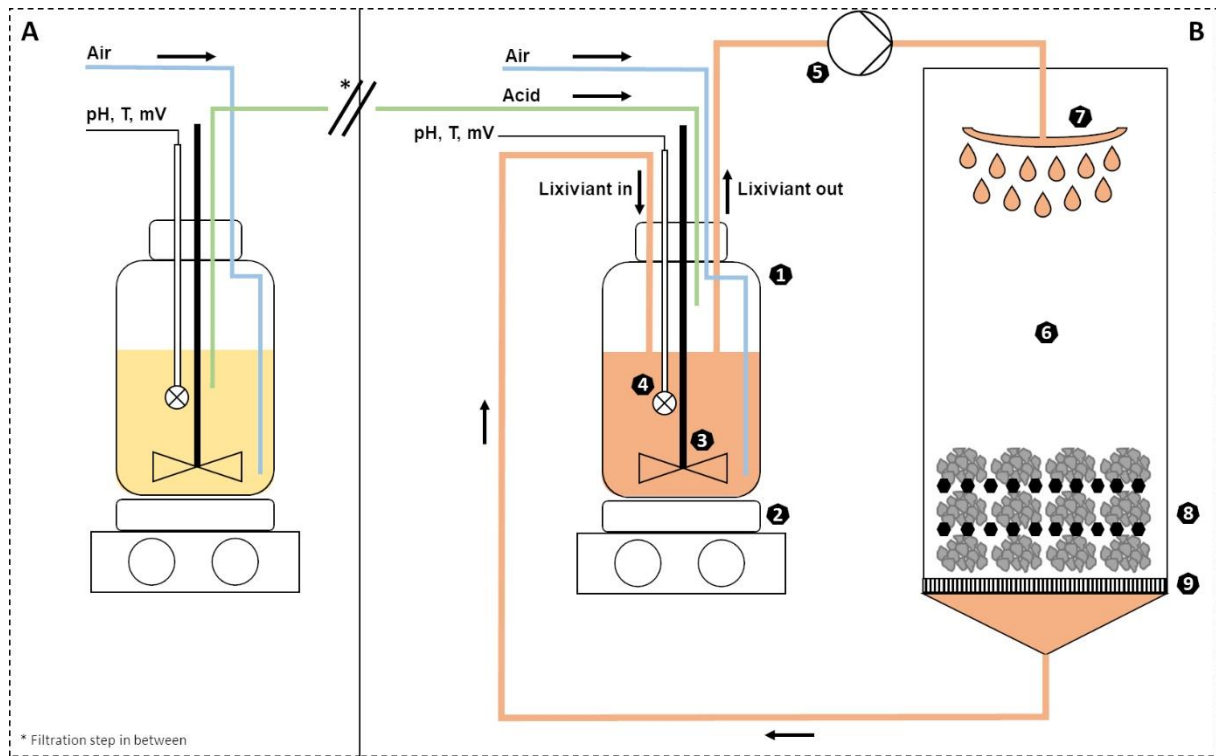
temperatures, 30 and 37 °C. 10 mL (10% v/v) of the pure bacterial preculture was inoculated in 90 mL minimal culture medium with the corresponding  $S^0$  concentration. 5 mL of each bacterium was used to inoculate the combined culture. The 250-mL Erlenmeyer flasks were cultured under shaking at 150 rpm in biological triplicates for up to four weeks. Samples for pH, OD and sulfate measurements were collected periodically every 2-3 days. An abiotic blank containing minimal culture medium with  $S^0$  only was run in parallel. Prior to pH and sulfate measurements, samples were filtrated through a 0.2  $\mu$ m CHROMAFIL® Xtra Nylon filter (Macherey-Nagel, Düren, Germany) to remove bacteria and sulfur.

#### 4.3.4. Sulfuric acid reactor and heap-bioleaching system

Following the batch experiments, biological sulfuric acid production was tested in a continuous stirred tank reactor (CSTR) with a working volume of 1.5 L (Fig. 1A). The stirred tank reactor consisted of a 2 L wide-necked vessel operated at a constant stirring speed of 250 rpm and aerated with 60 L h<sup>-1</sup> of pressurized air. An RCT basic magnetic stirring and heating plate (IKA, Staufen, Germany) was used to maintain constant stirring and temperature. The same  $S^0$  concentrations tested in the batch experiments were also tested in the stirred tank reactor. Experiments were run for four weeks in batch mode or three weeks in semi continuous mode. Samples for pH, OD and sulfate measurements were collected periodically every 2-3 days. In semi-continuous mode, 2/3 of the reactor content were removed after three weeks and replaced by fresh minimum medium with  $S^0$ . The spent medium containing sulfuric acid was filtered through a 0.2  $\mu$ m filter before subsequent pH control in the heap bioleaching reactor.

The heap bioleaching system consisted of a 2 L stirred tank reactor, connected to a sprinkling system that rinsed the heap from the top of the apparatus (Fig. 1B). 250 g of

the incineration slag (25% w/v) were piled into the heap reactor. In the stirred tank reactor, pH was automatically controlled by an M800 transmitter (Mettler Toledo, Vienna Austria), connected to a pH electrode and a TEKNA EVO acid pump (Hennlich, Suben, Austria). For constant stirring (250 rpm) and temperature control (30 °C), the stirred tank reactor was operated with a magnetic stirring plate similar to that used for the sulfuric acid reactor. Furthermore, the reactor was aerated with 60 L h<sup>-1</sup> of pressurized air. A peristaltic pump LA900 (Landgraf HLL, Langenhagen, Germany) with a speed of 40 mL min<sup>-1</sup> was used to circulate the lixiviant from the stirred tank reactor to the heap. The effect of pH on leaching efficiency was investigated by conducting experiments at pH 2.4 and 2.0. pH was controlled using chemically or biologically produced sulfuric acid. Before starting the recirculation over the heap system, *A. ferrooxidans* was precultured in the stirred tank reactor for one week to ensure an active growth of sufficient biomass (1 g L<sup>-1</sup> Fe<sup>2+</sup> and 10 g L<sup>-1</sup> S<sup>0</sup>). After pre-cultivation, the lixiviant was pumped over the heap system for one week, followed by a 3-4 days regeneration phase, during which new iron (1 g L<sup>-1</sup> Fe<sup>2+</sup>) and sulfur (10 g L<sup>-1</sup>) were added to promote bacterial growth and sulfuric acid generation. In total, heap bioleaching included one week of pre-cultivation, three weeks of recirculation, and two regeneration phases in between. Samples were collected every 2-3 days from both reactor systems and processed as described above.



**Figure 1:** Process scheme of the reactor system used for biological sulfuric acid production (A) and the heap-bioleaching system for the biological leaching of waste incineration slag (B). The heap bioleaching system consisted of a stirred tank reactor (1) constantly agitated and the temperature was regulated by a magnetic stirring plate (2). Inside the reactor, a magnetic stirring bar (3) was used to agitate the lixiviant, and a pH electrode (4) was connected to the process control transmitter. The lixiviant was pumped over the heap by a peristaltic pump (5) and dispersed by a sprinkling system (7) at the top of the heap (6). Inside the heap system, the coarse incineration slag was mixed with plastic spacers in between to prevent the clogging of the heap (8). After rinsing the heap, the lixiviant was filtered through a sieve plate (9), pumped back into the stirred tank reactor and recirculated continuously. Biogenic sulfuric acid was filtered through a 0.2  $\mu\text{m}$  filter before using (\*).

#### 4.3.5. Calculation of total acidity and leaching efficiency

Sulfate concentrations and pH values for the combination of microbially produced sulfate and bisulphate with protons/hydronium ions ( $\text{H}_3\text{O}^+$ ) were used to calculate the total acidity produced by sulfur oxidizers (Pakostova and Johnson, 2019).

$$\text{Total acidity [M]} = [\text{SO}_4^{2-}] * p_1 + [10^{-\text{pH}}] \quad (3)$$

The coefficient  $p_1$  was calculated using the Henderson-Hasselbalch equation and reflects the relative amount of bisulphate present:

$$\log \frac{[\text{SO}_4^{2-}]}{[\text{HSO}_4^-]} = \text{pH} - \text{pK}_a \quad (4)$$



The  $pK_a$  value is temperature-dependent and shows a value of  $\sim 1.99$  at  $30\text{ }^{\circ}\text{C}$  (Marshall and Jones, 1966).

The sulfate concentration was measured by a spectrophotometric method using barium chloride (Tabatabai, 1974) with a protocol modification to allow use in 96-well plates (Kremser et al., 2021).

The leaching efficiency was calculated as previously described by the ratio of the metal concentration in the lixiviant to the metal concentration in the untreated incineration slag (Kremser et al., 2020). Furthermore, the leaching efficiency was calculated by comparing the metal concentration in the untreated sample with the concentration in the biologically leached sample after heap bioleaching. Metal concentrations in lixiviants and untreated slag were measured by inductively coupled plasma mass spectrometry (ICP-MS) according to a previously described protocol (Kremser et al., 2020).

#### 4.3.6. Nucleic acid extraction and quantification

For DNA extraction, 10 ml of homogeneous sample was centrifuged at  $1,500\text{ }g$  for 1 min to remove sulfur particles, followed by centrifugation at  $12,000\text{ }g$  for 30 min. The cell pellets were stored at  $-80\text{ }^{\circ}\text{C}$  before use. Bacterial DNA was extracted using the QIAamp® BiOstic® Bacteremia DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of DNA extracts, PCR products, and plasmids was determined by measuring in a Qubit 4.0 fluorometer (ThermoFisher Scientific, USA). DNA extraction

#### 4.3.7. Quantitative real-time PCR and 16S rRNA gene amplicon sequencing

##### qPCR quantification

The available genomes of *A. caldus* and *A. thiooxidans* were assigned using MAUVE software, and regions that occurred uniquely in each species were selected and analyzed using BLAST. The GCD22\_01356 gene encoding an OmpA-like domain-containing protein was selected for quantification of *A. thiooxidans* using primer pairs with sequences 5'-GCTATTGCCTGCTTCCTTGC-3' (Athiox-Fwd) and 5'-ACCGTGGACATTGACCTGAC-3' (Athiox-Rev). The Acaty\_c0044 gene encoding an extracellular matrix protein PelG was selected for quantification of *A. caldus* using primer pairs with sequences 5'-CTTGCGCTTTTGGGTACTC-3' (Acaldus-Fwd) and 5'-AACGTTGCCCAAGAGAAAAG-3' (Acaldus-Rev). Primers were designed in Primer3 software and finally checked using Primer BLAST. Extracted DNA was amplified using QuantiFast SYBR® Green PCR Kit (Qiagen, Hilden, Germany) and the LightCycler® 480 (Roche, Basel, Switzerland). The qPCR reactions were performed in a total volume of 12.5 µL containing 2X master mix, 1 µM of each primer, and template DNA. Purified PCR products of linearized plasmids carrying the species-specific gene were used as standards for qPCR. For each set of reactions, a seven-point serial decimal dilution of the respective standard was performed in triplicate to generate the threshold cycle (Ct) standard curve as a function of gene copy number. A temperature gradient was used to determine the optimum annealing temperature for each primer pair. The final cycling conditions consisted of an initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 10 s, and annealing/extension for 30 s at 60 °C. After each qPCR run, melting curves were constructed with the following parameters: one cycle of 95 °C for 5 s and 60 °C for 1 min, followed by a temperature ramping up to 95 °C in 0.3 °C steps. The specificity of each primer pair for the target

species was evaluated based on the efficiency of qPCR assays and melting curves. In addition, the amplification products were analyzed on an agarose gel to confirm the absence of nonspecific products.

#### 16S rRNA gene amplicon sequencing

The highly variable V4 region was amplified with unique bar code primers and sequenced as described previously (DOI 10.3390/bios11060170). Briefly, PCR amplification was performed using Platinum II Taq Hot-Start DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA), as follows: initial DNA denaturation step at 94 °C for 3 min, 35 cycles of DNA denaturation at 94 °C for 45 s, annealing at 52 °C for 60 s with a 50% thermal ramp, extension at 72 °C for 90 s, and a final extension step at 72 °C for 10 min. The library was purified by AMPure XP beads (Beckman Coulter, Brea, CA, USA) and sequenced using a MiniSeq System (Illumina, San Diego, CA, USA) with MiniSeq Mid Output Kit (300 cycles). Raw fastq reads were processed in R software (v4.0.3) using the open-source package DADA2 (v1.16.0).

### 4.4. Results and discussion

#### 4.4.1. Biogenic sulfuric acid production

The generation of biogenic sulfuric acid by pure and combined cultures of *A. thiooxidans* and *A. caldus* was tested at two different temperatures and three different S<sup>0</sup> concentrations. Among the parameters monitored were optical density (Fig. 2), sulfate concentration and pH (Table 1), and calculated total acidity (Fig. 2). At 30 °C, the combined culture supplemented with 3.0 and 6.0% S<sup>0</sup> showed an almost linear increase in total acidity over the four weeks of cultivation reaching a maximum of around 0.66 M in the presence of 6.0% S<sup>0</sup> (Fig. 2C). During the four-week cultivation, significant differences in the monitored parameters were found between the three S<sup>0</sup> concentrations. By comparing the pure culture of *A. thiooxidans* grown at the optimal

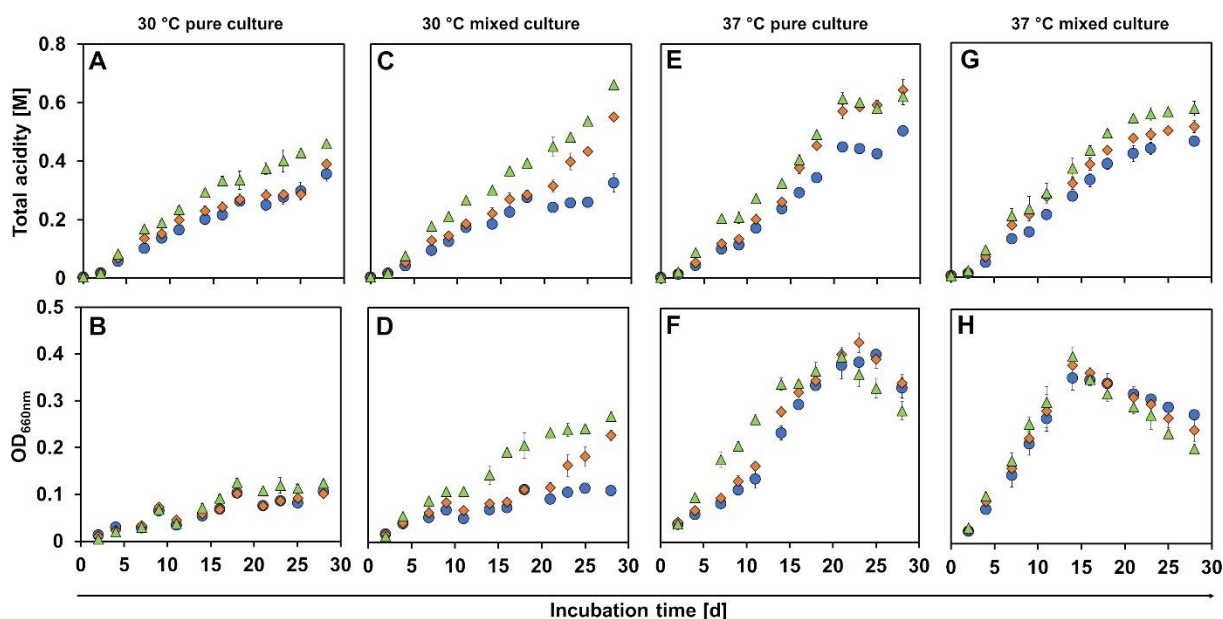
temperature of 30 °C with the combined culture, the pH decreases and the increase in total acidity was lower, reaching a minimum pH of 0.78 and a maximum acidity of 0.46 M with 6.0% S<sup>0</sup> (Fig. 2A). The lower acidity was also reflected in lower cell growth after four weeks of cultivation than in the combined culture (Fig. 2B).

**Table 1:** pH and sulfate concentration after four weeks of cultivating a pure culture of *A. thiooxidans* and *A. caldus*, and mixed culture at different temperatures.

	<i>A. thiooxidans</i>			Combined culture			<i>A. caldus</i>			Combined culture		
Temperature	30 °C			30 °C			37 °C			37 °C		
Sulfur [g]	1.5	3.0	6.0	1.5	3.0	6.0	1.5	3.0	6.0	1.5	3.0	6.0
pH	0.78	0.78	0.79	0.82	0.67	0.56	0.60	0.61	0.60	0.75	0.66	0.61
SO <sub>4</sub> <sup>2-</sup> [M]	0.20	0.22	0.27	0.18	0.32	0.38	0.28	0.35	0.37	0.29	0.29	0.34

A second experiment with the combined bacterial culture and a pure culture of *A. caldus* was conducted at 37 °C. In contrast to the combined culture at 30 °C, a linear increase in acidity could only be observed over the first three weeks of cultivation, with no further increase in the following week. The maximum value of acidity obtained was 0.58 M, with 6.0% S<sup>0</sup> showing only minor differences between the different sulfur concentrations (Fig. 2G). The pure culture of *A. caldus* reached comparable values of total acidity (0.62 M) and pH (0.60) as obtained with the combined culture at 37 °C, while sulfur metabolization likewise stopped after three weeks (Fig. 2E). In contrast to the cultivation at 30 °C, both cultures (combined and pure) showed a decrease in the cell number after two (combined) and three weeks (pure) of cultivation at 37 °C, which is the reasons for the reduced acidity at the end of cultivation (Fig. 2F and H). The high cell numbers reached by the pure and combined cultures at 37 °C after two weeks may have covered the free sulfur particles with a biofilm, preventing the growth of non-adhered cells (Gourdon and Funtowicz, 1998). Consequently, the lower and constant increase in cell number by the combined culture at 30 °C favoured a continuous sulfate

and acidity production rate. The combined culture at 30 °C was used in stirred tank experiments for biogenic sulfuric acid production for the following reasons: i) the lower temperature of 30 °C enables a cheaper process in terms of heating energy cost, ii) more effective sulfur-oxidation of mixed acidophilic cultures has already been reported in previous studies (Pakostova and Johnson, 2019; Plumb et al., 2008).



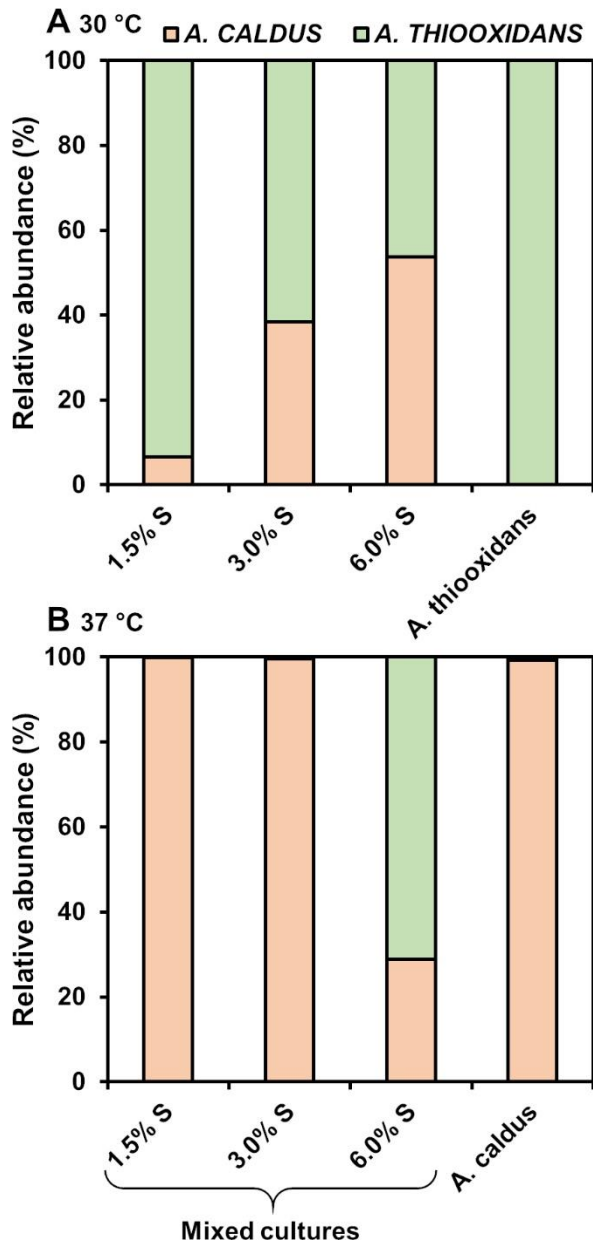
**Figure 2:** Biogenic sulfuric acid production of pure and combined cultures over 4 weeks of incubation. **A** and **B**, total acidity and cell growth of *A. thiooxidans* at 30 °C with 1.5% (blue circle), 3.0% (orange diamond) and 6.0% (green triangle)  $S^0$ . **C** and **D**, total acidity and cell growth of the combined culture of *A. thiooxidans* and *A. caldus* at 30 °C. **E** and **F**, total acidity and cell growth of *A. caldus* at 37 °C. **G** and **H**, total acidity and cell growth of the combined culture of *A. thiooxidans* and *A. caldus* at 37 °C. Error bars indicate the standard deviation (SD) of the biological triplicates.

#### 4.4.2. Population dynamics during biogenic sulfuric acid production

Quantitative PCR analysis was performed on pooled biological triplicates after four weeks of cultivation at 30 and 37 °C. As a reference and proof of culture purity, qPCR analysis of biological triplicates of *A. thiooxidans* and *A. caldus*, both supplemented with 3.0%  $S^0$ , were performed.  $S^0$  concentration showed an apparent effect on the mixed culture at 30 °C (Fig. 3A). The relative abundance of *A. thiooxidans* decreased with elevating  $S^0$  concentrations (93% > 61% > 46%), whereas the opposite trend was observed for *A. caldus*. Using a concentration of 6.0%  $S^0$ , an almost equal distribution of both species (46% *A. thiooxidans* and 54% *A. caldus*) was observed after four weeks

of cultivation at 30 °C. A higher  $S^0$  concentration seems to favour the growth of *A. caldus*, even at a suboptimal temperature. At a later stage of cultivation, the increased biomass can also generate heat. Unless the system is effectively cooled, the increased temperature may be favourable for *A. caldus*.

As expected, *A. caldus* was the dominant species under moderately thermophilic conditions (37 °C), accounting for almost 100% of the relative abundance (Fig. 3B). An increased growth rate of *A. caldus* under optimal temperature conditions resulted in overgrowing the *A. thiooxidans* culture. Surprisingly, *A. thiooxidans* was observed as the dominant species after four weeks of cultivation at the highest  $S^0$  concentration of 6.0%, accounting for around 70% relative abundance. One reason might be the difference in pH tolerance of both species. During bacterial sulfuric acid production, the pH dropped under 1.0, outside the growth optimum of *A. caldus*, and promotes *A. thiooxidans*, reported to grow down to pH 0.5 (Wang et al., 2019). Nevertheless, the results obtained by qPCR analysis underline the findings related to total acidity measurements, showing that a mixed culture of *A. thiooxidans* and *A. caldus* cultured at 30 °C with 6.0%  $S^0$  can be the optimal setting for biogenic sulfuric acid production in a CSTR.



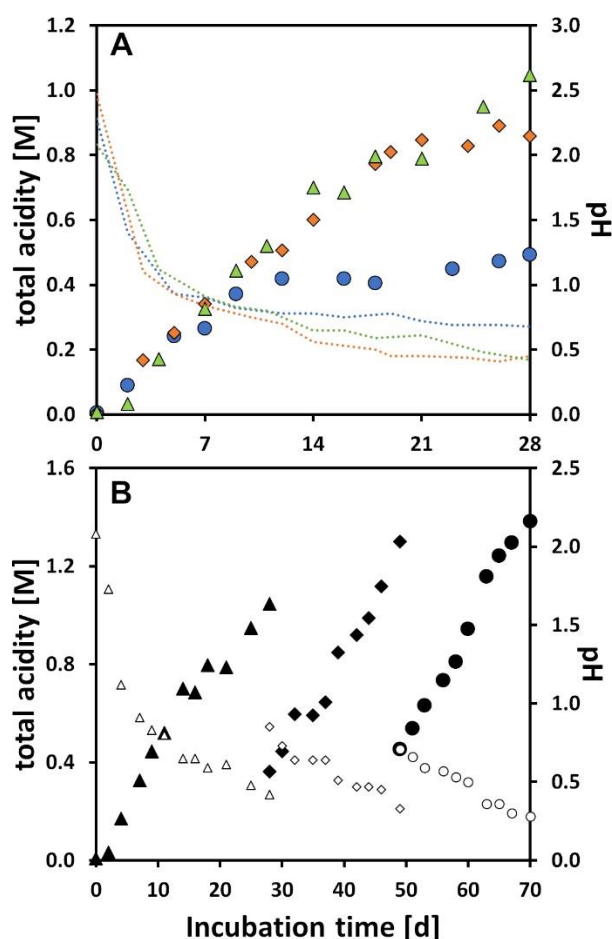
**Figure 3:** Relative abundance of *A. thiooxidans*, *A. caldus* at 37 °C. Results of the quantitative PCR analysis in the batch experiments after four weeks of cultivation using the combined culture at 30 °C (**A**) and 37 °C (**B**) with three different  $S^0$  concentrations, respectively. The relative abundance of both bacteria is shown in %, together with results from the pure cultures *A. thiooxidans* at 30 °C and *A. caldus* at 37 °C, both supplemented with 3.0%  $S^0$ .

#### 4.4.3. Biogenic acid production in CSTR

To identify the most suitable  $S^0$  concentration in the stirred tank reactor, three concentrations identical to those in batch experiments with the combined culture were tested at 30 °C. Due to better aeration of 60 L h<sup>-1</sup> and a higher stirring rate (250 rpm) in the reactor, the lowest concentration of 1.5%  $S^0$  leads to higher total acidity values

(0.49 M in Fig. 4A) in comparison to the batch experiments (0.33 M in Fig. 2C). Furthermore, the maximum acidity was reached even faster after two weeks of cultivation. This can be attributed to an increased bioavailability of sulfur due to more efficient agitation in the stirred tank reactor and a consequently better sulfur solubility. Nevertheless, compared to higher  $S^0$  concentrations (i.e., 3.0 and 6.0%), the amount of acidity produced was half (Fig. 4A). Thus, the maximum of total acidity values increased with higher  $S^0$  concentrations, reaching 0.9 and 1.05 M for 3.0 and 6.0%  $S^0$ , respectively (Fig. 4A). The minor differences in maximum acidity with 3.0 and 6.0%  $S^0$  can be attributed to the aggregation of sulfur particles due to hydrophobic interactions at concentrations greater than 5%, as previously reported (Gourdon and Funtowicz, 1998). To overcome this effect and to further increase the acidity production rate, a semi continuous experimental setup was tested with the highest sulfur concentration of 6.0%. Therefore, two-third of the produced biogenic acid were replaced by a fresh minimal culture medium with  $S^0$  after four weeks, following a reduction in process time to three weeks in the following two periods. As a result, a higher acidity of around 1.12 M was obtained after two weeks in the first period, reaching a maximum of appr. 1.3 M after three weeks (Fig 4B). In the second period of semi continuous acid production, the same trend could be observed reaching a higher total acidity of 1.16 M after 14 days of incubation, ending up with a 1.38 M biogenic acid after three weeks. At the end of each period (I–III) of semi continuous cultivation, three-quarters of the mixed culture were dominated by *A. thiooxidans*, likely related to the low pH of down to 0.3 at which the growth of *A. thiooxidans* is more favored than *A. caldus*.





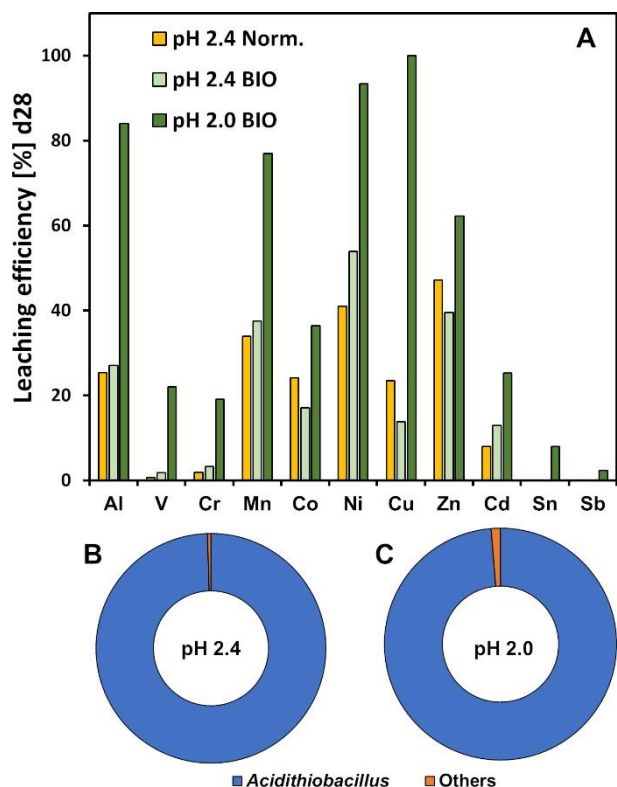
**Figure 4:** **A:** Continuous stirred tank production of biogenic sulfuric acid with the combined culture of *A. thiooxidans* and *A. caldus* at 30 °C with three different  $S^0$  concentrations 1.5% (blue circle), 3.0% (orange diamond) and 6.0% (green triangle) in batch mode respectively. The symbols represent the total acidity generated, together with the decrease in pH (dotted lines) over the four weeks of cultivation. **B:** Semi-continuous production of biogenic acid in the CSTR with 6.0%  $S^0$  and the combined culture in three production periods I (black triangle), II (black diamond) and III (black circle) and the corresponding pH-values (blank symbols).

#### 4.4.4. Heap bioleaching using biogenic sulfuric acid for pH control

Factors such as low process costs, operation under environmentally friendly conditions, and the suitability for processing low-grade materials make heap bioleaching an attractive processing technique in different applications (Li et al., 2021). To verify the suitability of heap bioleaching for the efficient processing of waste incineration residuals, a system combining biogenic sulfuric acid production for pH control with a heap leaching reactor was assembled (Fig. 1). To compare the metal removal capacity of the biogenic acid regulated heap with normal sulfuric acid regulation, two experiments with 0.4 M biogenic sulfuric acid, spent medium from

bioreactors with 1.5% S<sup>0</sup> (pH 0.42) and 0.4 M chemically produced sulfuric acid, both at a pH control of 2.4, were performed. The results obtained show that the same leaching efficiencies in the range of 14 – 54% can be achieved for metals such as Cu, Zn, Ni, Mn, Co and Cd using the biogenic sulfuric acid (Fig. 5A). Moreover, the acid consumption for pH control was comparable, comprising 0.04 mL 18 M H<sub>2</sub>SO<sub>4</sub> per gram slag of commercial and 0.05 mL g<sup>-1</sup> of biogenic acid, respectively. In the blank experiment, 0.07 mL g<sup>-1</sup> sulfuric acid was consumed to adjust pH 2.4 using only water and 0.4 M sulfuric acid. At pH 2.0 and pH adjustment with 1.12 M biogenic sulfuric acid (spent medium of the first semi continuous cultivation with 6% S<sup>0</sup>), the leaching efficiency of all tested metals increased, reaching maximum values of 99, 93, 77, 62, 36 and 25 for Cu, Ni, Mn, Zn, Co, and Cd, respectively, after three weeks of heap bioleaching (Fig. 5A). The acid consumption rate increased with lower pH to 0.12 mL g<sup>-1</sup> at pH 2.0. The conducted blank experiment, using only water and pH-regulation, resulted in metal extraction efficiencies below 10%, highlighting the need and efficiency of bioleaching bacteria. Comparable studies on heap-bioleaching of municipal solid waste bottom ash, operated at pH 2, reached lower leaching efficiencies for Cu (44%) and Zn (53%) after a total leaching time of twenty weeks (Mäkinen et al., 2019).

The 16S metagenomic analysis of the cell samples at the end of the pH 2.4 and 2.0 heap bioleaching experiments revealed that 98 – 99% of the bacteria in the lixiviant account for the *Acidithiobacillus* sp. (Fig. 5B and C). By performing an nucleotide sequence scan with the sequence accounting for 99% of the reads via BLASTN (BLASTN 2.12.0+, U.S. National Medicine Library) (Zhang et al., 2000), *Acidithiobacillus ferrooxidans* was identified to be the major contributor with a query cover of 100%. This indicates that the heap bioleaching system can be operated under non-sterile conditions, without risking a contamination.



**Figure 5:** Bioleaching efficiencies of the heap-bioleaching system regulated with normal sulfuric acid (A, orange) and biogenic sulfuric acid (A, light green) at pH 2.4 and with biogenic acid at pH 2.0 (A, dark green). **B and C:** Results of the 16S metagenomic analysis of the bacteria sample at the end of the pH 2.4 (B) and 2.0 (C) bioleaching experiment showing the relative abundance of *Acidithiobacillus* spp.(blue) and others (orange)

#### 4.4.5. Economical assessment

By estimating the retail cost of sulfuric acid with the market value of the metals released (Table 2), heap bioleaching appears to be an economically attractive method (Li et al., 2021). At pH 2.0, the value of the metals released, calculated from the corresponding leaching efficiency, is more than five times the cost (in USD \$) of the required sulfuric acid. Considering the actual price of USD 90/t (echemie.com, 2021) and the required amount of around 221 kg of concentrated sulfuric acid per ton of incineration residue to maintain pH 2.0, 19.9 USD are required. On the other hand, metals with a value of around 135 USD can be generated. One has to consider that the sulfuric acid price is strongly fluctuating reaching for example around 60 USD/t in North America or over

200 USD/t in Asia at the time this publication was written (chemanalyst.com, 2021). Nevertheless, already the value of metals such as aluminium and copper which were biologically extracted by 84 and 99% respectively, exceed the potential costs for purchasing the required amount of sulfuric acid. Overall, around 70% of the potential total value incorporated in one ton of incineration slag could be recovered via bio-extraction in the heap bioleaching system.

Furthermore, the aim of this study was to produce the required sulfuric acid in a biological and cheap way from waste elemental sulfur. This can contribute to further maximize the generated value, by minimizing costs for purchasing and transporting commercial sulfuric acid and enables the operator to be independent from the price fluctuations. To further increase the leaching efficiency and the corresponding liberated value, possible adjustments according lower pH-values, higher ferric iron concentration and higher recirculation rate of the lixiviant might be applied. Therefore, a careful economic assessment of the scale-up parameters needs to be performed to find a balance between science and engineering (Petersen, 2010).

**Table 6:** Metal concentrations of waste incineration slag and the corresponding potential value of the incorporated metals. With the achieved leaching efficiencies at pH 2.0, the potential recovery in kg metal and USD \$ were calculated and summed up as potential total value and potential value recovered via heap-bioleaching.

Element	Market price USD t <sup>-1</sup>	Concentration in slag kg t <sup>-1</sup>	Potential value USD t <sup>-1</sup>	Bioleaching efficiency %	Potential recovery kg t <sup>-1</sup>	Recovered value USD t <sup>-1</sup>
Co	45195 <sup>a</sup>	0.089	4.02	56	0.05	2.26
Cd	2300 <sup>c</sup>	0.008	0.02	88	0.007	0.02
Sn	32918 <sup>d</sup>	0.17	5.6	27	0.05	1.65
V	19621 <sup>e</sup>	0.032	0.6	22	0.01	0.2
Al	2446 <sup>a</sup>	42.0	102.7	84	35.3	86.3
Cr	8400 <sup>b</sup>	0.23	1.9	19	0.04	0.31
Fe	500 <sup>a</sup>	54.5	27.3	n.d.	n.d.	n.d.
Mn	2500 <sup>b</sup>	0.96	2.4	77	0.74	1.85
Ni	17958 <sup>a</sup>	0.15	2.7	93	0.14	2.5
Zn	2936 <sup>a</sup>	4.63	13.6	68	3.15	9.25
Cu	9992 <sup>a</sup>	3.14	31.4	99	3.11	31.08
Total			192.24			135.4

<sup>a</sup>London Metal exchange (5.5.2021), <sup>b</sup>SMM, <sup>c</sup>Statista.com (30.06.2021), <sup>d</sup>lme.com (30.06.2021),

<sup>e</sup>vanadiumprice.com (30.06.2021)

#### 4.5. Conclusion

The integration of externally produced biogenic sulfuric acid for pH control of heap bioleaching systems can provide a cheap, effective, and environmentally friendly processing of low-grade artificial ores such as incineration residuals. Implementation directly in municipal waste incineration plants would provide benefits such as reduced transport costs for incineration residuals and sulfuric acid, possible value generation from extracted metals, and easy temperature control in the nearby incineration facility. This study demonstrated the efficient generation of biogenic sulfuric acid by a combined culture of sulfur-oxidizing *Acidithiobacillus* spp., which can be successfully applied for pH control in the heap bioleaching system. Using this system, it was possible to effectively leach metals such as Cu, Ni, Zn, Cr, V, and Al from incineration bottom ash within three weeks without the need for commercially produced sulfuric

acid. The findings highlight a new application field of bioleaching, combining the two most crucial bioleaching mechanisms.

#### **4.6. Acknowledgements**

This work was supported by the European fund for regional development, under the program Interreg V-A Austria-Czech Republic, project ATCZ183, IRAS (Innovative Recycling technology for Ashes and Slags). Slag samples were kindly provided by the EVN Abfallverwertung NÖ GmbH (Dürnrohr, Austria). Furthermore, the authors want to thank Cicely Warne for proofreading the manuscript. Open access funding provided by University of Natural Resources and Life Sciences Vienna (BOKU).

#### **4.7. References**

- Abramov, S., He, J., Wimmer, D., Lemloh, M.L., Muehe, E.M., Gann, B., Roehm, E., Kirchhof, R., Babechuk, M.G., Schoenberg, R., Thorwarth, H., Helle, T., Kappler, A., 2018. Heavy metal mobility and valuable contents of processed municipal solid waste incineration residues from Southwestern Germany. *Waste Manag.* 79, 735–743. <https://doi.org/10.1016/j.wasman.2018.08.010>
- Blasenbauer, D., Huber, F., Lederer, J., Quina, M.J., Blanc-Biscarat, D., Bogush, A., Bontempi, E., Blondeau, J., Chimenos, J.M., Dahlbo, H., Fagerqvist, J., Giro-Paloma, J., Hjelm, O., Hyks, J., Keaney, J., Lupsea-Toader, M., O’Caollai, C.J., Orupöld, K., Pająk, T., Simon, F.G., Svecova, L., Šyc, M., Ulvang, R., Vaajasaari, K., Van Caneghem, J., van Zomeren, A., Vasarevičius, S., Wégner, K., Fellner, J., 2020. Legal situation and current practice of waste incineration bottom ash utilisation in Europe. *Waste Manag.* 102, 868–883. <https://doi.org/10.1016/j.wasman.2019.11.031>
- chemanalyst.com, 2021. Sulfuric acid price overview [accessed 30.06.2021]. URL <https://www.chemanalyst.com/Pricing-data/sulphuric-acid-70>
- Coherent Market Insights, 2021. Global Sulfuric Acid Market to Surpass 324.1 Million Tons by 2027.
- echemie.com, 2021. Sulfuric Acid Market [accessed 30.06.2021]. URL

- [https://www.echemi.com/productsInformation/pid\\_Rock19440-sulfuric-acid.html](https://www.echemi.com/productsInformation/pid_Rock19440-sulfuric-acid.html)
- European Parliament and Council, 2008. Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 on waste and repealing certain directives (Waste framework. LexUriServ. do 3–30.  
[https://doi.org/2008/98/EC.; 32008L0098](https://doi.org/2008/98/EC.;32008L0098)
- Eurostat Waste Statistics, 2017. Municipal waste statistics 1–6.  
<https://doi.org/10.2785/408702>
- EVN Abfallverwertung, 2017. EVN Abfallverwertung Niederösterreich, MVA Dürnrohr Umwelterklärung 2017.
- Gomes, H.I., Funari, V., Dinelli, E., Soavi, F., 2020. Enhanced electrodialytic bioleaching of fly ashes of municipal solid waste incineration for metal recovery. *Electrochim. Acta* 345, 136188. <https://doi.org/10.1016/j.electacta.2020.136188>
- Gourdon, R., Funtowicz, N., 1998. Kinetic model of elemental sulfur oxidation by *Thiobacillus thiooxidans* in batch slurry reactors. *Bioprocess Eng.* 18, 241–249. <https://doi.org/10.1007/pl00008985>
- Gu, T., Rastegar, S.O., Mousavi, S.M., Li, M., Zhou, M., 2018. Advances in bioleaching for recovery of metals and bioremediation of fuel ash and sewage sludge. *Bioresour. Technol.* 261, 428–440.  
<https://doi.org/10.1016/j.biortech.2018.04.033>
- Khandelwal, H., Dhar, H., Thalla, A.K., Kumar, S., 2019. Application of life cycle assessment in municipal solid waste management: A worldwide critical review. *J. Clean. Prod.* 209, 630–654. <https://doi.org/10.1016/j.jclepro.2018.10.233>
- King, M.J., Davenport, W.G., Moats, M.S., 2013. Costs of sulfuric acid production. *Sulfuric Acid Manuf.* 357–362. <https://doi.org/10.1016/b978-0-08-098220-5.00032-0>
- Krebs, W., Brombacher, C., Bosshard, P.P., Bachofen, R., Brandl, H., 1997. Microbial recovery of metals from solids. *FEMS Microbiol. Rev.* 20, 605–617. [https://doi.org/10.1016/S0168-6445\(97\)00037-5](https://doi.org/10.1016/S0168-6445(97)00037-5)
- Kremser, K., Gerl, P., Pellis, A., Guebitz, G.M., 2021. A new bioleaching strategy for the selective recovery of aluminum from multi-layer beverage cans. *Waste*

- Manag. 120, 16–24. <https://doi.org/10.1016/j.wasman.2020.11.012>
- Kremser, K., Thallner, S., Strbik, D., Spiess, S., Kucera, J., Vaculovic, T., Vsiansky, D., Haberbauer, M., Mandl, M., Guebitz, G.M., 2020. Leachability of metals from waste incineration residues by iron- and sulfur-oxidizing bacteria. *J. Environ. Manage.* 111734. <https://doi.org/10.1016/j.jenvman.2020.111734>
- Li, J., Yang, H., Tong, L., Sand, W., 2021. Some Aspects of Industrial Heap Bioleaching Technology: From Basics to Practice. *Miner. Process. Extr. Metall. Rev.* 00, 1–19. <https://doi.org/10.1080/08827508.2021.1893720>
- Mäkinen, J., Salo, M., Soini, J., Kinnunen, P., 2019. Laboratory Scale Investigations on Heap (Bio)leaching of Municipal Solid Waste Incineration Bottom Ash. *Minerals* 9, 290. <https://doi.org/10.3390/min9050290>
- Marshall, W.L., Jones, E. V., 1966. Second dissociation constant of sulfuric acid from 25 to 350° evaluated from solubilities of calcium sulfate in sulfuric acid solutions. *J. Phys. Chem.* 70, 4028–4040. <https://doi.org/10.1021/j100884a045>
- Moya-Beltrán, A., Beard, S., Rojas-Villalobos, C., Issotta, F., Gallardo, Y., Ulloa, R., Giaveno, A., Degli Esposti, M., Johnson, D.B., Quatrini, R., 2021. Genomic evolution of the class Acidithiobacillia: deep-branching Proteobacteria living in extreme acidic conditions. *ISME J.* <https://doi.org/10.1038/s41396-021-00995-x>
- Moya, D., Aldás, C., López, G., Kaparaju, P., 2017. Municipal solid waste as a valuable renewable energy resource: A worldwide opportunity of energy recovery by using Waste-To-Energy Technologies. *Energy Procedia* 134, 286–295. <https://doi.org/10.1016/j.egypro.2017.09.618>
- Ñancucheo, I., Rowe, O.F., Hedrich, S., Johnson, D.B., 2016. Solid and liquid media for isolating and cultivating acidophilic and acid-tolerant sulfate-reducing bacteria. *FEMS Microbiol. Lett.* 363, 1–17. <https://doi.org/10.1093/femsle/fnw083>
- Pakostova, E., Johnson, D.B., 2019. Microbial generation of sulfuric acid from granular elemental sulfur in laboratory-scale bioreactors. *Hydrometallurgy* 190, 105152. <https://doi.org/10.1016/j.hydromet.2019.105152>
- Petersen, J., 2010. Modelling of bioleach processes: Connection between science and engineering. *Hydrometallurgy* 104, 404–409. <https://doi.org/10.1016/j.hydromet.2010.02.023>



- Plumb, J.J., McSweeney, N.J., Franzmann, P.D., 2008. Growth and activity of pure and mixed bioleaching strains on low grade chalcopyrite ore. *Miner. Eng.* 21, 93–99. <https://doi.org/10.1016/j.mineng.2007.09.007>
- Steudel, R., 2003. Elemental Sulfur und Sulfur-Rich Compounds II, *Topics in Current Chemistry*. Springer Berlin Heidelberg, Berlin, Heidelberg. <https://doi.org/10.1007/b11909>
- Tabatabai, M.A., 1974. A Rapid Method for Determination of Sulfate in Water Samples. *Environ. Lett.* 7, 237–243. <https://doi.org/10.1080/00139307409437403>
- Wang, R., Lin, Jian Qiang, Liu, X.M., Pang, X., Zhang, C.J., Yang, C.L., Gao, X.Y., Lin, C.M., Li, Y.Q., Li, Y., Lin, Jian Qun, Chen, L.X., 2019. Sulfur oxidation in the acidophilic autotrophic *Acidithiobacillus* spp. *Front. Microbiol.* 10, 1–20. <https://doi.org/10.3389/fmicb.2018.03290>
- Zhang, X., Tang, Y., Qu, S., Da, J., Hao, Z., 2015. H<sub>2</sub>S - Selective Catalytic Oxidation : Catalysts and Processes 1067. <https://doi.org/10.1021/cs501476p>
- Zhang, Z., Schwartz, S., Wagner, L., Miller, W., 2000. A greedy algorithm for aligning DNA sequences. *J. Comput. Biol.* 7, 203–214. <https://doi.org/10.1089/10665270050081478>

## 5. Stirred-tank and heap-bioleaching of shredder-light-fractions (SLF) by acidophilic bacteria

Published in: Journal of Hydrometallurgy, 20 May 2020, Volume 193,  
<https://doi.org/10.1016/j.hydromet.2020.105315>

**Klemens Kremser<sup>a</sup>, Sophie Thallner<sup>b,c</sup>, Herta Schoen<sup>c</sup>, Stefan Weiss<sup>c</sup>, Christine Hemmelmaier<sup>a</sup>, Wolfgang Schnitzhofer<sup>c</sup>, Alexia Aldrian<sup>d</sup>, Georg M. Guebitz<sup>a,c\*</sup>**

<sup>a</sup>University of Natural Resources and Life Sciences Vienna BOKU, Dept. of Agrobiotechnology, IFA-Tulln, Inst. of Environmental Biotechnology, Konrad-Lorenz-Straße 20, 3430 Tulln and der Donau, Austria

<sup>b</sup>K1-MET GmbH, Stahlstraße 14, 4020 Linz, Austria

<sup>c</sup>Austrian Centre of Industrial Biotechnology, Konrad-Lorenz-Straße 20, 3430 Tulln and der Donau, Austria

<sup>d</sup>Montanuniversität Leoben, Chair of Waste Processing Technology and Waste Management, Franz-Josef-Str. 18, 8700 Leoben, Austria

**Keywords:** Automobile Shredder Residue, sulfur and iron oxidation, *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, biohydrometallurgy, metal recovery

## 5.1. Abstract

End-of-life vehicles are usually crushed in large scaled shredders in order to increase the surface area for further processing. During the shredding process, the so-called shredder-light-fraction (SLF), containing mainly plastics and other synthetic polymers, is discharged. However, this fraction still contains up to 20% metals such as iron (Fe), copper (Cu), nickel (Ni) and zinc (Zn), making them an interesting substrate for bioleaching and source of high-value metal traces. To develop a bioleaching process for metal recovery from shredded fractions, two experimental approaches including a heap- and a stirred-tank reactor were tested. The acidophilic, iron- and sulfur-oxidizing bacteria *Acidithiobacillus ferrooxidans* and the iron-oxidizing bacteria *Leptospirillum ferrooxidans* were investigated as pure and co-culture for their ability to solubilize trace metals. *A. ferrooxidans* proved to be the more suitable bioleaching organism resulting in leaching efficiencies of up to 100% for Zn and Ni in batch experiments. Furthermore, in the stirred-tank reactor using *A. ferrooxidans* about 100, 80 and 55% of Cu, Zn and Ni have been recovered from 50 g l<sup>-1</sup> of the SLF, respectively. Bioleaching of 3000 g SLF in the heap-reactor resulted in a recovery of about 100% Zn, 60% Cu and ~ 55% Ni after 33 days without the need of external sulfuric acid addition for pH regulation. The high leaching efficiencies in both experimental setups confirmed the potential of bioleaching for a sustainable metal recovery from shredder residues (i.e. SLF) making it a profitable, eco-friendly alternative to conventional chemical leaching processes.

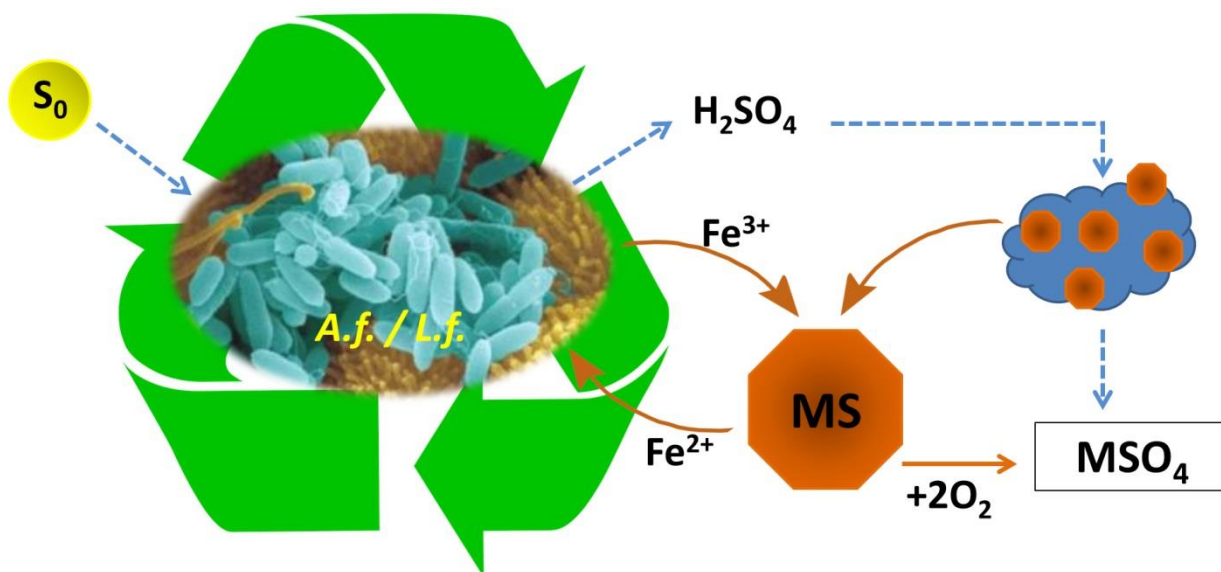
## 5.2. Introduction

In the European Union (EU) every year 7 – 9 million tons of end-of-life vehicles (ELV) are produced corresponding to approximately 1.5 – 30 kg per capita (EUROSTAT, 2010), which makes the automotive sector a major contributor to waste generation. By 2015, EU-member states committed to recover and recycle ELV by 85 to 95% (EU DIRECTIVE 2000/53, 2000; EU DIRECTIVE 2005/64/EC, 2005). However, in order to reach high recycling rates, new strategies are necessary to develop ways to use and valorize all waste fractions of ELV.

ELV waste is usually crushed in big shredders in order to increase the surface area for further treatment. During the shredding process the SLF arises, accounting for approximately 25% (w/w) of the whole waste fraction. Related to the heavy metal content of the fractions, SLF is normally declared as hazardous waste and therefore commonly discarded and landfilled as the heavy metal content inhibits the option of thermal combustion (Margarido and Nogueira, 2011). Although it mainly consists of 20 – 45% plastics, 5 – 25% rubber and 5 – 30% textiles/fibers, still 5 - 20% metals such as Fe, Cu, Ni and Zn are present (Cheminfo Services, 2014). Depending on the origin of the waste, there are significant differences in the concentration of valuable metals. Fractions which arise during the shredding of waste electronic and electrical equipment (WEEE's) for example still contain up to 60% metals (Witne and Phillips, 2001) whereas residual waste consists only of around 5% metals (FhG-IBP, 2014). Therefore, SLF present a valuable source for metals, which would normally end up on landfills.

In order to develop a simple, efficient and environmentally friendly process for metal recovery, microbial leaching, also called “biomining or bioleaching” was proposed. The term “biomining” was first introduced by Temple et al. in 1951 by discovery of

*Acidithiobacillus ferrooxidans* in iron, copper and manganese rich environments (Temple and Colmer, 1951). The concept of using bacteria for bio oxidation of low grade copper and gold ores came up in the 1970s resulting in the first pilot plant in South Africa (Van Aswegen et al., 1988). In general, the recovery of metals from waste is carried out by acidification with  $H_2SO_4$  and  $HCl$ , leading to low pH values between 1.5 – 2.0 at which many heavy metals present, are solubilized. The acidification is normally done by inorganic and organic acids termed as chemical leaching (Oliver and Carey, 1976; Sreekrishnan and Tyagi, 1996). Bioleaching bacteria like *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* are extremely acidophilic (pH < 3) and mesophilic (30 °C) organisms, oxidizing Fe(II) ions and/or inorganic sulfur compounds for energy generation (Rohwerder et al., 2003). The main action of both bacteria is the dissolution of metal-sulfides (MS). Non-soluble metal-sulfides (MS) are solubilized by the thiosulfate pathway, whereas for acid-soluble MS the polysulfide pathway is responsible (Schippers and Sand, 1999). Moreover, *A. ferrooxidans* is both, an iron- and sulfur-oxidizing bacterium, producing sulfuric acid during the oxidation of elemental sulfur and other sulfur compounds (Kupka et al., 2009). This leads to a decrease in pH, which further facilitates the solubility of MS (Fig. 1).



**Fig. 1:** Schematic presentation of the bioleaching process involving *A. ferrooxidans* and *L. ferrooxidans* (adapted from (Schipper and Sand, 1999)). The production of sulfuric acid by sulfur-oxidizing bacteria (blue, dashed pathway) leads to the dissolution of the waste matrix surrounding the metal-sulfides (MS) and facilitates the production of soluble metal-sulfates. The oxidation of Fe(II) to Fe(III) by iron-oxidizing bacteria (orange pathway) by thiosulfate- and polysulfide pathway leads to the dissolution of acid-soluble and acid non-soluble MS.

Acidophilic bacteria like *A. ferrooxidans* and *L. ferrooxidans* have already been successfully used in the metal recovery from waste electronic and electrical equipment, allowing recovery of 85 - 95% copper within 120 h (Bas et al., 2013; Xiang et al., 2010a). In a different approach, up to 80 and 90% of Ni and Zn, respectively, were recovered by using sewage sludge and different industrial waste ashes and slags as substrate for bioleaching experiments (Funari et al., 2017; Kaksonen et al., 2011; Pathak et al., 2009). Most of these bioleaching experiments for metal recovery were carried out in stirred-tank reactors making it a well-controllable process. On the other hand, the bioleaching of low grade copper and uranium ores for example is already applied in industrial scale by using heap-bioleaching-systems (Pradhan et al., 2008; Wang et al., 2017).

In this study the potential of bioleaching for metal recovery from shredder-light-fractions (SLF) from automobile residues with different acidophilic bacteria was investigated for the first time. Furthermore, two different reactor setups were evaluated

for their potential of cost-efficient metal recovery considering subsequent use in an industrial scale.

### **5.3. Materials and methods**

All chemicals were of analytical grade unless otherwise specified and purchased from Sigma-Aldrich (Sigma-Aldrich, Vienna, Austria). Deionized water was used for the preparation of cultivation media and stock solutions. For pH measurements, a Mettler Toledo S220 pH meter with a combined glass electrode was used. Cell number determination was done by counting bacteria cells in a Neubauer improved hemocytometer with 0.01 mm depth (BRAND GmbH, Wertheim, Germany).

#### **5.3.1. Shredder-light-fraction (SLF) samples**

Two different samples (Shredder-fraction “alt” SA [2013] and shredder-fraction “neu” SN [2014]) of shredder-light-fractions were obtained directly from the local waste processing company RMVG (Eisenerz, Austria) and used for further treatment. To obtain a representative and homogenized sample (sample taking according ÖNORM S 2137 and ÖNORM EN 14899), a total amount of six SLF samples were taken directly at the processing facility over a period of two months in two different years (2013 and 2014) and combined to the two samples SA and SN. The grain size of the waste particles was in the range of 0 – 8 mm. For batch and up-scale experiments, particles were milled to 1 mm in order to increase the surface area. Prior to use, both samples were dried at 60 °C for up to one week to remove remaining water from the samples resulting in a dry matter (DM) of 84.0 and 83.3% for SA and SN. The metal composition of both samples was determined *via* inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500 cx, after a microwave-assisted total digestion using a mixture of HF, HNO<sub>3</sub> and HCl according to ÖNORM EN 13656).

### 5.3.2. Bacteria and growth media

Two mesophilic bacteria, *A. ferrooxidans* (DSMZ 583) and *L. ferrooxidans* (DSMZ 2705), obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany) were cultivated in the following culture media. The medium for *A. ferrooxidans* contained 33.3 g l<sup>-1</sup> FeSO<sub>4</sub> x 7 H<sub>2</sub>O, 0.4 g l<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.4 g l<sup>-1</sup> MgSO<sub>4</sub> x 7 H<sub>2</sub>O, 0.4 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub> and was adjusted to pH 1.4 with concentrated sulfuric acid. *L. ferrooxidans* was cultivated in a medium containing 950 ml of solution A (147 mg l<sup>-1</sup> CaCl<sub>2</sub> x 2 H<sub>2</sub>O, 132 mg l<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 53 mg l<sup>-1</sup> MgCl<sub>2</sub> x 6 H<sub>2</sub>O and 27 mg l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>), 50 ml of solution B (20 g l<sup>-1</sup> FeSO<sub>4</sub> x 7 H<sub>2</sub>O) and 1 ml of a trace element solution (62 mg l<sup>-1</sup> MnCl<sub>2</sub>, 68 mg l<sup>-1</sup> ZnCl<sub>2</sub>, 64 mg l<sup>-1</sup> CoCl<sub>2</sub> x 6 H<sub>2</sub>O, 31 mg l<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 10 mg l<sup>-1</sup> Na<sub>2</sub>MoO<sub>4</sub> and 67 mg l<sup>-1</sup> CuCl<sub>2</sub> x 2 H<sub>2</sub>O). The pH of the *L. ferrooxidans* medium was adjusted to pH 1.8 with concentrated sulfuric acid. For the cultivation of both bacteria together, a slightly modified 9K media (Silvermann and Lundgren, 1959) at pH 1.4, 30 g l<sup>-1</sup> FeSO<sub>4</sub> x 7 H<sub>2</sub>O and addition of 1 ml trace element solution, was used. For the cultivation of both bacteria, 250 ml Erlenmeyer flasks containing 90 ml of media were inoculated with 10 ml bacteria pre-culture and incubated at 30 °C and 115 rpm for one week.

### 5.3.3. Bioleaching batch tests

Batch tests were carried out as biological duplicates in 250 ml Erlenmeyer flasks containing 108 ml culture media and 1% DM of the shredder-light-fraction (SLF, 1% w/v) prior to inoculation. Flasks were inoculated with 12 ml (10% v/v) of a one week old, actively growing culture (~ 10<sup>8</sup> Cells ml<sup>-1</sup>) and incubated at 30 °C and 115 rpm for 2 weeks. In order to determine the difference between microbial bioleaching activity and chemical leaching, one blank per SLF containing only medium and the SLF was run in parallel. All experiments, bacteria and blanks, were performed without externally



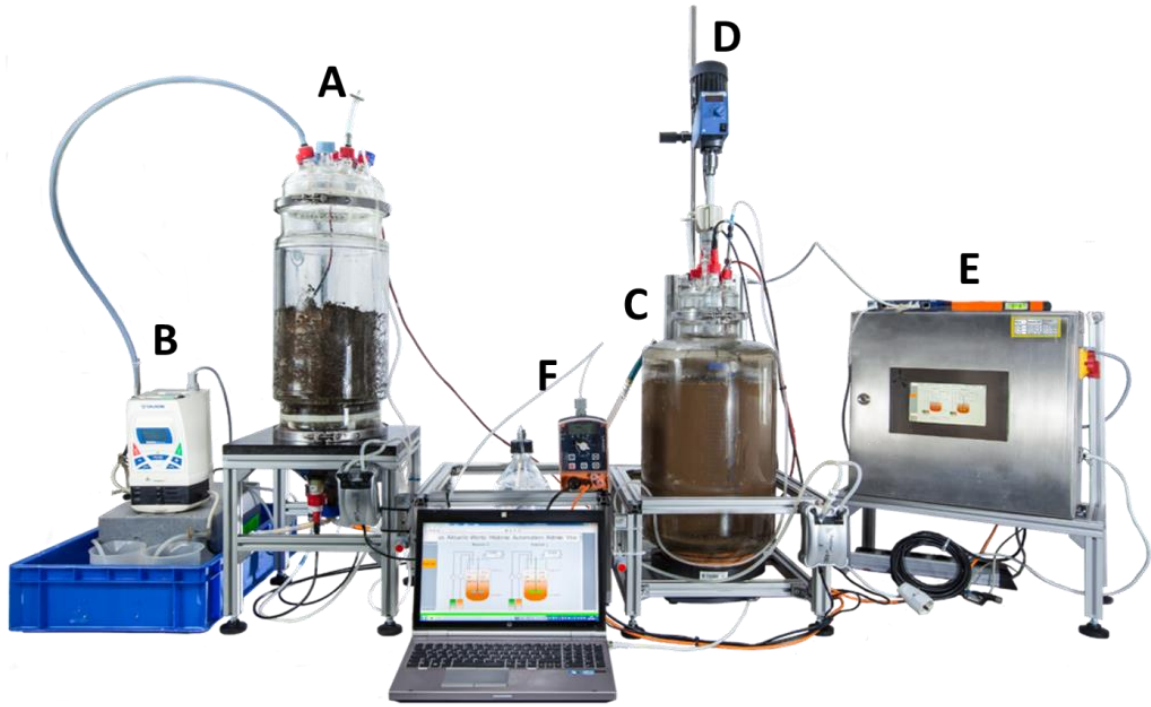
addition of sulfuric acid to adjust the pH throughout the two weeks of incubation. To test the effect of substrate-concentration, different amounts of SLF (10, 30 and 80 g l<sup>-1</sup>) were added in a further approach under the same conditions as described above. At the start and end of the experiments samples were taken for ICP-MS analysis of the metal content and pH was measured. Prior to ICP- and pH-measurements, samples were filtrated through a CHROMAFIL® Xtra Nylon filter (Macherey-Nagel GmbH, Germany) with a pore size of 0.45 µm to remove particles and precipitates.

#### 5.3.4. Tank and heap-bioleaching experiments

For the scale up experiments, two different reactor systems including a continuous stirred-tank reactor [CSTR] and heap reactor for continuous metal recovery were investigated. The CSTR consisted of a 25 l glass tank with a double glass jacket for temperature regulation *via* a connected water bath (Fig. 2C). Temperature, pH and redox potential (mV vs. Ag/AgCl reference) were controlled and regulated during the whole process. To ensure a proper aeration and mixing of the system, a stirring speed of 150 rpm and an air flow of 20 l h<sup>-1</sup> were set. For monitoring the parameters and automatic regulation of the pH (1.8) *via* dosage of 1 M sulfuric acid, an AwiFlex Series 7 process control system (AWITE Bioenergie GmbH, Langenbach, Germany) was used. Prior to the addition of SLF, 10% (v/v) inoculum was used to inoculate the 20-l working volume of the reactor in order to perform a one-week pre-cultivation to reach 10<sup>8</sup> cells ml<sup>-1</sup>. The CSTR was run in semi-continuous mode while the SLF concentration was increased (10 – 100 g l<sup>-1</sup>) after every two weeks and concomitantly replacing 3/4 of the incubation mixture (15 l) with fresh medium. Removing of the spent media and SLF was done *via* a connected peristaltic pump, whilst the reactor content was stirred continuously to provide a proper homogenization. After emptying 3/4 of the reactor content, the amount of SLF corresponding to the different loading rates and

15 l fresh media were added. The remaining 5 l of each loading rate were used as inoculum for the next incubation step with increased SLF concentration. In this way, *A. ferrooxidans* was adapted to rising concentrations of the SLF. Samples of the leachate and the spent SLF were taken before and after each addition of SLF and media. Prior to ICP-measurements, liquid samples were filtrated as stated in section 2.3.. SLF samples were centrifuged in an Eppendorf 5920R centrifuge (Eppendorf, Vienna, Austria) at maximum speed (3700 rpm) for 30 minutes. The supernatant was removed and prior to analysis, samples were dried at 60 °C for up to 1 week.

For the simulation of a heap-bioleaching-system, a 20 l glass reactor based system with a sieve plate at the bottom, a pump for circulation of the leachate and a sprinkling system at the top was constructed (Fig. 2A). The pH and redox sensors were installed in the sink of the reactor and the pH (1.8) was automatically controlled *via* dosage of 1 M sulfuric acid. 3000 g of the SLF were piled in the reactor with plastic spacers in between the layers in order to prevent the clogging of the material. To start the heap-system, 6 l of the one-week pre-culture from the CSTR was used for inoculation. The solution was circulated with a circulation rate of approximately 3 l h<sup>-1</sup> to rinse the SLF from the top. The temperature was set to 30 °C and regulated *via* a double glass jacket and the connected water bath. Samples of the untreated SLF were taken out of the 3000 g at the beginning of the experiment. After 33 days of incubation the whole reactor content was removed and mechanically homogenized *via* stirring. Samples were taken directly from the homogenized 3000 g SLF. Prior to ICP analysis, samples after 33 days were treated in the same way as described above.



**Fig. 2:** Picture of the reactor setup used for bioleaching experiments to evaluate the potential in industrial scale application showing the heap-reactor (A) with the connected pump for recirculation of the leachate (B) and the continuous-stirred-tank reactor CSTR (C) with the stirrer at the top (D). Automatic Awite process control system is shown on the right side (E) and pH regulation via dosage of 1 M sulfuric acid is done by the pump (F). Regulation of the temperature at 30 °C with a connected water bath (not shown).

### 5.3.5. Calculation of the leaching efficiency

For the calculation of the leaching efficiency, the amount of metals leached out of the SLF was set in relation to the metal content in the untreated SLF fraction:

$$L_{eff}[\%] = \frac{c_s * V_s}{c_F * M_F} * 100$$

The concentration of the metal in the leachate ( $c_s$ ) was multiplied by the volume of the batch ( $V_s$ ) to reveal the total amount of metal in the liquid phase. This concentration was set in relation to the concentration in the original SLF ( $c_F$ ) and the total mass used for the leaching experiments ( $M_F$ ).

## **5.4. Results and discussion**

### **5.4.1. Characterization of the original shredder-light-fraction**

Table 1 shows the metal content of the two untreated, original SLF which were sampled in two different years. Both fractions showed relatively high amounts of Al, Ba, Cu, Fe, Mn, Ti and Zn, which are common metals used in the automotive industry and therefore part of the shredded waste. By comparing the results analyzed by ICP-MS analyses, quite high variations have been noticed between the two samples obtained from the same site but in different years. The iron content in the SN fraction for example is nearly the double compared to the SA fraction, whilst in contrast the Zn concentration is only the half. Variations can also be found in the concentrations of the other metals, but not to such a high extent. The composition of waste treated in waste processing facilities is depended on different factors including the origin, the season and the location of the site, making it almost impossible to generate a homogenous mixture over several years. Since the recovery of valuable Cu, Ni, and Zn was the main task of the study, analyses and bioleaching optimization was focused on these metals.

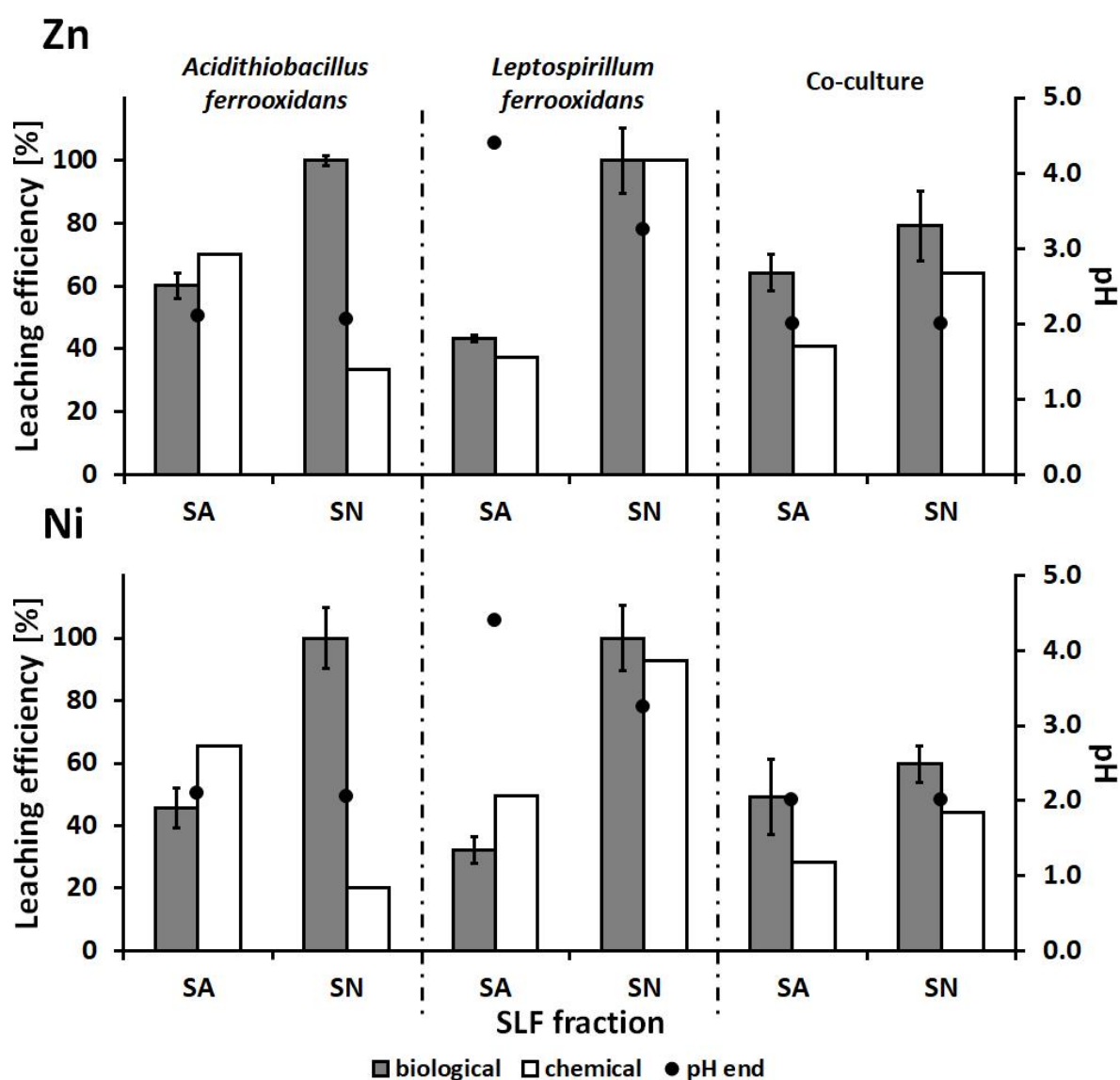
**Tab. 1:** ICP-MS analysis to determine the metal content of the two different shredder-light-fractions (SLF). Metals in high concentrations written in bold letters.

	SLF		Limit of detection
	SA	SN	
	[mg kg <sup>-1</sup> ]	[mg kg <sup>-1</sup> ]	[mg kg <sup>-1</sup> ]
<b>Al</b>	29300	20600	2.5
As	22	19	2.5
<b>Ba</b>	4900	2370	0.5
Cd	39	26	0.5
Co	67	52	0.25
Cr	500	700	5.0
<b>Cu</b>	9900	8420	0.5
<b>Fe</b>	95400	160000	25
Li	33	44	0.25
<b>Mn</b>	2800	4540	2.5
Mo	150	62	2.5
Ni	510	570	2.5
Pb	2700	1110	0.5
Sb	150	150	0.5
Sn	270	250	0.5
<b>Ti</b>	4100	3190	0.25
V	34	44	0.25
<b>Zn</b>	13100	6040	5.0

#### 5.4.2. Bioleaching efficiencies by pure bacterial cultures

Comparing the leaching efficiencies of both cultures (Fig. 3), it is obvious that bioleaching efficiencies depend on both the organisms used and the substrate (i.e. the SLF). *A. ferrooxidans*, was able to leach Zn and Ni out of the SA fraction by up to 60% and 48%, respectively, showing no significant difference to the chemical metal extraction process. In contrast, the SN fraction with *A. ferrooxidans* showed complete bioleaching efficiencies, up to 100% for Ni and Zn, which outcompeted the chemical leaching (70 and 80%, respectively - see Fig. 3). Enhanced bioleaching efficiencies might be caused by the double amount of iron in the SN fraction, compared to the SA substrate (Tab. 1). Iron as the main energy source for both bacteria boosts the leaching performance in the SN fraction. The positive impacts of iron for bioleaching were shown

before (Rohwerder et al., 2003). This assumption is supported by the work of Bas et al. who discovered that a higher initial Fe(II) concentration facilitates a faster and more efficient extraction of copper from low grade scrap circuit boards (Bas et al., 2013). *A. ferrooxidans* maintained a low pH around 2 during the whole incubation with both SLF caused by its ability to produce sulfuric acid from the waste substrate's sulfur compounds. The production of sulfuric acid by sulfur oxidizing bacteria was previously reported and is a major part of the bioleaching mechanism (Johnson, 1998).



**Fig. 3:** Chemical (white) and biological (grey) leaching efficiency for Zinc (Zn) and Nickel (Ni) by pure cultures of *A. ferrooxidans* and *L. ferrooxidans* and a co-culture of both for two different shredder-light-fractions (SLF). pH values for each set up and SLF are shown as black dots on the secondary axis.

*L. ferrooxidans* showed comparably low biological and chemical metal extraction for the SA fraction. For both metals, the leaching efficiency was below 50%, showing no difference between microbial action and chemical extraction. The lower bioleaching efficiency of *L. ferrooxidans* for the SA fraction compared to *A. ferrooxidans* is in agreement with previous results, indicating that *L. ferrooxidans* shows lower growth rates on Fe(II) and oxidizes Fe(II) slower (Hallmann et al., 1992). In case of the other fraction (SN), Zn and Ni were extracted up to 100% by both possible extraction ways. Physical conditions and inhomogeneity of the raw material might explain the limitation of the bioleaching efficiency. The pH values for both SLF samples were significantly higher (4.5 and 3.2) compared to the pure *A. ferrooxidans* culture and the co-culture (Fig. 3). Alkaline components of waste released during incubation, might explain the pH increase during the experiment. Additionally, *L. ferrooxidans* is not able to lower the pH by the production of sulfuric acid out of sulfur compounds (Hallmann et al., 1992).

Comparability of both substrates is limited due to differences in the metal contents, especially regarding to the iron concentration. To control this in further experiments, the metal content of the solid SLF before and after leaching experiments was measured by ICP-MS analyses. Concentrations of each metal fraction in the solid matter were then used for the calculation of leaching efficiencies.

#### 5.4.3. Leaching efficiency of a bioleaching co-culture

Mixed cultures containing different acidophilic bacteria like *A. ferrooxidans*, *A. thiooxidans* and *L. ferrooxidans* were previously used to efficiently leach metals (Akinci and Guven, 2011; Falco et al., 2003; Qiu et al., 2005). To test the bioleaching performance of a co-culture consisting of *A. ferrooxidans* and *L. ferrooxidans* on both shredder-light-fractions (SLF), a series of batch tests was performed. In all tests,

biological leaching led to higher efficiencies than simple chemical leaching while pH values at the end were comparable to the ones with the pure *A. ferrooxidans* culture (Fig. 3). The SA fraction could be biologically leached more efficient (Zn 65% and Ni 50%) with the co-culture compared to the individual bacteria (Fig. 3). Mixed bacterial cultures are able to tolerate higher heavy metal concentrations (Hallmann et al., 1992), explaining increased bioleaching efficiency for the SA fraction. A different trend was observed for the SN fraction, where the biological leaching efficiency decreased to 80 and 60% for Zn and Ni, respectively (Fig. 3). Especially for Ni this reflects a reduction of about 20% and 40% compared to the pure cultures of *A. ferrooxidans* and *L. ferrooxidans*. In contrast to the pure cultures, the microbial leaching efficiency of the co-culture was higher for the SA fraction. However, a complete metal recovery was not reached, like for both pure cultures with the SN fraction.

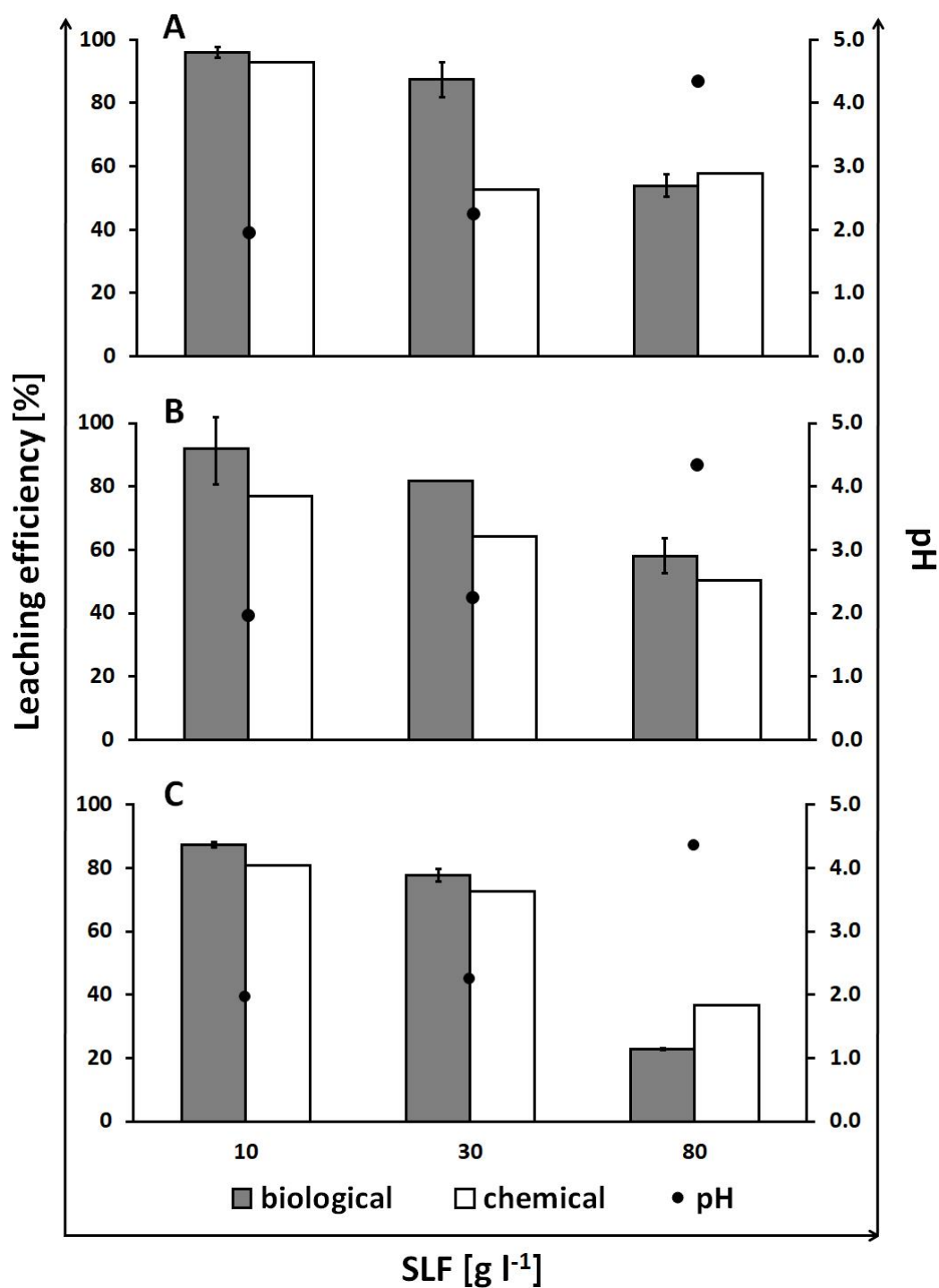
Comparing the leaching efficiencies of the different setups according to the media used, there are different factors which might have an influence on the performance. The pH (pH 1.4), as well as the concentration of  $\text{FeSO}_4 \times 7 \text{ H}_2\text{O}$  ( $30 \text{ g l}^{-1}$ ) were identical for both, the pure *A. ferrooxidans* and the co-culture media. As these two factors contribute the most to the leaching performance, the differences in bioleaching compared to chemical leaching within the batch tests is clearly related to the bacterial action (Fig. 3). The medium used for *L. ferrooxidans* had a comparably low pH (pH 1.8) but a lower  $\text{FeSO}_4 \times 7 \text{ H}_2\text{O}$  concentration ( $20 \text{ g l}^{-1}$ ). As described above, the main difference between *L. ferrooxidans* and *A. ferrooxidans* is the missing ability of *L. ferrooxidans* to metabolize sulfur compounds to produce sulfuric acid, lowering the pH. During the batch experiments the pH was not adjusted externally, which makes the results in terms of bioleaching performance comparable among the different setups.



#### 5.4.4. Influence of substrate concentration

In a next step, the influence of the substrate concentration (SN fraction) on bioleaching with *A. ferrooxidans* was investigated. Batch tests were performed with concentrations ranging from 10 – 80 g l<sup>-1</sup>. Moreover, the copper concentration was measured in addition to Ni and Zn, since Cu is one of the most prominent and well-studied metals in bioleaching processes (Sajjad et al., 2018; Schnell, 1997; Xiang et al., 2010b, 2010a). By comparing the leaching efficiencies of Cu, Ni and Zn (Fig. 4 A, B and C), a clear effect of the substrate concentration and pH was observed. A low substrate concentration (10 g l<sup>-1</sup>) resulted in the highest leaching efficiencies of 96, 92 and 87% for Cu, Ni and Zn, respectively. Maintaining a constant pH of around 2 in the experiments is essential to achieve well performing metal recovery. Minor differences between chemical and biological leaching for both fractions might be an effect of the low SLF concentration. At lower substrate concentrations, presence of alkaline components in the waste substrate is also limited, leading only to an insignificant increase of the pH in samples without bacteria. Therefore, the chemical leaching has a preferential performance over the microbial treatment at low substrate concentrations. By increasing the substrate concentration to 30 g l<sup>-1</sup>, the difference between biological and chemical leaching was evident (Fig. 4A, B). The bioleaching efficiency of all metals was equal for the lower SLF concentration, but the chemical leaching rate for Cu and Ni was about 30 and 15% less, respectively. For *A. ferrooxidans*, a constant pH of approximately 2.0 could be maintained, whereas the pH of the flasks without bacteria was increasing. Rising pH values resulted in reduced chemical leaching rates. By further increasing the substrate concentration up to 80 g l<sup>-1</sup>, the effect of the alkaline character of the SLF become apparent, leading to high pH of about 4.5. The bacteria were not able to maintain the required strong acidic pH at

these high substrate concentrations. Hence, bioleaching efficiency decreased to 54% and 58% for Cu and Ni, respectively, and to around 23% in the case of Zn (Fig. 4A, B and C). In summary, high substrate concentrations in biological and chemical leaching processes led to similar results, indicating that SLF concentrations between 10 – 30 g l<sup>-1</sup> to be most suitable for bioleaching in lab-scale applications.



**Fig. 4:** Chemical (white) and biological (grey) leaching efficiency of Cu (A), Ni (B) and Zn (C) with a pure culture of *A. ferrooxidans* at increasing concentrations of the SN substrate. pH values in dependency of the substrate concentration are shown as black dots on the secondary axis.

#### 5.4.5. CSTR and heap-bioleaching

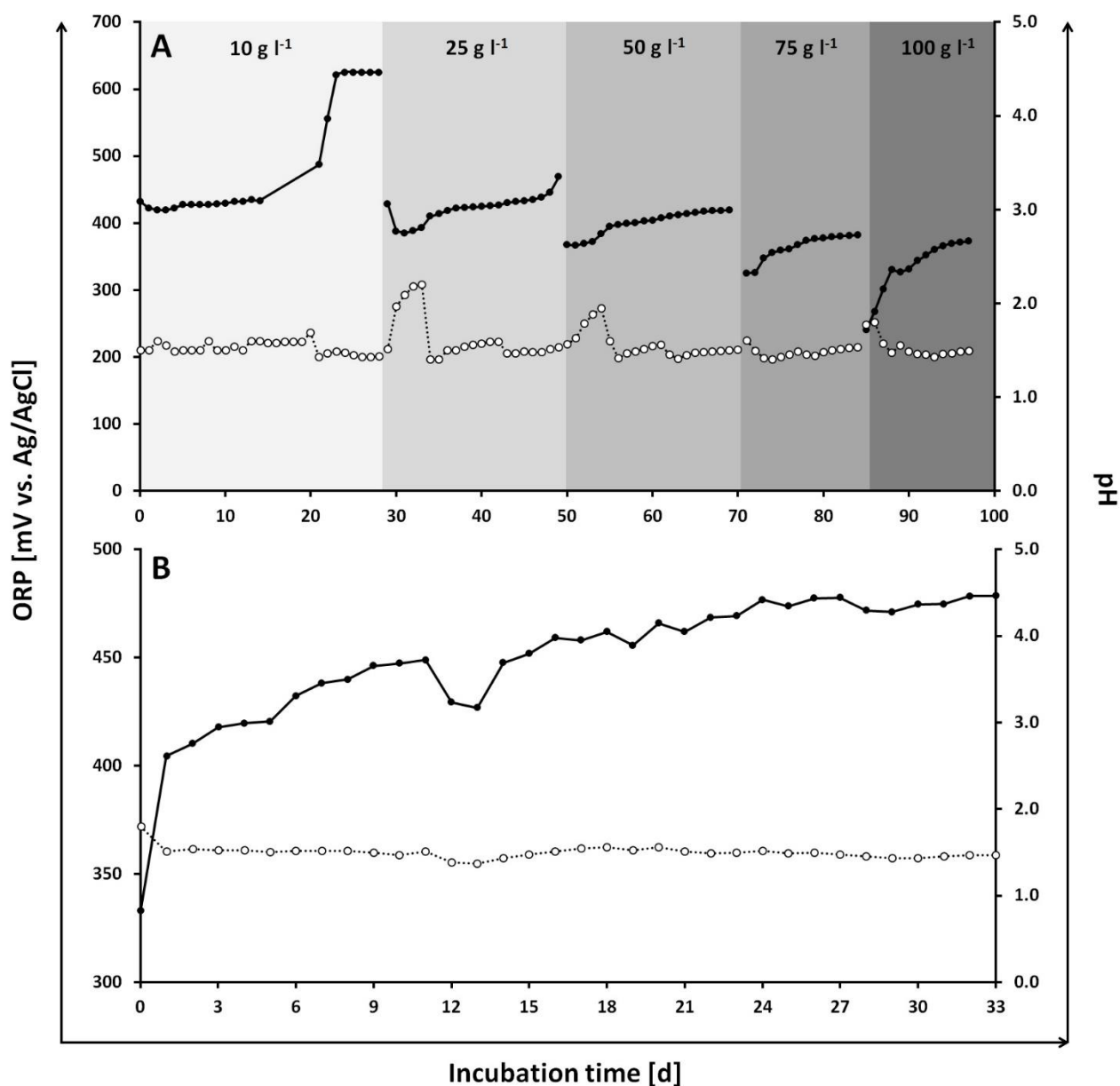
Leaching with *A. ferrooxidans* at 10 – 100 g l<sup>-1</sup> of the SN fraction in the CSTR was performed in a continuous mode. The substrate concentration was gradually increased

and 3/4 of the media used was replaced by fresh media after 2 – 4 weeks. At 10 g l<sup>-1</sup> substrate concentration, the redox potential as well as the pH showed no distinct differences within the first two weeks of incubation. Between week two and four, the pH in the reactor increased and was adjusted accordingly by addition of 1 M H<sub>2</sub>SO<sub>4</sub>. As explained before, higher concentrations of alkaline components in the shredder-light-fraction (SLF) increased the pH, and required active pH control. The increase of redox potential at 10 and 25 g l<sup>-1</sup> SLF is therefore related to the addition of sulfuric acid (Fig. 5A). The redox potentials, given in Figure 6 revealed minor changes at 25, 50 and 75 g l<sup>-1</sup> (i.e. 428 to 469, 367 to 419 and 325 to 382 mV), increasing with each loading rate (Fig. 5A). The lowest redox potential was observed at the highest substrate concentration of 100 g l<sup>-1</sup>. The lower redox potential at the beginning of the higher substrate concentrations (i.e. 50, 75 and 100 g l<sup>-1</sup>) is related to the addition of fresh media (high Fe<sup>2+</sup> concentration), higher amounts of SLF (more iron) and lower cell numbers after replacement of 2/3 leaching solution. This results are in agreement with findings of Santos et al. who investigated the change of the redox potential by addition of ferrous ion with and without bacteria (Santos et al., 2017). Ferrous ion in combination with no or low bacteria concentration leads to a lower redox potential favouring the oxidation of Fe<sup>2+</sup> to Fe<sup>3+</sup>. When the bacterial growth reaches a higher density, the redox potential increases, which facilitates the oxidation of Cu<sup>2+</sup> and other metals (Fig. 5A).

Cell numbers were counted throughout the whole 97 days of the CSTR incubation period at least at the beginning and at the end of each loading rate cycle (Fig. S1, supplementary). Starting with around 1.4 x 10<sup>8</sup> Cells ml<sup>-1</sup> at 10 g l<sup>-1</sup>, cell numbers decreased within the first week of incubation. After 1 week the bacteria adapted to the substrate and accelerated growth, which resulted in an increasing cell number after 4 weeks of leaching experiment. The same trend could be observed at 25 g l<sup>-1</sup> showing

a slightly higher cell number after 3 weeks of incubation ( $\sim 1.3 \times 10^8$  Cells  $\text{ml}^{-1}$ ). At higher SLF concentrations ( $> 50 \text{ g l}^{-1}$ ) cell growth was slower and the cell number maintained constant or decrease at the end of each increased loading rate cycle, which might be related to the shorter leaching time, combined with the higher heavy metals concentrations at higher loading rates. Interestingly, cell numbers at the introduction of 75 and  $100 \text{ g l}^{-1}$  of substrate were higher compared to lower concentrations of  $50 \text{ g l}^{-1}$  indicating a faster growth. The induced growth of *A. ferrooxidans* could be explained by the adaptation of the culture to  $10 - 50 \text{ g l}^{-1}$  SLF during the previous leaching cycles. Using such preadapted cells facilitates the growth and bioleaching efficiency which is in agreement with previous studies on bioleaching (Li and Ke, 2001). Moreover, the variation of counted cells in the hemocytometer mixed with fine particles in higher concentrated solutions can be easily misinterpreted as bacteria, which gives further possible explanations for increased cell numbers at higher loading rates.

The bioleaching experiment in the heap-reactor was running for 33 days in total with a substrate concentration of  $500 \text{ g l}^{-1}$ . Similar to the stirred-tank reactor (CSTR) redox potential, pH values and cell numbers were monitored throughout the whole period. The redox potential increased continuously during the 33 days of incubation, reaching a higher final value compared to the CSTR experiments (Fig. 5B). Furthermore, *A. ferrooxidans* could be maintained at a constantly low pH of around 1.5 without the need of active regulation by sulfuric acid addition (data not shown). Cell counts were monitored regularly in the reactor liquid. Lower cell numbers in the liquid, compared to the CSTR, derive from high cell numbers immobilised within the heap (Fig. 5B).



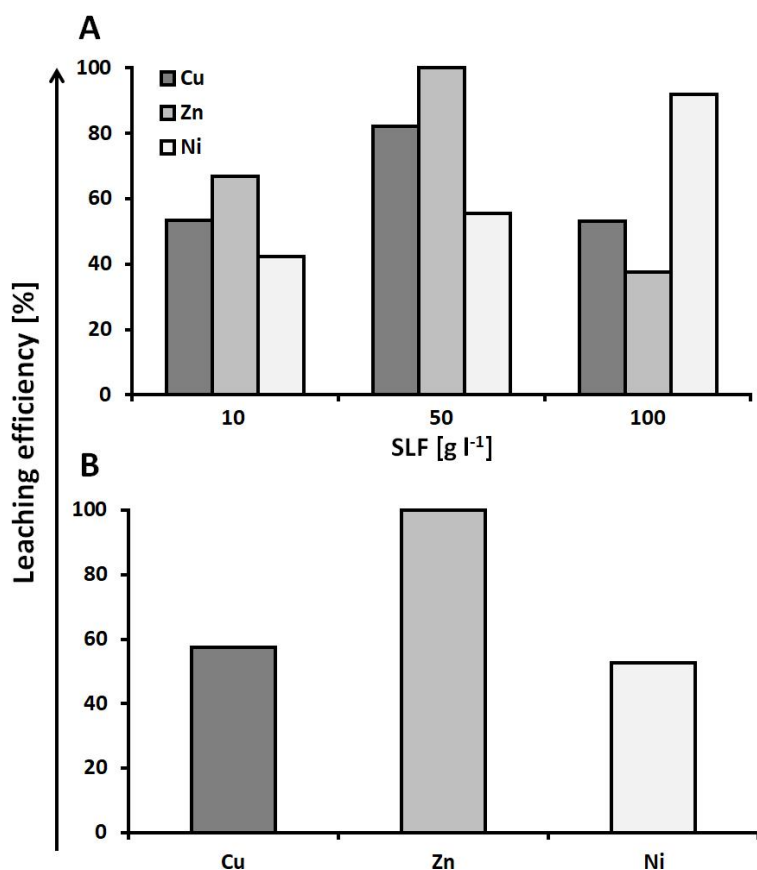
**Fig. 5:** Bioleaching of 10 – 100 g l<sup>-1</sup> shredder-light-fraction (SLF) by *A. ferrooxidans* over 97 days in continuous stirred-tank reactor (CSTR) (A) and bioleaching of 3000 g SLF with *A. ferrooxidans* in the heap-bioleaching system (B). The change of the redox potential (black line) and the pH (dotted line) is shown for both reactor types over the whole incubation time (97 days in CSTR and 33 days in heap).

#### 5.4.6. CSTR and heap-bioleaching efficiency

In the CSTR, leaching efficiencies of *A. ferrooxidans* were calculated for Cu, Zn and Ni at three different substrate concentrations. Outstanding results of 80 and 100% leaching efficiency for Cu and Zn were obtained at 50 g l<sup>-1</sup> for the SN fraction. At 10 g l<sup>-1</sup>, the leaching process was not as efficiently as compared to 50 g l<sup>-1</sup>, considering that freshly grown cultures were not performing as well as adapted cultures in later stages

of the experiment. In contrast, for the 50 g l<sup>-1</sup> approach the *A. ferrooxidans* bacteria have already been adapted to 10 and 25 g l<sup>-1</sup> SLF respectively. Different results in leaching efficiencies of Cu, Zn and Ni were obtained at the highest substrate concentration of 100 g l<sup>-1</sup>. In accordance to batch tests, the leaching efficiencies at higher substrate concentrations decreased for Cu and Zn to around 50 and 35%, respectively. In contrast, nearly 95% of Ni was leached at a substrate concentration of 100 g l<sup>-1</sup> (Fig. 6A). In particular for Ni, the stirred-tank leaching is significantly more efficient than batch experiments. Sufficient aeration, constant stirring and continuous adaptation of the pre-culture, throughout the previous incubations with 10 - 75 g l<sup>-1</sup>, may explain the higher nickel leaching efficiency in the CSTR.

The leaching efficiencies of *A. ferrooxidans* for Cu, Zn and Ni in the heap-reactor system were calculated after 33 days of incubation resulting in around 60, 100 and 55%, respectively. Comparing heap and CSTR efficiencies for all three metals, reveals that copper could be leached to a higher extent in the heap-system, while zinc could be leached to almost 100% in both reactor systems. The leaching efficiency for nickel in the heap was comparable to data obtained with 10 and 50 g l<sup>-1</sup> in the CSTR, but could not reach the 95% leaching rate, which was calculated for a substrate concentration of 100 g l<sup>-1</sup> in the stirred-tank reactor (Fig. 6B). Despite the fact that the leaching efficiency for Ni was lower in the heap-system, the process feasibility in terms of material input and operation costs is still lower compared to the stirred-tank reactor system.



**Fig. 6:** Leaching efficiencies of *A. ferrooxidans* for Cu, Zn and Ni at 10, 50 and 100  $\text{g l}^{-1}$  SLF in the continuous stirred-tank reactor CSTR (**A**) and for 3000 g SLF in the heap-bioleaching system (**B**)

## 5.5. Conclusion

This study has demonstrated the high potential of bioleaching for recovery of metals from shredder-light-fractions, which are commonly discarded and unutilised landfilled. In addition, different microbial leaching processes were compared and two different experimental setups for possible industrial scale application were tested.

The SN fraction was efficiently leached by *A. ferrooxidans*. The higher iron content compared to the SA fraction facilitated the growth of the organism and leaching performance. Leaching efficiencies ranging from 80 – 100 % were seen for a pure culture of *A. ferrooxidans* for Cu, Zn and Ni in the continuous stirred-tank reactor, while optimal substrate concentrations were in the range of 10 – 50  $\text{g l}^{-1}$ . For higher substrate concentrations, pH control was necessary. Furthermore, a simple heap-bioleaching



system was successfully maintained for 33 days, showing a promising leaching performance for all three tested metals. Cu and Zn have been recovered by up to 60 and 100 %, respectively, without any need of pH regulation.

## **5.6. Acknowledgements**

The project SHLauBi2 was financially supported by the FFG. SLF samples were kindly provided by the company partner RMVG and ICP metal analysis were carried out by Dr. Alexia Aldrian from the Montan-University Leoben.

## **5.7. References**

- Akinci, G., Guven, D.E., 2011. Bioleaching of heavy metals contaminated sediment by pure and mixed cultures of *Acidithiobacillus* spp. *Desalination* 268, 221–226. <https://doi.org/10.1016/j.desal.2010.10.032>
- Bas, A.D., Deveci, H., Yazici, E.Y., 2013. Bioleaching of copper from low grade scrap TV circuit boards using mesophilic bacteria. *Hydrometallurgy* 138, 65–70. <https://doi.org/10.1016/j.hydromet.2013.06.015>
- Cheminfo Services, 2014. Background Study on the Content of Shredder Residue.
- EU DIRECTIVE 2000/53, 2000. Dir. 2000-53-EC of the EPAOTC of 18 September 2000 on end-of-life vehicles. *Off. J. Eur. Communities* 4, 42–46. <https://doi.org/10.1016/j.jclepro.2010.02.014>
- EU DIRECTIVE 2005/64/EC, 2005. Directive 2005/64/EC of the European Parliament and of the Council of 26 October 2005 on the type-approval of motor vehicles with regard to their reusability, recyclability and recoverability and amending Council Directive 70/156/EEC. *Off. J. Eur. Union* 10–27. <https://doi.org/ISSN1725-2555>
- EUROSTAT, 2010. Environmental statistics and accounts in Europe 2010.
- Falco, L., Pogliani, C., Curutchet, G., Donati, E., 2003. A comparison of bioleaching of covellite using pure cultures of *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans* or a mixed culture of *Leptospirillum ferrooxidans*

and *Acidithiobacillus thiooxidans*. *Hydrometallurgy* 71, 31–36.

[https://doi.org/10.1016/S0304-386X\(03\)00170-1](https://doi.org/10.1016/S0304-386X(03)00170-1)

FhG-IBP, 2014. D 2.2 Waste Profiling.

Funari, V., Mäkinen, J., Salminen, J., Braga, R., Dinelli, E., Revitzer, H., 2017. Metal removal from Municipal Solid Waste Incineration fly ash: A comparison between chemical leaching and bioleaching. *Waste Manag.* 60, 397–406.

<https://doi.org/10.1016/j.wasman.2016.07.025>

Hallmann, R., Friedrich, A., Koops, H., Pommerening-Röser, A., Rohde, K., Zenneck, C., Sand, W., 1992. Physiological characteristics of *thiobacillus ferrooxidans* and *leptospirillum ferrooxidans* and physicochemical factors influence microbial metal leaching. *Geomicrobiol. J.* 10, 193–206.

<https://doi.org/10.1080/01490459209377920>

Johnson, D.B., 1998. Biodiversity and ecology of acidophilic microorganisms. *FEMS Microbiol. Ecol.* 27: 307–317. 27. <https://doi.org/10.1007/s00394-015-1034-7>

Kaksonen, A.H., Lavonen, L., Kuusenaho, M., Kolli, A., Närhi, H., Vestola, E., Puhakka, J.A., Tuovinen, O.H., 2011. Bioleaching and recovery of metals from final slag waste of the copper smelting industry. *Miner. Eng.* 24, 1113–1121.

<https://doi.org/10.1016/j.mineng.2011.02.011>

Kupka, D., Liljeqvist, M., Nurmi, P., Puhakka, J.A., Tuovinen, O.H., Dopson, M., 2009. Oxidation of elemental sulfur, tetrathionate and ferrous iron by the psychrotolerant *Acidithiobacillus* strain SS3. *Res. Microbiol.* 160, 767–774.

<https://doi.org/10.1016/j.resmic.2009.08.022>

Li, H. mei, Ke, J. jun, 2001. Influence of  $\text{Ni}^{2+}$  and  $\text{Mg}^{2+}$  on the growth and activity of  $\text{Cu}^{2+}$ -adapted *Thiobacillus ferrooxidans*. *Hydrometallurgy* 61, 151–156.

[https://doi.org/10.1016/S0304-386X\(01\)00167-0](https://doi.org/10.1016/S0304-386X(01)00167-0)

Margarido, F., Nogueira, C.A., 2011. Characterisation of automotive shredder residue 1–5.

Oliver, B.G., Carey, J.H., 1976. Acid solubilization of sewage sludge and ash constituents for possible recovery. *Water Res.* 10, 1077–1081.

[https://doi.org/10.1016/0043-1354\(76\)90039-7](https://doi.org/10.1016/0043-1354(76)90039-7)

- Pathak, A., Dastidar, M.G., Sreekrishnan, T.R., 2009. Bioleaching of heavy metals from sewage sludge: A review. *J. Environ. Manage.* 90, 2343–2353.  
<https://doi.org/10.1016/j.jenvman.2008.11.005>
- Pradhan, N., Nathsarma, K.C., Srinivasa Rao, K., Sukla, L.B., Mishra, B.K., 2008. Heap bioleaching of chalcopyrite: A review. *Miner. Eng.* 21, 355–365.  
<https://doi.org/10.1016/j.mineng.2007.10.018>
- Qiu, M.Q., Xiong, S.Y., Zhang, W.M., Wang, G.X., 2005. A comparison of bioleaching of chalcopyrite using pure culture or a mixed culture. *Miner. Eng.* 18, 987–990.  
<https://doi.org/10.1016/j.mineng.2005.01.004>
- Rohwerder, T., Gehrke, T., Kinzler, K., Sand, W., 2003. Bioleaching review part A: *Appl. Microbiol. Biotechnol.* 63, 239–248. <https://doi.org/10.1007/s00253-003-1448-7>
- Sajjad, W., Zheng, G., Zhang, G., Ma, X., Xu, W., Khan, S., 2018. Bioleaching of copper - and zinc - bearing ore using consortia of indigenous iron - oxidizing bacteria. *Extremophiles* 22, 851–863. <https://doi.org/10.1007/s00792-018-1042-7>
- Santos, A.L.A., Arena, F.A., Benedetti, A.V., Bevilaqua, D., 2017. Effect of redox potential on chalcopyrite dissolution imposed by addition of ferrous ions. *Eclet. Quim.* 42, 40–50. <https://doi.org/10.26850/1678-4618eqj.v42.1.2017.p40-50>
- Schippers, A., Sand, W., 1999. Bacterial leaching of metal sulfides proceeds by two indirect mechanisms via thiosulfate or via polysulfides and sulfur. *Appl. Environ. Microbiol.* 65, 319–321.
- Schnell, H.A., 1997. Bioleaching of Copper. *Biomining* 21–43.  
[https://doi.org/10.1007/978-3-662-06111-4\\_2](https://doi.org/10.1007/978-3-662-06111-4_2)
- Silvermann, M.P., Lundgren, D.G., 1959. Studies on the chemoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. I. An improved medium and a harvesting procedure for securing high cell yields. *J. Bacteriol.* 77, 642–647.
- Sreekrishnan, T.R., Tyagi, R.D., 1996. A comparative study of the cost of leaching out heavy metals from sewage sludges. *Process Biochem.* 31, 31–41.

- Temple, K.L., Colmer, A.R., 1951. The autotrophic oxidation of iron by a new bacterium, *thiobacillus ferrooxidans*. *J. Bacteriol.* 62, 605–611.
- Van Aswegen, P.C., Haines, A.K., Marais, H.J., 1988. Design and operation of a commercial bacterial oxidation plant at Fairview. *Randol Perth Gold* 88 144–147.
- Wang, X., Liu, Y., Sun, Z., Li, J., Chai, L., Min, X., Guo, Y., Li, P., Zhou, Z., 2017. Heap bioleaching of uranium from low-grade granite-type ore by mixed acidophilic microbes. *J. Radioanal. Nucl. Chem.* 314, 251–258.  
<https://doi.org/10.1007/s10967-017-5406-8>
- Witne, J.Y., Phillips, C. V., 2001. BWitne, J. Y., & Phillips, C. V. (2001). Bioleaching of Ok Tedi copper concentrate in oxygen- and carbon dioxide-enriched air. *Minerals Engineering*, 14(1), 25–48. [https://doi.org/10.1016/S0892-6875\(00\)00158-8](https://doi.org/10.1016/S0892-6875(00)00158-8)bioleaching of Ok Tedi copper concentrate in oxy. *Miner. Eng.* 14, 25–48. [https://doi.org/10.1016/S0892-6875\(00\)00158-8](https://doi.org/10.1016/S0892-6875(00)00158-8)
- Xiang, Y., Wu, P., Zhu, N., Zhang, T., Liu, W., Wu, J., Li, P., 2010a. Bioleaching of copper from waste printed circuit boards by bacterial consortium enriched from acid mine drainage. *J. Hazard. Mater.* 184, 812–818.  
<https://doi.org/10.1016/j.jhazmat.2010.08.113>
- Xiang, Y., Wu, P., Zhu, N., Zhang, T., Liu, W., Wu, J., Li, P., 2010b. Bioleaching of copper from waste printed circuit boards by bacterial consortium enriched from acid mine drainage. *J. Hazard. Mater.* 184, 812–818.  
<https://doi.org/10.1016/j.jhazmat.2010.08.113>

## **6. A new bioleaching strategy for the selective recovery of aluminum from multi-layer beverage cans**

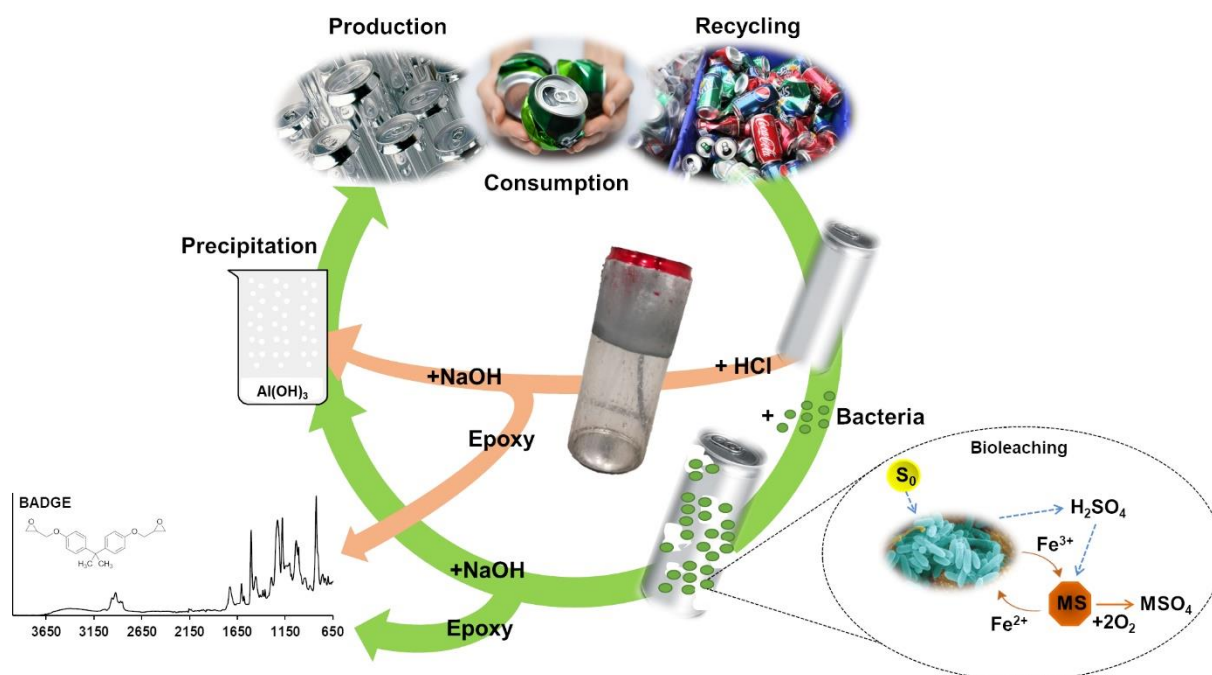
Published in: Journal of Waste Management, 01 February 2021, Volume 120, <https://doi.org/10.1016/j.wasman.2020.11.012>

**Klemens Kremser, Patrick Gerl, Alessandro Pellis\*, Georg M. Guebitz**

University of Natural Resources and Life Sciences Vienna BOKU, Dept. of Agrobiotechnology, IFA-Tulln, Inst. of Environmental Biotechnology, Konrad-Lorenz-Straße 20, 3430 Tulln and der Donau, Austria

**Keywords:** Used beverage cans, bioleaching, selective aluminum recovery, multi-layer materials

## Graphical abstract



### 6.1. Abstract

Used beverage cans (UBC's) represent one of the largest sources for secondary aluminum production worldwide. Beverage cans are one of the most frequently produced multi-layer packaging materials made of aluminum with an inner epoxy resin coating to prevent direct contact of food and aluminum surface. In the common way of UBC's recycling, the whole can is re-melted, resulting in the burning and loss of the inner epoxy coating. The use of acidophilic bacteria for the biological leaching of metals has already been well studied, but until now their applications for the selective separation of metal-containing multilayer materials has not been investigated. In this study, the three bioleaching bacteria: *A. ferrooxidans*, *A. thiooxidans* and *A. caldus* were explored to selectively leach the aluminum from the epoxy layer, resulting in leaching efficiencies of around 92% after three weeks of incubation. Surface characterization of the epoxy layer after bioleaching application revealed that the nature of the epoxy resin was unchanged, which could allow for recycling. The

dissolved aluminum was afterwards selectively precipitated from the lixiviants at pH=6.5, resulting in aluminum hydroxide precipitation efficiencies of almost 100%. The high leaching efficiencies and the selective precipitation shows the significant potential of acidophilic bacteria in the separation and recycling of multi-layer materials.

## **6.2. Introduction**

The production of primary aluminum from bauxite faces disadvantages such as a high energy demand, the generation of around 120 million tons of highly alkaline red-mud *per year*, the release of large amounts of carbon dioxide (CO<sub>2</sub>) and the emission of perfluorocarbons (PFC), thus contributing to environmental pollution and the greenhouse effect (Das and Green, 2010; Milačič et al., 2012).

On the other hand, secondary aluminum produced from aluminum scrap has important advantages such as a reduced energy demand (5% of the energy compared to the primary aluminum production chain) and saves up to 230 000 tons/year of alloying elements such as Cu, Fe, Mn, Mg and Zn in Europe. The emission of acidic compounds, dioxins and furans can also be reduced by cleaning of the exhaust gases in the refiners and remelters (Buxmann et al., 2006).

As the global production and demand of aluminum is increasing, the recycling and production of secondary aluminum also needs to be increased (International Aluminum Institute, 2009). The global packaging market consist of around 15% metal packaging materials, of which, metal cans are expected to reach a marked value of 61.37 billion US \$ by 2025 (Deshwal and Panjagari, 2020; Mordor Inteligence, 2019). In 2014, around 364.4 billion cans were produced worldwide, with aluminum cans in beverage and soda industry accounting for the major share (Deshwal and Panjagari, 2020). With more than 220 billion consumed beverage cans *per year*, used beverage cans (UBC's) present a major source for secondary aluminum recycling (Škúrková and Ingaldi,

2014). Globally, around 70% of UBC's are collected annually with different waste management systems can be re-melted and re-used (International Aluminum Institute, 2009). The aluminum-alloy number 3004, used for the production of beverage cans mainly consist of 95.6 – 98.2% Al, 1.0 – 1.5% Mn and 0.8 – 1.3% Mg (The Aluminium Association, 2015). Aluminum is the non-ferrous metal most commonly used in the packaging industry, as well as for the manufacture of a wide variety of supplies in construction and transportation industry, due to their lightweight, strength and durability characteristics.

To avoid the direct contact between food and the aluminum surface of the can, different chemicals can be used for the coating of the internal surfaces (Council of Europe, 2009), such as amino resin cross-linked coatings and bisphenol A-diglycidyl ether (BADGE) or bisphenol F-diglycidyl ether (BFDGE) polymerization products (Lord, 2005; Magami and Guthrie, 2012). Since there is no common way to separate the polymer layer from the aluminum surface on the beverage can during the recycling and re-melting process, the plastic is commonly incinerated, leading to the loss of resources and the possible emission of toxic gases (Turner et al., 2015). On the other hand, research was already performed for recycling and re-use of epoxy resins, e.g. in the repolymerization and production of recycled resin (Dang et al., 2002; Kuang et al., 2018), as well as in the production of dynamic crosslinked polymers with reshaping and thermal-healing abilities (Mai et al., 2019).

Beverage cans are only an example for many existing metal-containing multi-layer materials. In fact, there are many different packaging materials consisting of different mixtures of fibers, polymers and metals (e.g. aluminum). Examples for these kind of materials are food packaging (beverage cartons and coffee packaging) and pharmaceutical blisters, among others (Mumladze et al., 2019). In the recycling of this



kind of materials, it is crucial to separate the single layers in order to allow recycling of all valuable components such as fibers. Common ways in the recycling of these materials involve chemical and physical methods (Kaiser et al., 2018; Zawadiak, 2017), e.g. the in Europe frequently applied re-melting of aluminum scrap (e.g. UBC's) in a rotary furnace, which is accompanied with a metal loss of 5 – 8% (Capuzzi and Timelli, 2018). For the before mentioned separation of aluminum from multi-layer packaging materials, a delamination is crucial. Several techniques are reported, involving the use of acids (e.g. Nitric acid or sulfuric acid) to dissolve the binding adhesive in between the layers or the plastic component itself (Mukhopadhyay, 2001; Patel et al., 2016). Other methods are focusing on the separation by selective dissolution of the aluminum layer *via* alkali or acid solutions (Kaiser et al., 2018; Lee et al., 2006). Nevertheless, the high specificity and mild process conditions of biological methods such as bioleaching or enzymatic separation has not been well investigated in this context.

In this study, we propose the use of acidophilic bacteria for the biological leaching (bioleaching) of the aluminum, resulting in the separation of metal and polymer before reprocessing. The use of bacteria for the solubilization of different metals is already applied in several processes for the recovery of metals from secondary resources such as sewage sludge, waste-shredder light fractions and other industrial residues (Kremser et al., 2020a; Mishra and Rhee, 2010, 2014; Pathak et al., 2009). Most of the bacteria used are extremely acidophilic mesophiles (pH<3), which generate energy by bacterial oxidation of ferrous iron ( $\text{Fe}^{2+}$ ) and reduced inorganic sulfur compounds (RISCs) (Quatrini and Johnson, 2018; Rohwerder et al., 2003). The leaching of metal oxides from solid materials by bacteria is mainly facilitated by the bacterial production of inorganic and organic acids (e.g.  $\text{H}_2\text{SO}_4$  and  $\text{HCl}$ ) and the secretion of complexing agents.

Testing the feasibility of bioleaching for recycling aluminum from UBC's was the objective of this study. For this, an iron- and sulfur-oxidizing bacterium (*Acidithiobacillus ferrooxidans*) and two sulfur-oxidizing bacteria (*Acidithiobacillus thiooxidans* and *Acidithiobacillus caldus*) were used as models for other multilayer packaging materials. With this in mind, the selective precipitation of aluminum from the lixiviant was also assessed and the purity of the resulted precipitate was evaluated.

### **6.3. Material and methods**

All chemicals were of analytical grade unless otherwise specified and purchased from Sigma-Aldrich (Sigma-Aldrich, Vienna, Austria). Deionized water was used for the preparation of cultivation media and stock solutions. Cell numbers were determined by counting bacterial cells in a Neubauer improved hemocytometer with 0.01 mm depth (BRAND GmbH, Wertheim, Germany) under an Olympus BX43 optical microscope (Olympus, Hamburg, Germany). For pH measurements, a Mettler Toledo S220 pH meter with a combined glass electrode (Mettler-Toledo GmbH, Vienna, Austria) was used.

#### **6.3.1. Aluminum can**

Aluminum cans for soft drinks of 330 mL capacity were obtained from a local supermarket, emptied and used for all experiments. In order to avoid interference in analysis of the epoxy resins, prior to leaching experiments, the paint was manually removed by sanding the cans with sandpaper until the aluminum layer was fully exposed (Fig. 1A). After removal of the paint, 1 cm<sup>2</sup> samples were cut and washed 3 times by dipping them alternately in ultra-pure water and ethanol (75% v/v). Following the washing step, aluminum pieces were dried in an oven set at 60 °C before being used.

### 6.3.2. Acid dissolution of aluminum cans

A 330 mL aluminum can (after removal of the paint) was submerged in 500 mL of 10% HCl for 3 h. After the process, the resulting material was washed three times with ultra-pure water and dried for 48 h at room temperature prior to FT-IR and SEM analysis.

### 6.3.3. Bacteria and growth media

The following bacteria were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany) and cultivated in their corresponding culture media recommended by DSMZ; *Acidithiobacillus ferrooxidans* DSM 583 (Medium 70), *Acidithiobacillus thiooxidans* DSM 504 (Medium 35) and *Acidithiobacillus caldus* DSM 9466 (Medium 670 with strain-specific modifications). Bacteria were cultivated in 250-mL Erlenmeyer flasks in a volume of 100 mL at 150 rpm and 30 °C (*A. ferrooxidans* and *A. thiooxidans*) or 37 °C (*A. caldus*) until a desired cell-density of approximately  $1 \times 10^8$  cells mL<sup>-1</sup> was reached.

### 6.3.4. Bioleaching batch-tests

Batch tests with the selected bacteria were carried out in biological triplicates. One abiotic blank for each set-up containing the corresponding culture media only, was run in parallel. Flasks with 100 mL of the bacterial cultures were pre-cultivated for approximately one week to reach the desired cell-density. Sulfur-oxidizing bacteria (*A. thiooxidans* and *A. caldus*) were pre-cultivated with 1 g (10% w/v) of elemental sulfur to ensure an effective oxidation of sulfur to produce sulfuric acid (H<sub>2</sub>SO<sub>4</sub>), resulting in the decrease of the pH value. Iron- and sulfur-oxidizing bacterium (*A. ferrooxidans*) was pre-cultivated without sulfur to ensure a complete oxidation of ferrous-iron (Fe<sup>2+</sup>) to ferric-iron (Fe<sup>3+</sup>) serving as strong oxidizing agent. To start the biological leaching after pre-cultivation, 4 pieces (~100 mg) of aluminum flakes were

added to each flask (including abiotic controls) under the laminar flow hood. Additionally, 1 g (10% w/v) elemental sulfur was added to flasks containing *A. ferrooxidans* to also facilitate the production of  $\text{H}_2\text{SO}_4$ . Leaching experiments were conducted for up to 3 weeks at 150 rpm and the corresponding temperatures (see section 2.2). Samples were taken in regular intervals (every 2-3 days) and filtrated through a CHROMAFIL® Xtra Nylon filter (Macherey-Nagel GmbH, Düren, Germany) with a pore size of  $0.2\ \mu\text{m}$  to remove bacteria and sulfur prior to analytical measurements. The removed volume was replaced by ultra-pure water.

#### 6.3.5. Iron-determination

To determine the amount and the ratio of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$ , *A. ferrooxidans* lixiviant samples were analyzed using the Hach-Lange LCK 320 iron-test (Hach-Lange GmbH, Vienna, Austria), which determines the  $\text{Fe}^{2+}$  concentration by measuring the absorbance at 485 nm of an orange-red complex formed with 1.10-phenanthroline on a Hach-Lange DR 3900 spectrophotometer.

#### 6.3.6. Sulfate measurement

For the measurement of produced sulfate due to the oxidation of elemental sulfur, a spectrophotometrically method using barium chloride was used (Tabatabai, 1974). To use this method in 96-well plates, modifications of the original procedure were done. A standard curve was prepared by serially diluting a  $1\ \text{g L}^{-1}\ \text{SO}_4^{2-}$  stock solution (0.37 g  $\text{Na}_2\text{SO}_4$  in 250 mL  $\text{ddH}_2\text{O}$ ) with  $\text{ddH}_2\text{O}$ . For the barium chloride solution, 0.19 g gelatin were added to 62.5 mL  $\text{ddH}_2\text{O}$  and heated until the gelatin dissolves. After cooling the solution on ice, 1.25 g  $\text{BaCl}_2 \times 2\text{H}_2\text{O}$  were put to the reagent and dissolved under stirring. Prior to use, the barium chloride solution was mixed (1:1) with 0.5 M HCl. For measurement, 250  $\mu\text{L}$  of standard or unknown sample were mixed with 50  $\mu\text{L}$  of barium chloride reagent and mixed. 250  $\mu\text{L}$  of this mixture were put in 96-well plates

and the absorbance was measured at 420 nm on a Tecan infinite M200 pro plate reader spectrophotometer.

#### 6.3.7. Aluminum determination

The aluminum concentration in the lixiviant was spectrophotometrically measured using the Spectroquant® aluminum test (Merck KGaA, Darmstadt, Germany), in which aluminum ions react with chromazurol S to form a blue-violet complex. The absorbance of the formed complex was afterwards measured at 550 nm. For the preparation of a standard curve, a 100 mg L<sup>-1</sup> Al stock solution was prepared (1.76 g KAl(SO<sub>4</sub>)<sub>2</sub> in 1 L ultra-pure water) and serially diluted. Measurements of standards and unknown samples were conducted after suitable dilution in 96-well plates in a plate reader.

#### 6.3.8. Surface characterization

Fourier transformed infrared (FT-IR) spectroscopy was used to determine the nature of the epoxy resin before and after biological and chemical leaching. Therefore, polymer samples were folded two times and measured between 4000 and 650 cm<sup>-1</sup> on a Perkin Elmer Spectrum 100 FT-IR Spectrometer (PerkinElmer, Traiskirchen, Austria). After measurement, spectra were normalized at the suitable reference peak at 1184 cm<sup>-1</sup> (Chike et al., 1993). All samples were acquired using 30 scans and a resolution of 4 cm<sup>-1</sup>.

For the investigation of surface changes before and after leaching experiments, pictures of the samples were taken on a Hitachi TM3030 scanning electron microscope (SEM). Prior to measurements, the epoxy surface was sputter coated with platinum (4 nm) using a Leica EM ACE600 sputter coater. Changes of the aluminum surface were also determined using energy-dispersive X-ray spectroscopy (EDS).

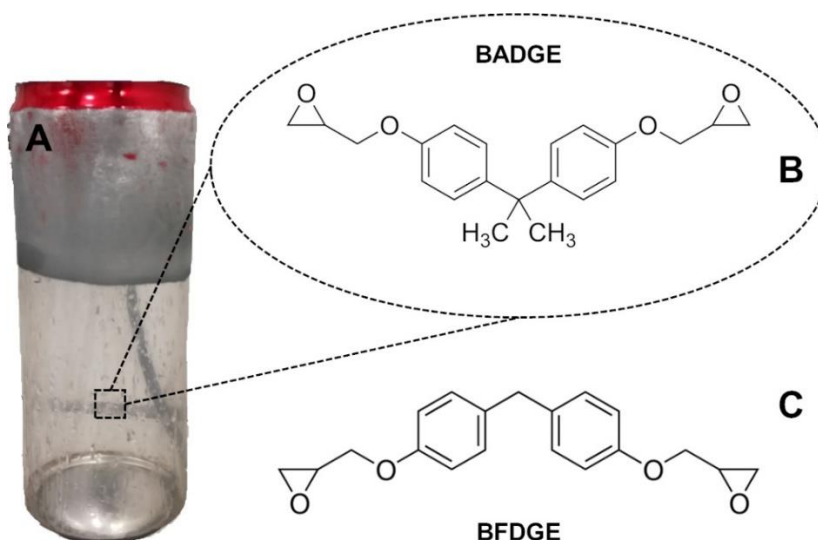
#### 6.3.9. Aluminum recovery

To test the recovery of aluminum from the lixiviant after biological and chemical leaching, iron and aluminum were selectively precipitated at pH=3.5 (Fe) and pH=6.5 (Al) with a 1 M NaOH solution to form  $\text{Fe}(\text{OH})_2$  and  $\text{Al}(\text{OH})_3$  (Wei et al., 2005). In experiments with *A. ferrooxidans*, iron as part of the medium, made it necessary to precipitate the iron first, followed by another precipitation step for aluminum. Aluminum from lixiviants leached with *A. thiooxidans* and *A. caldus* could directly be precipitated at pH=6.5. Before precipitation, all lixiviants were filtrated through a CHROMAFIL® Xtra Nylon filter with a pore size of 0.2  $\mu\text{m}$ . After precipitation, samples were filtrated through a Durapore® PVDF filter membrane with a pore size of 0.45  $\mu\text{m}$  to remove precipitates. Samples of the flow-through were collected and iron- and aluminum-concentrations were measured as mentioned in material and methods section 2.5 and 2.6 to determine the recovery efficiency by precipitation. To calculate the precipitation efficiency, three times 20 mL of the resulting lixiviants were used to measure the aluminum concentration before and after precipitation. The average values of the aluminum concentrations were calculated and shown together with the resulting standard deviation (SD).

### 6.4. Results and discussion

#### 6.4.1. Acid dissolution and characterization of UBC

In order to separate and characterize the two different layers (i.e. aluminum and epoxy) of the used beverage can, a 330 mL aluminum can was leached for 3 h in HCl, resulting in complete dissolution of the outer aluminum layer (Fig. 1A). The remaining polymer surface of the inner layer (Fig. 1A) was used to determine the type of coating (Fig. 1B and C) via FT-IR measurement.

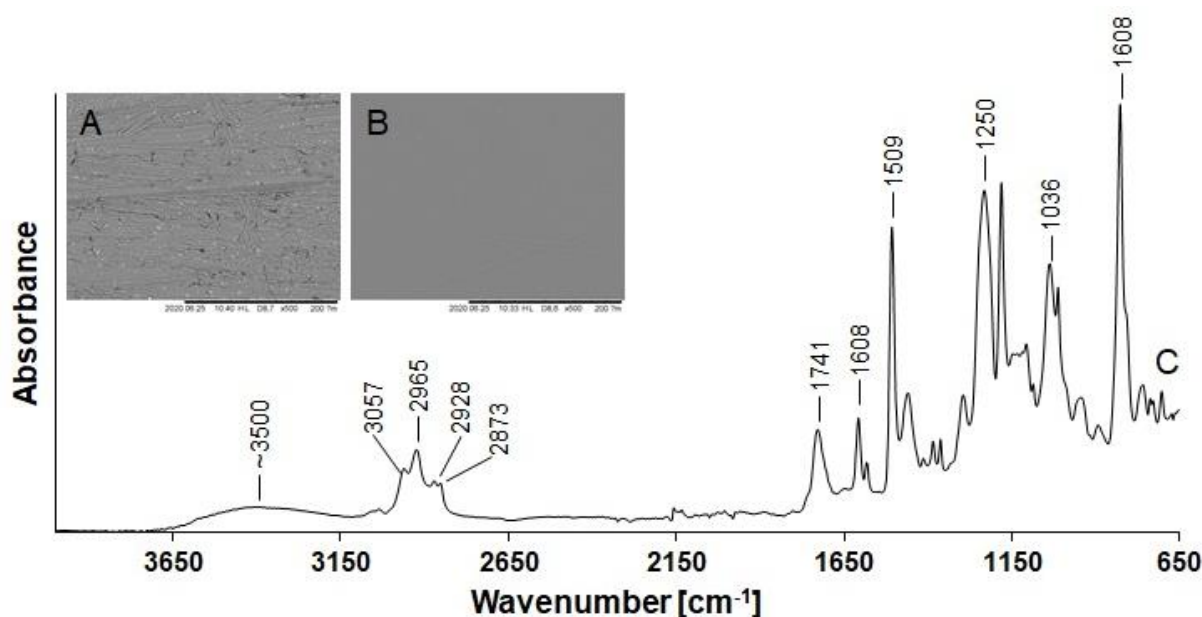


**Figure 1:** Picture of the acid leached aluminum can revealing the two different layers of aluminum (A, upper part) and epoxy resin (A, lower part). Chemical structures on the right represent two of the most frequently used monomers for the production of the inner coatings: namely derivatives of bisphenol A-diglycidyl ether (B) and bisphenol F-diglycidyl ether (C)

FT-IR analysis of the coating confirmed BADGE as the used coating type. The characteristic bands at  $\approx 3500\text{ cm}^{-1}$  (OH stretching),  $3057\text{ cm}^{-1}$  (CH stretching of oxirane ring),  $2965 - 2873\text{ cm}^{-1}$  (CH stretching of  $\text{CH}_2$  and CH of aromatic and aliphatic),  $1608\text{ cm}^{-1}$  (C=C stretching of aromatic rings),  $1509\text{ cm}^{-1}$  (C-C stretching of aromatic rings),  $1036\text{ cm}^{-1}$  (C-O-C stretching of ethers),  $915\text{ cm}^{-1}$  (C-O stretching of oxirane group),  $831\text{ cm}^{-1}$  (C-O-C stretching of oxirane group) and  $772\text{ cm}^{-1}$  ( $\text{CH}_2$  rocking) are clearly visible in figure 2. Furthermore, the band at  $1771\text{ cm}^{-1}$  which is specific for the C=O of ester groups, underlines the identification of BADGE in the inner layer of the aluminum can (González et al., 2012; Tudorachi and Mustata, 2020).

The findings clearly identified a present epoxy layer on the inside of the aluminum can. Towards common ways of recycling (e.g. melting and reprocessing) this polymer layer is incinerated which is accompanied with the loss of the epoxy resin. The chemical leaching by HCl resulted in the separation of aluminum and epoxy layer. As the use of highly concentrated, chemically produced acids (e.g. 3 M HCl) does not present a more environmentally-friendly recycling alternative to the melting of UBC's, the idea of

biological leaching by microbially produced organic acids (e.g.  $\text{H}_2\text{SO}_4$ ) and strong oxidizing agents (e.g.  $\text{Fe}^{3+}$ ) was considered in this study.



**Figure 2:** SEM image of the aluminum (A) and epoxy surface (B) of the untreated beverage can together with the FT-IR spectra of the epoxy surface (C) including labels for the characteristic peaks of bisphenol A-diglycidyl ether (BADGE).

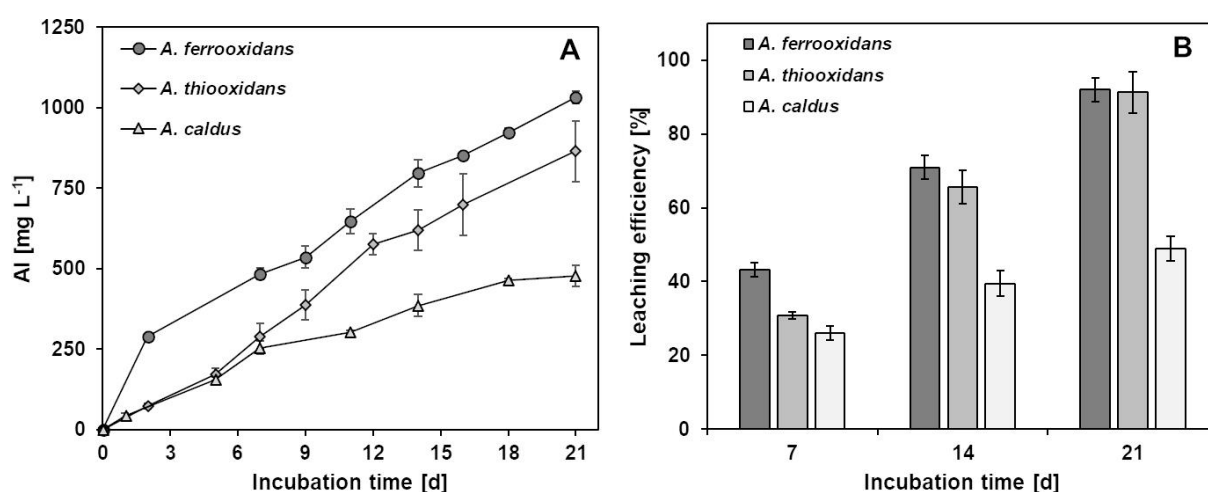
#### 6.4.2. Bioleaching of the aluminum layer

##### 6.4.2.1. *Acidithiobacillus ferrooxidans*

The three different acidophilic bacteria mentioned in section 2.3. were tested for their ability to dissolve the outer layer of aluminum within a total time of 3 weeks. The iron- and sulfur-oxidizing bacterium (*A. ferrooxidans*) achieved around 43% of the aluminum within the first week, following a continuous increase in the dissolved aluminum concentration over the remaining time. After 3 weeks, around 1031 mg L<sup>-1</sup> of aluminum was present in the lixiviant, corresponding to a leaching efficiency of 92% (Fig. 3A, B). The leaching of aluminum in the experiment with *A. ferrooxidans* was mainly driven by two different factors, which are the high amount of strong oxidizing agent ( $\text{Fe}^{3+}$ ) after one-week pre-cultivation with a ferrous-iron containing medium under acidic conditions (pH<2), due to pH adjustment of the medium. Measurements of the ferrous-iron



concentration within the first week of pre-cultivation showed that  $\text{Fe}^{2+}$  was already completely oxidized to  $\text{Fe}^{3+}$  after 2 days of growth ( $\text{Fe}^{2+} = 3.48 \text{ g L}^{-1}$  at the beginning and  $0.18 \text{ g L}^{-1}$  at day 2). The low pH value of the medium also facilitated a relatively high leaching yield of the abiotic BLANK (i.e. around 60% after 3 weeks, respectively). Regarding the pH values of the abiotic BLANK and *A. ferrooxidans* experiments (Fig. 4A), only minor differences were observed. Due to the added elemental sulfur, the pH value in the medium of *A. ferrooxidans* was slightly decreased from pH 2 to 1.7 until the end of the 3 weeks incubation. The sulfate concentration remains constantly high throughout the whole incubation time (Fig. 4D), which was more likely due to the high amount of sulfuric acid and ferrous sulfate added to the culture medium instead of bacterial oxidation.



**Figure 3:** Dissolved aluminum concentration over 3 weeks bioleaching of the beverage cans (A) with the 3 different bacteria *A. ferrooxidans*, *A. thiooxidans* and *A. caldus* and the resulting leaching efficiencies (B). Error bars indicate the standard deviation (n=3)

#### 6.4.2.2. *Acidithiobacillus thiooxidans*

The sulfur-oxidizing bacterium *A. thiooxidans* showed slower leaching of aluminum within the first week of incubation ( $\approx 31\%$ ) when compared to *A. ferrooxidans* ( $\approx 43\%$ ). Nevertheless, the leaching efficiencies were comparable after 2 and 3 weeks resulting in an almost total dissolution of aluminum after 3 weeks ( $864 \text{ mg L}^{-1}$ ,  $91\%$ ) (Fig. 3A,B).

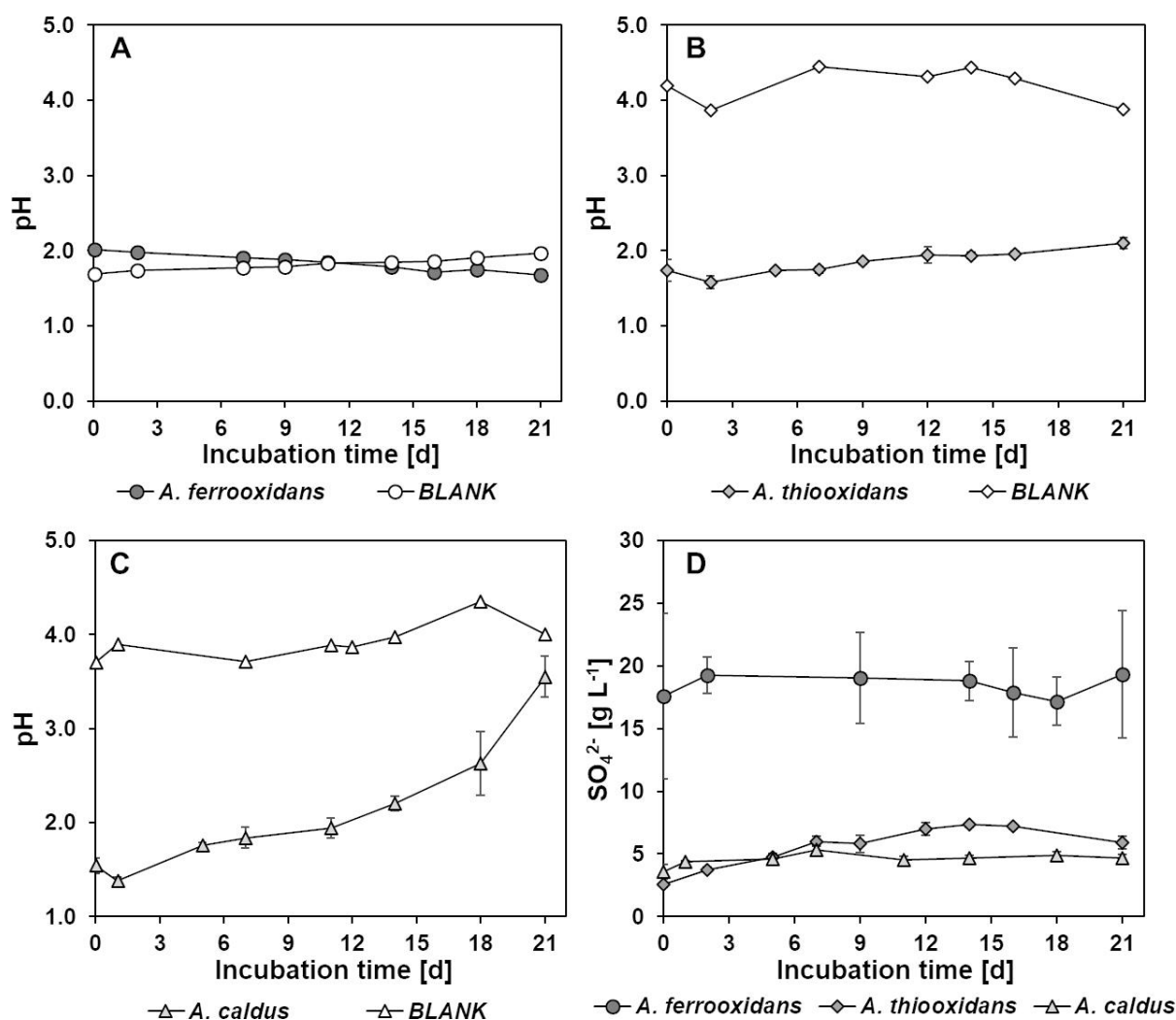
The advantage of *A. thiooxidans*, compared to the used iron-oxidizing bacterium, is the fast and efficient metabolization of RISCs to produce sulfuric acid. Therefore, it is not necessary to adjust the medium pH value resulting in a saving of chemicals and therefore in the potential reduction of process costs. Another advantage of using sulfur-oxidizing bacteria is the fact, that no addition of ferrous-iron is necessary, resulting in an easier recovery of the dissolved aluminum from the lixiviant (see section 3.4.). By comparing the pH-values of the abiotic BLANK and experiments containing *A. thiooxidans* (Fig. 4B), it is obvious, that the bacteria were able to maintain a constant low pH ( $\text{pH} \approx 2$ ) over the whole incubation time, facilitating the dissolution of the aluminum layer. In comparison, the pH-value of the BLANK remained between 4 and 4.5. The efficient metabolization of RISCs is also reflected by the concentration of sulfate measured in the experiment. *A. thiooxidans* could constantly increase the sulfate concentration over the first two weeks, reaching its maximum at day 14 with around  $7.3 \text{ g L}^{-1}$  (Fig. 4D).

#### 6.4.2.3. *Acidithiobacillus caldus*

The second sulfur-oxidizing bacterium, *A. caldus* showed aluminum leaching efficiencies comparable to *A. thiooxidans* after 1 week of bioleaching ( $\approx 32\%$ ). Nevertheless, the concentration of dissolved aluminum increased only insignificant over the remaining 3 weeks of incubation, reaching its maximum at day 21 of around  $477 \text{ mg l}^{-1}$  which corresponds to approximately 49% aluminum leaching efficiency (Fig. 3A, B). The low leaching efficiencies are also reflected by the increase in pH value over the 3 weeks of incubation. In contrast to *A. ferrooxidans* and *A. thiooxidans*, the pH values of the experiments containing *A. caldus* were strongly increasing after 1 week, reaching their maximum with around 3.6 after 3 weeks (Fig. 4C). At this high pH values, an effective metal dissolution is no longer feasible. This is also reflected by the constant

sulfate concentration throughout the incubation time (Fig. 4D). *A. caldus* did not manage to increase the amount of sulfate within the three weeks of incubation. The effect of pH increase could also be observed to a minor extent in experiments with the other sulfur-oxidizing bacterium *A. thiooxidans*.

The increase in pH, as well as the lower leaching efficiencies for the sulfur-oxidizing bacteria after 1 week may result from a combination of different factors, like a reported faster growth and higher resistance of iron-oxidizing bacteria against elevated metal concentrations (Bas et al., 2013; Shafikh and Ade, 2014). Comparing the heavy metal tolerance of *A. caldus* and *A. ferrooxidans*, it is already reported that the iron-oxidizing bacterium *A. ferrooxidans* shows significant higher heavy metal tolerances (Navarro et al., 2013). Therefore, an increased aluminum concentration in the lixiviant may have resulted in growth inhibition of *A. caldus*, leading to the increase in pH and the lower leaching efficiencies within 3 weeks of bioleaching. On the other hand, Fischer et al. showed that growth of *A. thiooxidans* is only affected to a minor extent by higher aluminum concentrations (Fischer et al., 2002), explaining that the pH-value was only slightly increasing over the 3 weeks bioleaching experiment without affecting the bioleaching efficiency. To our knowledge, this was the first-time bioleaching was applied for the selective separation of aluminum from multilayer packaging materials. Therefore, a comparison of aluminum leaching efficiencies achieved with other aluminum-containing waste materials was provided in the supplementary material (Table S1). The overall extraction yields achieved by *A. ferrooxidans* and *A. thiooxidans* on UBC's were higher compared to other waste materials.

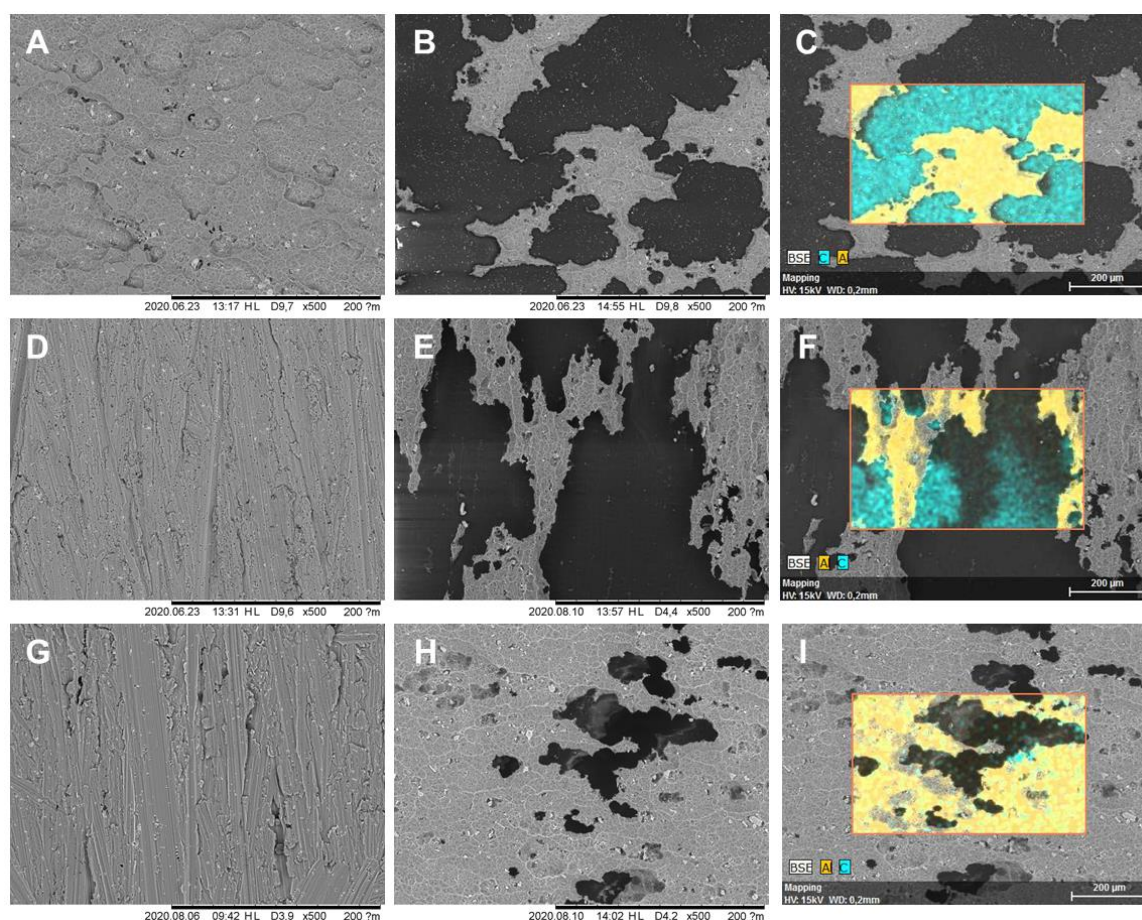


**Figure 4:** pH values of the 3 bacterial experiments with *A. ferrooxidans* (A), *A. thiooxidans* (B) and *A. caldus* (C) and the corresponding BLANK values. The sulfate concentrations throughout the incubation time are shown in D. Error bars indicate the standard deviation (n=3)

#### 6.4.3. Surface characterization

The difference in aluminum leaching efficiency was also reflected by surface characterization via SEM and EDX analysis. The low pH value of the mineral medium used for *A. ferrooxidans* resulted in the partly dissolution of the aluminum layer also in the BLANK experiment (Fig. 5A), whereas in the leaching experiments with the sulfur-oxidizing bacteria, the aluminum surface remained unaffected (Fig. 5D, G) when compared to the SEM image of the original surface in figure 2. Within 3 weeks of bacterial leaching, a major part of the aluminum layer was dissolved by *A. ferrooxidans* (Fig. 5B) and *A. thiooxidans* (Fig. 5E), leaving the epoxy surface untouched. In the

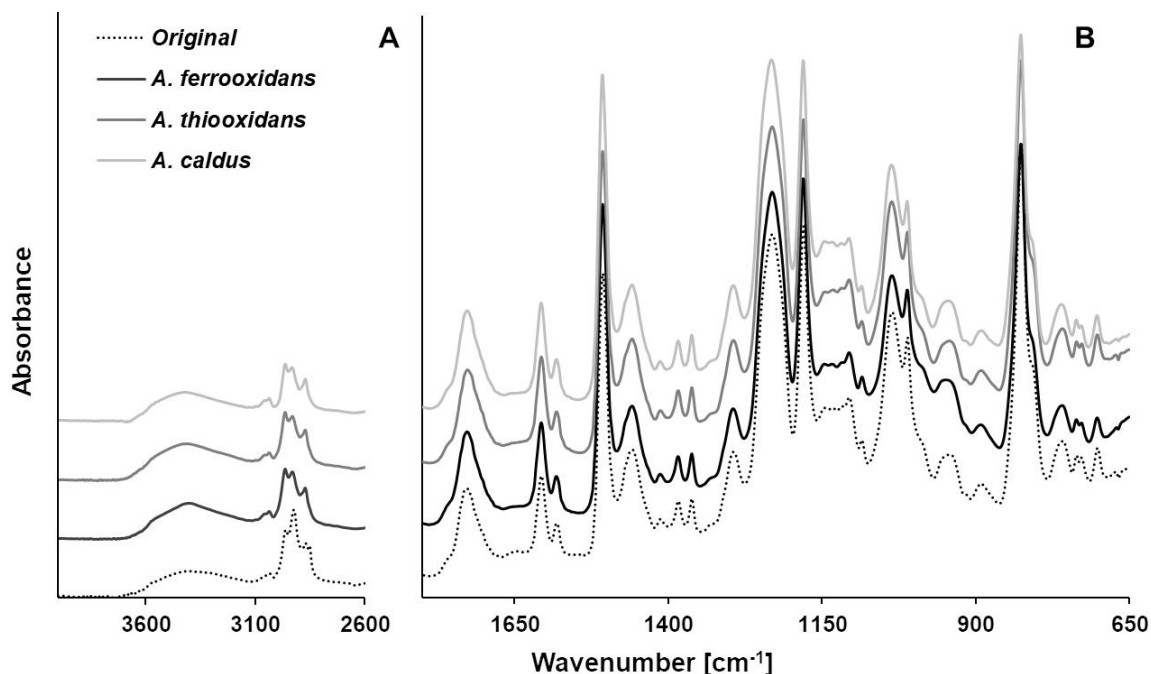
case of *A. caldus*, the lower leaching efficiency calculated according the dissolved aluminum concentration is in agreement with the surface picture in figure 5 (Fig. 5H). Compared to the BLANK (Fig. 5G), *A. caldus* was also able to leach aluminum from the surface, but not to such a high extend as *A. ferrooxidans* and *A. thiooxidans*. The results were further confirmed by elemental analysis (EDX), showing the difference in the aluminum and epoxy layer after bacterial leaching (Fig. 5C, F and I).



**Figure 5:** SEM images of the aluminum layer leached by *A. ferrooxidans* (A-C), *A. thiooxidans* (D-F) and *A. caldus* (G-I). Pictures A, D and G represent the BLANKs after 3 weeks of incubation with the corresponding mineral medium only. Pictures B, E and H show the aluminum surface after 3 weeks of bacterial leaching. EDX analysis of the samples after bacterial leaching (C, F and I) confirmed the presence of aluminum (yellow) and the epoxy layer (blue).

To confirm the integrity of the epoxy layer after biological leaching, FT-IR analysis of the polymer surface were performed and compared to the original, untreated epoxy surface. Figure 6 shows the recorded spectra of the original, and the three bacterial leached samples after 3 weeks of incubation. All peaks, characteristic for BADGE (see

section 3.1.) where clearly visible and remained unchanged over the bacterial incubation. This indicates a high potential for selective metal recovery by bacteria, without changing the nature of the epoxy resin.



**Figure 6:** FT-IR spectra of the epoxy surface before (original) and after bacterial dissolution of the aluminum layer by *A. ferrooxidans*, *A. thiooxidans* and *A. caldus*. The two characteristic fingerprint regions of BADGE are shown in A (4000 – 2600  $\text{cm}^{-1}$ ) and B (1800 – 650  $\text{cm}^{-1}$ ).

#### 6.4.4. Aluminum precipitation and recovery

The resulting lixiviants after 3 weeks of bacterial leaching were tested for selective precipitation of the dissolved aluminum for recovery as aluminum hydroxide. In the case of *A. ferrooxidans*, the iron in the medium made the aluminum precipitation more complicated and less effective. It was necessary to first selectively precipitate the iron at  $\text{pH}=3.5$ , leading to a loss in aluminum concentration of around 22% after the first precipitation. Nevertheless, the remaining aluminum in the lixiviants could afterwards be precipitated with an efficiency of almost 100%. This high efficiency was also reached in the selective aluminum precipitation from the lixiviants of the sulfur-oxidizing bacteria, without the need of a first precipitation step (Tab. 1).

**Table 1:** Aluminum concentrations in the lixiviants of the three bacteria *A. ferrooxidans*, *A. thiooxidans* and *A. caldus* before and after the selective precipitation and the resulting precipitation efficiencies.

Bacteria	Al in lixiviant $\pm$ SD [mg L <sup>-1</sup> ]	Al after precipitation $\pm$ SD [mg L <sup>-1</sup> ]	Precipitation efficiency [%]
<i>A. ferrooxidans</i>	1031.05 $\pm$ 19.4	0.3 $\pm$ 0.15	99.96
<i>A. thiooxidans</i>	863.99 $\pm$ 94.9	0.1 $\pm$ 0.01	99.99
<i>A. caldus</i>	476.62 $\pm$ 33.4	N/A	100

\*Al precipitation at pH=6.5 with 1 M NaOH; SD is the standard deviation (n=3)

The precipitated aluminum hydroxide may afterwards be again integrated in the production of beverage cans by converting it to aluminum oxide via calcination or can be used in other aluminum materials. Furthermore, aluminum hydroxide finds applications in different other fields, for example as a precursor for different aluminum products (i.e.  $\text{Al}_2(\text{SO}_4)_3$ ,  $\text{AlCl}_3$ , zeolite and others) or as a very prominent fire retardant filler for polymers and as smoke suppressant (Hudson et al., 2000; Hull et al., 2011).

## 6.5. Conclusion

In this study, the potential application of bioleaching bacteria for the selective separation of multi-layer packaging materials from beverage cans as models were successfully demonstrated. The high leaching efficiencies of *A. ferrooxidans* and *A. thiooxidans* lead to an almost complete dissolution of the aluminum layer within 3 weeks without affecting the epoxy resin. In contrast to *A. ferrooxidans*, the sulfur-oxidizing bacterium (*A. thiooxidans*) did not require pH adjustment of the medium or addition of ferrous iron, making it the most promising and effective candidate for future studies with other multi-layer materials. In fact, similar incubations times like used here are routinely applied in well-established large-scale heap-bioleaching systems in order to further reduce process costs. Such systems are already applied, e.g. in the extraction of copper from low grade ores. Furthermore, the selective, simple and efficient precipitation of the dissolved aluminum makes the recovery economically attractive especially for aluminum recovery from mixed waste-streams or multi-layer

materials where collection systems are not established. The selective removal and recovery of the aluminum layers would also allow recycling of other components of multi-layer materials

## **6.6. Acknowledgements**

Funding: This work was supported by the Austrian Science Fund (FWF) through the Erwin Schrödinger Program [grant agreement: J4014-N34 – Dr. Alessandro Pellis].

## **CRedit author statement**

**Klemens Kremser:** Conceptualization, Methodology, Writing – Original Draft, Data Curation, Visualization. **Patrick Gerl:** Investigation, Formal analysis. **Alessandro Pellis:** Conceptualization, Methodology, Writing – Review & Editing, Supervision. **Georg M. Guebitz:** Supervision, Writing – Review & Editing

## **Declaration of interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## **6.7. References**

- Bas, A.D., Deveci, H., Yazici, E.Y., 2013. Bioleaching of copper from low grade scrap TV circuit boards using mesophilic bacteria. *Hydrometallurgy* 138, 65–70.  
<https://doi.org/10.1016/j.hydromet.2013.06.015>
- Buxmann, K., Furrer, P., Gerber, J., Glimm, S., Morrison, J., Schäfer, J., Vigeland, P., 2006. Aluminium Recycling in Europe. *Eur. Alum. Assoc.* 52.
- Capuzzi, S., Timelli, G., 2018. Preparation and melting of scrap in aluminum recycling: A review. *Metals (Basel)*. 8. <https://doi.org/10.3390/met8040249>
- Chike, K.E., Myrick, M.L., Lyon, R.E., Angel, S.M., 1993. Raman and near-infrared studies of an epoxy resin. *Appl. Spectrosc.* 47, 1631–1635.  
<https://doi.org/10.1366/0003702934334714>



- Council of Europe, 2009. Coatings intended to come into contact with foodstuffs, Version 3 - 12.02.2009.
- Dang, W., Kubouchi, M., Yamamoto, S., Sembokuya, H., Tsuda, K., 2002. An approach to chemical recycling of epoxy resin cured with amine using nitric acid. *Polymer (Guildf)*. 43, 2953–2958. [https://doi.org/10.1016/S0032-3861\(02\)00100-3](https://doi.org/10.1016/S0032-3861(02)00100-3)
- Das, S.K., Green, J.A.S., 2010. Aluminum industry and climate change-Assessment and responses. *Jom*. <https://doi.org/10.1007/s11837-010-0027-5>
- Deshwal, G.K., Panjagari, N.R., 2020. Review on metal packaging: materials, forms, food applications, safety and recyclability. *J. Food Sci. Technol.* 57, 2377–2392. <https://doi.org/10.1007/s13197-019-04172-z>
- Fischer, J., Quentmeier, A., Gansel, S., Sabados, V., Friedrich, C.G., 2002. Inducible aluminum resistance of *Acidiphilium cryptum* and aluminum tolerance of other acidophilic bacteria. *Arch. Microbiol.* 178, 554–558. <https://doi.org/10.1007/s00203-002-0482-7>
- González, M.G., Cabanelas, J.C., Baselga, J., 2012. Applications of FTIR on Epoxy Resins - Identification, Monitoring the Curing Process, Phase Separation and Water Uptake. *Infrared Spectrosc. - Mater. Sci. Eng. Technol.* 2. <https://doi.org/10.5772/36323>
- Hudson, K.L., Misra, C., Perrotta, A.J., Wefers, K., Williams, F.S., 2000. Aluminum Oxide. *Ullmann's Encycl. Ind. Chem.* 8, 255–271. <https://doi.org/10.1002/14356007.a01>
- Hull, T.R., Witkowski, A., Hollingbery, L., 2011. Fire retardant action of mineral fillers. *Polym. Degrad. Stab.* 96, 1462–1469. <https://doi.org/10.1016/j.polymdegradstab.2011.05.006>
- International Aluminum Institute, 2009. Global Aluminium Recycling : A Cornerstone of Sustainable Development. *Int. Alum. Inst.* 1–36.
- Kaiser, K., Schmid, M., Schlummer, M., 2018. Recycling of polymer-based multilayer packaging: A review. *Recycling* 3. <https://doi.org/10.3390/recycling3010001>
- Kremser, K., Thallner, S., Schoen, H., Weiss, S., Hemmelmaier, C., Schnitzhofer, W.,

- Aldrian, A., Guebitz, G., 2020. Stirred-tank and heap-bioleaching of shredder-light-fractions (SLF) by acidophilic bacteria. *Hydrometallurgy*.  
<https://doi.org/https://doi.org/10.1016/j.hydromet.2020.105315>
- Kuang, X., Zhou, Y., Shi, Q., Wang, T., Qi, H.J., 2018. Recycling of Epoxy Thermoset and Composites via Good Solvent Assisted and Small Molecules Participated Exchange Reactions. *ACS Sustain. Chem. Eng.* 6, 9189–9197.  
<https://doi.org/10.1021/acssuschemeng.8b01538>
- Lee, Y.C., Kim, M.J., Lee, H.C., 2006. A Recycling Method of Multilayer Packaging Film Waste. EP1683829 A1 26.
- Lord, A.W., 2005. Packaging materials. *Chem. Eng. News* 21, 341–352.  
<https://doi.org/10.1021/cen-v021n008.p600>
- Magami, S., Guthrie, J., 2012. Amino resin cross-linked can coatings. *Surf. Coat. Int* 95, 64–73.
- Mai, V.D., Shin, S.R., Lee, D.S., Kang, I., 2019. Thermal healing, reshaping and ecofriendly recycling of epoxy resin crosslinked with Schiffbase of vanillin and hexane-1,6-diamine. *Polymers (Basel)*. 11, 1–13.  
<https://doi.org/10.3390/polym11020293>
- Milačič, R., Zuliani, T., Ščančar, J., 2012. Environmental impact of toxic elements in red mud studied by fractionation and speciation procedures. *Sci. Total Environ.* 426, 359–365. <https://doi.org/10.1016/j.scitotenv.2012.03.080>
- Mishra, D., Rhee, Y.-H., 2010. Current research trends of microbiological leaching for metal recovery from industrial wastes. *Curr Res Technol Educ Top. Appl Microbiol Microb Biotechnol* 2, 1289–1292.
- Mishra, D., Rhee, Y.H., 2014. Microbial leaching of metals from solid industrial wastes. *J. Microbiol.* 52, 1–7. <https://doi.org/10.1007/s12275-014-3532-3>
- Mordor Intelligence, 2019. Metal Cans Market - Growth, Trends, and Forecast (2020 - 2025), <https://www.mordorintelligence.com>. Accessed on 27th of October 2020.
- Mukhopadhyay, A., 2001. Process of de-lamination of multi-layer laminated packaging industrial refuse 1, 10–13.
- Mumladze, T., Tatariants, M., Rimšaitė, A., Yousef, S., Denafas, G., 2019. Recycling

- of Multilayer Packaging Foils by using Different Organic Solvent. Iran. J. Energy Environ. 10, 38–42.
- Navarro, A.C., Von Bernath, D., Jerez, A.C., 2013. Heavy Metal Resistance Strategies of Acidophilic Bacteria and Their Acquisition: Importance for Biomining and Bioremediation. Biol. Res. 46, 363–371.
- Patel, K.M., Vaviya, M.M., Patel, M.H., 2016. Process for Recovering Low-Density Polyethylene from Flexible Packaging Material. WO15159301 A3 21.
- Pathak, A., Dastidar, M.G., Sreekrishnan, T.R., 2009. Bioleaching of heavy metals from sewage sludge: A review. J. Environ. Manage. 90, 2343–2353.  
<https://doi.org/10.1016/j.jenvman.2008.11.005>
- Quatrini, R., Johnson, D.B., 2018. Microbiomes in extremely acidic environments: functionalities and interactions that allow survival and growth of prokaryotes at low pH. Curr. Opin. Microbiol. 43, 139–147.  
<https://doi.org/10.1016/j.mib.2018.01.011>
- Rohwerder, T., Gehrke, T., Kinzler, K., Sand, W., 2003. Bioleaching review part A: Appl. Microbiol. Biotechnol. 63, 239–248. <https://doi.org/10.1007/s00253-003-1448-7>
- Shafikh, S., Ade, A., 2014. Research Article METAL RESISTANCE IN INDIGENOUS THIOBACILLUS FERROOXIDANS AND PSEUDOMONAS FLUORESCENS 44–46.
- Škúrková, K.L., Ingaldi, M., 2014. Recycling process of the aluminium cans as an example of the renewable material sources. Adv. Mater. Res. 1001, 103–108.  
<https://doi.org/10.4028/www.scientific.net/AMR.1001.103>
- Tabatabai, M.A., 1974. A Rapid Method for Determination of Sulfate in Water Samples. Environ. Lett. 7, 237–243.  
<https://doi.org/10.1080/00139307409437403>
- The Aluminium Association, 2015. International Alloy Designations and Chemical Composition Limits for Wrought Aluminum and Wrought Aluminum Alloys With Support for On-line Access From: Aluminum Extruders Council Use of the Information. Alum. Assoc. Arlington, Virginia 31.

- Tudorachi, N., Mustata, F., 2020. Curing and thermal degradation of diglycidyl ether of bisphenol A epoxy resin crosslinked with natural hydroxy acids as environmentally friendly hardeners. *Arab. J. Chem.* 13, 671–682.  
<https://doi.org/10.1016/j.arabjc.2017.07.008>
- Turner, D.A., Williams, I.D., Kemp, S., 2015. Greenhouse gas emission factors for recycling of source-segregated waste materials. *Resour. Conserv. Recycl.* 105, 186–197. <https://doi.org/10.1016/j.resconrec.2015.10.026>
- Wei, X., Viadero, R.C., Buzby, K.M., 2005. Recovery of iron and aluminum from acid mine drainage by selective precipitation. *Environ. Eng. Sci.* 22, 745–755.  
<https://doi.org/10.1089/ees.2005.22.745>
- Zawadiak, J., 2017. Tetra Pak Recycling – Current Trends and New Developments. *Am. J. Chem. Eng.* 5, 37. <https://doi.org/10.11648/j.ajche.20170503.12>

## 7. General conclusion

This thesis investigated the applicability of acidophilic bacteria for the biological extraction of metals with a high economic importance in small scale batch and upscaling stirred-tank and heap-bioleaching systems. Additionally, different waste materials were characterized according to their physicochemical properties including particle size, concentrations of heavy and valuable metals and leaching behaviour of these metals. Using the environmentally friendly and effective method of bioleaching, can contribute to exploit new recycling ways for urban mining of artificial ores, closing the material cycle of depleting metal resources and contributing to the goal of a circular economy.

Waste incineration residuals were found to contain high concentrations of valuable metals. Common treatment techniques like landfilling or their use as additives in construction materials result in the loss of valuable metal resources. By applying different acidophilic, iron- and sulfur-oxidizing bacteria, metals incorporated in these residues could be bio-extracted and recovered. Nevertheless, there are only few reports in literature about the use of incineration residues as valuable substrates for bioleaching applications. In fact, their application in concrete and construction industries was investigated previously, resulting in the loss of valuable metal resources. As natural ore grades are declining, the urgent need to consider these materials as artificial ores for urban mining is obvious. The iron- and sulfur oxidizing bacterium *Acidithiobacillus ferrooxidans* was found to gain the highest bioleaching efficiencies under the tested conditions. Furthermore, the addition of nutrients such as ferrous iron and elemental sulfur, in combination with a low media pH were found to be key factors in the efficient metal extraction. Metals like Cu, Zn, Ni, Mn and Cr could be extracted by almost 100%, highlighting the great potential of bioleaching in the treatment of waste.

Sulfuric acid is a common additive in media preparation for bioleaching experiments and is often used in the pH-regulation of different bioleaching operations. Nevertheless, the production of sulfuric acid requires a very high energy input and the transportation of huge amounts of concentrated acid can pose a risk to human and the environment. The biological production of sulfuric acid has not yet been investigated in detail, but can provide a more energy efficient alternative for application in in-situ bioleaching processes such as heap bioleaching systems. Sulfur-oxidizing bacteria such as *Acidithiobacillus thiooxidans* and *Acidithiobacillus caldus* are very effective in the metabolization and oxidation of reduced inorganic sulfur compounds. Therefore, the application of both bacteria in the generation of a biologically produced sulfuric acid was tested. Using a combined culture of both, a concentrated sulfuric acid could successfully be produced within three weeks. This acid was further used to regulate the pH-value of a heap-bioleaching system in which metals from incineration slag could be extracted by an iron-oxidizing bacterium. In this way, a cheap and biological production of sulfuric acid was developed and combined with another effective and cheap bioleaching technique. Both findings can contribute to make the bioleaching of waste residuals an economically interesting, cheap and effective treatment process.

Shredder light fractions, generated during the shredding and treatment of end-of-life vehicles and waste electric and electronic equipment (WEEE), present another interesting waste substrate for bioleaching applications. However, there are only very few studies on the use of these residues in bioleaching applications. Even the concentrations of certain metals are higher than natural ore grades, these residues are deposited or incinerated. With increasing consumption of electronic devices and the resulting increase in WEEE, these residues need to be considered as valuable artificial ores for secondary metal recycling. Using the iron-oxidizing bacterium *A. ferrooxidans*,

incorporated metals like Cu, Zn and Ni were successfully bio-extracted with high efficiencies. The application of two different reactor systems, i.e. a 20 L stirred tank reactor and a 3 kg heap-bioleaching system, provided a first proof of concept that the method of bioleaching can also be applied in potential industrial scale. Heap-bioleaching was found to provide a cheap, but still effective way to treat bigger amounts of waste substrate in a reasonable time, making it an economically interesting process.

Using bioleaching for the selective recovery and recycling of metallization layers within multilayer packaging waste provides a completely new and innovative area of application. Used beverage cans as a model material consist of both, a plastic layer on the inside of the can and an aluminium layer for structural integrity and light barrier. Being able to selectively dissolve and separate the aluminium from the plastic layer provides a breakthrough in the recycling of more complex multilayer materials such as liquid packaging boards. By testing three different acidophilic bacteria, two out of three were found to effectively leach almost 100% of the aluminium within 21 days of incubation. The successful application of the bacteria in the recovery and recycling of aluminium from multilayer materials can contribute to increase the recycling rates of all different materials used in multilayer packaging strengthening again the idea of zero waste and a circular economy. Future studies should focus on new alternatives to or improvement of already existing recycling technologies to tackle all different waste materials. Therefore, bioleaching experiments in bigger scale need to be performed to proof their applicability in an industrial scale. This might help to close the material cycle loop of different metals and polymers.

## 8. Appendix

### 8.1. Publications

1. Quartinello F, **Kremser K**, Vecchiato S, et al. Increased Flame Retardancy of Enzymatic Functionalized PET and Nylon Fabrics via DNA Immobilization. *Front Chem.* 2019;7. doi:10.3389/fchem.2019.00685
2. **Kremser K**, Thallner S, Schoen H, et al. Stirred-tank and heap-bioleaching of shredder-light-fractions (SLF) by acidophilic bacteria. *Hydrometallurgy.* 2020;193:105315. doi:10.1016/j.hydromet.2020.105315
3. **Kremser K**, Thallner S, Strbik D, et al. Leachability of metals from waste incineration residues by iron- and sulfur-oxidizing bacteria. *J Environ Manage.* 2021;280:111734. doi:10.1016/j.jenvman.2020.111734
4. **Kremser K**, Gerl P, Pellis A, Guebitz GM. A new bioleaching strategy for the selective recovery of aluminum from multi-layer beverage cans. *Waste Manag.* 2021;120:16-24. doi:10.1016/j.wasman.2020.11.012
5. Spiess, S., Kucera, J., Seelajaroen, H., Sasiain, A., Thallner, S., **Kremser, K.**, Novak, D., Guebitz, G.M., Haberbauer, M., 2021. Impact of carbon felt electrode pretreatment on anodic biofilm composition in microbial electrolysis cells. *Biosensors* 11. <https://doi.org/10.3390/bios11060170>
6. Quartinello F, **Kremser K**, Schoen H, Tesei D. Together Is Better : The Rumen Microbial Community as Biological Toolbox for Degradation of Synthetic Polyesters. 2021;9(July):1-11. doi:10.3389/fbioe.2021.684459



## **8.2. Oral presentations as presenting author**

1. **Klemens Kremser**, Sophie Thallner, Sabine Spieß, Jiri Kucera, Marianne Haberbauer, Georg M. Guebitz - Microbial recovery of metals from waste incineration ashes and slags, *Recy & DepoTech 2020, 18 – 20 November 2020, Leoben, Austria*

2. **Klemens Kremser**, Herta Schoen, Sophie Thallner, Sabine Spieß, Marianne Haberbauer, Jiri Kucera, Georg M. Guebitz - Recovery of valuable metals from waste incineration residues by iron- and sulfur-oxidizing bacteria, *18th International Sysmposium on Waste Management and Sustainable Landfilling, 11 – 15 October 2021, Cagliari, Italy*

## **8.3. Poster presentations as presenting auhtor**

1. **Klemens Kremser**, Stefan Weiss, Sophie Thallner, Christine Hemmelmair, Wolfgang Schnitzhofer, Georg M. Guebitz - Lab-scale tank and heap bioleaching of light shred fractions by *Acidithiobacillus ferrooxidans*, *ECCE 12 & ECAB 5, 15 – 19 September 2019, Florence, Italy*

2. **Klemens Kremser**, Melanie Maltschnig, Herta Schön, Jiri Kucera, Georg M. Guebitz - Recovery of metals from recycled materials: Combining biological sulfuric acid production with bioleaching applications, *efb 2021 virtual conference, 10 – 14 May 2021*

#### **8.4. Acknowledgements**

This thesis would not exist without support and contribution of many different people:

My supervisor Prof. Georg Gübitz offered me great support and gave me the opportunity to work at the Institute of Environmental Biotechnology. He always had an open ear and encouraged me to pursue new ideas and concepts for my research. He further gave me the possibility to attend scientific conferences to promote and present my findings.

My colleagues Felice and Oskar, for fruitful scientific discussions and real friendships that developed during my time at the Institute.

My office colleagues Miguel and Cicely for scientific input and a nice working environment.

Our two secretaries, Elke and Petra, who helped me with all kinds of administration and bureaucratic issues.

My students Patrick, Dorina, Melanie and Herta who did an amazing job in the lab and contributed to this thesis with their work.

Our great technician team, comprising Marion, Robert and Marcus for their support during my research.

All the people at IFA who made my PhD a joyful experience, especially the nice Italian community.

Last but not least I want to thank my family and especially Theres for supporting me throughout the whole period of my PhD and before.

**Statutory declaration**

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used source.

X

---

Klemens Kremser