

**Statistical optimization of parameters for bioleaching of mine wastes from São Domingos Mine, Portugal**

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## List of Abbreviations

**Agjrs:** Argeno-jarosite  
**Alu:** Alunite  
**AMD:** Acid Mine Drainage  
**Ang:** Anglesite  
**Ann:** Annite  
**ANOVA:** Analysis of Variance  
**BBD:** Box–Behnken Design  
**Bdn:** Beudanite  
**CCD:** Central Composite Design  
**CI:** Confidence Interval  
**Coq:** Coquimbite  
**CP:** Crushed Pyrite (used as sample code prefix)  
**DoE:** Design of Experiments  
**DRC:** Democratic Republic of the Congo  
**DSM:** *Deutsche Sammlung von Mikroorganismen* (German Collection of Microorganisms and Cell Cultures)  
**EU:** European Union  
**FFD:** Full Factorial Design  
**HBS:** Basal Salts Medium  
**ICP-OES:** Inductively Coupled Plasma – Optical Emission Spectroscopy  
**IPB:** Iberian Pyrite Belt  
**ISC:** Inorganic Sulfur Compounds  
**ISO:** International Organization for Standardization  
**Jrs:** Jarosite  
**Kln:** Kaolinite  
**LCA:** Life Cycle Assessment  
**LNEG:** *Laboratório Nacional de Energia e Geologia* (National Laboratory of Energy and Geology)  
**Minitab:** Minitab Statistical Software (version 22)  
**ML:** Machine Learning  
**OFAT:** One-Factor-At-a-Time  
**ORP:** Oxidation-Reduction Potential  
**PA:** Pyrite Ash (used as sample code prefix)  
**pH:** Potential of Hydrogen (measure of acidity/basicity)  
**Py:** Pyrite  
**Qz:** Quartz  
**RFR:** Random Forest Regression  
**RISC:** Reduced Inorganic Sulfur Compounds  
**RSM:** Response Surface Methodology  
**SD-BIO-1:** Slag sample from São Domingos (sample 1)  
**SD-BIO-2:** Pyrite ash sample from São Domingos (sample 2)

**SD-BIO-3:** Crushed pyrite sample from São Domingos (sample 3)

**SL:** Slag (used as sample code prefix)

**SLA:** Mixed culture of *Sulfobacillus thermosulfidooxidans*, *Leptospirillum ferriphilum*, and *Acidithiobacillus caldus*

**TAL QUAL:** "As is" in Portuguese (used for untreated sample)

**TEA:** Techno-Economic Assessment

**TQO:** Thiosulfate: quinone oxidoreductase (enzyme involved in sulfur oxidation)

**TSD:** Thiosulfate dehydrogenase (enzyme involved in sulfur oxidation)

**VMS:** Volcanogenic Massive Sulfide

**VSC:** Volcanic-Sedimentary Complex

**XRD:** X-ray Diffraction

**XRF:** X-ray Fluorescence

## Abstract

While the extraction of metals is essential for sustainable energy technologies, they pose environmental challenges due to the toxic materials used in their production and the hazardous waste they generate. The presence of waste containing metals and organic compounds on Earth is increasing, and Europe holds large amounts of metals locked up in industrial process residues, such as tailings, metallurgical sludges, slags, dust, and ashes. The waste or secondary material may contain critical raw materials and base metals with application in the energetic transition. These factors highlight the urgent need for effective recycling practices to manage mine waste and recover valuable secondary raw materials, addressing both environmental and economic challenges.

This thesis investigates the optimal conditions for effective bioleaching of pyrite mine wastes and modern slags from the Achada do Gamo Sulfur factory in the São Domingos Mine area, focusing particularly on Manganese and Zinc recovery to address sustainability concerns. Using Response Surface Methodology (RSM), the study evaluates the impact and relationship between pulp density, initial pH, and initial ferrous iron concentration evaluated through a Box-Behnken design (BBD). Direct bioleaching experiments were conducted on altered and crushed pyrite, pyrite ashes, and modern slags using a thermophilic microbial culture consisting of *Sb. thermosulfidooxidans*, *L. ferriphilum*, and *At. caldus* (SLA), which utilized elemental sulfur and ferrous iron as energy sources, driving the oxidation of ferrous to ferric iron and the conversion of sulfur to sulfuric acid in the leaching medium.

Results showed that SLA culture is less effective bioleaching in pyrite wastes than slag, although ORP measurements indicated microbial iron oxidation for all materials. This study successfully optimized the recovery of metals from slag samples, indicating a maximum efficiency of metal solubilization under the conditions of 2.32% (w/v) solid load, pH 1.90, and ferrous iron supplementation of 60 mM. The maximum metal recovery achieved was 86.9% Zn, 81.4% Mn, 65% Cu, and 66.4% Co.

For crushed pyrite, under the conditions of pH 1.66, 54 mM supplementation of iron and 2% (w/v) pulp density was estimated to achieve moderate to high recovery of Mn (>53.8%) and Zn (>8.0%). However, the values obtained were not within the 95% confidence interval and the null hypothesis could not be rejected, indicating the proposed model does not apply to the practical application; consequently, an optimized condition was not defined. The application of the same conditions obtained in the model for crushed pyrite was applied to pyrite ash material, which did not result in satisfactory results. Hence, to better recover metals from pyrite ashes and crushed pyrite, further optimization is needed.

## 1. Introduction

### 1.1. Background and Relevance

The presence of waste containing metals and organic compounds on Earth is increasing, potentially damaging the environment and human health irreversibly or causing impacts that last for generations. For instance, the negative impacts on human health include cancer, organ disorders, immunodeficiency, neurological and psychological disorders (Naseri et al., 2023).

Waste is carried across countries, and its long-term pollution of land and aquatic ecosystems is considered one of the main threats to biodiversity loss and the integrity of the environment. Furthermore, it is estimated that 90% of all biodiversity loss is caused by land use and the consumption of related resources (UNEP, 2024).

The production of metals forms undesirable products, including solids, liquids, and gases. These products may be released directly during the extraction and processing of mineral resources or indirectly during the use of raw materials and utilities, such as in the production of reagents, explosives, and electricity (Norgate, Jahanshahi & Rankin, 2007).

Often, the chemical and physical processing of ores to extract metals requires significant amounts of energy. Decades of excessive mining caused a global decline in high-grade ores. With their depletion, there is a decrease in the supply of commercially valuable metals using traditional strategies such as hydro- and pyrometallurgy. As the grade lowers, more energy, water, and chemicals are needed, more waste is produced, and more greenhouse gas emissions are released to access and extract target metals (Norgate, Jahanshahi & Rankin, 2007).

Community concerns and regulatory aspects require metal processing companies and other businesses from the raw materials value chain to reduce their environmental impacts. However, achieving this goal is often difficult due to the large number of feed, by-product, waste, and energy streams associated with most metal production (Norgate, Jahanshahi & Rankin, 2007).

Europe holds large amounts of metals locked up in industrial process residues, such as tailings, metallurgical sludges, slags, dust, and ashes. (Spooren et al, 2020). These materials are called secondary resources or secondary raw materials, as they can be identified as materials and products that can be used as raw materials by simple reuse or via recycling and recovery (European Commission, 2020). The waste or secondary material contains critical raw materials and base metals such as Zn, Pb, and Cu at grades that are becoming competitive with the decreasing grades of primary ores.

Strategic minerals such as Co, Mn, and Cu, and other valuable metals known for their properties and applications in the energetic transition, can be found in those waste streams. Nowadays, the European Union (EU) relies heavily on imports to meet the necessity of domestic raw materials. Co is concentrated only in a few countries, including politically insecure suppliers (Alves et al., 2018). Around 55% of cobalt is mined in the Democratic Republic of the Congo (DRC), viewed as politically unstable, thereby reducing the certainty of access to supply (Alves et al., 2018). Concurrently, it is estimated that DRC will remain the most relevant source of cobalt in the future (Alves et al., 2018). Furthermore, the EU is fully dependent on imports of battery-grade manganese (European Union, 2025). Therefore, the urge to recover these metallic elements from solid waste and wastewater has become more critical

not only to prevent their negative impact on human health and the environment but also to secure nations' economic activities (Naseri et al., 2023).

Waste reprocessing can be an alternative to the reduction of imports as well as a way of promoting cleaner production, an initiative to minimize waste generation and pollution while increasing the product efficiency (Tranvik et al., 2017). There are several options to recover metals from residues; the traditional methods include pyrometallurgy and hydrometallurgy. However, they were commonly designed for high-grade ores, decreasing the efficiency for waste and also for processing low-grade ores (Komnitsas, 2019). Pyrometallurgical methods consist of ore or metallic waste treatment at high temperatures, aiming to convert them to raw materials or intermediate compounds for additional refining (Singh & Schwan, 2019). However, pyrometallurgical processes present high energy consumption and release of hazardous gases, such as dioxin and furans. Hydrometallurgy promotes the treatment with aqueous solutions, such as strong acids and alkalis, to recover metals (Singh & Schwan, 2019). The drawback of these processes is the use of hazardous chemicals and the formation of unwanted byproducts, as well as high water consumption (Naseri et al., 2023).

An alternative to those methods is called biomining, which is a biotechnological application that promotes interest in metal recovery from ores or wastes with the aid of microorganisms. These microorganisms can remove the metal ions from solid wastes and transfer them into a solution (Naseri et al., 2023). A wide range of microorganisms can be employed, namely autotrophic and heterotrophic bacteria, archaea, and fungi. Furthermore, they are capable of biofilm formation and production of extracellular polymers (Vera et al., 2022).

Biomining is subdivided into two processes: bioleaching, a conversion of an insoluble valuable metal into a soluble form by means of microorganisms (Schippers et al., 2013), in which the target metal is solubilized from the mineral matrix (e.g. Zn from sphalerite); and biooxidation, in which the target metal trapped as microscopic particles within the mineral (e.g. As from arsenopyrite) loses electrons (is oxidized) and hence is brought into solution by means of microorganisms. The term biooxidation is commonly employed when the solubilized metals are not the target to be recovered (Vera et al., 2013).

Among the advantages of bioleaching, it is possible to highlight that it is a less energy-intensive, more environmentally friendly process and has lower cost with moderate to high efficiency when compared to the pyro- and hydrometallurgical processes (Vera et al., 2022). With this in mind, if bioleaching is proven to be economically feasible and scalable, it turns out as a preferred method to recover valuable metals from waste and low-grade ores (Naseri et al., 2023). Yet, cost-effective mass production of biolixiviants with heterotrophic microorganisms can be a challenge (Spooren et al, 2020).

## **1.2. São Domingos, Portugal**

Iberian Pyrite Belt (IPB) extends 250 km in a WNWSE direction, from the Marateca area on the Portuguese Atlantic coast, southeast of Lisbon (Alentejo region), to the Seville area in Spain (Andalusia region) (Tornos, 2006). IPB represents one of the most relevant metallogenic provinces for Volcanogenic Massive Sulfide (VMS) deposits worldwide, including world-class deposits as well as medium-sized deposits such as the São Domingos

Mine (XTRACT Project, 2025). The area faces cool to mild, rainy winters and dry and warm summers because of its warm Mediterranean climate.

About 5 kilometers from the Spanish border, in the northern region of the IPB, is the São Domingos (37°40'10"N, 7°29'52"W) deposit (GeoHack Toolforge, n.d.), an abandoned copper mine (Figure 1.2).

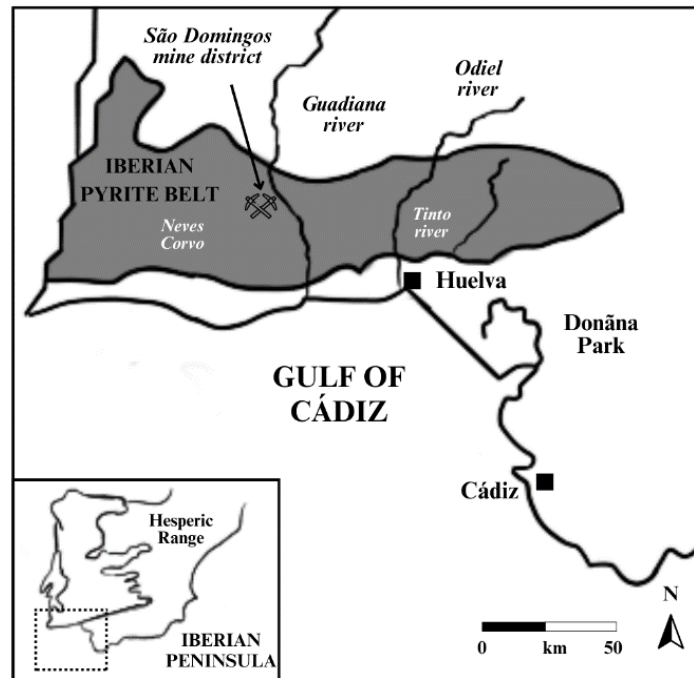


Figure 1.1. The geographic location of the Iberian Pyrite Belt with a highlight to the São Domingos mine district. Adapted from: Álvarez-Valero et al., 2008.

IPB is a complex geologic sequence, which includes volcanic and sedimentary rocks from the Devonian period, and forms the basis of the volcanic-sedimentary complex (VSC) in the region (Albardeiro, 2023). The middle series, known as VSC, is primarily composed of felsic volcanic and subvolcanic rocks, and hosts the extensive sulfide deposits of the IPB. Near the top of the VSC is the São Domingos pyrite orebody. The outcropping area was formed by a unique vertical mass of cupriferous pyrite associated with zinc and lead sulfides, elongated in an E-W direction (XTRACT Project, 2025). Pyrite, sphalerite, chalcopyrite, galena, arsenopyrite, and sulfosalts compose the majority of the mineral assemblage found in this single sub-vertical body of massive sulfides (Álvarez-Valero et al., 2008).

### 1.3. Ore processing in the São Domingos Mine

São Domingos Mine has a long-time span history of mining exploitation. During the Roman era, the superficial and oxidized part of the vertical massive sulfide lens was intensely exploited (Matos et al., 2012). Intermittent mining of the metals such as Au, Ag, Cu, Sn, Pb, and Fe in IPB occurred for over 5000 years, which has provided in total of 14.6 Mt Cu, 13.0 Mt Pb, 34.9 Mt Zn, 46100 t Ag, and 880 t Au (Leistel et al., 1997).

It was mined on a commercial scale from 1859 to 1966 for copper and sulfur production, from pyrite (Johnson, 2006). IPB orebody was exploited to a depth of 120 m below the

topographic surface as an open pit mine and from this depth down to 420 m (XTRACT Project, 2025).

The first products were extracted by incineration processes, then the richest nodules underwent a fusion process (XTRACT Project, 2025). The 2% cut-off Cu ore was separated into four piles: fine material and poor material. Then, the richest and coarse grained material was crushed into 5 cm pieces.  $\text{Cu} < 2\%$  was accumulated in piles for easy circulation of air, and the temperature was controlled (XTRACT Project, 2025). Then, the low-grade Cu products underwent leaching in cementation tanks. Piles were leached with water to avoid spontaneous combustion and the formation of sulfuric acid (Gaspar, 1998).

The ore was exported for sulfuric acid production; the leaching products had 14% of Cu and were lidded to the tanks to decant particles and sent to cementation tanks. The iron used in the process was about 20000 t a year. From the 4 Mt of copper mineralization extracted from 1870 to 1887, 334575 t were transported to the treatment facilities in Achada do Gamo, and 378320 t of washed product and 85046 t of copper concentrate were exported (XTRACT Project, 2025).

The mentioned mine management had implications for the present-day dumps (XTRACT Project, 2025). At the end of the 20<sup>th</sup> century, many mines closed, including São Domingos. Although mining activity has ceased, the large-scale exploitation occasioned the production of enormous waste dumps, approximately  $200 \times 106 \text{ m}^3$  of massive sulfide-bearing waste rock piles, dumps, tailings, and flooded pits (Pérez-López et al., 2008). Waste mining material either concentrated or dispersed in the area, is still enriched in hazardous elements such as Zn, Pb, Sb, Cu, As, Hg, and Cd. These waste materials leached by rainwater and stream waters can have highly acid-generating potential, resulting in acid mine drainage (AMD), due to sulfide oxidation and Fe hydrolysis, which increases dissolution and mobility of elements (XTRACT, 2025). They constitute hot spots for water, soil, and plant contamination. Present-day mine dumps cover large areas and were mapped accordingly to tailings composition, which is estimated to be around 5 Mt of tailings and mineral spoil are stored at the abandoned mine (Johnson, 2006).

When processing it from the São Domingos mining area, there were seven main groups of solid wastes generated based on the processing type: Modern slag, roman slag, iron hat material, metal volcanic rocks and shales, shales, finely crushed sulfide ore, and roasted sulfide ore (Matos et al., 2011). Roman slags are connected to the exploitation of gossan and supergene-enrichment zones according to Pérez-Lopez et al. (2008). While the 20<sup>th</sup> century mine exploitation is responsible for modern slags, sulfide roasted ashes, brittle pyrite ore, coarse blocks of pyrites, smelting ashes, and dumps (Pérez-Lopez et al. 2008), which are considered processing waste (Álvarez-Valero, 2008).

#### **1.4. Optimization of bioleaching**

To make the bioleaching processes scalable and feasible, it is necessary to have in mind that the percentage of metal bio-recovery is affected by different variables, including the substrates and their respective concentrations, pulp density, initial ferrous iron concentration,  $\text{O}_2$  and  $\text{CO}_2$  concentrations, pH, temperature, inoculum density, the waste's particle size, solid-

to-liquid ratio, bioleaching duration, and shaking speed (Naseri et al., 2023). The interaction between these variables significantly impacts the bioleaching rate, which, in turn, determines the efficiency of metal dissolution and the overall recovery process (Lujan-Moreno et al., 2018). Furthermore, the selection of the ideal conditions is also dependent on the microorganisms employed in the bioleaching. For example, *S. thermosulfidooxidans*, *At. caldus* and *L. ferriphilum* dominate at temperatures between 40 °C and 60 °C (Vera et al., 2022).

Investigating all the factors involved in the process requires many experiments, which are costly and time-consuming, and reaction time optimization is essential in bioleaching (Rastegar, 2014). Therefore, one of the most crucial challenges in using the bioleaching process to extract metals from solid wastes is the proper selection of experimental conditions (Lujan-Moreno et al., 2018).

In order to identify the ideal conditions for bioleaching, two optimization methods are mainly employed: one-factor-at-a-time (OFAT) and Design of Experiment (DoE). OFAT is the conventional method, in which one factor is analyzed while the others are maintained constant throughout the experiments (Naseri et al., 2023). In the literature, several factors were investigated following the OFAT method to obtain the optimal parameters for pyrite waste recovery from 2000 to 2024. The main varied parameters identified were temperature, pulp density, and pH. Different factors were also monitored, such as dissolved oxygen, redox potential, amount of elemental sulfur, nutrient concentration, and dissolved salts.

However, the OFAT approach does not consider possible interactions between the factors. DoE, on the other hand, consists of a systematic method to define the relationships between several input factors affecting a process and the output variables, known as responses (Naseri et al., 2023). It can improve the process efficiency and sustainability, lowering production costs, and improving the understanding of the input and output connection in the process (Xu, 2004).

Therefore, OFAT can be employed when a new system is being investigated and there is no prior knowledge about the significant interactions between the factors, or even if there is enough information to confirm that the interaction among the factors is negligible. (Naseri et al., 2023). The use of OFAT designs allows researchers to discover faster if a factor has an effect on the experiment outcome because the information is received continuously, as it is not necessary to wait for the results of an entire experiment (Qu & Wu, 2005). DoE is more appropriate when studying the optimization of a process to find the best combination of factor levels to study the effects of multiple factors simultaneously, which is relevant to industry-scale optimizations.

In order to optimize parameters in bioleaching, the design of experiments can be employed, as it is capable of identifying the interaction between parameters, reducing the total number of experiments, which results in less material consumption and fewer laboratory experiments. DoE was previously used to evaluate the optimum response of heavy metals' recovery rates in studies on solid waste bioleaching (Naseri et al., 2023).

To employ DoE, techniques such as Response Surface Methodology (RSM) are used. According to Lujan-Moreno et al. (2018), RSM has drawn extensive attention, especially in the design, optimization, and modeling of the bioleaching processes. It consists of a statistical method to model the relationships between several explanatory variables and one or more

response variables. RSM includes optimization to adjust the settings of factorial variables, with the goal of achieving the best possible outcome by minimizing, maximizing or setting a target response.

There are several ways to design experiments in RSM, for example, Full Factorial Design (FFD), Central Composite Design (CCD), and Box-Behnken Design (BBD). CCD is the most used RSM that considers 5 levels of each factor's surface and is a highly rotational method. CCD (Ghelich et al., 2019), which is also called the Box–Wilson design, can be defined as an experimental design to obtain as much information as possible about a process from a small number of experiments. The benefits of CCD are an estimation of the nonlinearity of dependent variables, maximum information with minimal experimental result data, and reduction of the number of experiments to predict quadratic terms in the second-order model.

Box-Behnken Design is one type of RSM that always uses three levels per input variable and fits the output (expected response) to a quadratic model (LaPanse et al., 2021). Box-Behnken designs are used to generate higher-order response surfaces using fewer required runs than a normal factorial technique (Rao & Kumar, 2012). As can be seen in Figure 1.1, the cube includes a central point and the midpoints of its edges, represented by blue dots. Each of these points corresponds to an experimental measurement.

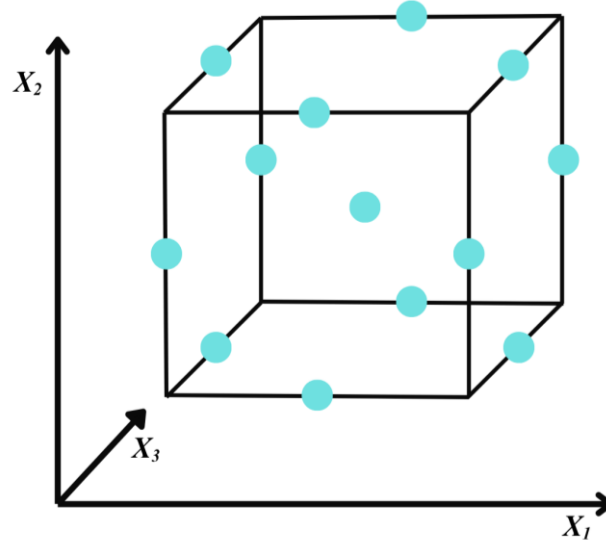


Figure 1.2. Box-Behnken Design cube. Adapted from Ferreira et al., 2017.

A full factorial design is composed of two or more factors with specific values and includes experiments for all possible combinations of these values across all the factors. The Full Factorial Design may not be viable for a large number of factors because of the complexity and costs, and it is advised for initial screening; however, it shows limitations in providing an understanding of higher-order interactions (Jankovic et al., 2021).

Each of them is more suitable for different scenarios. For instance, CCD is useful for determining optimal conditions for several factors when the factors to be explored are beyond the initial levels to refine responses. It consists of factorial and axial points extending beyond factorial limits and center points. It generates response surface equations, optimal factor settings for the response, and insight into curvature and regions of maximum and minimum. CCD optimizes formulations by examining quadratic interactions through  $\alpha$  points that may exceed practical limits.

The BBD is useful when extreme factor levels, high or low, can be impractical. It requires fewer experiments than CCD for the same number of factors. BBD consists of combinations at the midpoint of factor ranges and center points. BBD avoids extreme factor levels, making it practical but less efficient for studying higher-order interactions. Furthermore, it generates a response surface equation and optimal factor settings; however, it provides less insight into the extreme factor effects when compared to CCD. Both CCD and BBD excel in optimization, but CCD is more detailed, while BBD is more time and cost-efficient (Narukulla et al., 2024).

Several software tools can be used to implement DoE, enabling researchers to optimize bioleaching processes and other experimental systems efficiently. Popular options include Minitab, Design-Expert, JMP, MATLAB, R, and Python. Once the design is implemented, the comparison of these models with real-time experimental data is critical to validate the reliability and applicability of the findings. First, statistical metrics like the coefficient of determination ( $R^2$ ), adjusted  $R^2$ , and predicted  $R^2$  are used to assess how well the model fits the data. High  $R^2$  values indicate that the model explains a significant portion of the variability in the response, while the adjusted  $R^2$  accounts for the number of predictors, ensuring the model is not overfitting.

Additionally, analysis of variance (ANOVA) is performed to test the statistical significance of the model and individual factors. A significant p-value in ANOVA confirms that the model or factors have a relevant impact on the response variable. Further, response surface plots, contour plots, and residual plots are valuable for visualizing how closely experimental data align with the predicted response surface.

After the model is done, it needs to be validated by conducting confirmatory experiments at the optimal settings. If the results obtained match the predicted values, the model is reliable and can be tested for other waste streams. On the other hand, if discrepancies are found between predicted and observed data, the model needs to be further refined by incorporating other factors, increasing the number of replicates, and more. When the statistical validation is crossed with the experimental data, it is possible to provide the optimal bioleaching conditions with resource efficiency and scalability.

#### **1.4.1. Minitab Statistical Software**

Minitab is a well-known statistical software developed in 1972. It provides a structured and efficient approach to statistical analysis and is used by statistical experts, practitioners, engineers, and students worldwide in research and industry (Alin, 2010). It allows graphs, calculations, charts, basic statistics, experiment design, multivariate analysis, hypothesis testing, regression analysis, and more.

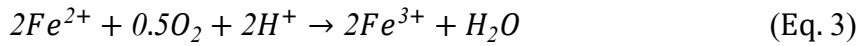
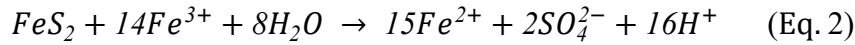
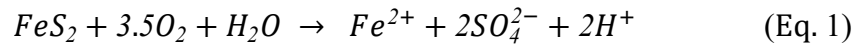
Minitab has a user-friendly display for data analysis, improvement of quality improvement, and process optimization of processes. For processing optimization, Minitab is commonly associated with the Six Sigma method, a strategy used in quality management and improvement to identify flaws and reduce errors and discrepancies in production processes (Ilin & Bohlen, 2023), which allows data-driven decision-making.

In DoE, Minitab allows assessing the effects of several variables on a system response and provides a list of experiments that should be performed to identify the optimal conditions for increasing performance. Furthermore, it also facilitates data visualization through graphs and provides statistical tests such as t-tests and ANOVA.

## 2. Theory and Background Knowledge

### 2.1. Oxidation Mechanism

Bacteria involved in biomining oxidize the ferrous ions and inorganic sulfur compounds (ISCs) in ores to produce ferric ions and protons (Vera et al., 2022). Because pyrite's valence bands are derived from the metal atoms' orbitals, it can only undergo chemical oxidation in aqueous solution when it comes into contact with dissolved oxygen (Equation 1) or ferric iron (Equation 2). The formation of ferric iron in the presence of dissolved oxygen is seen in Equation 3.



To oxidize the pyrite, both ferric ions and oxidized sulfur species should be replenished. In nature, different bacteria can oxidize elemental sulfur and ferrous iron, such as *Sulfobacillus thermosulfidooxidans* (iron and sulfur oxidizer), *Acidithiobacillus caldus* (sulfur oxidizer), and *Leptospirillum ferriphilum* (iron oxidizer).

In the bioleaching process, microorganisms can remove metal ions in two mechanisms, indirect or direct bioleaching. In the indirect, bacteria have no physical contact with the solid waste surface, which is dissolved indirectly through the production of metabolites. In the direct method, bacteria are in contact with the particles. Thus, metals become soluble due to electrochemical reactions (Naseri et al., 2022).

Equation 1 illustrates the direct method, in which bacteria use dissolved oxygen as an electron acceptor and use enzymes to oxidize sulfur or iron. In the indirect method, pyrite undergoes chemical degradation after the bacteria convert soluble ferrous iron to ferric iron, as indicated by Equation 3.

Therefore, both mechanisms are important for pyrite oxidation, taking place at the same time in the log phase. Furthermore, the direct mechanism is most dominant in the lag phase in which the bacteria are adapting (Yu et al., 2001). When studying *A. ferrooxidans*, Rodríguez et al. (2003) concluded that pyrite oxidation is initiated by direct mechanisms (Equation 1) and when the cells release metabolites and ferric ions to the solution, the indirect mechanisms dominate (Equation 3).

Moreover, the pathway to oxidize pyrite consists of the thiosulfate mechanism (Figure 2.1). In the thiosulfate mechanism, the microbial process taking place involves the oxidation of iron sulfides to form thiosulfate ( $S_2O_3^{2-}$ ). Ferric ions attack metal sulfides and are reduced to ferrous ions. In this process, the acidophilic iron-oxidizing bacteria (indicated in blue) attach to the surface of the iron sulfide mineral or waste particles and initiate the oxidation process

by transferring electrons from the iron sulfide to their electron transport chain. During the electron transfer, intermediate sulfur compounds are formed, such as sulfur (S) and polysulfides ( $S_x^{2-}$ ). The intermediate sulfur compounds are further oxidized by the bacteria to form thiosulfate ( $S_2O_3^{2-}$ ), which involves the incorporation of oxygen ( $O_2$ ) as the terminal electron acceptor.

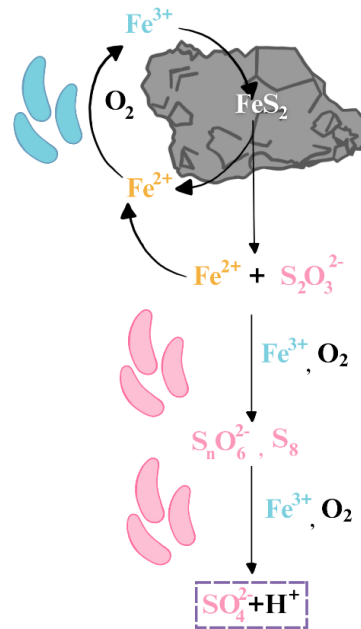


Figure 2.1. Schematic of the thiosulfate mechanism pathway in bioleaching of pyrite. The microorganisms represented in blue are iron (II) oxidizing bacteria, while the pink ones consist of sulfur compound oxidizing bacteria. Adapted and modified from: Schippers & Sand, 1999.

As a result, metal cations and sulfur compounds are released and solubilized in water. Further, the iron (II) is oxidized again to iron (III) ions by bacteria such as *Sulfobacillus thermosulfidooxidans* and *Leptospirillum ferriphilum* in acidic solutions (Vera et al., 2022).

## 2.2. Microorganisms

Studies often employ mixed microbial cultures in bioleaching, as the synergistic interactions among different species can enhance metal solubilization efficiency (Vera, 2022). These microbial communities adapt dynamically to changes in the leaching environment, maintaining process effectiveness over time. Mixed cultures for bioleaching typically include chemoautotrophic microorganisms which derive energy from inorganic compounds and directly contribute to metal solubilization, with no need of supplementation of organic substrates to support community growth (Jones & Santini, 2023).

Among the bacteria employed in bioleaching, *Acidithiobacillus caldus* is well known for the ability to oxidize elemental sulfur and a variety of reduced inorganic sulfur compounds (RISCs). It uses energy and electrons derived from sulfur oxidation for carbon dioxide fixation and other anabolic processes (Hallberg, 1994). As a result, it became an important player and one of the most common autotrophic bacteria in mixed cultures and single cultures to recover

metals from a variety of waste streams (Naseri et al, 2023). *A. caldus* is a Gram-negative, acidophilic, and sulfur-oxidizing species (Hallberg, 1994).

Another relevant microorganism is *Sulfobacillus thermosulfidooxidans*, a Gram-positive, non-motile, rod-shaped, endospore-forming, and moderately thermophilic acidophile. It can be employed to oxidize ferrous iron, RISCs such as tetrathionate, thiosulfate, and elemental sulfur, as well as sulfide. Besides the oxidizing properties, bacteria like *S. thermosulfidooxidans* are found to be highly tolerant to metal concentrations (Li et al., 2020).

*Leptospirillum* is a mesophilic, vibrioid-shaped, iron-oxidizing and Gram-negative bacterium with properties that allow it to thrive in leaching habitats and, compared with *Acidithiobacilli*, it can resist 10 times higher ferric ion concentrations (Sand et al., 1992). *Leptospirilli* attaches to metal sulfides fast and firmly which is crucial for biofilm formation in mixed cultures (Sand et al., 1992). They are pre-colonizers that help other microorganisms attach to substrata (Li et al., 2020). *L. ferriphilum* is an iron-oxidizing bacterium, therefore, when oxidizing pyrite as a pure culture, it causes the accumulation of sulfur in the medium and surface of the mineral (Vardanyan & Akopyan, 2003). To improve their performance, it is advisable to associate with sulfur-oxidizing bacteria such as *S. thermosulfidooxidans* and *A. caldus*. *Sulfobacillus* and *Leptospirillum* occur frequently in leaching systems, and the bioleaching performance using a mixed culture is reported to be better than that of a pure culture of *S. thermosulfidooxidans* (Li et al., 2020). Different studies suggest that consortia containing the three species are efficient for bioleaching too (Hong et al., 2019).

### 3. Task and Objective

The objective of this thesis is to identify the optimal conditions for effective bioleaching of crushed and altered pyrite from heap dumps in part affected by leaching processes, modern slags from Achada do Gamo Sulfur factory, and pyrite ashes - native sulfur and black layers from São Domingos Mine.

Using pyrite wastes and modern slag, the study evaluates the impact and relationship between pulp density, initial pH, initial ferrous iron concentration, and initial inoculum evaluated through BBD. This approach will establish near-optimal conditions in a cost-effective way, which will be validated in the laboratory with bioleaching experiments by employing a consortium of microorganisms composed of *Leptospirillum ferriphilum*, *Sulfobacillus thermosulfidooxidans*, and *Acidithiobacillus caldus*.

Therefore, this thesis aims to investigate the most favorable bioleaching conditions to assess and maximize the efficiency for the recovery of valuable metals in sustainable resource recovery solutions.

## 4. Materials and methods

### 4.1. Experimental design and optimization by Response Surface Methodology

In order to employ the experiment design and RSM in this study, Minitab Statistical Software 22 was used. 7 steps were employed for optimization (Naseri et al., 2023) of pyrite waste and modern slag bioleaching as shown in Figure 4.1: (1) goal definition, (2) selection of

factors that have relevant effects on the system through screening literature work, (3) choice of type of Response Surface Methodology, in this case, Box-Behnken Design, (4) running experiments and collecting results, (5) fit the model to the experimental data, (6) model confirmation using Analysis of Variance (ANOVA) and plotting graphs, (7) determination of optimal conditions.

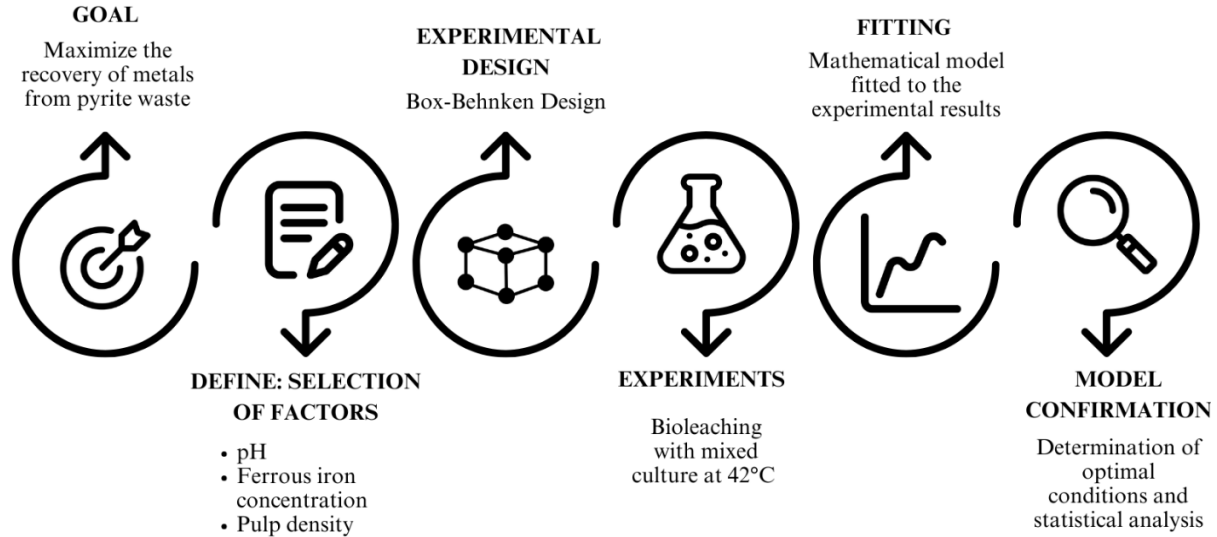


Figure 4.1. Successive steps involved in optimizing the bioleaching process using Response Surface Methodology.

#### 4.1.1. Box-Behnken Design

A three-factor three-level Box-Behnken design with 9 center points and 3 replicates was used to identify the best conditions for mine waste bioleaching. The experiment aimed to determine the relationship between pulp density, initial ferrous iron concentration, and pH with respect to metal recovery. Each factor was tested in three levels: low, medium, and high values represented as -1, 0, and 1, respectively, which correspond to real values as listed in Table 4.1 and Table 4.2. The real values were chosen based on preliminary tests using the same materials, and the factor values were codified according to equation 4. The coded data were then analyzed using Minitab Software to simplify calculations and allow uniform comparison among the factors. The three variables (pH, initial ferrous iron concentration, and pulp density) were labeled as  $X_1$ ,  $X_2$ , and  $X_3$ , respectively.

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (\text{Eq. 4})$$

Where  $x_i$  is the coded value of an independent variable,  $X_i$  is the real value of an independent variable,  $X_0$  is the real value of an independent variable at the central point, and  $\Delta X_i$  is the step change value.

Instead of testing all possible combinations, Minitab picks a smart subset of the combinations. BBD starts with a two-level factorial design by choosing important factor combinations, then a third level is added and arranges the tests using an incomplete block design so that not all combinations are tested, but enough to obtain an accurate quadratic model. This makes BBD efficient, having fewer experiments while still capturing complex effects.

The selected experimental points are on a cube at an equal distance from the central point. BBD has a high ability to approximate second-order response surfaces.

Table 4.1. Box–Behnken design with three variables and three levels for crushed pyrite (SD-BIO-3) bioleaching optimization.

| Sample Code | pH             |                     | [Fe (II)] (mM)     |                     | Pulp density (% w/v) |                     |
|-------------|----------------|---------------------|--------------------|---------------------|----------------------|---------------------|
|             | X <sub>1</sub> | Code X <sub>1</sub> | X <sub>2</sub> (%) | Code X <sub>2</sub> | X <sub>3</sub> (%)   | Code X <sub>3</sub> |
| CP1         | 1.5            | -1                  | 10                 | -1                  | 3                    | 0                   |
| CP2         | 2.5            | 1                   | 10                 | -1                  | 3                    | 0                   |
| CP3         | 1.5            | -1                  | 60                 | 1                   | 3                    | 0                   |
| CP4         | 2.5            | 1                   | 60                 | 1                   | 3                    | 0                   |
| CP5         | 1.5            | -1                  | 35                 | 0                   | 2                    | -1                  |
| CP6         | 2.5            | 1                   | 35                 | 0                   | 2                    | -1                  |
| CP7         | 1.5            | -1                  | 35                 | 0                   | 4                    | 1                   |
| CP8         | 2.5            | 1                   | 35                 | 0                   | 4                    | 1                   |
| CP9         | 2              | 0                   | 10                 | -1                  | 2                    | -1                  |
| CP10        | 2              | 0                   | 60                 | 1                   | 2                    | -1                  |
| CP11        | 2              | 0                   | 10                 | -1                  | 4                    | 1                   |
| CP12        | 2              | 0                   | 60                 | 1                   | 4                    | 1                   |
| CP13        | 2              | 0                   | 35                 | 0                   | 3                    | 0                   |
| CP14        | 2              | 0                   | 35                 | 0                   | 3                    | 0                   |
| CP15        | 2              | 0                   | 35                 | 0                   | 3                    | 0                   |
| CP16        | 1.5            | -1                  | 10                 | -1                  | 3                    | 0                   |
| CP17        | 2.5            | 1                   | 10                 | -1                  | 3                    | 0                   |
| CP18        | 1.5            | -1                  | 60                 | 1                   | 3                    | 0                   |
| CP19        | 2.5            | 1                   | 60                 | 1                   | 3                    | 0                   |
| CP20        | 1.5            | -1                  | 35                 | 0                   | 2                    | -1                  |
| CP21        | 2.5            | 1                   | 35                 | 0                   | 2                    | -1                  |
| CP22        | 1.5            | -1                  | 35                 | 0                   | 4                    | 1                   |
| CP23        | 2.5            | 1                   | 35                 | 0                   | 4                    | 1                   |
| CP24        | 2              | 0                   | 10                 | -1                  | 2                    | -1                  |
| CP25        | 2              | 0                   | 60                 | 1                   | 2                    | -1                  |
| CP26        | 2              | 0                   | 10                 | -1                  | 4                    | 1                   |
| CP27        | 2              | 0                   | 60                 | 1                   | 4                    | 1                   |
| CP28        | 2              | 0                   | 35                 | 0                   | 3                    | 0                   |
| CP29        | 2              | 0                   | 35                 | 0                   | 3                    | 0                   |
| CP30        | 2              | 0                   | 35                 | 0                   | 3                    | 0                   |
| CP31        | 1.5            | -1                  | 10                 | -1                  | 3                    | 0                   |
| CP32        | 2.5            | 1                   | 10                 | -1                  | 3                    | 0                   |
| CP33        | 1.5            | -1                  | 60                 | 1                   | 3                    | 0                   |
| CP34        | 2.5            | 1                   | 60                 | 1                   | 3                    | 0                   |
| CP35        | 1.5            | -1                  | 35                 | 0                   | 2                    | -1                  |
| CP36        | 2.5            | 1                   | 35                 | 0                   | 2                    | -1                  |
| CP37        | 1.5            | -1                  | 35                 | 0                   | 4                    | 1                   |
| CP38        | 2.5            | 1                   | 35                 | 0                   | 4                    | 1                   |
| CP39        | 2              | 0                   | 10                 | -1                  | 2                    | -1                  |
| CP40        | 2              | 0                   | 60                 | 1                   | 2                    | -1                  |
| CP41        | 2              | 0                   | 10                 | -1                  | 4                    | 1                   |
| CP42        | 2              | 0                   | 60                 | 1                   | 4                    | 1                   |
| CP43        | 2              | 0                   | 35                 | 0                   | 3                    | 0                   |
| CP44        | 2              | 0                   | 35                 | 0                   | 3                    | 0                   |
| CP45        | 2              | 0                   | 35                 | 0                   | 3                    | 0                   |

Table 4.2. Box–Behnken design with three variables and three levels for slag (SD-BIO-1) bioleaching optimization.

| Sample Code | pH             |                     | [Fe (II)] (mM)     |                     | Pulp density (%w/v) |                     |
|-------------|----------------|---------------------|--------------------|---------------------|---------------------|---------------------|
|             | X <sub>1</sub> | Code X <sub>1</sub> | X <sub>2</sub> (%) | Code X <sub>2</sub> | X <sub>3</sub> (%)  | Code X <sub>3</sub> |
| SL1         | 1              | -1                  | 10                 | -1                  | 3                   | 0                   |
| SL2         | 2              | 1                   | 10                 | -1                  | 3                   | 0                   |
| SL3         | 1              | -1                  | 60                 | 1                   | 3                   | 0                   |
| SL4         | 2              | 1                   | 60                 | 1                   | 3                   | 0                   |
| SL5         | 1              | -1                  | 35                 | 0                   | 2                   | -1                  |
| SL6         | 2              | 1                   | 35                 | 0                   | 2                   | -1                  |
| SL7         | 1              | -1                  | 35                 | 0                   | 4                   | 1                   |
| SL8         | 2              | 1                   | 35                 | 0                   | 4                   | 1                   |
| SL9         | 1.5            | 0                   | 10                 | -1                  | 2                   | -1                  |
| SL10        | 1.5            | 0                   | 60                 | 1                   | 2                   | -1                  |
| SL11        | 1.5            | 0                   | 10                 | -1                  | 4                   | 1                   |
| SL12        | 1.5            | 0                   | 60                 | 1                   | 4                   | 1                   |
| SL13        | 1.5            | 0                   | 35                 | 0                   | 3                   | 0                   |
| SL14        | 1.5            | 0                   | 35                 | 0                   | 3                   | 0                   |
| SL15        | 1.5            | 0                   | 35                 | 0                   | 3                   | 0                   |
| SL16        | 1              | -1                  | 10                 | -1                  | 3                   | 0                   |
| SL17        | 2              | 1                   | 10                 | -1                  | 3                   | 0                   |
| SL18        | 1              | -1                  | 60                 | 1                   | 3                   | 0                   |
| SL19        | 2              | 1                   | 60                 | 1                   | 3                   | 0                   |
| SL20        | 1              | -1                  | 35                 | 0                   | 2                   | -1                  |
| SL21        | 2              | 1                   | 35                 | 0                   | 2                   | -1                  |
| SL22        | 1              | -1                  | 35                 | 0                   | 4                   | 1                   |
| SL23        | 2              | 1                   | 35                 | 0                   | 4                   | 1                   |
| SL24        | 1.5            | 0                   | 10                 | -1                  | 2                   | -1                  |
| SL25        | 1.5            | 0                   | 60                 | 1                   | 2                   | -1                  |
| SL26        | 1.5            | 0                   | 10                 | -1                  | 4                   | 1                   |
| SL27        | 1.5            | 0                   | 60                 | 1                   | 4                   | 1                   |
| SL28        | 1.5            | 0                   | 35                 | 0                   | 3                   | 0                   |
| SL29        | 1.5            | 0                   | 35                 | 0                   | 3                   | 0                   |
| SL30        | 1.5            | 0                   | 35                 | 0                   | 3                   | 0                   |
| SL31        | 1              | -1                  | 10                 | -1                  | 3                   | 0                   |
| SL32        | 2              | 1                   | 10                 | -1                  | 3                   | 0                   |
| SL33        | 1              | -1                  | 60                 | 1                   | 3                   | 0                   |
| SL34        | 2              | 1                   | 60                 | 1                   | 3                   | 0                   |
| SL35        | 1              | -1                  | 35                 | 0                   | 2                   | -1                  |
| SL36        | 2              | 1                   | 35                 | 0                   | 2                   | -1                  |
| SL37        | 1              | -1                  | 35                 | 0                   | 4                   | 1                   |
| SL38        | 2              | 1                   | 35                 | 0                   | 4                   | 1                   |
| SL39        | 1.5            | 0                   | 10                 | -1                  | 2                   | -1                  |
| SL40        | 1.5            | 0                   | 60                 | 1                   | 2                   | -1                  |
| SL41        | 1.5            | 0                   | 10                 | -1                  | 4                   | 1                   |
| SL42        | 1.5            | 0                   | 60                 | 1                   | 4                   | 1                   |
| SL43        | 1.5            | 0                   | 35                 | 0                   | 3                   | 0                   |
| SL44        | 1.5            | 0                   | 35                 | 0                   | 3                   | 0                   |
| SL45        | 1.5            | 0                   | 35                 | 0                   | 3                   | 0                   |

The experimental designs for RSM of crushed pyrite (SD-BIO-3) and slag (SD-BIO-1) are shown in Tables 4.1 and 4.2, respectively. The response for the generated model is set as Bioleaching (%) after 9 days.

## 4.2. Materials Required

### 4.2.1. Salt solutions for media

For all cultivation experiments, the basal salt medium HBS was used at pH 1.0, 1.5, 2.0 or 2.5, and its composition is detailed in Table 4.3. To prepare the medium, a solution of 50-fold concentrate was made by adding the components as described by Nancucheo et al. (2016) (Table 4.4). Another necessary component is the trace element solution; the composition is presented in Table 4.5. Furthermore, a 1 M solution of  $\text{Fe}^{2+}$  with a pH of 2.0 was prepared using Iron (II) sulfate heptahydrate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) and deionized water. The pH levels of all solutions were adjusted and maintained using  $\text{H}_2\text{SO}_4$  using a Knick 766 pH-Meter.

Table 4.3. Composition of basal salts medium (HBS).

| Component                     | Volume [mL] |
|-------------------------------|-------------|
| 50x Basal Salts Solution      | 20          |
| 1000x Trace Elements Solution | 1           |
| Double-distilled water        | 979         |

Table 4.4. Composition of 50x Basal Salt Solution medium (Nancucheo et al., 2016).

| Component                                            | Concentration [g/L] |
|------------------------------------------------------|---------------------|
| $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$  | 7.5                 |
| $(\text{NH}_4)_2\text{SO}_4$                         | 22.5                |
| KCl                                                  | 2.5                 |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$            | 25                  |
| $\text{KH}_2\text{PO}_4$                             | 2.5                 |
| $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ | 0.7                 |

Table 4.5. Composition of 1000x Trace Elements Solution.

| Component                                             | Concentration [g/L] |
|-------------------------------------------------------|---------------------|
| $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$             | 10                  |
| $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$             | 1                   |
| $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$             | 1                   |
| $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$             | 1                   |
| $\text{Cr}(\text{SO}_4)_3 \cdot 15\text{H}_2\text{O}$ | 0.5                 |
| $\text{H}_3\text{BO}_3$                               | 0.6                 |
| $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$   | 0.5                 |
| $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$             | 1                   |
| $\text{Na}_2\text{SeO}_4 \cdot 10\text{H}_2\text{O}$  | 1                   |
| $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$    | 0.1                 |
| $\text{NaVO}_3$                                       | 0.1                 |

#### 4.2.2. Inoculum Preparation

For the direct-bioleaching experiments, a mixed culture of iron- and sulfur-oxidizing acidophiles and thermophiles was used, consisting of *Sb. thermosulfidooxidans*, *L. ferriphilum*, and *At. caldus* (SLA), which are summarized in Table 4.6. The culture initial inoculum was obtained from the Microbiology and Biohydrometallurgy research group of Freiberg University of Mining and Technology culture collection.

Table 4.6. Bacteria mixed culture used for bioleaching experiments.

| Bacteria                                                        | Name | T [°C] |
|-----------------------------------------------------------------|------|--------|
| <i>Sulfobacillus thermosulfidooxidans</i> DSM 9293 <sup>T</sup> |      |        |
| <i>Leptospirillum ferriphilum</i> DSM 14647                     | SLA  | 42     |
| <i>Acidithiobacillus caldus</i> DSM 8584                        |      |        |

The cultures used were pre-adapted to a 3% (w/v) of crushed pyrite, pyrite ash, or slag in HBS medium with S and Fe (Table 4.7). These adapted cultures served as the inoculum for the bioleaching experiments of this study. The pre-adapted cultures were incubated at 42 °C at a stirring rate of 120 rpm for 7 days before their first use.

Moreover, the elemental sulfur added to the culture media was tyndallized to prevent microorganism contamination. The process involved elemental sulfur heating at 112 °C for 20 minutes, three times with a 24-hour interval in between each heating session.

Table 4.7. Composition of pre-adapted culture media at pH 2.0.

| Component                    | Concentration or volume |
|------------------------------|-------------------------|
| Waste from São Domingos Mine | 3% (w/v)                |
| HBS-Media                    | 1x                      |
| Elemental Sulfur             | 1% (w/v)                |
| Iron (II) – Ferrous Sulfate  | 20 mM                   |
| Inoculum                     | 10% (v/v)               |

#### 4.2.3. Mineralogical and chemical characterization of mine waste samples

The material was collected in three different locations at the São Domingos Mine region (Figure 4.2), where SD-BIO-1 is the source of slag, from Achada do Gamo Sulfur Factory (Figure 4.3A), SD-BIO-2 (pyrite ash) was collected from an area of native sulfur and black layers (Figure 4.3B), while SD-BIO-3 (crushed pyrite) was collected from an area partially affected by leaching processes (Figure 4.3C). All samples were collected, characterized, and provided by the *Laboratório Nacional de Energia e Geologia* (LNEG), Portugal.



Figure 4.2. Location of the 3 samples from São Domingos mine provided by the *Laboratório Nacional de Energia e Geologia* (LNEG).



Figure 4.3. (A) Appearance of sampling area in Achada do Gamo Sulfur Factory for slag (SD-BIO-1). (B) Appearance of sampling area with native sulfur and black layers for pyrite ashes (SD-BIO-2). (C) Appearance of sampling area for crushed pyrite SD-BIO-3 heap dumps.

Prior to the bioleaching experiments, the characterization done by LNEG was done via X-ray diffraction (XRD) analysis of the bulk sample and preliminary chemical quantification by portable X-ray fluorescence (XRF) analysis of the bulk sample (Table 4.8).

Table 4.8. Phase identification of SD-BIO-1, SD-BIO-2, and SD-BIO-3.

| Sample Reference | Mineral Data Bank N° | Phase Identification                                              | +++ | ++       | +   |
|------------------|----------------------|-------------------------------------------------------------------|-----|----------|-----|
| SD-BIO-1         | 262                  | Amorphous                                                         |     |          |     |
| SD-BIO-2         | 259                  | Agrs? (vtg) + Ang + Bdn + Gp + Jrs (vtg) + Qz                     | Gp  | Ang, Bdn | Qz  |
| SD-BIO-3         | 257                  | Alu (vtg) + Ang + Ann (vtg) + Coq (vtg) + Kln (vtg) + Py + Qz + S | Qz  | Py, S    | Ang |

\* **Notes:** Agjrs – Argenojarosite ( $\text{AgFe}^{3+}_3(\text{SO}_4)_2(\text{OH})_6$ ); Alu – Alunite ( $\text{KAl}_3(\text{SO}_4)_2(\text{OH})_6$ ); Ang – Anglesite ( $\text{PbSO}_4$ ); Ann – Annite ( $\text{KFe}^{2+}_3(\text{AlSi}_3\text{O}_{10})(\text{OH})_2$ ); Bdn – Beudanite ( $\text{PbFe}_3(\text{AsO}_4)(\text{SO}_4)(\text{OH})_6$ ); Coq – Coquimbite ( $\text{AlFe}_3(\text{SO}_4)(\text{H}_2\text{O})_{12} \cdot 6\text{H}_2\text{O}$ ); Gp – Gypsum ( $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ ); Pyrite ( $\text{FeS}_2$ ); Qz – Quartz ( $\text{SiO}_2$ ); S – Sulfur ( $\text{S}_8$ ).

**Abbreviations:** +++: phase well represented; ++: well represented; +: poorly represented; vtg: vestigial content; ?: dubious identification.

Table 4.9. Elemental composition table of SD-BIO-1 TAL QUAL (slag), SD-BIO-2 TAL QUAL (pyrite ashes) and SD-BIO-3 TAL QUAL (crushed pyrite).

| Element | SD-BIO-1 TAL QUAL<br>(slag) [ppm] | SD-BIO-2 TAL QUAL<br>(pyrite ashes) [ppm] | SD-BIO-3 TAL QUAL<br>(crushed pyrite) [ppm] |
|---------|-----------------------------------|-------------------------------------------|---------------------------------------------|
| Al      | 6758                              | 3043                                      | 20124                                       |
| Ca      | 23692                             | 67797                                     | 1786                                        |
| Fe      | 498305                            | 130221                                    | 168740                                      |
| K       | 2476                              | 11292                                     | 16334                                       |
| Mg      | -                                 | 6326                                      | -                                           |
| Mn      | 635                               | 1163                                      | 108                                         |
| P       | 620                               | -                                         | -                                           |
| Ti      | -                                 | 3431                                      | 2511                                        |
| Si      | 91077                             | 58958                                     | 191110                                      |
| Ag      | -                                 | 103                                       | -                                           |
| As      | 312                               | 50167                                     | 748                                         |
| Ba      | -                                 | 624                                       | 225                                         |
| Bi      | -                                 | 7503                                      | 254                                         |
| Cd      | 28                                | -                                         | -                                           |
| Co      | 390                               | -                                         | 64                                          |
| Cu      | 4389                              | 1884                                      | 3989                                        |
| Ga      | -                                 | -                                         | 33                                          |
| Hg      | -                                 | 700                                       | 260                                         |
| La      | -                                 | -                                         | 27                                          |
| Mo      | 16                                | 11                                        | 21                                          |
| Nb      | -                                 | -                                         | 29                                          |
| Nd      | -                                 | -                                         | 103                                         |
| Pb      | 2633                              | 238009                                    | 40050                                       |
| Rb      | -                                 | 305                                       | 115                                         |
| S       | 18068                             | 160275                                    | 194647                                      |
| Sb      | 129                               | 18660                                     | 1253                                        |
| Sc      | 41                                | 309                                       | 35                                          |
| Se      | -                                 | 539                                       | 269                                         |
| Sn      | 233                               | 20462                                     | 849                                         |
| Sr      | 40                                | 219                                       | 189                                         |
| Th      | 25                                | 83                                        | 15                                          |
| U       | 21                                | -                                         | -                                           |
| Zn      | 17004                             | 2991                                      | 1981                                        |
| Zr      | 147                               | 620                                       | 436                                         |

The elemental composition of the slag sample (SD-BIO-1) is presented in Table 4.9. The material is characterized by particle size  $< 250 \mu\text{m}$  and a high iron (Fe) content (498305 ppm). Silicon (Si) and calcium (Ca) were also present in substantial amounts at 91077 ppm and 23692 ppm, respectively. Notably, the slag contained significant concentrations of heavy

metals such as copper (Cu, 4389 ppm), zinc (Zn, 17004 ppm), and lead (Pb, 2633 ppm), as well as sulfur (S, 18068 ppm), suggesting the presence of residual sulfide phases.

The pyritic ash sample (SD-BIO-2) was generated through the roasting of pyrite ores, although specific roasting parameters (e.g., temperature and equipment) were not available, the particle size was smaller than 250  $\mu\text{m}$ . The chemical composition of this material is shown in Table 4.9. The ashes show relevant concentrations of iron (Fe, 130221 ppm), sulfur (S, 160275 ppm), and calcium (Ca, 67797 ppm), consistent with the thermal decomposition of sulfide minerals. The material also contains high levels of lead (Pb, 238009 ppm), arsenic (As, 50167 ppm), and tin (Sn, 20462 ppm), indicating the potential environmental risk and economic interest for metal recovery. Other notable elements include aluminum (Al, 3043 ppm), silicon (Si, 58958 ppm), and antimony (Sb, 18660 ppm).

The crushed pyrite sample (SD-BIO-3 TAL QUAL) had undergone partial leaching and exhibited a particle size of less than 250  $\mu\text{m}$ . The elemental composition of this material is presented in Table 4.9. The sample is characterized by high concentrations of iron (Fe, 168740 ppm), sulfur (S, 194647 ppm), and silicon (Si, 191110 ppm), which are typical of pyritic materials. Moreover, the sample contains elevated levels of lead (Pb, 40050 ppm), copper (Cu, 3989 ppm), and arsenic (As, 748 ppm), highlighting both potential environmental risks and the possibility of resource recovery.

Other elements of interest include aluminum (Al, 20124 ppm), antimony (Sb, 1253 ppm), and zinc (Zn, 1981 ppm). These results show compositional similarities to pyrite ashes, reflecting their shared origin from the weathering or processing of pyritic ores.

### **4.3. Bioleaching Experiments**

#### **4.3.1. Bioleaching of crushed pyrite**

This study was conducted at the laboratory scale, and the experiments were carried out based on the design provided by Minitab software for BBD. For this purpose, the inoculum was set as 10% (v/v) and elemental sulfur at 1% (w/v). Several batches of 100 mL total volume with different factor combinations were performed for until 9 days. The pH of the samples was studied within the range of 1.5-2.5, pulp density from 2-4% (w/v), and  $\text{Fe}^{2+}$  concentration from 10-60 mM.  $\text{H}_2\text{SO}_4$  was used to adjust the pH of the medium. Samples were withdrawn at regular intervals to monitor the cell growth, pH, ORP,  $\text{Fe}^{2+}$ , and  $\text{Fe}^{3+}$  concentration. After the analysis of the experimental data, the conditions that showed the best recovery were confirmed in 100 mL experiments. Throughout the experiments, the incubation temperatures were maintained at 42 °C for the SLA culture to ensure optimal growth and bioleaching activity.

#### **4.3.2. Bioleaching of slag**

The slag bioleaching tests followed the same experimental setup and design methodology as for crushed pyrite. The tested pH range was slightly lower (1.0-2.0), while pulp density and  $\text{Fe}^{2+}$  concentration remained the same (2-4% and 10-60 mM, respectively). Optimal conditions identified by the design were also confirmed in 100 mL batches under 42 °C incubation with SLA culture.

### 4.3.3. Bioleaching of pyrite ashes

Given the chemical similarity between pyrite ashes and crushed pyrite, experiments for this material were conducted only under the optimized conditions previously determined for crushed pyrite using response surface methodology (RSM).

## 4.4. Analytical Methods

### 4.4.1. Sampling and Sample Preparation

During the incubation of the experiments, 1 mL from each of the shaking flasks was collected on days 0, 2, 4, 7, and 9 at an interval of approximately two days, and added to microcentrifuge tubes of 1.5 mL. From this, 45  $\mu$ L was taken to evaluate cell growth and viability under a microscope. The remaining was centrifuged for 2 minutes at a maximum speed of 15000 rpm using an Eppendorf 5424 Centrifuge. After centrifugation, 500  $\mu$ L of the supernatant was pipetted to a new tube with 5  $\mu$ L concentrated  $\text{HNO}_3$  (63%) to stabilize the metals in their dissolved ionic form and stored for ICP-OES analysis. The remaining volume was used for pH and ORP measurements.

### 4.4.2. Cell viability

Cell counting was done under a Zeiss ICS KF2 microscope equipped with a 40x 0.65 Ph2 CP-Achromat  $\infty$ /0.17 objective lens and a 10x ocular lens, providing phase contrast imaging. 45  $\mu$ L was placed in a slide measuring 76  $\times$  26 mm (Knittel Glasbearbeitungs GmbH), and the slide cover 18 $\times$ 18 mm (DWK Life Sciences GmbH) was placed on top of it. The plate was rinsed with ethanol and dried with a tissue each time after use, and the cover was disposed of.

### 4.4.3. Ferrozine Assay

The methodology for ferrozine assay was followed according to Braunschweig et al (2012) to quantify the ferrous iron and total iron. The ferrozine reagent solution was composed of deionized water, 11.9 g/L HEPES (Carl Roth GmbH, Pufferan®, 238.31 g/mol), 1 g/L ferrozine (Ferospectral, Merck KGaA), and it was adjusted with 2M KOH solution to pH 4.7.

Before the ferrozine assay with the bioleaching samples, a calibration curve (Figure 4.4) was made using dilutions from 0.1 to 1 mM of an Iron (II) Sulfate Heptahydrate (Carl Roth GmbH+Co., Karlsruhe) stock solution. The iron solutions were prepared and 12  $\mu$ L pipetted to a 96-well plate (Greiner Bio-One). After that, 228  $\mu$ L of ferrozine solution was added, and the samples were analyzed using a SpectraMax M2<sup>e</sup> Molecular spectrophotometer device running on SoftMax Pro 6.2.1 with a wavelength of 562 nm and shaken for 5 seconds.

For the bioleaching samples, dilutions 1:100 were prepared to ensure the concentration was within the calibration curve range, in case ferrous iron concentration was higher than 1 M. After dilution, 12  $\mu$ L of the diluted sample was taken and mixed with 228  $\mu$ L ferrozine solution in one well for each sample with the purpose of measuring the absorbance to calculate the

concentration of iron (II). The next step was adding ascorbic acid to the diluted samples remaining and incubating for 20 minutes at room temperature.

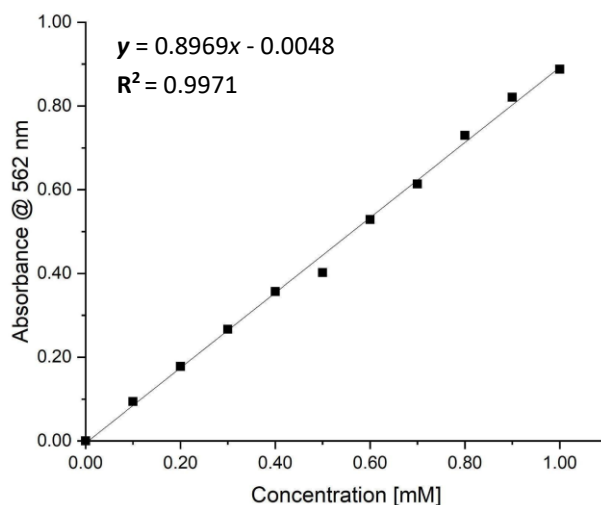


Figure 4.4. Calibration curve for ferric, ferrous, and total iron quantification.

The samples were vortexed using a mixer (Vortex-Genie™ 2, Scientific Industries) every 5 minutes and added extra ascorbic acid such that a small amount of ascorbic acid remained undissolved at the end of 20 minutes. 12  $\mu$ L of the ascorbic acid-treated samples were combined with 228  $\mu$ L of ferrozine solution in new wells, this time to quantify total iron. The plate with all the samples was taken to the spectrophotometer, with a wavelength of 562 nm, and shaken for 5 seconds.

The obtained absorbance results were multiplied by 100 and used to calculate iron (II) and total iron concentration using the regression equation presented in equation 5. To determine the ferric iron content, the concentration of iron (II) was subtracted from the total iron concentration as shown in equation 6.

$$y = 0.8969x - 0.0048 \text{ (Eq. 5)}$$

Where  $y$  is the concentration of Iron (II) and  $x$  is the absorbance value recorded at 562 nm.

$$[Total Fe] - [Fe^{2+}] = [Fe^{3+}] \text{ (Eq. 6)}$$

#### 4.4.4. ORP and pH measurements

For the pH measurement, a Mettler Toledo electrode Inlab® Semi-Micro (pH range 0 to 12, temperature range 0 – 100 °C) was used, and for the ORP, a Mettler Toledo electrode Inlab® Redox-Micro was used (Ag electrode) (Temperature range 0 – 100 °C, 3 mol/L KCl). Before each measurement, the pH meter was calibrated using standard buffer solutions. Since ORP electrodes cannot be calibrated, their potential was verified daily using a standardized

468 mV solution, and any deviation from this value was recorded and used to correct the measured Eh values.

#### 4.4.5. ICP-OES

Samples were periodically collected to monitor metal release from the pyritic material and slag using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, SPECTRO ARCOS FHX3X). To ensure accurate quantification within the detection limits of the instrument, measurements were performed at various dilutions (1:50, 1:100, 1:500) in deionized water with 3% HNO<sub>3</sub> as the diluent. For crushed pyrite and pyrite ashes experiments, the final dilution was set at 1:50, while for slag samples 1:100.

Bioleaching efficiency for each target metal was calculated by comparing the amount released into solution with the total metal content in the original mine waste (Eq. 7).

$$\text{Bioleaching Efficiency of Metals \%} = \frac{C_l}{\left(\frac{C_w}{1000}\right)} \times 100\% \quad (\text{Eq. 7})$$

Where  $C_l$  is the concentration of metal in the leach liquor (mg/g) measured by ICP-OES,  $C_w$  is the concentration of metal in the mine waste (ppm) (Table 4.11). Recoveries for all tests were calculated using this equation.

The experimental results were fitted with a second-order polynomial equation by multiple regression analysis. To predict the optimal point, the full quadratic equation model was expressed according to equation 8:

$$y = \beta_0 + \sum_{i=1}^3 \beta_i x_i + \sum_{i=1}^3 \beta_{ii} x_i^2 + \sum_{i=1}^3 \sum_{j=i+1}^3 \beta_{ij} x_i x_j \quad (\text{Eq. 8})$$

Where  $y$  is the predicted response by the model, and  $\beta_0, \beta_i, \beta_{ii}$ , and  $\beta_{ij}$  are constant regression coefficients of the model;  $x_i, x_i^2$ , and  $x_i x_j$  represent the linear, quadratic, and interactive terms of the coded independent variables, respectively.

By solving the obtained polynomial equation, the optimal values of each three factors were determined. Furthermore, the coefficient of determination ( $R^2$ ) was used to evaluate the accuracy and quality of the full quadratic equation. Analysis of variance (ANOVA) and Response Surface Regression were used to analyze the experimental data.

Moreover, contour plots were constructed to visualize the optimum conditions for pyrite waste bioleaching and the interactive effects of significant variables on the response. The significant  $\alpha$ -level of 5% was used for the analysis.

## 5. Results

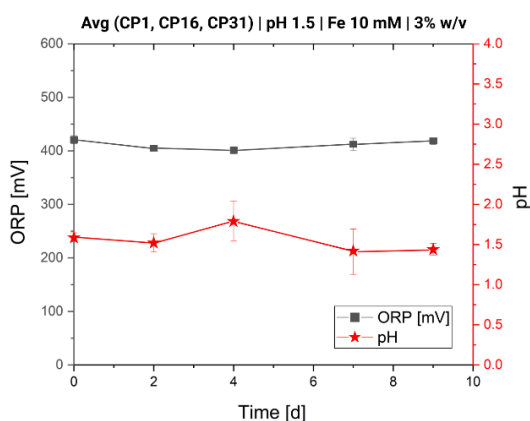
### 5.1. Direct bioleaching of crushed pyrite (SD-BIO-3) under different combinations of pH, Fe (II) and pulp density

The leaching experiments of crushed pyrite with SLA bacterial culture were conducted at 42 °C, with ferrous iron supplementation at 10 mM, 35 mM, or 60 mM, pH 1.5, 2.0, or 2.5, and pulp density of 2%, 3% or 4% (w/v). In all flasks, cells were observed from day 2 onwards, including an abiotic control. After 9 days, it was observed a color change and turbidity increase in experiments when compared to the abiotic control, occasioned by microorganism growth and biological activity.

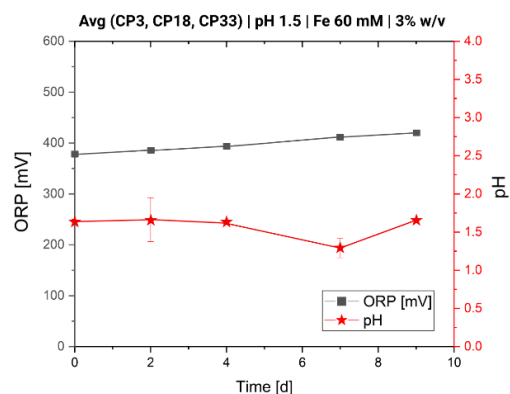
### 5.1.1. pH and ORP Changes During Crushed Pyrite Optimization Experiments

In Figure 5.1, the average values of pH and ORP for each experiment are shown. Initial (day 0) pH average values after inoculation ranged between 1.60 and 1.64 for experiments with HBS medium set at pH 1.5 (Figure 5.1A, 5.1B, 5.1C, 5.1D).

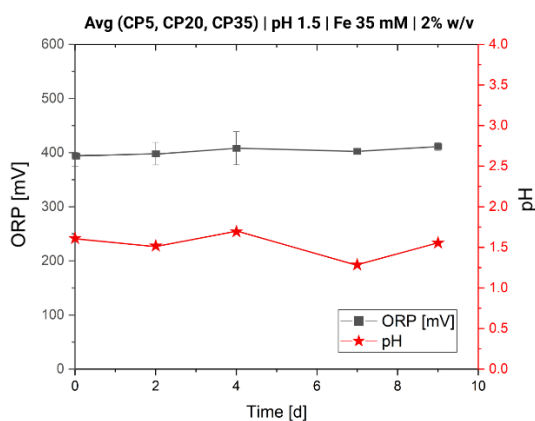
A



B



C



D

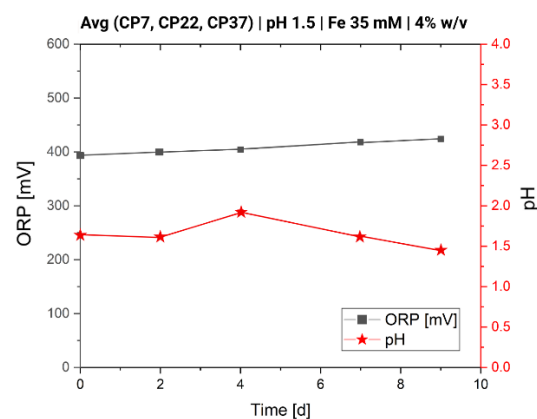
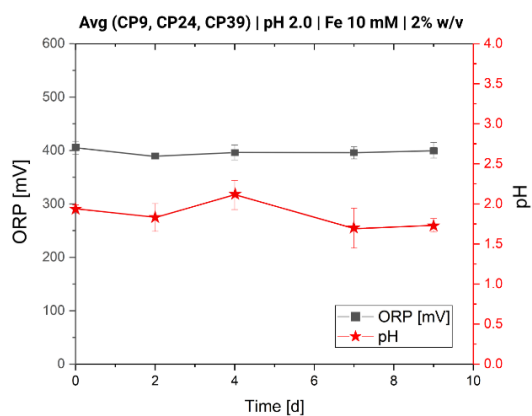


Figure 5.1. pH and ORP over time for the bioleaching of crushed pyrite carried out using starting media of pH 1.5, at 42 °C, and SLA culture. (A) Average of CP1, CP16, CP31 with Fe (II) 10 mM and 3% (w/v) solid load. (B) Average of CP3, CP18, CP33 with Fe (II) 60 mM and 3% (w/v) solid load. (C) Average of CP5, CP20, CP35 with Fe (II) 35 mM and 2% (w/v) solid load. (D) Average of CP7, CP22, CP37 with Fe (II) 35 mM and 4% (w/v) solid load.

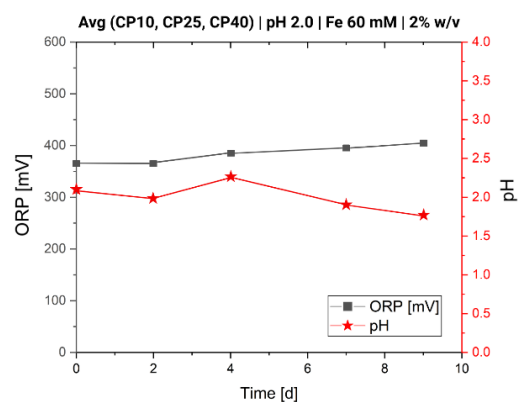
On day 4 it was observed a small increase in the pH and after 9 days, the pH slightly decreased again, varying from 1.40-1.65. Initial ORP values ranged from approximately 378-417 mV before incubation. By the end of the experiment, redox potential average values had

gradually increased to a range between 407 and 424 mV, indicating a very slight increase in microbial activity.

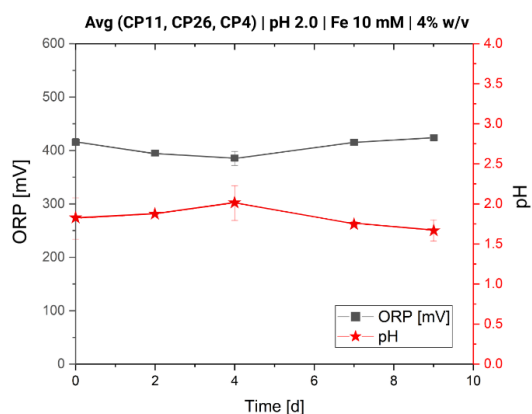
A



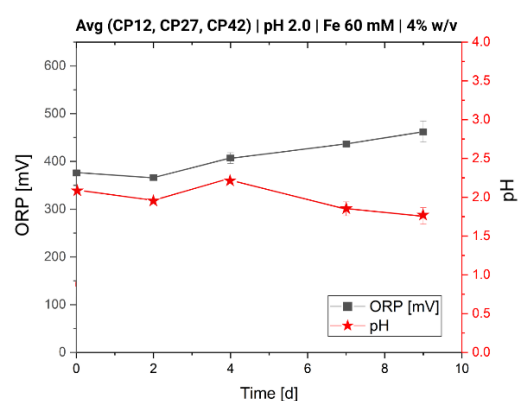
B



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E

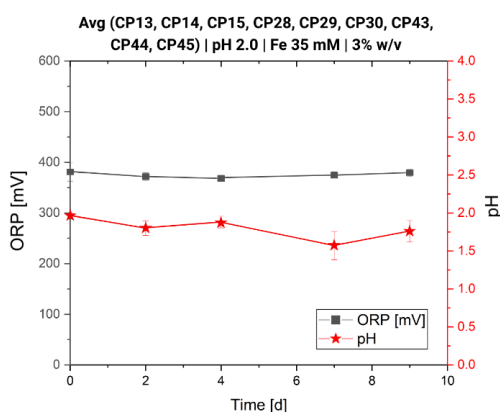
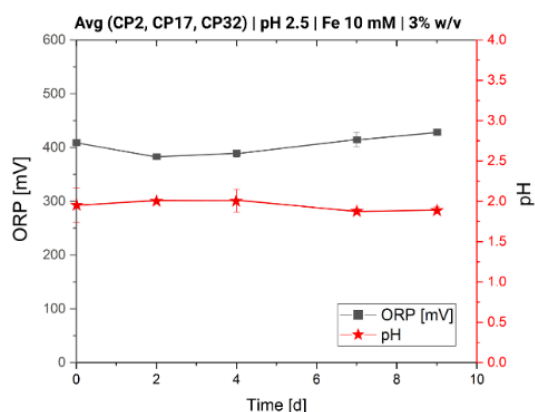
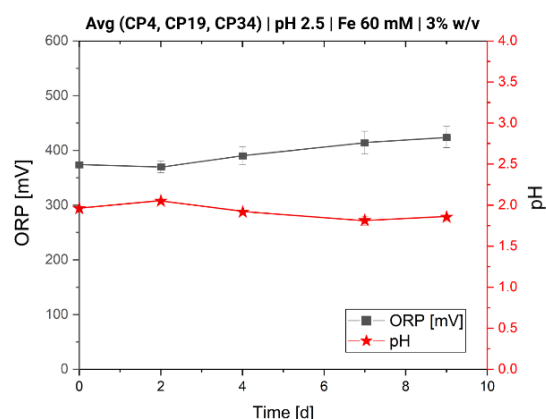


Figure 5.2. Monitoring of pH and ORP over incubation time for the bioleaching of crushed pyrite carried out using starting media HBS pH 2.0, at 42 °C, and SLA culture, and addition of 1% (w/v) sulfur. (A) Average of CP9, CP24, CP39 with Fe (II) 10 mM and 2% w/v solid load. (B) Average of CP10, CP25, CP40 with Fe (II) 60 mM and 2% w/v solid load. (C) Average of CP11, CP26, CP41 with Fe (II) 10 mM and 4% (w/v) solid load. (D) Average of CP12, CP27, CP42 with Fe (II) 60 mM and 4% (w/v) solid load. (E) Control - Average of CP13, CP14, CP15, CP28, CP29, CP30, CP43, CP44, CP45 with Fe 35 mM and 3% (w/v) solid load.

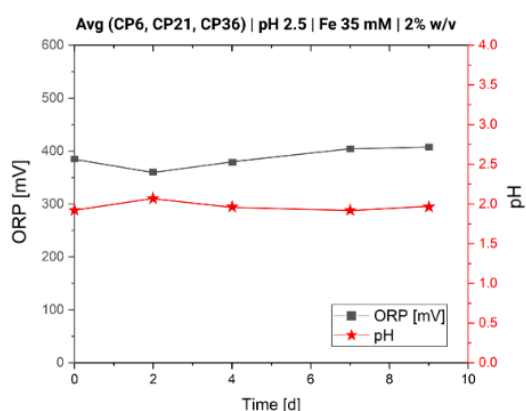
A



B



C



D

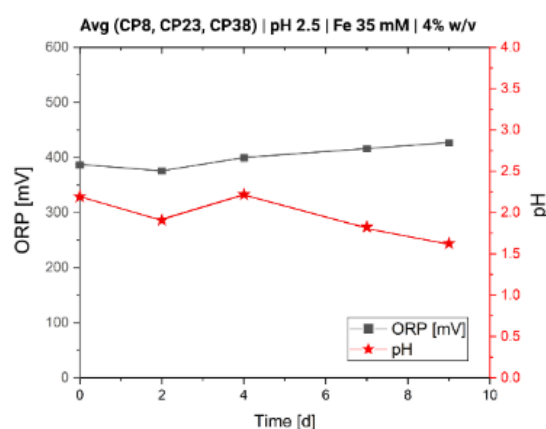


Figure 5.3. Monitoring of pH and ORP over incubation time for the bioleaching of crushed pyrite carried out using starting media HBS pH 2.5, at 42 °C and SLA culture, and addition of 1% (w/v) sulfur. (A) Average of CP2, CP17, CP32 with Fe (II) 10 mM and 3% (w/v) solid load. (B) Average of CP4, CP19, CP34 with Fe (II) 60 mM and 3% (w/v) solid load. (C) Average of CP6, CP21, CP36, Fe 35 mM and 2% (w/v) solid load. (D) Average of CP8, CP23, CP38 with Fe (II) 35 mM and 4% (w/v) solid load.

For the experiments using medium HBS media adjusted to pH 2.0, the initial pH values varied from 1.81 and 2.09, decreasing to values between 1.63 and 1.76. Moreover, ORP values increased from a range of 366-404 mV on the initial day to a range of 400-423 mV on the last day, as shown in Figure 5.2 (A, B, C, D). At pH 2.0, the abiotic control with iron at 35 mM (5.2E), a decrease in pH was shown from pH 1.96 on the first day and 1.76 in the last one. However, the ORP average value remained nearly the same during the time interval, varying from 376 to 379 mV on the last sampling day.

For the experiments using medium HBS media adjusted to pH 2.5, the initial pH varied from 1.83 to 2.18. On day 9, conditions at 10 mM Fe (II) + 3% (w/v) solid load (CP2-17-32) (Figure 5.3A) and 35 mM + 2% (w/v) (CP6-21-36) (Figure 5.3C) had a small increase in pH to 1.89 and 1.97, respectively. Contrasting, CP8-23-38 (35 mM Fe (II) + 4% (w/v)) (Figure 5.3D) and CP4-19-34 (60 mM + 3% (w/v)) (Figure 5.3B) had a decrease to 1.62 and 1.86 over time, respectively. Moreover, ORP values increased from a range of 386-414 mV on the initial day to 407-429 mV on the last day, as shown in Figure 5.3 (A, B, C, D).

### 5.1.2. Total Iron, Ferrous Iron, and Ferric Iron Concentration Determination for Crushed Pyrite

The bioleaching experiments revealed distinct patterns in the behavior of  $\text{Fe}^{2+}$ , total iron, and  $\text{Fe}^{3+}$  across biotic and abiotic conditions.

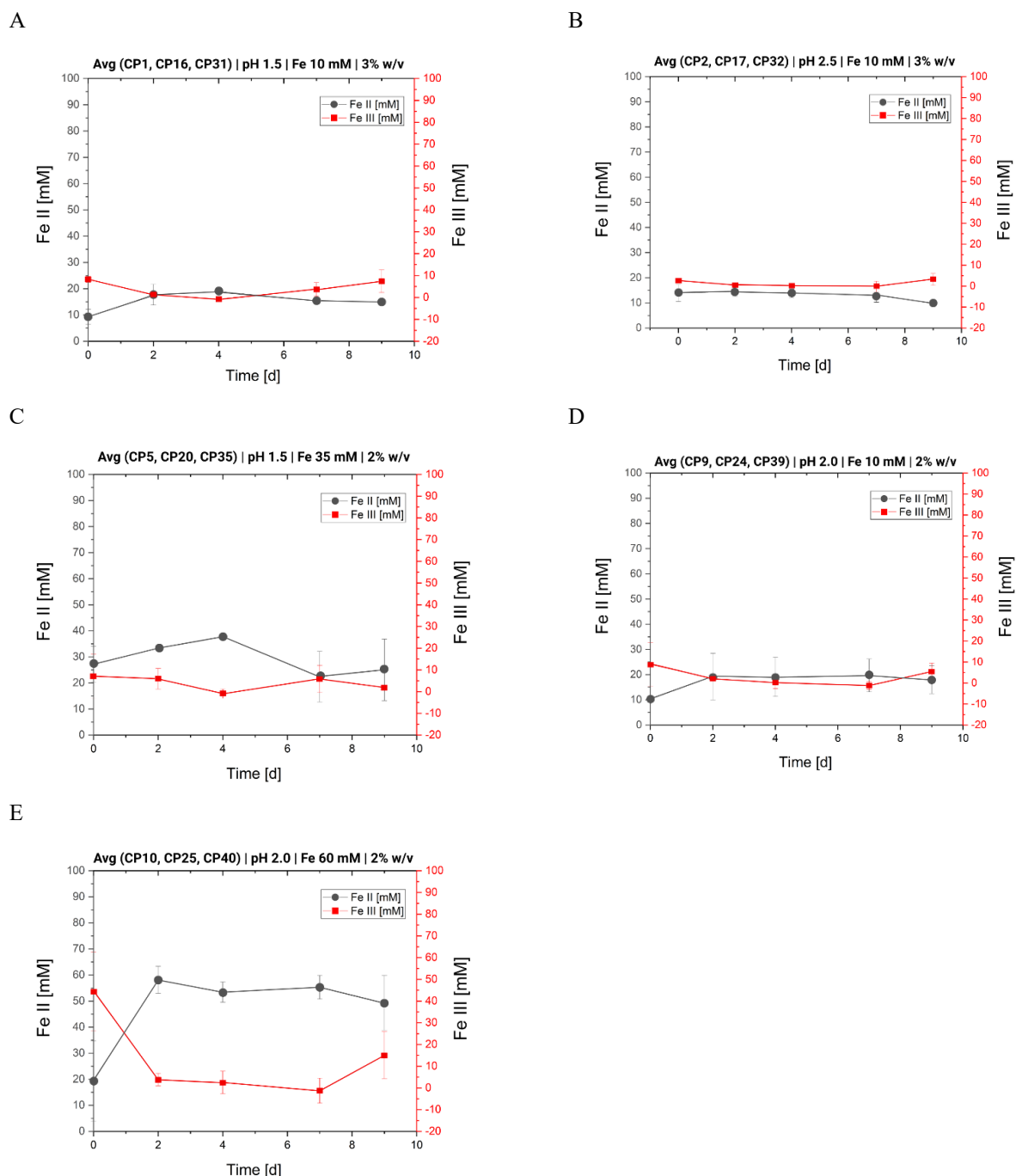


Figure 5.4. Monitoring of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  over incubation time for the bioleaching of crushed pyrite carried out using starting at 42 °C, SLA culture, and the addition of 1% (w/v) sulfur. (A) pH 1.5, Fe (II) 10 mM and 3% (w/v) (CP1-16-31). (B) pH 2.5, Fe (II) 10 mM and 3% (w/v) (CP2-17-32). (C) pH 1.5, Fe (II) 35 mM and 2% (w/v) (CP5-20-35). (D) pH 2.0, Fe (II) 10 mM and 2% (w/v) (CP9-24-39). (E) pH 2.0, Fe 60 mM and 2% (w/v) (CP10-25-40).

It was observed a decrease in concentration of  $\text{Fe}^{2+}$  in solution by the end of 9 days for majority of tested conditions (shown in Annex A1), except for an increase of  $\text{Fe}^{2+}$  in solution

between start and end dates for the conditions: pH 1.5, Fe (II) 10mM, 3% (w/v) (CP1-16-31) (Figure 5.4A); pH 1.5, Fe (II) 35 mM, 2% (w/v) (CP5-20-35) (Figure 5.4C); pH 2.0, Fe (II) 10 mM, 2% (w/v) (CP9-24-39) (Figure 5.4D) and pH 2.0, Fe (II) 60 mM, 2% (w/v) (CP10-25-40) (Figure 5.4E). For  $\text{Fe}^{3+}$  concentrations, the majority of the bioleaching experiments showed an increase after 9 days. However, abiotic control and conditions pH 2.5, with Fe (II) 10 mM and 3% (w/v) (CP2-17-32) (Figure 5.4B), pH 2.0, Fe (II) 10mM and 2% (w/v) (CP9-24-39) (Figure 5.4D), and pH 2.0, Fe (II) 60 mM and 2% (w/v) (CP10-25-40) (Figure 5.4E) had the amount in solution reduced.

Although 60 mM of ferrous iron was added to CP10-25-40 (Figure 5.4E), only 19 mM was detected on day 0 using the ferrozine assay. However, by the second measurement on day 2, the ferrous concentration had increased to 58 mM, aligning more closely with the expected value. Similarly, an unexpectedly high ferric iron concentration of 44 mM was measured on day 0, which subsequently dropped to 3.8 mM on day 2. These fluctuations suggest a likely measurement error during the first analysis, possibly due to sample handling, interference, or timing inconsistencies in the assay procedure.

### 5.1.3. ICP-OES Analysis for Crushed Pyrite Samples

As observed in Figure 5.5, Mn (Figure 5.5A) and Zn (Figure 5.5B) had significant recovery; there was variation in the recovery of metals across all experiments. Other metals measured can be seen in Annex A2. The highest Mn bioleaching efficiency ( $\sim 100\%$ ) was observed in (CP10-25-40). However, when compared last and first day of experiments, both days showed nearly same values.

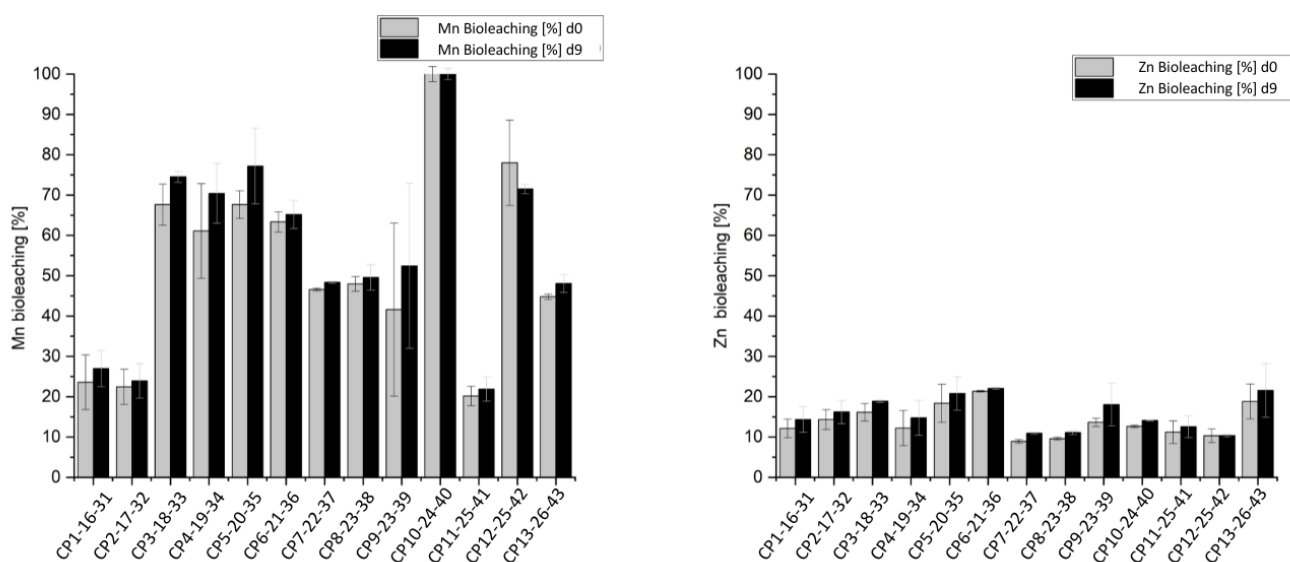


Figure 5.5. (A) Mn bioleaching efficiency on day 0 and day 9. (B) Zn bioleaching efficiency on day 0 and day 9.

For Zn, CP5-20-35 and CP6-21-36 showed the highest bioleaching percentage, 20.8% and 22% respectively. Even so, compared to the abiotic control CP13-28-43, 21.5% the results were too similar.

#### 5.1.4. Response Surface Methodology For Crushed Pyrite Samples

After seeing which metals were being leached, the Response Surface Regression (RSR) models for those are presented, focusing on factors that significantly affect metal recovery and the overall reliability of the models. For the other analyzed elements, see Annex A3. The presence of Mn in the liquor is significantly influenced by ferrous iron concentration, with a strong positive effect (Coef = +21.36,  $p < 0.05$ ), pulp density, with a strong negative effect (Coef = -17.76,  $p < 0.05$ ) and Pulp density<sup>2</sup> has a positive quadratic effect with  $p < 0.05$ , suggesting a U-shaped response and an ideal range of pulp density.

For Mn recovery, pH, pH<sup>2</sup> and the interaction of pH with ferrous iron did not show an effect, with a p-value of pH ( $p = 0.595$ ), suggesting it does not influence Mn recovery much within this range, as can be seen in the Pareto Chart (Figure 5.6). In Annex A3 is present the entire data extracted from the regression.

The model explains a moderate portion of the variance in Mn recovery with a  $R^2 = 61.34\%$ , and Predicted  $R^2 = 31.3\%$  indicates a lower reliability when predicting unseen data. Moreover, some data points show large residuals, which could indicate outliers or model limitations.

Based on the calculated values of the regression coefficients, a polynomial regression model equation that fitted 61.34% of the variation in the data was proposed as follows:

$$\text{Mn Bioleaching (\%)} = 226 + 57 \text{ pH} - 0.44 [\text{Fe II}] (\text{mM}) - 141.2 \text{ Pulp density (\%)} - 20.3 \text{ pH} \times \text{pH} + 0.00953 [\text{Fe II}] (\text{mM}) \times [\text{Fe II}] (\text{mM}) + 17.07 \text{ Pulp density (\%)} \times \text{Pulp density (\%)} - 0.021 \text{ pH} \times [\text{Fe II}] (\text{mM}) + 6.6 \text{ pH} \times \text{Pulp density (\%)} + 0.223 [\text{Fe II}] (\text{mM}) \times \text{Pulp density (\%)} \quad (\text{Eq. 9})$$

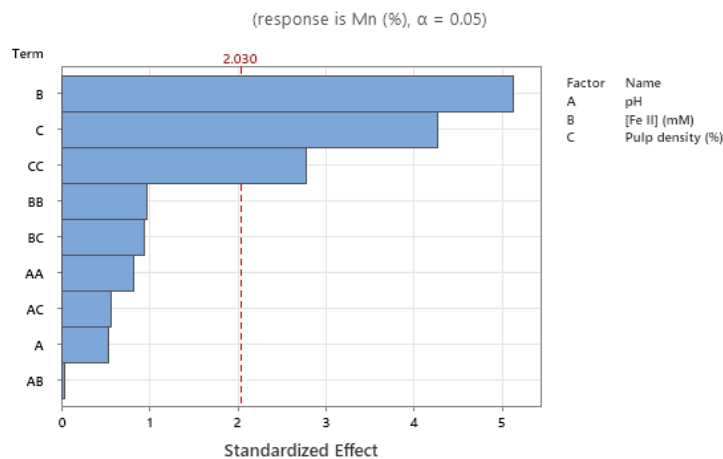


Figure 5.6. Pareto Chart of the standardized effects of pH, ferrous iron initial concentration, and pulp density on the bioleaching of crushed pyrite for Mn recovery.

For Zn bioleaching efficiency, linear pH, Fe(II), and all interaction terms are non-significant ( $p > 0.05$ ). On the other hand, pulp density (%) has a negative effect (Coef = -3.75,  $p = 0.000$ ), Fe (II)<sup>2</sup> and Pulp density<sup>2</sup> have significant quadratic effects ( $p = 0.007$  and  $p = 0.010$ , respectively), suggesting a nonlinear influence on Zn bioleaching. Zn recovery is mostly affected by pulp density, with a clear negative linear and nonlinear (quadratic) relationship (Figure 5.7).

The obtained model explains less than half of the variability ( $R^2 = 47.84\%$ ); moreover, the predicted  $R^2 = 21.3\%$  indicates low predictive power. The polynomial regression model equation that fitted 47.84% of the variation in the data was proposed as follows:

$$\begin{aligned} \text{Zn Bioleaching (\%)} = & -42.5 + 29.9 \text{ pH} + 0.619 [\text{Fe II}] (\text{mM}) + 19.6 \text{ Pulp density (\%)} - 6.06 \text{ pH} \times \text{pH} - 0.00637 \\ & [\text{Fe II}] (\text{mM}) \times [\text{Fe II}] (\text{mM}) - 3.81 \text{ Pulp density (\%)} \times \text{Pulp density (\%)} - 0.120 \text{ pH} \times [\text{Fe II}] (\text{mM}) - \\ & 0.55 \text{ pH} \times \text{Pulp density (\%)} + 0.0171 [\text{Fe II}] (\text{mM}) \times \text{Pulp density (\%)} \quad (\text{Eq. 10}) \end{aligned}$$

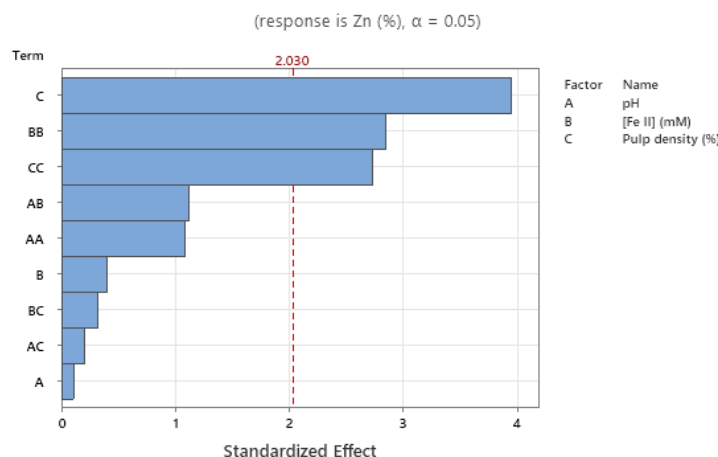


Figure 5.7. Pareto Chart of the standardized effects of pH, ferrous iron initial concentration, and pulp density on the bioleaching of crushed pyrite for Zn recovery.

In order to optimize the bioleaching of interest metals, a response optimization model was done, with the main goal being to reach 100% of Mn and the maximum possible from the other metals (Al, Zn, Sb, and Cu), while minimizing the recovery of Arsenic. The full model can be seen in Annex A4. The model uses desirability functions to balance all these goals simultaneously.

Table 5.1. Predicted Responses at optimal settings for crushed pyrite.

| Response:           | Goal         | Predicted value (%) | 95% CI*         | 95% PI**        |
|---------------------|--------------|---------------------|-----------------|-----------------|
| <b>Recovery (%)</b> |              |                     |                 |                 |
| Al                  | Maximize     | 3.572               | (3.336, 3.808)  | (3.031, 4.113)  |
| As                  | Minimize     | 6.69                | (2.74, 10.63)   | (-2.36, 15.73)  |
| Cu                  | Maximize     | 3.454               | (2.113, 4.795)  | (0.379, 6.529)  |
| Mn                  | Target: 100% | 99.84               | (79.76, 119.91) | (53.81, 145.86) |
| Sb                  | Maximize     | 2.483               | (2.242, 2.724)  | (1.930, 3.036)  |
| Zn                  | Maximize     | 18.56               | (13.98, 23.13)  | (8.07, 29.05)   |

\*CI (confidence interval), \*\*PI (prediction interval)

The model found the best trade-off between all these responses at the following input settings of pH at 1.66162, ferrous iron addition of 53.9394 mM, and pulp density of 2% (w/v). A composite desirability of 0.67 (on a scale of 0 to 1) indicates a reasonably good trade-off across all six responses. In multi-response optimization, anything above 0.6 is typically considered acceptable, depending on the strictness of industrial constraints.

Operating at low pH (1.66), moderately high Fe (II) (~54 mM), and very low pulp density (2% w/v) yields the best compromise. These conditions likely enhance metal solubility and reduce co-precipitation. Mn recovery is nearly at target (99.84%), Zn and Cu are significantly improved under these conditions. As is reduced, though still non-negligible (6.69%). Some responses, especially As and Mn, show wide prediction intervals, indicating that while the mean prediction is favorable, real-world variability may lead to lower or higher values. This might call for additional validation experiments or tightened control in actual operations.

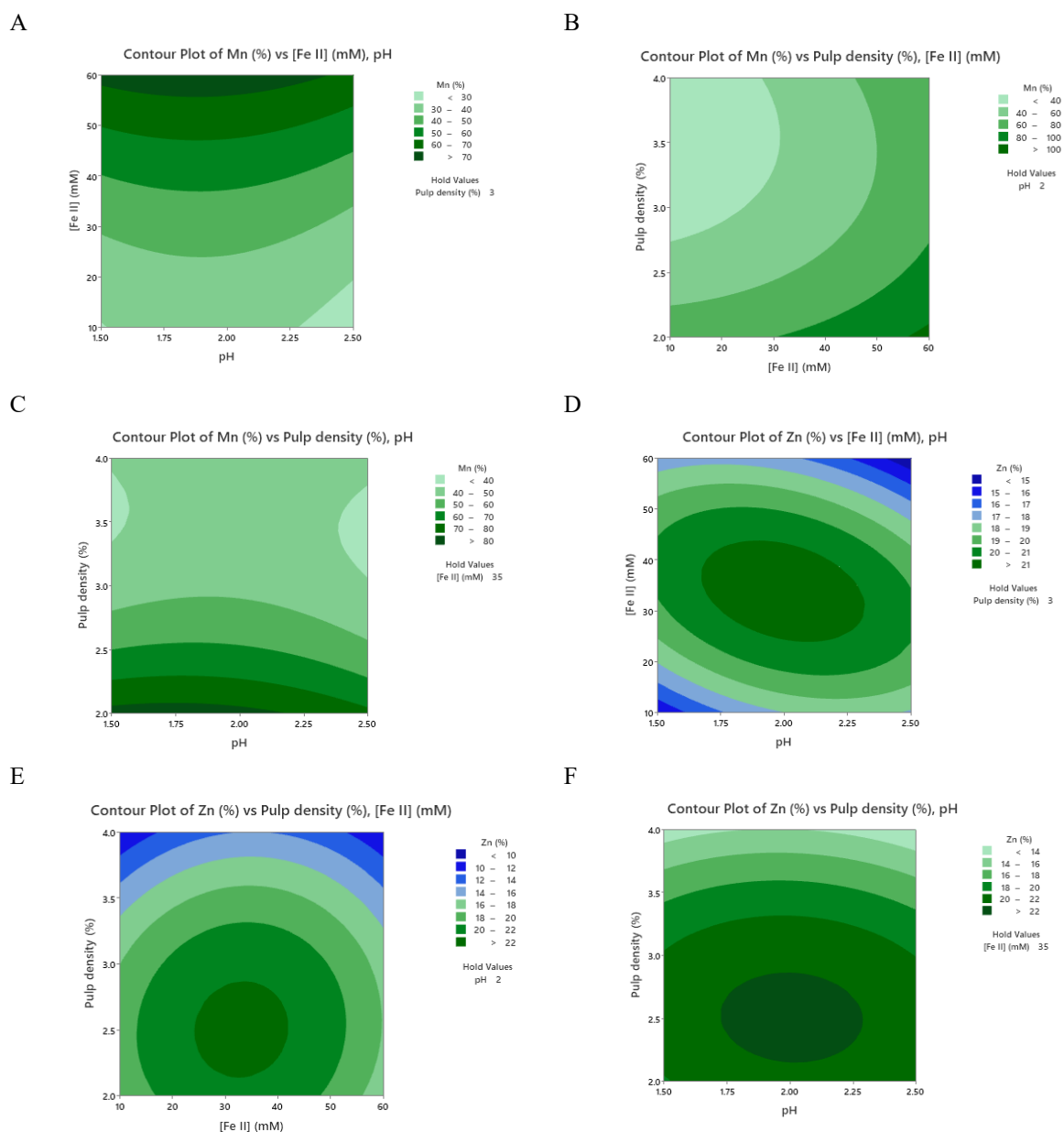


Figure 5.8. Contour plots for (A) Mn bioleaching efficiency with respect to ferrous iron and pH (pulp density fixed at its middle value). (B) Mn bioleaching efficiency with respect to ferrous iron and pulp density (pH fixed at its middle value). (C) Mn bioleaching efficiency with respect to pulp density and pH (ferrous iron concentration fixed at its middle value). (D) Zn bioleaching efficiency with respect to ferrous iron and pH (pulp density fixed at its middle value). (E) Zn bioleaching efficiency with respect to ferrous iron and pulp density (pH fixed at its middle value). (F) Mn bioleaching efficiency with respect to pulp density and pH (ferrous iron concentration fixed at its middle value).

Contour plots (Figure 5.8) show the behavior of Mn and Zn when varying the two out of the three factors, with one of the parameters held at the middle value. It is possible to observe that when the concentration of ferrous iron increases and pH is in the range of 1.5 to 2.25, it is obtained a higher bioleaching percentage of Mn (Figure 5.8A) is obtained.

When comparing Fe (II) and pulp density, the lower the pulp density and the higher the iron, the better the Mn recovery (5.8B). Furthermore, pH versus pulp density showed that the lower pulp density better recovery, in the range of pH 1.5 to 2.0 (Figure 5.8C). For Zn, best recoveries ranged from pH 1.75 to 2.25, while ferrous iron was 30 mM to 40 mM (Figure 5.8D). At the same ferrous iron range, pulp density around 2.5% showed the best Zn bioleaching (Figure 5.8E). When holding Fe (II) at 35 mM, the best recovery was present in the same pulp density range (2.5%) and pH 2.0. Contour plots for the other analyzed elements, as well as 3D surface response curves, are present in Annex A5, showing the combination of parameters and their respective impacts on metal bioleaching under the conditions tested.

#### 5.1.4.1. Confirmatory experiments

##### 5.1.4.1.1. Crushed Pyrite

To confirm the prediction done by the model, and therefore the applicability of the proposed second-order model for further optimization of mine waste bioleaching, confirmation at the predicted optimum level of the factors was carried out along with abiotic control. After 9 days, a color change and turbidity increase were observed in experiments when compared to abiotic control, occasioned by microorganism growth and biological activity (Figure 5.9).

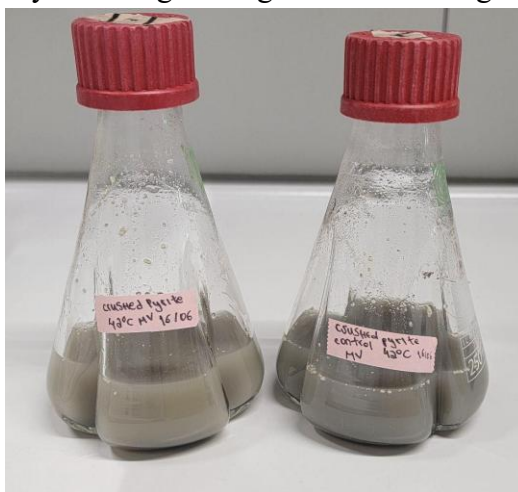


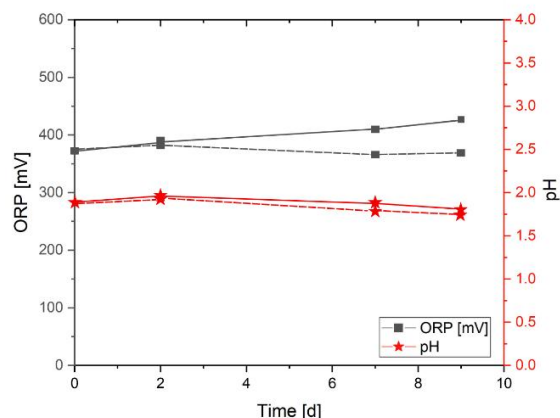
Figure 5.9. Crushed pyrite bioleaching on day 9 versus abiotic control on day 9 pH 1.66, 42°C, pulp density 2% (w/v), Fe (II) 54 mM, SLA culture and addition of 1% (w/v) sulfur.

In Figure 5.10A, the pH and ORP are shown over 9 days, where it was observed an increase in ORP, indicating microbial activity, and a decrease in pH. While in Figure 5.10B, the Fe (II) decreased over time, with an increase on day 2, while Fe (III) increased over time, reaching around 30 mM, which was not observed in the control, where no ferric iron was formed. This indicates that the microbial action led to the ferrous to ferric iron conversions for further metal mobilization.

The 95% confidence interval for Cu and As concentration after 9 days of incubation under the optimized conditions was obtained, on the other hand the 95% confidence interval

for the Al, Zn, Sb and Mn concentration was not obtained as it was expected between 53.81–145.86% for Mn, when the real value was 22.6%, expected a range of 3.031–4.113% and obtained 13.29% Al, expected 8.07–29.05% and obtained 1.85% Zn, expected 1.93–3.04% and obtained 16.4% Sb as can be seen in Figure 5.11.

A



B

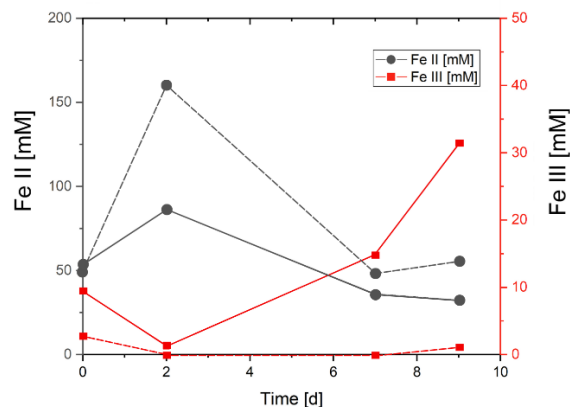


Figure 5.10. Monitoring of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  over incubation time for the bioleaching of crushed pyrite carried out using starting media HBS pH 1.66, 42 °C, pulp density 2% (w/v), Fe (II) 54 mM, and SLA culture and addition of 1% (w/v) sulfur. Dashed lines represent abiotic control, and solid lines represent biotic experiments. (A) ORP and pH over 9 days for bioleaching confirmatory experiments. (B) Ferrous versus ferric iron over 9 days for bioleaching confirmatory experiments.

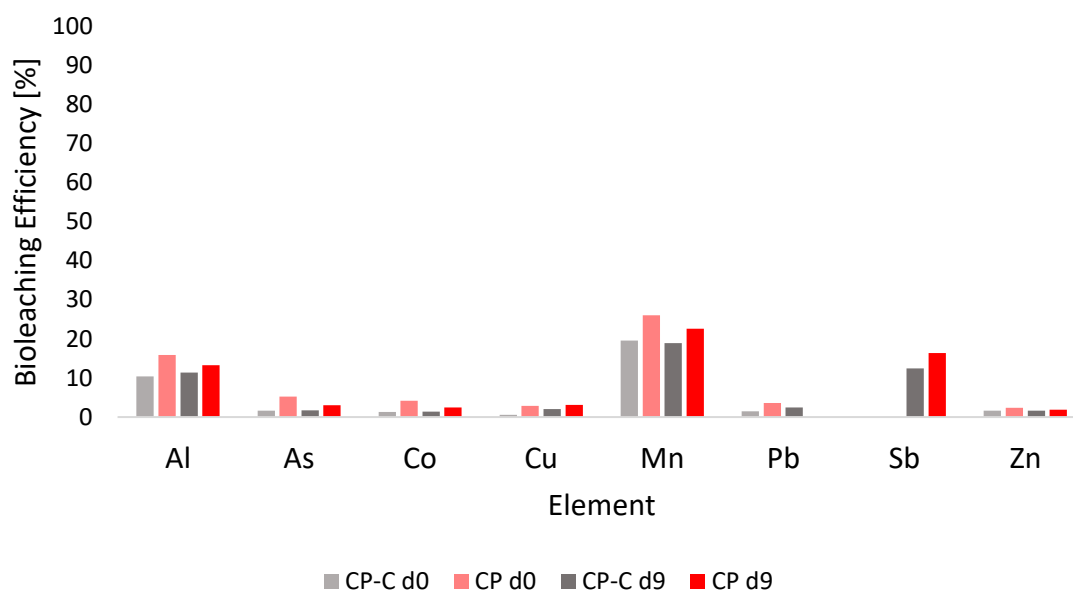


Figure 5.11. Bioleaching efficiency (%) of Al, As, Co, Cu, Mn, Pb, Sb, and Zn at day 0 and day 9. Light grey bars correspond to the abiotic control at day 0 (CP-C d0), and light red bars represent the biotic test at day 0 (CP d0). Dark grey bars indicate the abiotic control after 9 days (CP-C d9), while red bars represent the bioleaching efficiency in the biotic experiment after 9 days (CP d9).

Since the value predicted by the model is not within this interval, this cannot be taken as the confirmation of the suitability of the regression model for predictive purposes, especially considering the interest metals Zn and Mn, which showed great overprediction in the model.

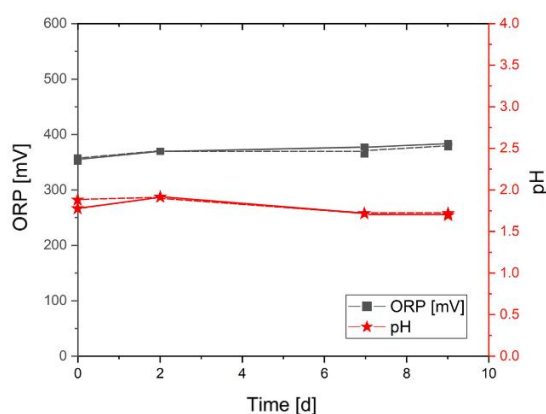
### 5.1.4.1.2. Pyrite ashes

As the composition of pyrite ashes is very similar to crushed pyrite (Table 4.10), the same optimized condition was tested together with an abiotic control, and after 9 days, no color difference was observed between the control and experiment (Figure 5.12).



Figure 5.12. Pyrite ashes confirmatory experiments at pH 1.66, 42 °C, pulp density 2% (w/v), Fe (II) 54 mM, and SLA culture and addition of 1% (w/v) sulfur.

A



B

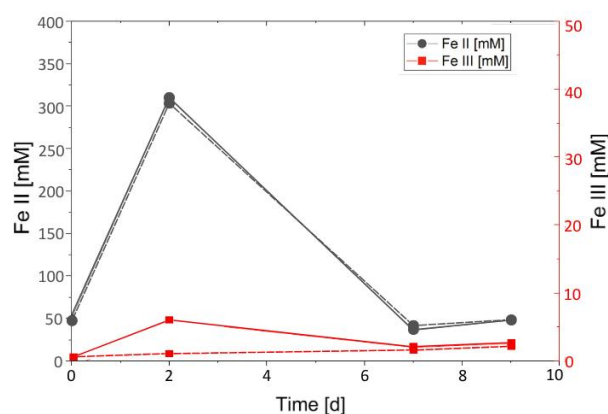


Figure 5.13. Monitoring of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  over incubation time for the bioleaching of pyrite ashes carried out using starting media HBS pH 1.66, 42 °C, pulp density 2% (w/v), Fe (II) 54 mM, and SLA culture and addition of 1% (w/v) sulfur. Dashed lines represent control, and solid lines the experiment. (A) ORP and pH over 9 days for bioleaching confirmatory experiments. (B) Ferrous versus ferric iron over 9 days for bioleaching confirmatory experiments.

Figure 5.13A shows that ORP slightly increases over time (from 355 mV to 384 mV). pH decreases from 1.77 to 1.71, but with no difference between the abiotic control and bioleaching experiment. When it comes to ferrous and ferric iron variation over time, the behavior of Fe (II) was almost identical in the control and bioleaching experiments, with a peak of ferrous iron on day 2 (Figure 5.13B). Differently, it was possible to see that on day 2, more

ferric iron was available in solution in the bioleaching than in the control. However, no other significant difference was observed between bioleaching and control experiments after 9 days.

When looking at the elements leached during the experiments, overall the metal recovery was extremely low, which shows that the model for crushed pyrite does not fit pyrite ashes even with similar compositions. Moreover, it is possible to observe that Mn and Sb had the highest leaching values; however, not higher than chemical control results (Figure 5.14). Therefore, probably Mn and Sb went into solution due to the pH, not necessarily the microbial activity.

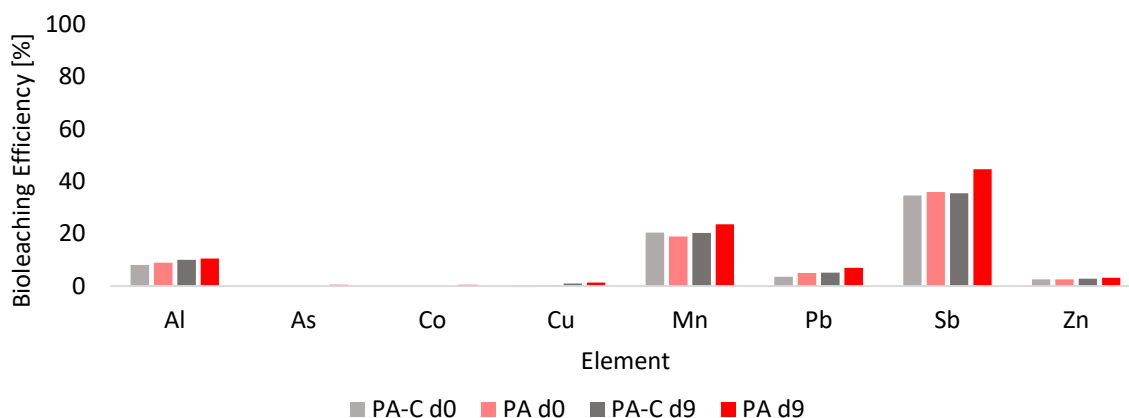


Figure 5.14. Bioleaching efficiency (%) of Al, As, Co, Cu, Mn, Pb, Sb, and Zn at day 0 and day 9. Light grey bars correspond to the abiotic control at day 0 (PA-C d0), and light red bars represent the biotic test at day 0 (PA d0). Dark grey bars indicate the abiotic control after 9 days (PA-C d9), while red bars represent the bioleaching efficiency in the biotic experiment after 9 days (PA d9).

## 5.2. Direct Bioleaching of Slag (SD-BIO-1) under different combinations of pH, Fe (II), and pulp density

The leaching experiments of slag with SLA bacterial culture were conducted at 42 °C, with ferrous iron supplementation at 10 mM, 35 mM, or 60 mM, pH 1.0, 1.5, or 2.0, and pulp density of 2%, 3% or 4% (w/v). The choice for a lower pH in slag than in crushed pyrite experiments was based on previous experiments done at the Microbiology and Biohydrometallurgy research group, which detected a substantial increase in the pH over time, making it not suitable for microbial growth and activity.

With this adjustment, in all flasks, cells were observed from day 2 onwards, including chemical control. Color and turbidity varied across 9 days, with the bioleaching experiment becoming more orange than the abiotic control.

### 5.2.1. pH and ORP Changes During Slag Bioleaching Experiments

Moreover, pH and redox potential showed an increase overtime for all experiments. In Figure 5.15, the average values of pH and ORP for each experiment are shown. Initial (day 0) pH average values after inoculation ranged between 0.80 and 0.97 for experiments with HBS medium set at pH 1.0 (Figure 5.15A, 5.15B, 5.15C, 5.15D). From day 2 onwards, pH was already around 1.5, and on the last day, it ranged from 1.48 to 1.60.

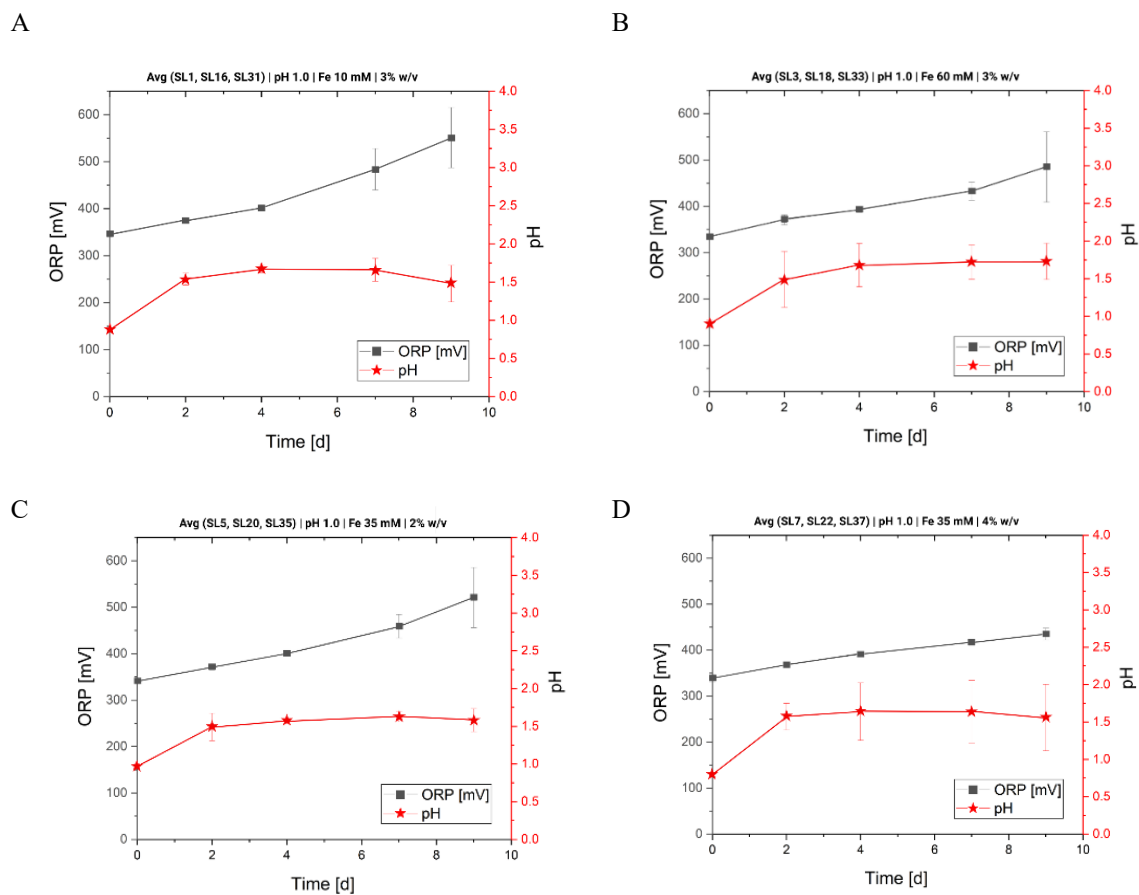


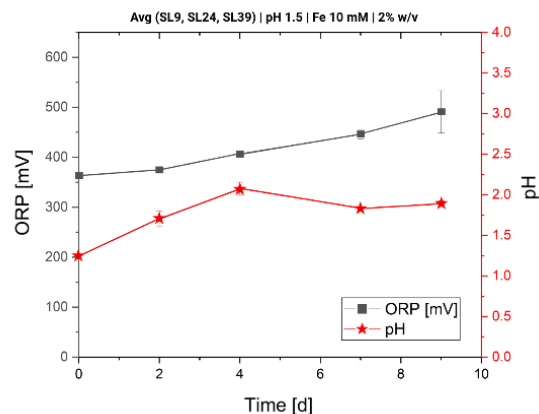
Figure 5.15. pH and ORP over time for the bioleaching of crushed pyrite carried out using starting media of pH 1.0, at 42 °C, and SLA culture. (A) Average of SL1, SL16, SL31 at pH 1.0 with Fe 10 mM and 3% (w/v) solid load. (B) Average of SL3, SL18, SL33 at pH 1.0, Fe 60 mM and 3% (w/v) solid load. (C) Average of SL5, SL20, SL35 at pH 1.0, Fe 35 mM and 2% (w/v) solid load. (D) Average of SL7, SL22, SL37 at pH 1.0, Fe 35 mM and 4% (w/v) solid load.

Initial ORP values ranged from approximately 335-346 mV before incubation. By the end of the experiment, redox potential average values had gradually increased to a range between 435 and 551 mV. For the experiments using medium HBS media adjusted to pH 1.5, the initial pH values varied from 1.03 and 1.27, increasing to values between 1.86 and 2.02. Moreover, ORP values increased from a range of 347-363 mV on the initial day to a range of 422-491 mV on the last day, as shown in Figure 5.16 (A, B, C, D).

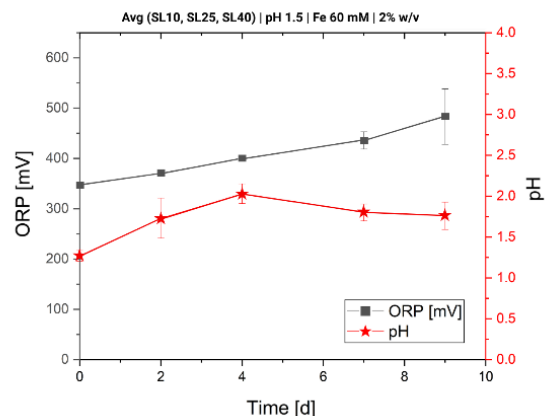
At pH 1.5, an increase in pH from 1.1 on day 0 to 2.17 on day 9 was observed on the abiotic control (iron at 35 mM, Figure 5.16). The ORP value stayed less than 400 mV during the time interval, varying from 333 to 392 mV on the last sampling day. This indicates that the microbial activity was low in the abiotic control. This is because of the chemical nature of the slag that increases the pH due to the release of alkaline substances like Ca, which increases the hydroxyl ions in the solution.

For the experiments using medium HBS media adjusted to pH 2.0, the initial pH after inoculation varied from 0.81 to 0.99, which all increased to a range of 1.73-1.81 over time, respectively. All experiments at pH 2 showed the peak of pH on day 2. Moreover, ORP values increased from a range of 377-401 mV on the initial day to 454-519 mV on the last day, as shown in Figure 5.17 (A, B, C, D).

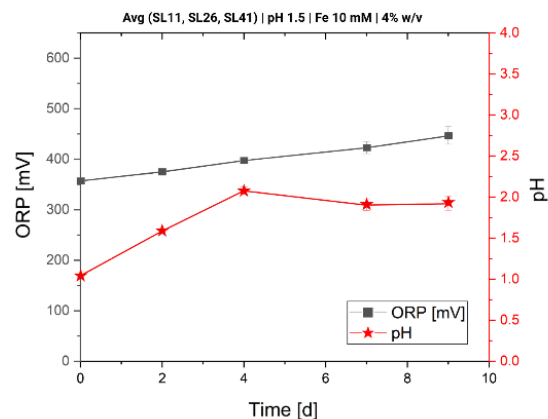
A



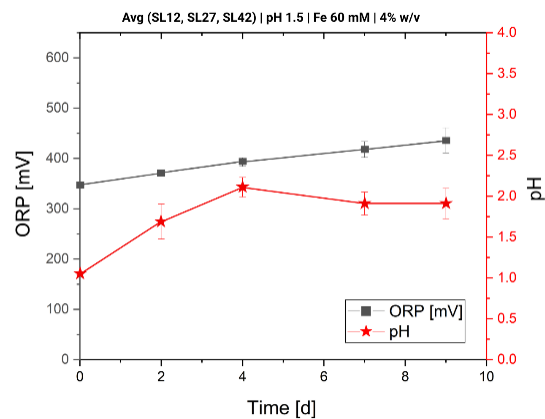
B



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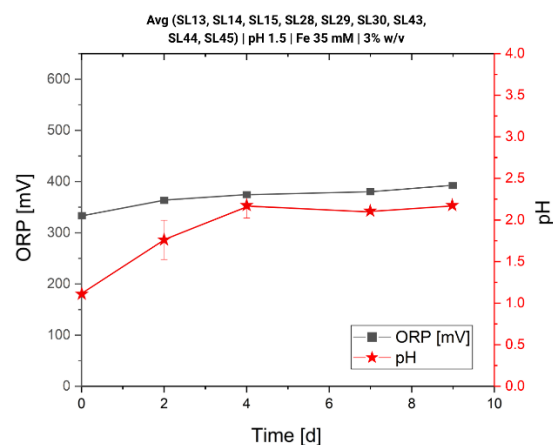
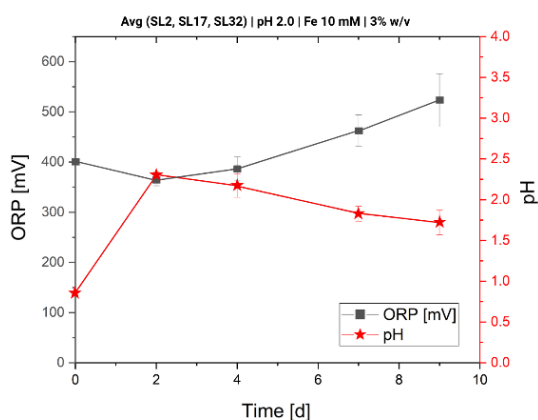
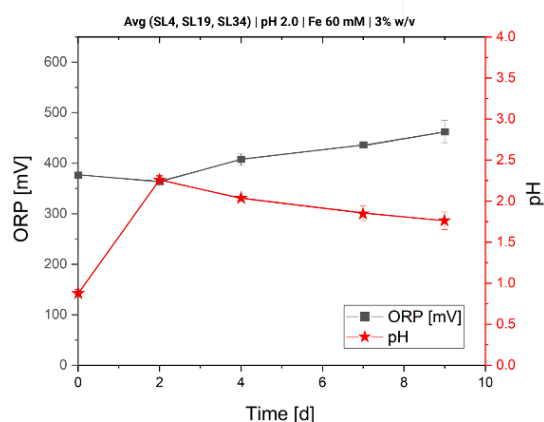


Figure 5.16. Monitoring of pH and ORP over incubation time for the bioleaching of slag was carried out using starting media HBS pH 1.5 at 42 °C and SLA culture, and addition of 1% (w/v) sulfur. (A) Average of SL9, SL24, SL39 at Fe (II) 10 mM and 2% (w/v) pulp density. (B) Average of SL10, SL25, SL40 at Fe (II) 60 mM and 2% (w/v). (C) Average of SL11, SL26, SL41 at Fe (II) 10 mM and 4% (w/v). (D) Average of SL12, SL27, SL42 at Fe (II) 60 mM and 4% (w/v). (E) Control – Average of SL13, SL14, SL15, SL28, SL29, SL30, SL43, SL44 and SL45 at Fe (II) 35 mM and 3% (w/v).

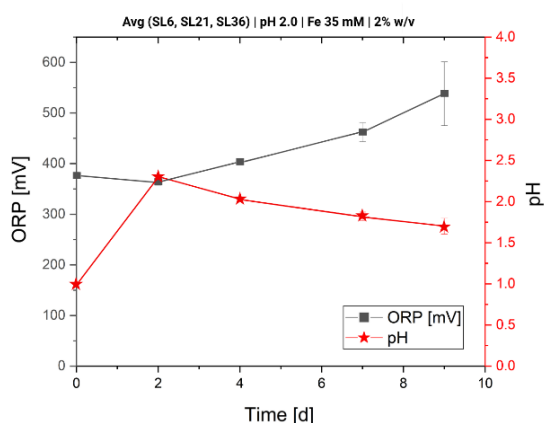
A



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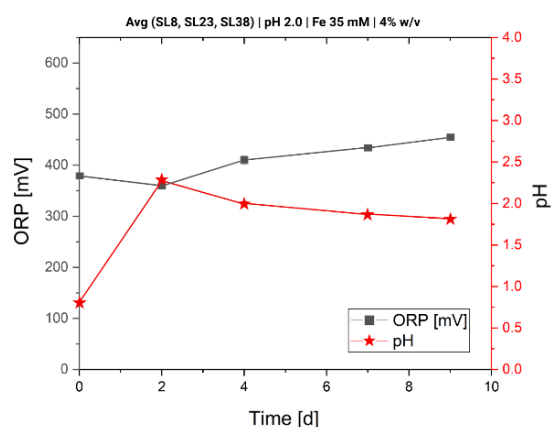
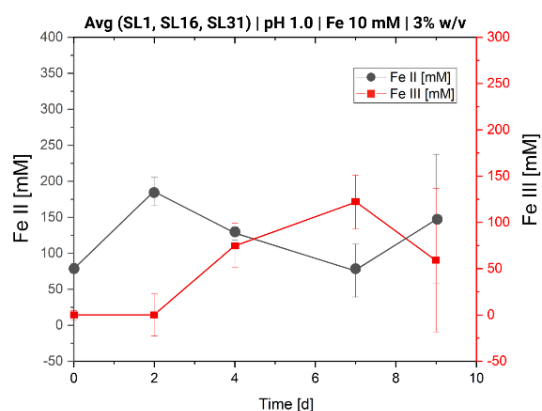


Figure 5.17. Monitoring of pH and ORP over incubation time for the bioleaching of slag was carried out using starting media HBS pH 2.0, at 42 °C, and SLA culture, and addition of 1% (w/v) sulfur. (A) Average of SL2, SL17, SL32 at Fe (II) 10 mM and 3% (w/v) pulp density. (B) Average of SL4, SL19, SL34 at Fe (II) 60 mM and 3% (w/v). (C) Average of SL6, SL21, SL36 at Fe (II) 35 mM and 2% (w/v). (D) Average SL8, SL23, SL38 at Fe (II) 35 mM and 4% (w/v).

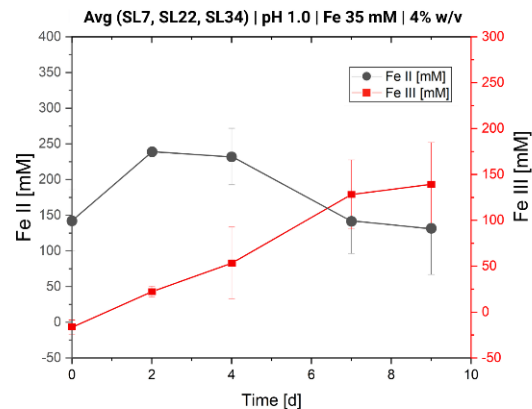
### 5.2.1. Total Iron, Ferrous Iron, and Ferric Iron Concentration Determination for Slag

The bioleaching experiments revealed that all experiments, including chemical control, by the last day had an increase in  $\text{Fe}^{3+}$  when compared to day 0 (shown in Annex A6). While most experiments showed a decrease of  $\text{Fe}^{2+}$  content in the leachate liquor (SL1-16-31, SL7-22-37, SL11-26-41, SL12-27-42) and Control - SL13-14-15-28-29-30-43-44-45 showed an increase, which can be seen in Figure 5.18. The amount of ferrous iron added to each flask was significantly more than measured in the first sampling. This suggests the release of more ferrous iron from the material, which could be released on the first day due to the extremely low pH.

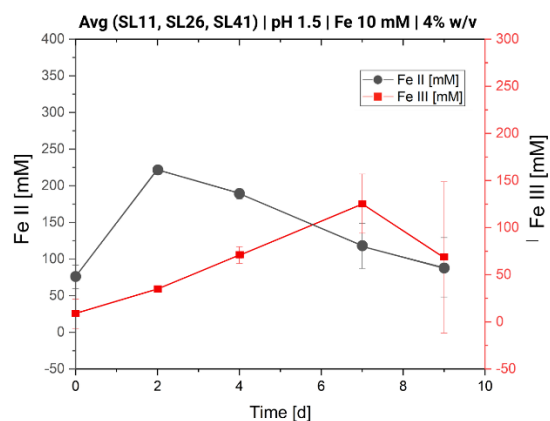
A



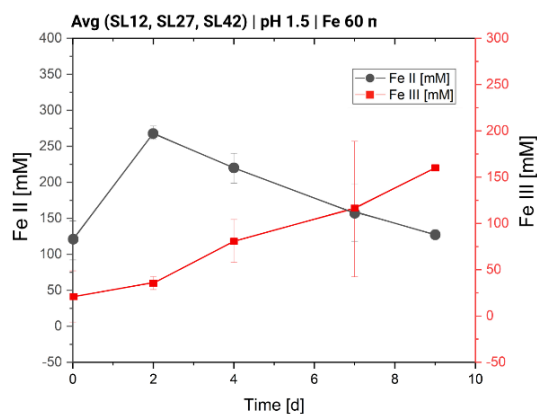
B



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D



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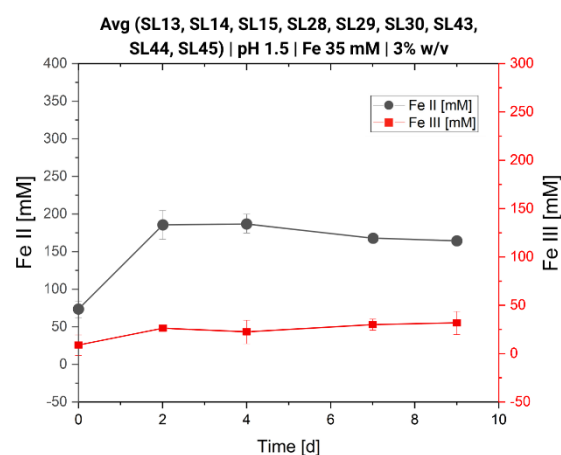


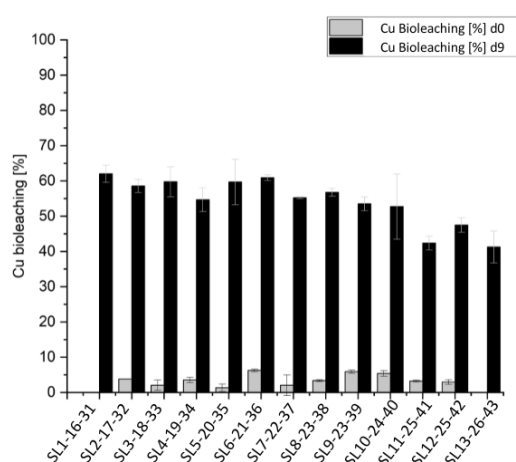
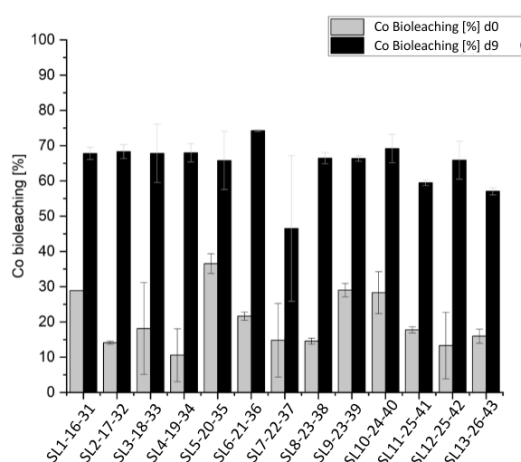
Figure 5.18. Monitoring of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  over incubation time for the bioleaching of slag, at 42 °C, SLA culture and addition of 1% (w/v) sulfur. (A) Average of SL1, SL16, SL31 at pH 1.0, Fe (II) 10 mM and 3% (w/v). (B) Average of SL7, SL22, SL37 at pH 1.0, Fe (II) 35 mM and 4% (w/v). (C) Average of SL11, SL26, SL4, at pH 1.5, Fe (II) 10 mM and 4% (w/v). (D) Average of SL12, SL27, SL42, at pH 1.5, Fe (II) 60 mM and 4% (w/v). (E) Control - Average SL13, SL14, SL15, SL28, SL29, SL30, SL43, SL44, SL45 at pH 1.5, Fe (II) 35 mM and 3% (w/v).

### 5.2.2. ICP-OES Analysis for Slag Samples

The metal recovery from slag material was calculated by Equation 7. Co, Cu, Mn, and Zn showed relevant recovery across different experiments (Figure 5.19); for the other metals measured, see Annex A7. The bioleaching on the last day was much higher than on day 0. Co bioleaching close to 60% in almost all conditions tested, and SL6-21-36 had 61%, the highest Co presence in liquor leach. Cu bioleaching varied from 40 to 60% on day 9. Both Mn and Zn showed higher bioleaching on day 0 when compared to Co and Cu. The final percentage of Mn and Zn in the liquor leach reached 85.8% in SL10-25-40 and 82.4% SL6-21-36, respectively.

A

B



C

D

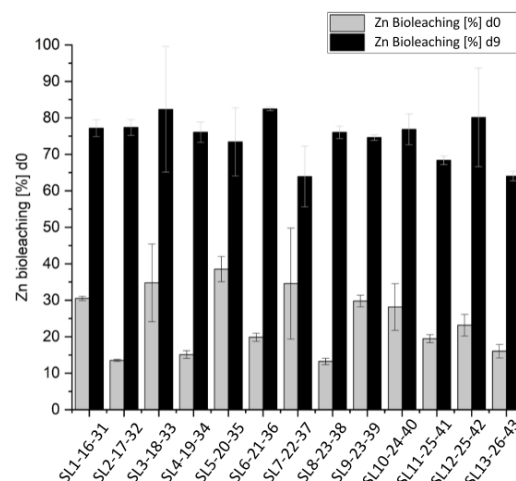
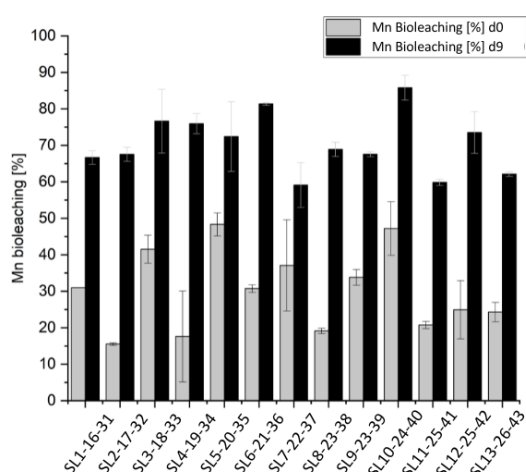


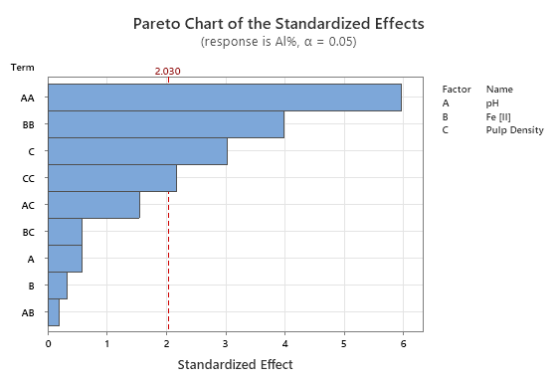
Figure 5.19. Efficiency of metals bioleaching on day 0 and day 9 of the experiment. (A) Co. (B) Cu. (C) Mn. (D) Zn.

### 5.2.3. Response Surface Methodology for Slag Samples

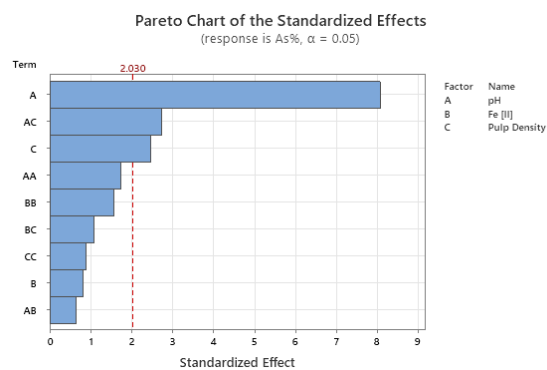
After seeing which metals were being leached, the Response Surface Regression (RSR) models for those are presented, focusing on factors that significantly affect metal recovery and the overall reliability of the models. For the other analyzed elements, check Annex A8. The behavior of Al is dominated by the quadratic terms, such as  $\text{pH}^2$ ,  $\text{Fe (II)}^2$ , and  $\text{pulp density}^2$ ; all these terms are highly significant with a  $p < 0.05$  (Figure 5.20A). It is also affected by the linear

term of pulp density. Bioleaching efficiency of As is very sensitive to pH, and it is also influenced by pulp density (Figure 5.20B). When it comes to the quadratic terms,  $\text{pH}^2$  and  $\text{Fe (II)}^2$  are significant as well. The presence of Co in the liquor is significantly influenced by the square of Fe (II) concentration, pulp density, and pH (Figure 5.20C). For Cu, the square of Fe (II), and pH, and pulp density were the most influential factors (5.20D). Mn was influenced by Fe (II) and the square of Fe (II), pH and the square of pH, pulp density and the square of pulp density (Figure 5.20E). Zn was mostly affected by the square of Fe (II) and of pH (Figure 5.20F).

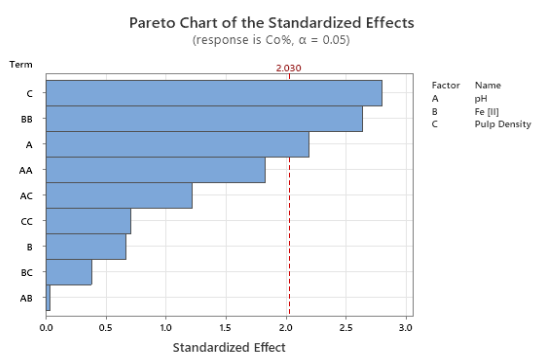
A



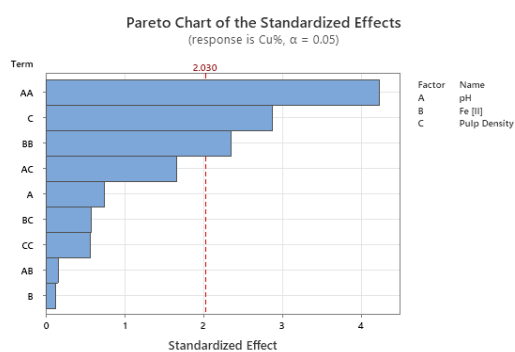
B



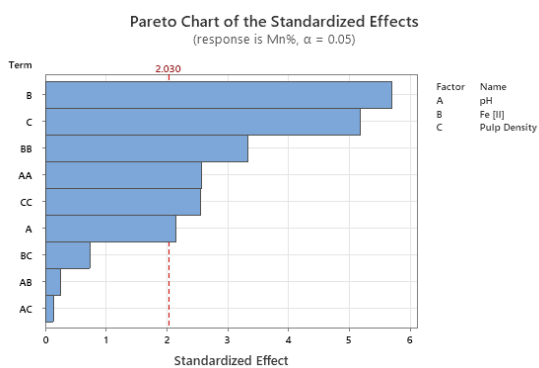
C



D



E



F

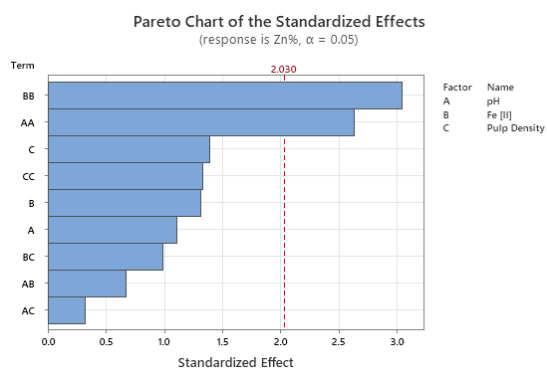


Figure 5.20. Pareto chart of the standardized effects. (A) Response is Al bioleaching efficiency after 9 days. (B) Response is As bioleaching efficiency after 9 days. (C) Response is Co bioleaching efficiency after 9 days. (D) Response is Cu bioleaching efficiency after 9 days. (E) Response is Mn bioleaching efficiency 9 days. (F) Response is Zn bioleaching efficiency after 9 days.

The model explains a good portion of the variance in Al ( $R^2 = 64.2\%$  and predicted  $R^2 = 37.1\%$ ), As ( $R^2 = 71.2\%$ ), and Mn ( $R^2 = 71.0\%$  and predicted  $R^2 = 48.9\%$ ) recovery and indicates good reliability when predicting unseen data. Zn ( $R^2 = 45.5\%$  and predicted  $R^2 = 1.69\%$ ), Co ( $R^2 = 41.2\%$  and predicted  $R^2 = 0\%$ ), and Cu ( $R^2 = 49.3\%$  and predicted  $R^2 = 11.7\%$ ) models are poor or weak in terms of fit and predictability. Moreover, some data points show large residuals, which could indicate outliers or model limitations.

Based on the regression coefficients, polynomial regression model equations that fitted the variation in the data for Al (Equation 11), As (Equation 12), Co (Equation 13), Cu (Equation 14), Mn (Equation 15), Zn (Equation 16) were proposed.

$$\text{Al Bioleaching (\%)} = 806 - 500.4 \text{ pH} - 3.22 \text{ Fe [II]} - 124.4 \text{ Pulp Density} + 146.0 \text{ pH} \times \text{pH} + 0.03902 \text{ Fe [II]} \times \text{Fe [II]} + 13.29 \text{ Pulp Density} \times \text{Pulp Density} + 0.090 \text{ pH} \times \text{Fe [II]} + 18.1 \text{ pH} \times \text{Pulp Density} + 0.137 \text{ Fe [II]} \times \text{Pulp Density} \quad (\text{Eq. 11})$$

$$\text{As Bioleaching (\%)} = 161.2 - 107.9 \text{ pH} - 0.324 \text{ Fe [II]} - 29.5 \text{ Pulp Density} + 15.21 \text{ pH} \times \text{pH} + 0.00548 \text{ Fe [II]} \times \text{Fe [II]} + 1.95 \text{ Pulp Density} \times \text{Pulp Density} + 0.109 \text{ pH} \times \text{Fe [II]} + 11.51 \text{ pH} \times \text{Pulp Density} - 0.0905 \text{ Fe [II]} \times \text{Pulp Density} \quad (\text{Eq. 12})$$

$$\text{Co Bioleaching (\%)} = 156.1 - 63.2 \text{ pH} - 0.775 \text{ Fe [II]} - 24.9 \text{ Pulp Density} + 17.87 \text{ pH} \times \text{pH} + 0.01032 \text{ Fe [II]} \times \text{Fe [II]} + 1.73 \text{ Pulp Density} \times \text{Pulp Density} - 0.007 \text{ pH} \times \text{Fe [II]} + 5.72 \text{ pH} \times \text{Pulp Density} + 0.0358 \text{ Fe [II]} \times \text{Pulp Density} \quad (\text{Eq. 13})$$

$$\text{Cu Bioleaching (\%)} = 222.6 - 156.4 \text{ pH} - 0.838 \text{ Fe [II]} - 29.0 \text{ Pulp Density} + 45.0 \text{ pH} \times \text{pH} + 0.00999 \text{ Fe [II]} \times \text{Fe [II]} + 1.50 \text{ Pulp Density} \times \text{Pulp Density} - 0.032 \text{ pH} \times \text{Fe [II]} + 8.44 \text{ pH} \times \text{Pulp Density} + 0.059 \text{ Fe [II]} \times \text{Pulp Density} \quad (\text{Eq. 14})$$

$$\text{Mn Bioleaching (\%)} = 144.3 - 45.4 \text{ pH} - 0.171 \text{ Fe [II]} - 29.7 \text{ Pulp Density} + 16.66 \text{ pH} \times \text{pH} + 0.00866 \text{ Fe [II]} \times \text{Fe [II]} + 4.16 \text{ Pulp Density} \times \text{Pulp Density} - 0.031 \text{ pH} \times \text{Fe [II]} + 0.41 \text{ pH} \times \text{Pulp Density} - 0.0456 \text{ Fe [II]} \times \text{Pulp Density} \quad (\text{Eq. 15})$$

$$\text{Zn Bioleaching (\%)} = 176.5 - 75.3 \text{ pH} - 0.855 \text{ Fe [II]} - 28.0 \text{ Pulp Density} + 26.3 \text{ pH} \times \text{pH} + 0.01218 \text{ Fe [II]} \times \text{Fe [II]} + 3.33 \text{ Pulp Density} \times \text{Pulp Density} - 0.129 \text{ pH} \times \text{Fe [II]} + 1.54 \text{ pH} \times \text{Pulp Density} + 0.0950 \text{ Fe [II]} \times \text{Pulp Density} \quad (\text{Eq. 16})$$

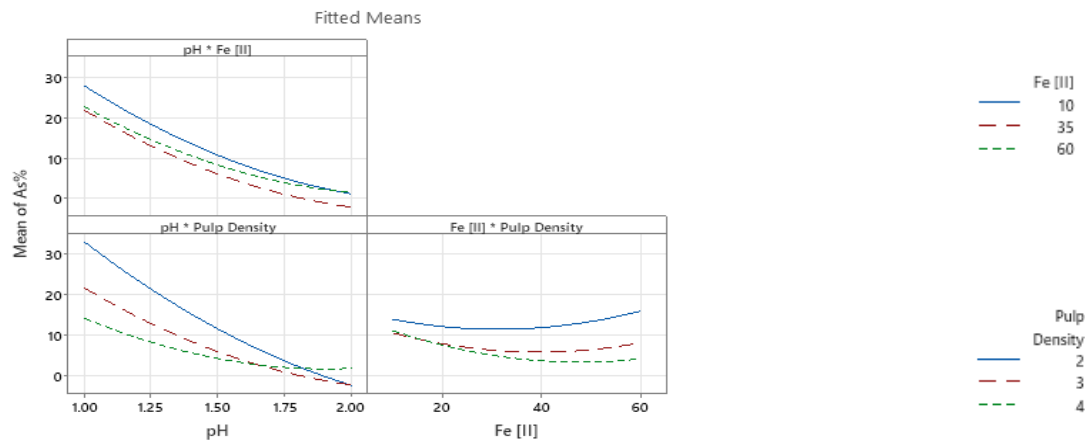


Figure 5.21. Interaction plot for As bioleaching efficiency.

No significant interactions were found between terms for Al, Co, Cu, Mn, and Zn. For As recovery, some interaction is present between pulp density and pH ( $p = 0.010$ ) (Figure 5.21). The interactions plot for all elements can be found in Annex A9.

In order to optimize the bioleaching of interest metals, a response optimization model was created, with the goal of reaching 100% of Al, Co, Cu, Mn, Zn, while minimizing the recovery of Arsenic. The full model can be seen in Annex A10. The resulting model indicated that operating at low pH (1.89), moderately high Fe (II) (60 mM), and low pulp density (2.32% w/v), the optimized bioleaching goals are likely to be met. These conditions are predicted to enhance metal solubility and reduce co-precipitation. Co, Mn, and Zn recoveries are close to the target under this model, with Co fit in 72.08%, Zn in 79.38% and Mn in 84.48%, while As is reduced to 4% (Table 5.2).

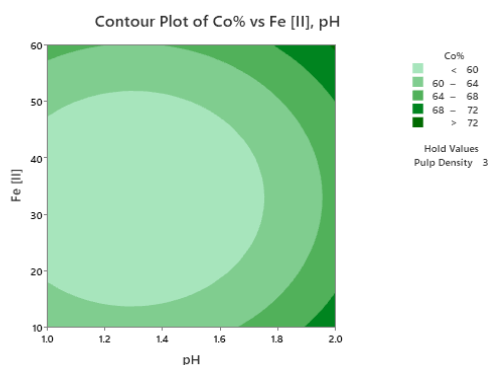
Table 5.2. Predicted Responses at optimal settings for slag.

| Response:<br>Recovery (%) | Goal         | Predicted value (%) | 95% CI*        | 95% PI**        |
|---------------------------|--------------|---------------------|----------------|-----------------|
| Al                        | Target: 100% | 221.2               | (200.7, 241.7) | (175.1, 267.3)  |
| As                        | Minimize     | 4.00                | (-3.33, 11.32) | (-12.48, 20.47) |
| Co                        | Target: 100% | 72.08               | (63.89, 80.28) | (53.66, 90.51)  |
| Cu                        | Target: 100% | 56.12               | (47.22, 65.02) | (36.10, 76.13)  |
| Mn                        | Target: 100% | 84.48               | (79.03, 89.93) | (72.23, 96.74)  |
| Zn                        | Target: 100% | 79.38               | (71.00, 87.77) | (60.52, 98.25)  |

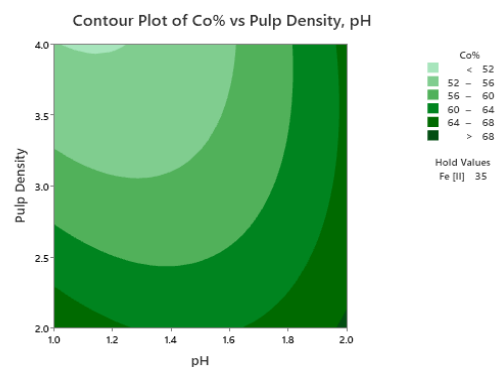
\*CI (confidence interval), \*\*PI (prediction interval)

Although Co and Cu show high concentration in the leachate solution, the model must be considered carefully due to the  $R^2$  values. The contour plots showed that Co is better recovered at high or low concentrations of iron (60 mM or 10 mM), at pH 2.0, when pulp density is held at the middle value (Figure 5.22A). When iron is in the middle value, best recovery is obtained at pH 2.0 and lowest pulp density (Figure 5.22B). Similarly, when pH is in the middle value, Co recovery is the highest at low pulp density and very high or low ferrous iron (Figure 5.22C). For Cu recovery, at pH 1.0 or 2.0 and the highest iron, more than 60% is recovered, at pulp density 3% (w/v) (Figure 5.22D).

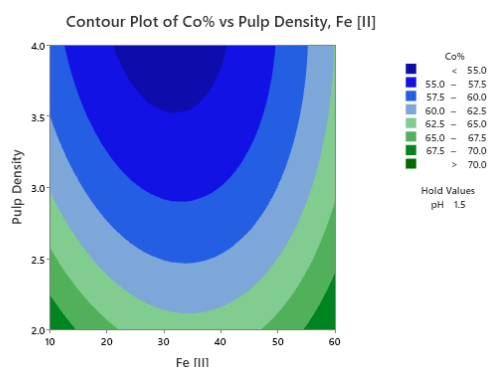
A



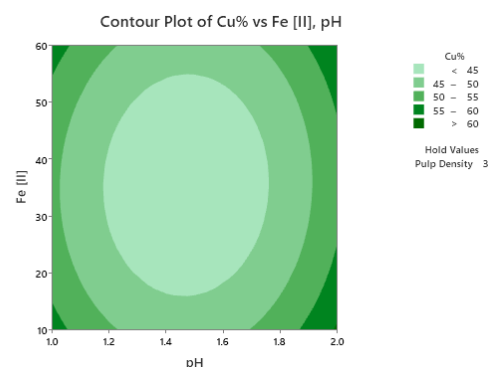
B



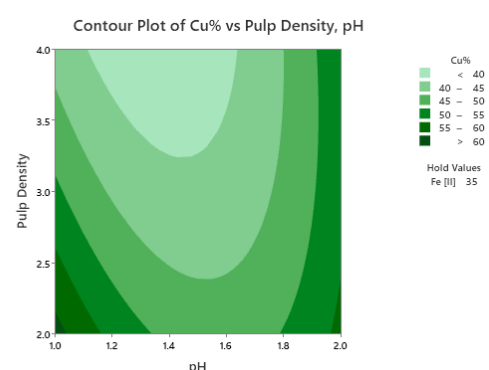
C



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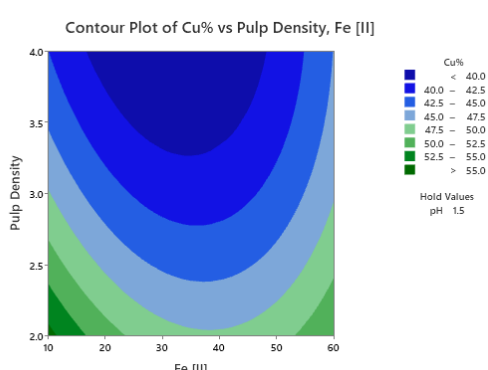


Figure 5.22. Surface contour plots for (A) Co recovery in leachate when pulp density is at the middle value. (B) Co recovery in leachate when Fe (II) is at the middle value. (C) Co recovery in leachate when pH is at the middle value. (D) Cu recovery in leachate when pulp density is at the middle value. (E) Cu recovery in leachate when Fe (II) is at the middle value. (F) Cu recovery in leachate when pH is at the middle value.

When ferrous iron is held at 35 mM, it confirms that lower pulp density and low or high ferrous iron will improve the recovery of copper (Figure 5.22E). When pH is at the middle value, the lowest pulp density performs the best with 10 mM or 60 mM ferrous iron (Figure 5.22F).

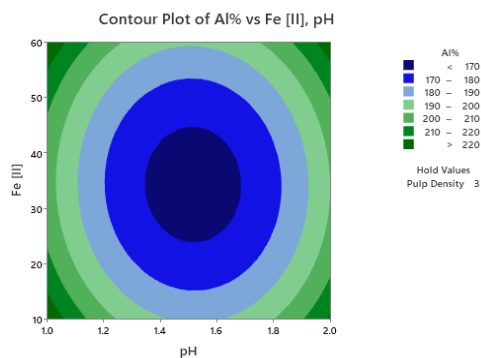
Considering the models with good or moderate fit, the contour plots show the behavior of each element when two out of three conditions vary. For all contour plots and 3D surface response curves, see Annex A11. The highest recovery of Al was achieved when the iron concentration was the highest or lowest tested, same for pH (Figure 5.23A). If Fe (II) is held at 35 mM, the best recovery is achieved when pH is 1.0 and pulp density is 2.0 (Figure 5.23B), and when the pH is held at 1.5, the best recovery is obtained at minimum or maximum iron concentration and lowest pulp density (Figure 5.23C). As the Al recovery in the experiments exceeded 100%, the model also showed a range from 170 to 230%.

The areas which it is possible to obtain the least As recovery (0%) are in pulp density set as the middle value, highest pH and Fe (II) from 20-50 mM, also in the region of pH 2.0, from pulp density 2-3.5% and Fe (II) at 35 mM (Figure 5.23E) and, when pH is held at 1.5, lowest recovery is found in higher pulp density 3.5-4.0 and higher iron concentration (30-60 mM) (Figure 5.23F).

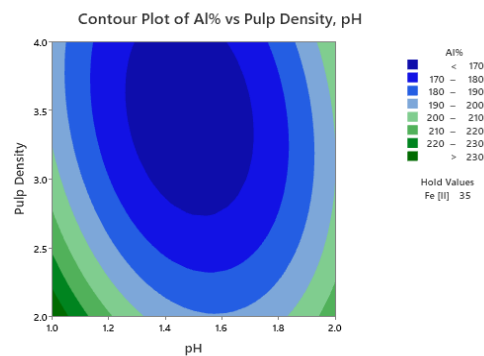
For Zn recovery, high pH and high iron promote recovery of around 80% according to the model, when pulp density is 3% (Figure 5.23J). When iron is held at 35 mM, pH 2.0, and

low pulp density remains the most suitable option, recovering up to 75% (Figure 5.23K). When pH is 1.5, low (10 mM) or high (60 mM) values promote recovery of 75% or more if pulp density is 2 % (w/v) (Figure 5.23L). Mn recovery was the best in higher iron concentration and using HBS media at pH 2.0, when the hold value for pulp density is 3% (w/v) (Figure 5.23G).

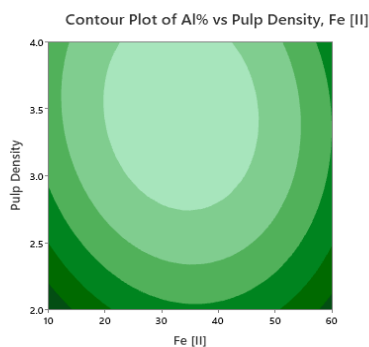
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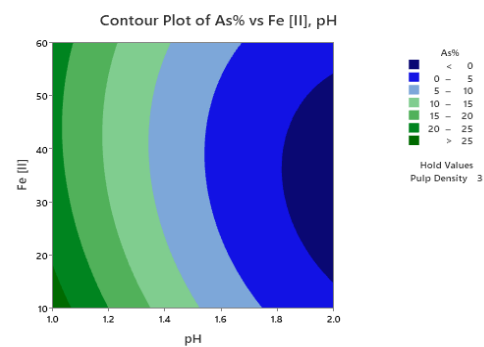
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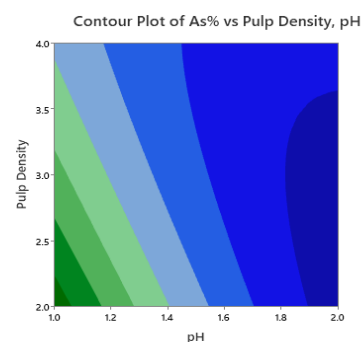
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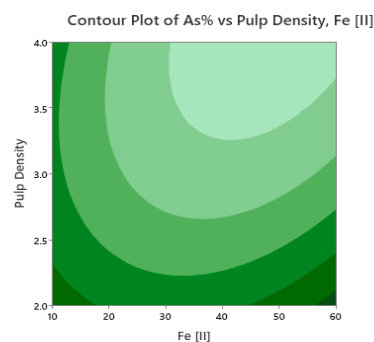
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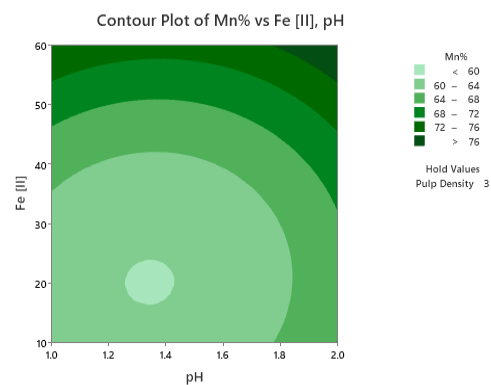
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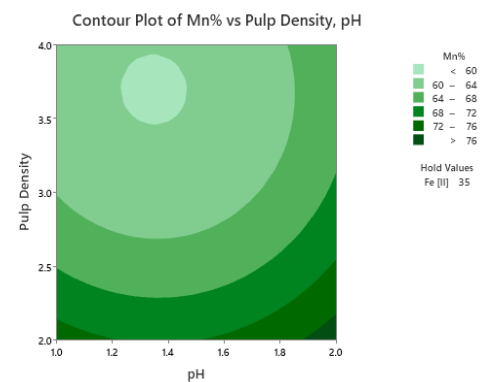
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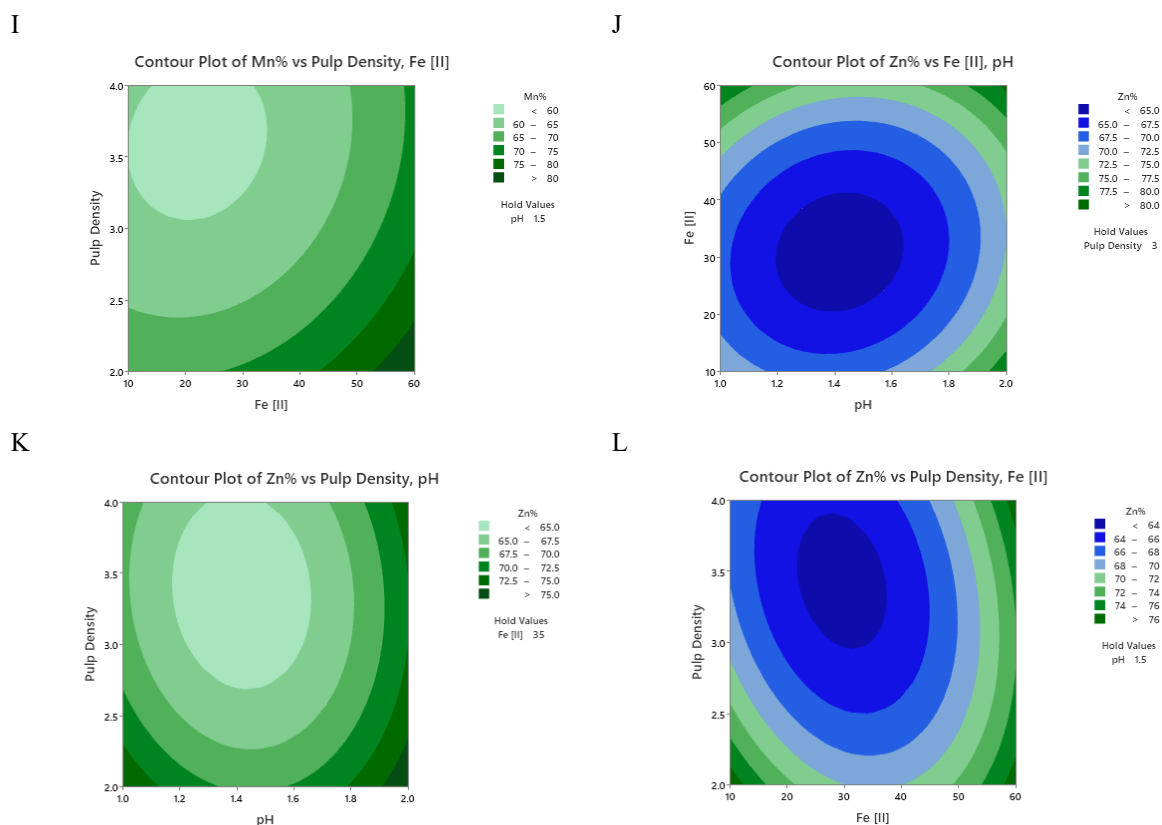


Figure 5.23. Contour plots for (A) Mn recovery with respect to ferrous iron and pH (pulp density fixed at its middle value). (B) Mn recovery with respect to ferrous iron and pulp density (pH fixed at its middle value). (C) Mn bioleaching recovery with respect to pulp density and pH (ferrous iron concentration fixed at its middle value). (D) Zn recovery with respect to ferrous iron and pH (pulp density fixed at its middle value). (E) Zn recovery with respect to ferrous iron and pulp density (pH fixed at its middle value). (F) Mn recovery with respect to pulp density % and pH (ferrous iron concentration fixed at its middle value).

Also, when ferrous iron is 35 mM, the best pH remains at 2.0, and pulp density 2% (w/v) (Figure 5.23H). When pH is held at 1.5, the best option is still high iron and low pulp density (Figure 5.23I).

### 5.2.3.1. Confirmatory Experiments of Slag

To confirm the prediction done by the model, and therefore the applicability of the proposed second-order model for further optimization of slag bioleaching, confirmation runs at the predicted optimum level of the factors were carried out along with abiotic controls. Color and turbidity varied over 9 days, with the bioleaching experiment becoming more orange than the abiotic control (Figure 5.24).

In Figure 5.25A, the pH and ORP are shown over 9 days, where it was observed an increase in ORP and pH is observed overall. Abiotic control had a pH increase higher than that of the experiment. Moreover, the increase in ORP was very low for the abiotic control. Both observations indicate that microbial action was negligible in the abiotic control, though a few cells were observed. In Figure 5.25B, the Fe (II) increased over time, reaching around 225 mM on the last day, and Fe (III) decreased over time, reaching 0 mM, in both the experiment and

the control. This is caused by the release of ferrous iron from the slag and, the use of ferric iron produced for the bioleaching process.

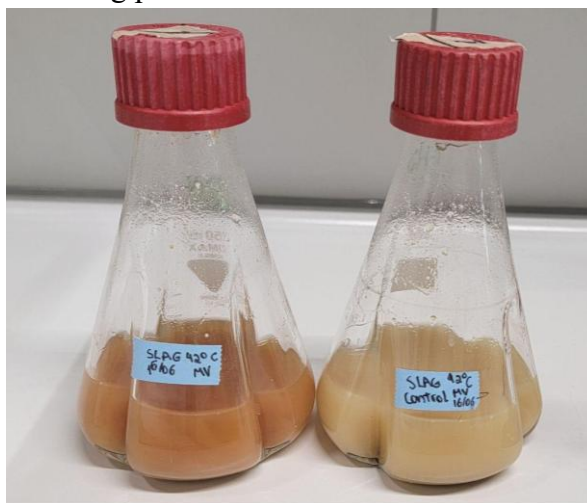
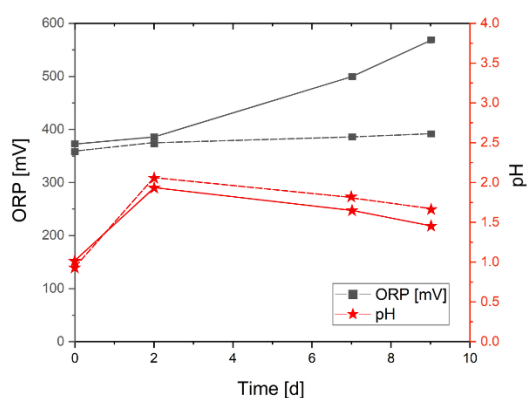


Figure 5.24. Slag bioleaching experiment on the 9th day versus abiotic control on the 9th day at pH 1.89, 42 °C, pulp density 2.32% (w/v), Fe (II) 60 mM, SLA culture and addition of 1% (w/v) sulfur.

A



B

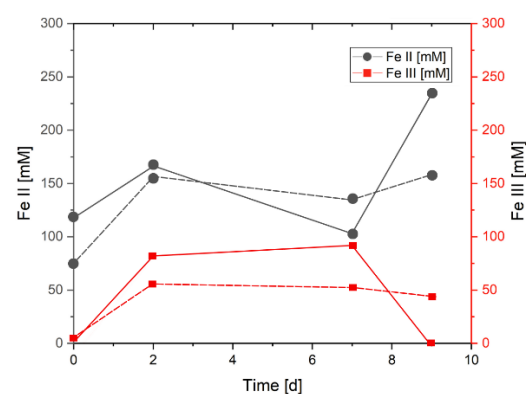


Figure 5.25. Monitoring of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  over incubation time for the bioleaching of slag carried out using starting media HBS pH 1.89, 42 °C, pulp density 2.32% (w/v), Fe (II) 60 mM, and SLA culture, and addition of 1% (w/v) sulfur. (A) ORP and pH over 9 days for bioleaching confirmatory experiments. (B) Ferrous versus ferric iron over 9 days for bioleaching confirmatory experiments.

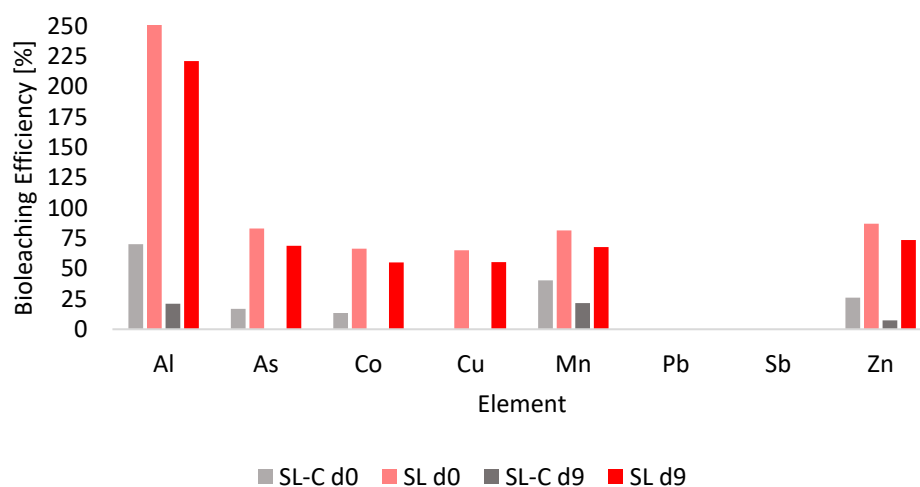


Figure 5.26. Bioleaching efficiency (%) of Al, As, Co, Cu, Mn, Pb, Sb, and Zn at day 0 and day 9. Light grey bars correspond to the abiotic control at day 0 (SL-C d0), and light red bars represent the biotic test at day 0 (SL d0). Dark grey bars indicate

the abiotic control after 9 days (SL-C d9), while red bars represent the bioleaching efficiency in the biotic experiment after 9 days (SL d9).

After confirmatory experiments, it was possible to see that Zn (86.9%), Mn (81.4%), Cu (65.0%), and Co (66.4%) are within the 95% confidence interval (Figure 5.26). For As, 83.0% was present in the leach liquor, when the maximum expected was 11.3%. Similarly, Al was underestimated, 258% in the confirmatory experiment versus a range between 200.7% and 241.7% expected. Although the values predicted for the model for Al and As are not within this interval, the model can be taken as confirmation of the suitability of the regression model for predictive purposes for the other metals, especially the interest metals Zn and Mn.

## 6. Discussion

### 6.1. Direct bioleaching of crushed pyrite (SD-BIO-3) and pyrite ashes (SD-BIO-2) under different combinations of pH, Fe (II), and pulp density

Bioleaching of waste materials differs considerably from conventional approaches used at a commercial scale for metal sulfide ores. Notably, mine waste is often pre-oxidized and may lack sufficient reduced sulfur to support microbial sulfur oxidation and sulfuric acid production (Kinnunen & Hedrich, 2023). Consequently, it was necessary to supplement the bioleaching flasks with elemental sulfur to stimulate microbial activity.

Iron supplementation also played a critical role, serving as a key energy source for the SLA consortium. This supplementation proved crucial in the proposed experiments. None of the biotic conditions supplemented with only 10 mM ferrous iron exhibited measurable iron oxidation. In contrast, experiments with higher ferrous iron concentrations showed significantly improved metal solubilization, highlighting the importance of iron availability in driving microbial metabolism.

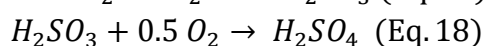
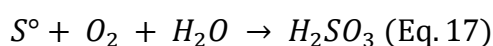
According to Li et al. (2020), the addition of iron in the bioleaching system at the beginning affects the bioleaching by *S. thermosulfidooxidans* and *L. ferriphilum* under autotrophic or rare organic nutrition conditions. It is noted that when there is no iron, the bioleaching reaches a plateau after a certain time; however, in the presence of iron, the bioleaching or at least the oxidization of RISCs continues.

Although earlier preliminary tests emphasized pH as a critical factor (Mokarian et al., 2022), within the tested range (pH 1.5 to 2.5), pH did not significantly influence metal recovery. The range tested was deliberately constrained and considered the growth restrictions of the microorganism consortia.

Microbial activity compatible with bioleaching and iron oxidation activity was confirmed in several experiments (CP3-18-33, CP4-19-34, CP6-21-36, CP7-22-37, CP8-23-38, and CP12-27-42) by observing cell growth, increasing redox potential, decreasing pH, and a shift from ferrous to ferric iron over a 9-day period. In contrast, the abiotic control exhibited little to no iron oxidation within the same timeframe. This behavior can be attributed to the absence of  $\text{Fe}^{2+}$  in the leaching media which restricted the microbial consortium of any energy source for their growth thereby indirectly affecting metal recovery (Panda, 2017). Hence, the observed redox trends support the conclusion that the SLA consortium effectively catalyzes the oxidation of Fe (II) to Fe (III), which subsequently acts as an oxidant in the bioleaching of crushed pyrite. When it comes to Fe (III), some values might be underrepresented, as it was

calculated by an indirect method, and errors might happen when total iron is being measured by adding ascorbic acid and measuring by spectrometry.

For both crushed pyrite and pyrite ashes optimization and confirmatory experiments, a gradual decrease in the pH was observed over the days. For example, at an initial pH of 2.0, the medium decreased to values between 1.63 and 1.76. This gives an indication of active sulfur oxidation by *Acidithiobacillus caldus* and *Sulfobacillus thermosulfidooxidans*, which contributed to lowering the pH of the system (Panda et al., 2017). The stepwise reaction of sulfur oxidation by the aforementioned microorganisms present in the bioleaching media is shown in Eqs. 17 and 18.



Even though sulfuric acid is produced in the process of bioleaching, for acid-insoluble minerals such as pyrite, the protons do not initiate breakdown. The bioleaching should proceed via the thiosulfate pathway which thiosulfate is generated via ferric iron oxidation and follows with cleaving Fe-S<sub>2</sub> bond. The thiosulfate is oxidized abiotically with ferric iron, via thiosulfate quinone oxidoreductase (TQO) and/or thiosulfate dehydrogenase (TSD) in sulfur-oxidizing microbes, species may then be oxidized to sulfate via biotic or abiotic reactions (Johns & Santini, 2023).

It is reported by Chen et al. (2012) that the sulfur oxidation system of *A. caldus* is divided into three subsystems, namely the Sox, the non-Sox sulfur, and the SOR. The sulfur oxidizing (Sox) enzyme system is responsible for the oxidation of sulfide, elemental sulfur, thiosulfate, and sulfite to sulfate, followed by electron transfer to cytochrome c. While SOR is able to catalyze the disproportionation of elemental sulfur, producing sulfite, thiosulfate, and sulfide. The non-Sox follows the S4I pathway of thiosulfate oxidation via the form of tetrathionate intermediates, in which thiosulfate is oxidized to tetrathionate by TQO; then, tetrathionate is hydrolyzed by tetrathionate hydrolase (TetH), yielding thiosulfate and other products.

Prior to ICP-OES analysis, a screening test was conducted to determine the optimal dilution and to identify the elements of interest. Only elements detected in both the screening and the bulk sample were included in the final analysis. Among the analyzed elements, Mn and Zn showed the highest solubilization rates.

Manganese exhibited consistent leaching across the different biotic conditions, consistent with its known mobility in acidic environments. Nearly 100% Mn recovery was achieved in CP-10-25-40, while other experiments, such as CP3-8-33, CP4-9-34, CP5-10-25, and CP12-27-42 showed more than 60% leaching after 9 days. Zinc leaching was most pronounced in CP5-20-35 and CP6-21-36, with recoveries of 20.8% and 22%, respectively. However, these values were very close to the abiotic control CP13-28-43 (21.5%), suggesting that Zn solubilization in these cases may not be strongly associated with biological activity.

Although Mn recovery approached 100% during the RSM optimization experiments, confirmatory experiments yielded significantly different results. This discrepancy may be attributed to Mn precipitation during confirmatory tests or contamination in the earlier samples used for RSM, leading to an overestimation.

The applied Box-Behnken design focusing on pH, initial Fe (II) concentration, and pulp density optimization showed that while experimental error could contribute, the model appears to suffer from systematic under- and over-predictions, indicating its limited ability to capture non-linear system behavior. Notably, the model severely underestimated Al leaching, likely due to interaction effects not captured in the design. A similar issue was observed with Sb: while the model predicted 1.93-3.04% leaching, the actual measured value was 16.4%. These mismatches point to limitations in the model's capacity to account for complex behaviors and interactions in bioleaching systems. Unlike the other quantified elements, for Cu and As, the model predictions fell within the 95% confidence intervals, though concentration in the leach liquor was low for these elements (Annex A2).

Moreover, the statistical model indicated that the three experimental variables acted independently, with no significant synergistic or antagonistic interactions observed. As can be seen by the p-values given by the two-way interaction in the analysis of variance and respective interaction plots (Annex A3).

## **6.2. Direct Bioleaching of Slag (SD-BIO-1) under different combinations of pH, Fe (II) and pulp density**

Slag waste exhibited significantly higher iron content compared to crushed pyrite and pyrite ashes, as can be seen in Table 4.9, which likely contributed positively to redox reactions that drive metal solubilization. Notably, even on the first day of the experiment, some flasks contained more ferrous iron than was initially added, suggesting that the slag itself released iron upon contact with the acidic medium in the shaking flasks.

Ferric iron is a chemical oxidant that breaks the bonds holding sulfide minerals together. Additionally,  $\text{Fe}^{3+}$  plays an important role in oxidizing released sulfide. Therefore, the regeneration of  $\text{Fe}^{3+}$  is critical to the bioleaching process. At low pH, ferrous iron is relatively stable, and abiotic oxidation is very slow. Thus, microorganisms are vital to the iron oxidation process in acidic environments.

Biological activity was evident in all tested conditions. Indicators such as changes in color, an increase of ferric iron concentrations, and a rise in oxidation-reduction potential (ORP) confirmed microbial oxidation. Cell growth and the progressive conversion of Fe (II) to Fe (III) over 9 days further supported the activity of the SLA consortium. In contrast, the abiotic control displayed little to no iron oxidation within the same timeframe, affirming the biological nature of the process. These results confirm that the SLA consortium effectively drives microbial iron oxidation, with ferric iron subsequently acting as a powerful oxidant in the slag bioleaching process.

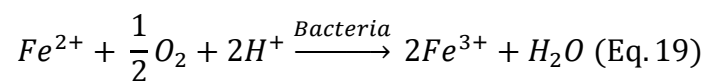
In these experiments, the buffering capacity of the slag led to a gradual pH increase, a behavior also observed in preliminary tests. To counteract this, sterile sulfuric acid was added before inoculation to lower the initial pH. The volume of acid added was proportional to the solid load and determined through previous acid consumption tests. Maintaining a low pH was crucial, as bioleaching reactions and associated microbial activity are acid-dependent. However, a thick residue layer — likely composed of silica — formed during the process (Figure 6.1).



Figure 6.1. Residue formed at the bottom of the shaking flask.

Research on amorphous FeO-SiO<sub>2</sub> slags reveals the formation of a passive layer of amorphous silica upon reacting with acid and precipitating during acidic leaching, which can prevent further reaction (Perederiy & Papangelakis, 2017). Therefore, this residue may hinder large-scale applications by forming a hard layer at the bottom of reactors and flasks at low pH, potentially reducing surface area and inhibiting microbial contact.

During Eh monitoring, shown in section 5.2.1, a gradual increase in the redox potential over the days was verified, reaching up to 551 mV. Compared to crushed pyrite and pyrite ashes bioleaching, ORP values were significantly higher in slag systems by the end of the experiment, indicating more favorable conditions for redox-driven metal release. This indicated the efficacy of the iron-oxidizing bacteria *S. thermosulfidooxidans* and *L. ferriphilum* that utilized the Fe<sup>2+</sup> iron source for their growth and metabolism. The primary role of these microorganisms is to regenerate the necessary Fe<sup>3+</sup> iron (Eq. 19) and maintain higher redox potential and promote faster biological dissolution of metals (Panda et al., 2017).



At pH 1.5, the abiotic control (Figure 5.17E) exhibited a gradual increase in pH throughout the experiments. This pH increase is attributable to the dissolution of alkaline phases in the slag, particularly those rich in Ca (23692 ppm) and K (2476 ppm), which can release hydroxyl ions into the leachate. In addition, the high Si (91077 ppm) content, as presented in Table 4.9, suggests the presence of silicate minerals, which can undergo slow weathering under acidic conditions, further contributing to pH neutralization. The release of these alkaline constituents is a common phenomenon in metallurgical slags, as Ca- and K-bearing phases react with the acidic solution to buffer the pH during bioleaching.

This chemical buffering is also supported by broader slag dissolution kinetics literature, where Ca is typically the most reactive alkaline species under acid attack and controls early pH neutralization behavior (De Windt et al., 2011). Preliminary results from Yang et al (2022) indicate that the Ca-bearing components and sulfate formation are the main cause of

neutralization in the leaching process. While Ca- and Mg- components contribute to the short-term neutralization, Si- and Al- components release alkaline materials in longer periods of time due to the low dissolution rate.

A Box-Behnken statistical model was employed to evaluate the effects of pH, Fe (II) concentration, and pulp density on metal bioleaching. The model demonstrated good predictive power for aluminum (Al), arsenic (As), and manganese (Mn). Importantly, interaction effects were found to be significant for As leaching, suggesting complex dependencies. Initially, the high concentration of lead (Pb) in the slag raised concerns in terms of toxicity. However, Pb was not detected in the leachates, likely due to its low solubility within the tested pH range. As a result, it was excluded from the optimization model. For cobalt (Co), copper (Cu), and zinc (Zn), the respective models showed limited predictive capability with low predicted  $R^2$  values.

To verify the reliability of predictions, adjusted  $R^2$  and predicted  $R^2$ , alongside the  $R^2$  are presented in Annex A7. As explained by Naseri et al. (2023), the adjusted  $R^2$  compares the explanatory power of regression models that contain different numbers of factors, and it increases if the new term improves the model more than would be expected by chance. The new data shows a regression model's ability of response, predicted  $R^2$ . It is also used for preventing model over-fitting.

To optimize the process, a multi-objective response optimization model was constructed with the goal of maximizing the recovery of Al, Co, Cu, Mn, and Zn, while minimizing As release. Minimization of As was done with the intention of reducing the need for complex downstream processing and detoxification of wastewater. If the presence of As in the solution is very high, it can result in the reduction of the purity of the other valuable products and also impact the operational costs.

According to the model (Annex A9), optimal bioleaching conditions were achieved at low pH (1.89), moderately high Fe (II) concentration (60 mM), and low pulp density (2.32% w/v). These conditions likely enhanced metal solubility and reduced co-precipitation.

Under these optimized parameters, predicted recovery rates for the target metals were set in at least 70% for the target metals. The confirmatory experiments supported the model's predictions for most target metals and validated the optimization model. The recovery results fell within the 95% confidence interval for Zn, Mn, Co, and Cu, while As and Al deviated significantly from the predictions (Annex A9). These discrepancies suggest that the model underestimated Al and As leaching, likely due to unmodeled interactions or non-linear effects. The Al bioleaching efficiency in the leach liquor solution surpassed 100% (Figure 5.18E), which can be explained by an error in the characterization of the material, due to experimental variability such as sampling error, evaporation effects, or matrix interference during analysis.

Confirmatory experiments showed higher concentrations of all target metals in solution under biotic conditions when compared to the abiotic conditions, even as early as day one. Furthermore, metal recovery percentages increased over the 9-day period across all biotic setups. Therefore, the consensus model was achieved and supported the utility of RSM for process optimization in slag bioleaching systems.

Other studies on the optimization of slag bioleaching systems have applied RSM successfully. Mirazimi & Rashchi (2011), optimized the recovery of vanadium from roasted Linz-Donawitz converter slag following CCD and achieved a maximum bioleaching of 90.58% of vanadium. Moreover, Ahmadi et al. (2017) improved the recovery yields of Fe and Cu from

converter slag by 26% and 33%, respectively using RSM, when compared to the abiotic leaching.

These findings demonstrate that the application of RSM provides a robust framework for optimizing bioleaching conditions in complex metallurgical residues such as slags. While minor deviations between predicted and experimental values for Al and As indicate that further refinement of the model may be warranted, the overall approach effectively captured the system behavior and provided actionable operational parameters.

## 7. Conclusion

The goal of this thesis was to investigate the effectiveness of using Response Surface Methodology through Box-Behnken design to optimize the extraction of valuable metals from different mine wastes from São Domingos mine. The direct leaching of pyrite wastes and modern slag demonstrated that microbial activity was efficiently producing sulfuric acid, highlighted by the reduction of pH and increase of ORP in the different conditions tested. Moreover, ferrozine tests confirmed the experiments in which iron oxidation was taking place. The addition of iron was critical to obtain the desired bioleaching, as it enhances microbial activity.

The bioleaching processes investigated in this study successfully optimized the recovery of metals from slag samples from São Domingos mine. A clear understanding of the affecting parameters on the optimization of this process was obtained. The results of the present study indicate that the maximum efficiency of metal solubilization was estimated under the conditions of 2.32% (w/v) solid load, pH 1.90, and ferrous iron supplementation of 60 mM by employing Box-Behnken Design. It was achieved 86.9% Zn, 81.4% Mn, 65% Cu and 66.4% Co, which are within the 95% confidence interval. Consequently, the null hypothesis was rejected, confirming that optimizing solid load, pH, and ferrous iron supplementation significantly enhances the efficiency of metal solubilization from slag.

For crushed pyrite, under the conditions of pH 1.66, 54 mM supplementation of iron and low pulp density (2% w/v) was estimated to achieve moderate to high recovery of Mn (>53.8%) and Zn (>8.0%). However, the values obtained were not within the 95% confidence interval and the null hypothesis could not be rejected, indicating the proposed model does not apply to the practical application; consequently, an optimized condition was not defined. More replicates of the confirmatory experiments for crushed pyrite should be done in order to confirm statistically that the model does not fit before discarding the model. Moreover, further optimization of crushed pyrite should be performed. It can be done by returning to the phase of screening of variables to determine again which parameters show high interactive effects on the responses.

The application of the optimized conditions obtained in the model for crushed pyrite was applied to pyrite ashes material, which did not result in satisfactory results. Hence, to better recover metals from pyrite ashes, a design of experiment focused on the material should be done, considering that metal removal using the bioleaching process can be affected by several environmental variables such as initial pH, pulp density, bacteria growth temperature, ferrous iron supplementation, and more.

RSM offers a powerful and efficient way to explore complex interactions among multiple variables, identify key factors, and optimize process parameters to maximize desired outcomes. The value of RSM in bioleaching research lies in its ability to systematically investigate and optimize various factors that influence bioleaching efficiency and selectivity.

This approach enables the identification of optimal operating conditions to efficiently explore interactive effects of several parameters, leading to enhanced leaching yields, reduced processing time, and improved resource utilization.

Regarding sustainability, RSM can contribute to developing environmentally friendly and economically viable bioleaching processes. By optimizing process parameters through RSM, researchers can reduce resource consumption, minimize waste generation, and maximize metal recovery, thereby promoting the circular economy and minimizing the environmental impact of mining and metal extraction. This study not only reinforces the applicability of RSM in biohydrometallurgical process design but also contributes to the development of environmentally responsible strategies for metal recovery from metal-containing residues.

## 8. Outlook and Recommendations

To enhance the optimization model for crushed pyrite bioleaching, it is recommended to re-run the center point experiments to resolve potential curvature issues in the response surface. Additionally, increasing the number of replicates in confirmatory experiments is advised to improve data reliability and reduce experimental variability. Before producing the response surface or contour plots again, the consensus model should be achieved, in order to avoid misinterpretation in the behavior of the experiment when changing the parameters (Igbani, 2020).

A notable anomaly was observed in aluminum recovery, where leaching exceeded the theoretical content of Al in the starting material. This discrepancy suggests the possibility of sample contamination or errors in the initial material characterization. Therefore, re-characterization of the bulk material is necessary to determine whether contamination occurred or if the discrepancy stems from incomplete or inaccurate compositional analysis.

Looking ahead, optimization strategies for bioleaching could benefit significantly from the integration of machine learning (ML). ML, as a branch of artificial intelligence, is capable of identifying complex, nonlinear relationships between input parameters and output responses. This capability allows for predictive modeling based on experimental data, enhancing decision-making in future process design (Mokarian et al., 2022). One particularly promising ML technique is Random Forest Regression (RFR), which has shown strong performance in capturing nonlinear patterns and improving predictive accuracy. RFR can serve as a powerful complement to traditional response surface methodology (RSM), offering robustness and flexibility in optimization tasks.

Due to time constraints, a detailed economic and environmental assessment of the proposed bioleaching strategies, particularly the optimized conditions for slag, was not conducted. However, such analysis is essential. The optimized process involves substantial consumption of ferrous iron, elemental sulfur, and sulfuric acid, which may influence both economic feasibility and environmental sustainability (Mokarian et al., 2022).

To evaluate the viability of implementing bioleaching at an industrial scale, a techno-economic assessment (TEA) is strongly recommended. TEA is a structured approach used to assess the economic performance of technologies through detailed modeling of production costs, capital and operating expenditures, and potential revenues (e.g., from recovered metals). Among the aspects that should be included, besides the use of reagents, is the limitation related to the low pulp density that reduces throughput, increases processing time, and can lead to higher operational costs due to the need for larger reactor volumes to handle the same mass of material. For reference, the work of Reed et al. (2016), as cited by Deng et al. (2020), exemplifies the application of TEA in evaluating bio-based technologies by incorporating all relevant cost and revenue components to estimate annual profitability.

Considering the environmental impact, a Life Cycle Assessment (LCA) can be conducted, and together with TEA, enabling assessment of the potential for applying bioleaching to the practical recovery of mine wastes. LCA allows the determination and comparison of the environmental impacts of processes or products. To assess and compare the new recovery technology regarding its environmental impacts, a standardized LCA method is used. It is based on the ISO 14040 series and allows the determination of the environmental impact of a product or process. LCA starting at an early stage of a process may help to increase its environmental friendliness (Falke & Höck, 2019).

For modern slag, the investigation of the residue layer composition could benefit the bioleaching efficiency in possible scale-up tests. Following such analysis, considerations for scale-up should be made, including re-optimization of process parameters to accommodate the challenges and constraints of pilot and industrial-scale operations. Incorporating newer data and operational conditions into future models will be critical for ensuring technical and economic success in bioleaching applications.

## References

- Ahmadi, S., Vafaie Sefti, M., Shadman, M. M., Azimi Dijvejin, Z., & Hosseini, H. (2017). The optimization of Cu and Fe bioleaching from converter slag using *Acidithiobacillus ferrooxidans*. *Journal of Advances in Environmental Health Research*, 5(3), 154-162.
- Albardeiro, L., Morais, I., Matos, J. X., Solá, R., Salgueiro, R., Pereira, Z., ... & Araújo, V. (2023). Time-space evolution of Iberian Pyrite Belt igneous activity: Volcanic and plutonic lineaments, geochronology, ore horizons and stratigraphic constraints. *Gondwana Research*, 121, 235-258.
- Alin, A. (2010). Minitab. *Wiley Interdisciplinary Reviews: Computational Statistics*, 2(6), 723-727.
- Álvarez-Valero, A. M., Pérez-López, R., Matos, J., Capitán, M. A., Nieto, J. M., Sáez, R., ... & Caraballo, M. (2008). Potential environmental impact at São Domingos mining district (Iberian Pyrite Belt, SW Iberian Peninsula): evidence from a chemical and mineralogical characterization. *Environmental Geology*, 55, 1797-1809.
- Alves Dias, P., Blagoeva, D., Pavel, C., & Arvanitidis, N. (2018). Cobalt: demand-supply balances in the transition to electric mobility. *Publications Office of the European Union*, 10, 97710.
- Chen, L., Ren, Y., Lin, J., Liu, X., Pang, X., & Lin, J. (2012). *Acidithiobacillus caldus* sulfur oxidation model based on transcriptome analysis between the wild type and sulfur oxygenase reductase-deficient mutant. *PLOS ONE* 7(9): e39470. <https://doi.org/10.1371/journal.pone.0039470>.
- Deng, S., Perez-Cardona, J., Huang, A., Yih, Y., Thompson, V.S., Reed, D.W., Jin, H. and Sutherland, J.W. (2020). Applying the design of experiments to evaluate the economic feasibility of rare-earth element recovery. *Procedia CIRP*, 90, pp.165-170.
- De Windt, L., Chaurand, P., & Rose, J. (2011). Kinetics of steel slag leaching: batch tests and modeling. *Waste Management*, 31(2), 225-235.
- European Commission. (2020). Circular economy action plan: For a cleaner and more Competitive Europe. Retrieved July 2025, from *Publications Office of the European Union*. <https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52020DC0098>.
- European Commission. (2023). Study on the Critical Raw Materials for the EU 2023. *Publications Office of the European Union*. <https://ec.europa.eu/newsroom/cipr/items/787007/en>.
- European Commission. (2025). Assessment of critical raw materials for strategic technologies and sectors in the EU: 2025 update. Retrieved July 2025, from *Publications Office of the European Union*. <https://webgate.ec.europa.eu/circabc-ewpp/d/d/workspace/SpacesStore/41471143-52cd-429f-9c76-c6359286d3aa/download>.
- Falke, A., & Höck, M. (2019). Ex-ante life cycle assessment of bioleaching in indium recovery from LCD-waste. *MRS Advances*, 4(35), 1949-1955.
- Ferreira, S. C., Bruns, R. E., Ferreira, H. S., Matos, G. D., David, J. M., Brandão, G. C., ... & Dos Santos, W. N. L. (2007). Box-Behnken design: An alternative for the optimization of analytical methods. *Analytica Chimica Acta*, 597(2), 179-186.

- Gaspar, O. (1998). História da Mineração dos Depósitos de Sulfuretos Maciços Vulcanogénicos da Faixa Piritosa Portuguesa. *Boletim de Minas*, 35(4).
- GeoHack Toolforge. (n.d.). São Domingos Mine. Retrieved July 2025, from [https://geohack.toolforge.org/geohack.php?pagename=S%C3%A3o\\_Domingos\\_Mine&params=37\\_40\\_08\\_N\\_007\\_29\\_38\\_W\\_region:PT\\_type:landmark](https://geohack.toolforge.org/geohack.php?pagename=S%C3%A3o_Domingos_Mine&params=37_40_08_N_007_29_38_W_region:PT_type:landmark).
- Ghelich, R., Jahannama, M. R., Abdizadeh, H., Torknik, F. S., & Vaezi, M. R. (2019). Central composite design (CCD)-Response surface methodology (RSM) of effective electrospinning parameters on PVP-B-Hf hybrid nanofibrous composites for synthesis of HfB<sub>2</sub>-based composite nanofibers. *Composites Part B: Engineering*, 166, 527-541.
- Goonan, T. G. (2012). Materials flow of Indium in the United States in 2008 and 2009 (No. 1377, pp. i-12). *U.S. Geological Survey*.
- Hallberg, K. B., & Lindström, E. B. (1994). Characterization of *Thiobacillus caldus* sp. nov., a moderately thermophilic acidophile. *Microbiology*, 140(12), 3451-3456.
- Hong, M., Wang, X., Wu, L., Fang, C., Huang, X., Liao, R., ... & Wang, J. (2019). Intermediates transformation of bornite bioleaching by *Leptospirillum ferriphilum* and *Acidithiobacillus caldus*. *Minerals*, 9(3), 159.
- Igbani, S., Appah, D., & Ogoni, H. A. (2020). The application of response surface methodology in Minitab 16, to identify the optimal, comfort, and adverse zones of compressive strength responses in ferrous oilwell cement sheath systems. *International Journal of Engineering and Modern Technology*, 6, 20-39.
- Ilin, M., & Bohlen, J. (2025). Six Sigma method. In *StatPearls*. StatPearls Publishing. <https://pubmed.ncbi.nlm.nih.gov/36943984>.
- Jankovic, A., Chaudhary, G., & Goia, F. (2021). Designing the design of experiments (DOE)—An investigation on the influence of different factorial designs on the characterization of complex systems. *Energy and Buildings*, 250, 111298.
- Johnson, D. B. (2006). Biohydrometallurgy and the environment: intimate and important interplay. *Hydrometallurgy*, 83(1-4), 153-166.
- Jones, S., & Santini, J. M. (2023). Mechanisms of bioleaching: iron and sulfur oxidation by acidophilic microorganisms. *Essays in Biochemistry*, 67(4), 685-699.
- Kinnunen, P., & Hedrich, S. (2023). Biotechnological strategies to recover value from waste. *Hydrometallurgy*, 222, 106182.
- Komnitsas, K. A. (2019). Editorial for Special Issue “Recent Advances in Hydro-and Biohydro metallurgy”. *Minerals*, 9(7), 424.
- LaPanse, A. J., Krishnan, A., & Posewitz, M. C. (2021). Adaptive Laboratory Evolution for algal strain improvement: Methodologies and applications. *Algal Research*, 53, 102122.
- Leistel, J. M., Marcoux, E., Thiéblemont, D., Quesada, C., Sánchez, A., Almodóvar, G. R., ... & Saez, R. J. M. D. (1997). The volcanic-hosted massive sulphide deposits of the Iberian Pyrite Belt Review and preface to the Thematic Issue: Review and preface to the Thematic Issue. *Mineralium Deposita*, 33, 2-30.
- Li, Q., Zhu, J., Li, S., Zhang, R., Xiao, T., & Sand, W. (2020). Interactions between cells of *Sulfobacillus thermosulfidooxidans* and *Leptospirillum ferriphilum* during pyrite bioleaching. *Frontiers in microbiology*, 11, 44.
- Lujan-Moreno, G. A., Howard, P. R., Rojas, O. G., & Montgomery, D. C. (2018). Design of

- experiments and response surface methodology to tune machine learning hyperparameters, with a random forest case study. *Expert Systems with Applications*, 109, 195-205.
- Matos, J. X., Pereira, Z., Rosa, C. J., Rosa, D., Oliveira, J. T., & Relvas, J. M. (2011). Late Strunian age: a key time frame for VMS deposit exploration in the Iberian Pyrite Belt. In *11th SGA Biennial Meeting: Let's Talk Ore Deposits*.
- Matos, J. X., Pereira, Z., Batista, M. J., & de Oliveira, D. P. S. (2012). Sao Domingos mining site-Iberian Pyrite Belt. In *9th International Symposium on Environmental Geochemistry*, Field Guidebook.
- Mirazimi, S. M. J., & Rashchi, F. (2011, November). Optimization of bioleaching of a vanadium-containing slag using RSM. In *Proceedings of the 7th International Chemical Engineering Congress & Exhibition, Kish, Iran* (pp. 21-24).
- Mishra, D., Kim, D. J., Ralph, D. E., Ahn, J. G., & Rhee, Y. H. (2008). Bioleaching of spent hydro-processing catalyst using acidophilic bacteria and its kinetics aspect. *Journal of Hazardous Materials*, 152(3), 1082-1091.
- Mokarian, P., Bakhshayeshi, I., Taghikhah, F., Boroumand, Y., Erfani, E., & Razmjou, A. (2022). The advanced design of bioleaching process for metal recovery: A machine learning approach. *Separation and Purification Technology*, 291, 120919.
- Narukulla, S., Bogadi, S., Tallapaneni, V., Sanapalli, B. K. R., Sanju, S., Khan, A. A., ... & Papadakis, M. (2024). Comparative study between the Full Factorial, Box–Behnken, and Central Composite Designs in the optimization of metronidazole immediate release tablet. *Microchemical Journal*, 207, 111875.
- Naseri, T., Pourhossein, F., Mousavi, S. M., Kaksonen, A. H., & Kuchta, K. (2022). Manganese bioleaching: an emerging approach for manganese recovery from spent batteries. *Reviews in Environmental Science and Bio/Technology*, 21(2), 447-468.
- Naseri, T., Beiki, V., Mousavi, S. M., & Farnaud, S. (2023). A comprehensive review of bio leaching optimization by statistical approaches: recycling mechanisms, factors affecting, challenges, and sustainability. *RSC Advances*, 13(34), 23570-23589.
- Norgate, T. E., Jahanshahi, S., & Rankin, W. J. (2007). Assessing the environmental impact of metal production processes. *Journal of Cleaner Production*, 15(8-9), 838-848.
- Panda, S., Akcil, A., Mishra, S., & Erust, C. (2017). Synergistic effect of biogenic Fe<sup>3+</sup> coupled to S<sup>0</sup> oxidation on simultaneous bioleaching of Cu, Co, Zn and As from hazardous Pyrite Ash Waste. *Journal of Hazardous Materials*, 325, 59-70.
- Perederiy, I., & Papangelakis, V. G. (2017). Why amorphous FeO-SiO<sub>2</sub> slags do not acid-leach at high temperatures. *Journal of Hazardous Materials*, 321, 737-744.
- Pérez-López, R., Álvarez-Valero, A. M., Nieto, J. M., Sáez, R., & Matos, J. X. (2008). Use of sequential extraction procedure for assessing the environmental impact at regional scale of the São Domingos Mine (Iberian Pyrite Belt). *Applied Geochemistry*, 23(12), 3452-3463.
- Qu, X., & Wu, C. J. (2005). One-factor-at-a-time designs of resolution V. *Journal of Statistical Planning and Inference*, 131(2), 407-416.
- Rao, J. S., & Kumar, B. (2012). 3D Blade root shape optimization. In *10<sup>th</sup> International conference on vibrations in rotating machinery* (pp. 173-188). Elsevier.
- Rastegar, S. O., Mousavi, S. M., Rezaei, M., & Shojaosadati, S. A. (2014). Statistical

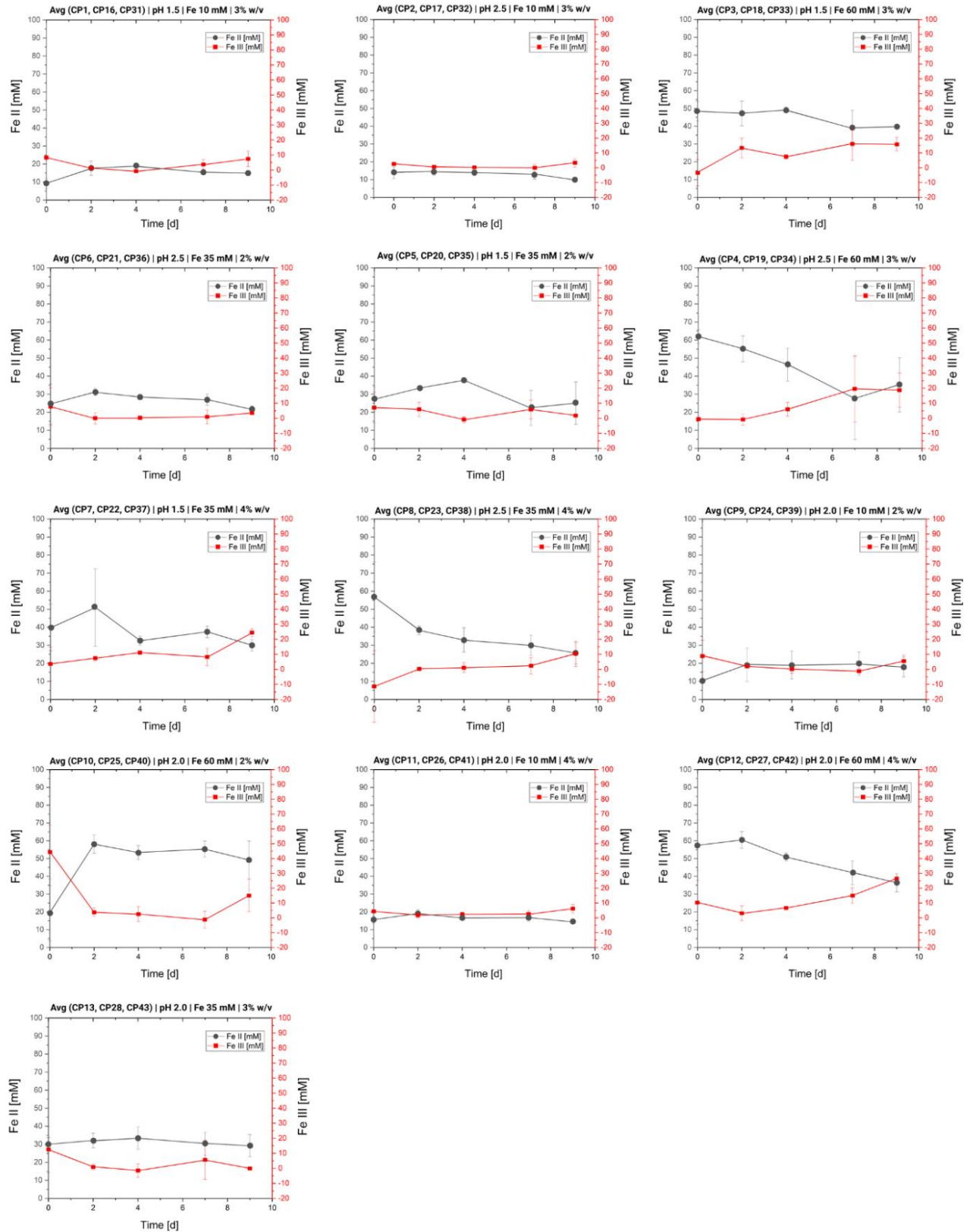
- evaluation and optimization of effective parameters in bioleaching of metals from molybdenite concentrate using *Acidianus brierleyi*. *Journal of Industrial and Engineering Chemistry*, 20(5), 3096-3101.
- Rawlings, D. E. (2005). Characteristics and adaptability of iron- and sulfur-oxidizing microorganisms used for the recovery of metals from minerals and their concentrates. *Microbial Cell Factories*, 4(1), 13.
- Reed, D. W., Fujita, Y., Daubaras, D. L., Jiao, Y., & Thompson, V. S. (2016). Bioleaching of rare earth elements from waste phosphors and cracking catalysts. *Hydrometallurgy*, 166, 34-40.
- Rodríguez, Y., Ballester, A., Blázquez, M. L., Gonzalez, F., & Muñoz, J. A. (2003). New information on the sphalerite bioleaching mechanism at low and high temperatures. *Hydrometallurgy*, 71(1-2), 57-66.
- Sand, W., Rohde, K., Sobotke, B., & Zenneck, C. (1992). Evaluation of *Leptospirillum ferrooxidans* for leaching. *Applied and environmental microbiology*, 58(1), 85-92.
- Schippers, A., & Sand, W. (1999). Bacterial leaching of metal sulfides proceeds by two indirect mechanisms: via thiosulfate or via polysulfides and sulfur. *Applied and environmental microbiology*, 65(1), 319-321.
- Schippers, A., Hedrich, S., Vasters, J., Drobe, M., Sand, W., & Willscher, S. (2013). Biomining: metal recovery from ores with microorganisms. *Geobiotechnology I: Metal-related Issues*, 1-47.
- Singh, S. P., & Schwan, A. L. (2011). Sulfur metabolism in plants and related biotechnologies. In M. Moo-Young (Ed.), *Comprehensive biotechnology* (2nd ed., pp. 257–271). Academic Press. <https://doi.org/10.1016/B978-0-08-088504-9.00268-3>.
- Spooren, J., Binnemans, K., Björkmalm, J., Breemers, K., Dams, Y., Folens, K., ... & Kinnunen, P. (2020). Near-zero-waste processing of low-grade, complex primary ores and secondary raw materials in Europe: technology development trends. *Resources, Conservation and Recycling*, 160, 104919.
- Tornos, F. (2006). Environment of formation and styles of volcanogenic massive sulfides: the Iberian Pyrite Belt. *Ore Geology Reviews*, 28, 259- 307. doi:10.1016/j.oregeorev.2004.12.005.
- Tranvik, E., Becker, M., Pålsson, B. I., Franzidis, J. P., & Bradshaw, D. (2017). Towards cleaner production—Using flotation to recover monazite from a heavy mineral sands zircon waste stream. *Minerals Engineering*, 101, 30-39.
- United Nations Environment Programme (UNEP). (2024). *Global Waste Management Outlook 2024: Beyond an age of waste – Turning rubbish into a resource*. Nairobi: United Nations Environment Programme.
- Vardanyan, N. S., & Akopyan, V. P. (2003). *Leptospirillum*-like bacteria and evaluation of their role in pyrite oxidation. *Microbiology*, 72, 438-442.
- Vaughan, D. J., & Lennie, A. R. (1991). The iron sulphide minerals: their chemistry and role in nature. *Science Progress (1933-)*, 371-388.
- Vera M, Schippers A, Sand W (2013a) Progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation-part A. *Applied Microbiology and Biotechnology*, 97(17), 7529–7541. <https://doi.org/10.1007/s00253-013-4954-2>
- Vera, M., Schippers, A., Hedrich, S., & Sand, W. (2022). Progress in bioleaching:

- fundamentals and mechanisms of microbial metal sulfide oxidation—part A. *Applied Microbiology and Biotechnology*, 106(21), 6933-6952.
- Wierzbicka-Wieczorek, M., Lottermoser, B. G., Kiefer, S., Sindern, S., Gronen, L., & Hensler, A. S. (2019). Indium distribution in metalliferous mine wastes of the Iberian Pyrite Belt, Spain–Portugal. *Environmental Earth Sciences*, 78, 1-14.
- XTRACT Project. (2023). *Pilot 3: São Domingos – Portugal*. Retrieved July, 2025, from <https://xtract-project.eu/pilot-3-sao-domingos-portugal/>
- Xu, T. J., & Ting, Y. P. (2004). Optimisation on bioleaching of incinerator fly ash by *Aspergillus niger*—use of central composite design. *Enzyme and microbial technology*, 35(5), 444-454.
- Yang, L., Tang, Y., Cao, D., Yang, M., He, Y., & Lu, C. (2022). Microscopic properties and neutralization mechanism of steel slag in acid mine drainage (AMD) remediation process.
- Yu, J. Y., McGenity, T. J., & Coleman, M. L. (2001). Solution chemistry during the lag phase and exponential phase of pyrite oxidation by *Thiobacillus ferrooxidans*. *Chemical Geology*, 175(3-4), 307-317.

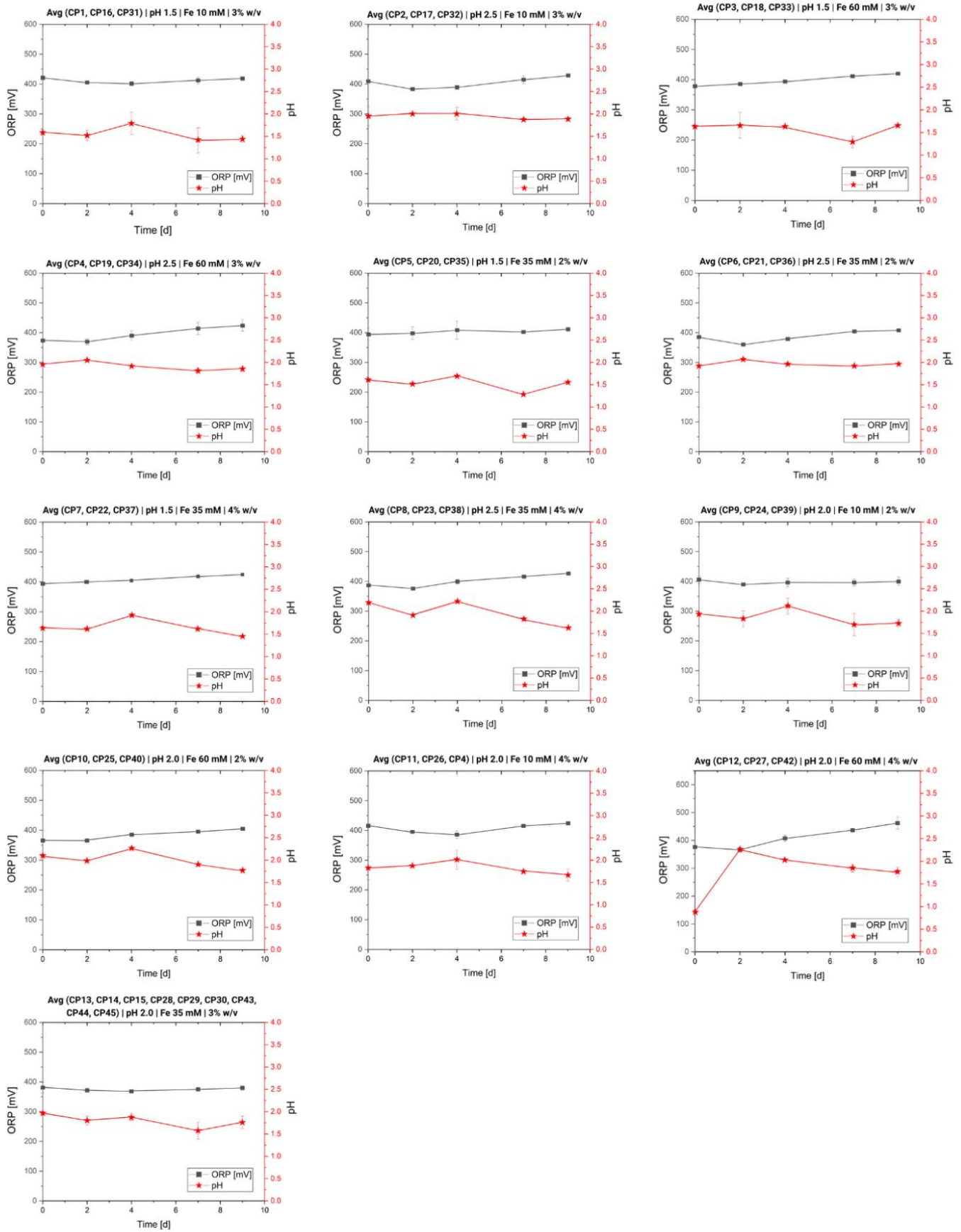
## Appendix

### Annex – A1

#### *Ferrous versus Ferric Iron for CP tests*

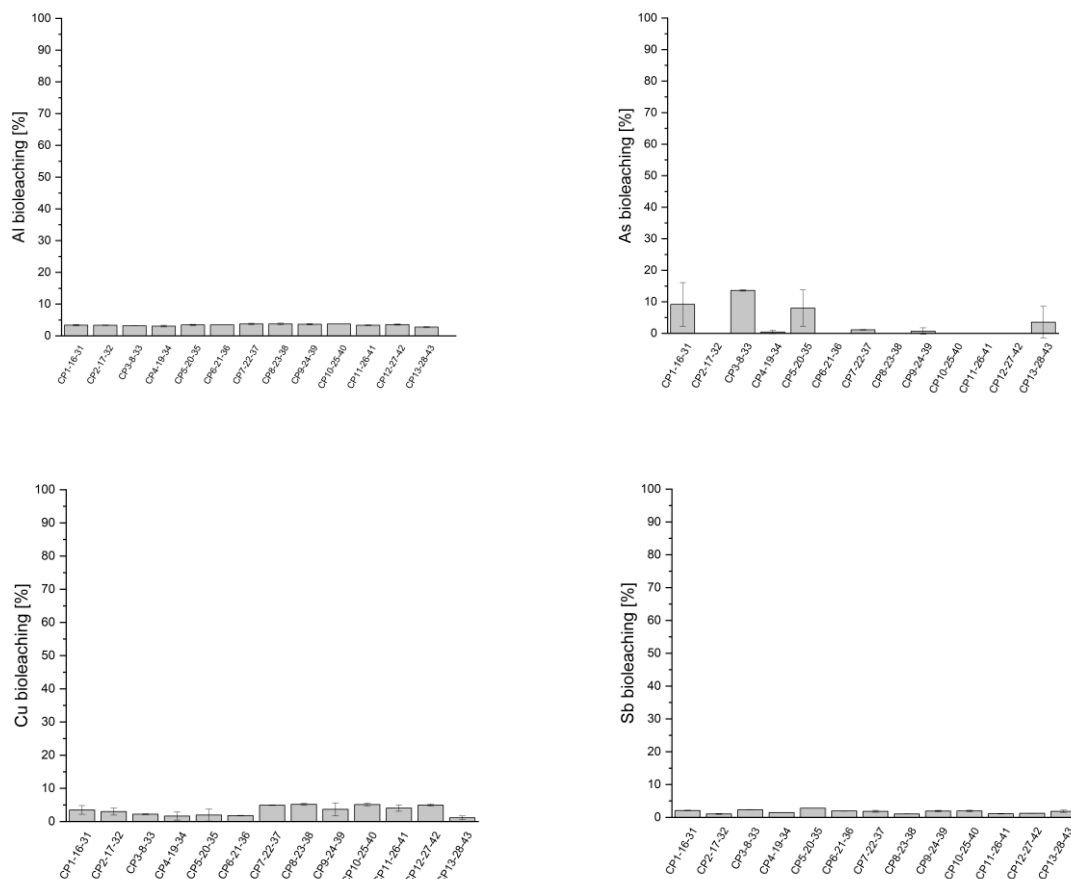


# *ORP versus pH for CP tests*



## Annex – A2

### *Bioleaching Efficiency of Metals (%) – day 9 for CP tests*



## Annex – A3

### *Response Surface Regression: Al (%) versus pH, [Fe II] (mM), Pulp density (%) for CP tests*

#### Coded Coefficients

| Term                              | Coef    | SE Coef | T-Value | P-Value | VIF  |
|-----------------------------------|---------|---------|---------|---------|------|
| Constant                          | 2.7433  | 0.0799  | 34.32   | 0.000   |      |
| pH                                | -0.0179 | 0.0489  | -0.37   | 0.717   | 1.00 |
| [Fe II] (mM)                      | -0.0204 | 0.0489  | -0.42   | 0.679   | 1.00 |
| Pulp density (%)                  | 0.0025  | 0.0489  | 0.05    | 0.960   | 1.00 |
| pH*pH                             | 0.2533  | 0.0720  | 3.52    | 0.001   | 1.01 |
| [Fe II] (mM)*[Fe II] (mM)         | 0.2367  | 0.0720  | 3.28    | 0.002   | 1.01 |
| Pulp density (%)*Pulp density (%) | 0.5892  | 0.0720  | 8.18    | 0.000   | 1.01 |
| pH*[Fe II] (mM)                   | -0.0217 | 0.0692  | -0.31   | 0.756   | 1.00 |
| pH*Pulp density (%)               | 0.0008  | 0.0692  | 0.01    | 0.990   | 1.00 |
| [Fe II] (mM)*Pulp density (%)     | 0.0242  | 0.0692  | 0.35    | 0.729   | 1.00 |

#### Model Summary

**S**      **R-sq**   **R-sq(adj)** **R-sq(pred)**  
 0.239782 70.05% 62.35% 48.36%

#### Analysis of Variance

| Source                            | DF | Adj SS  | Adj MS  | F-Value | P-Value |
|-----------------------------------|----|---------|---------|---------|---------|
| Model                             | 9  | 4.70650 | 0.52294 | 9.10    | 0.000   |
| Linear                            | 3  | 0.01786 | 0.00595 | 0.10    | 0.957   |
| pH                                | 1  | 0.00770 | 0.00770 | 0.13    | 0.717   |
| [Fe II] (mM)                      | 1  | 0.01000 | 0.01000 | 0.17    | 0.679   |
| Pulp density (%)                  | 1  | 0.00015 | 0.00015 | 0.00    | 0.960   |
| Square                            | 3  | 4.67599 | 1.55866 | 27.11   | 0.000   |
| pH*pH                             | 1  | 0.71089 | 0.71089 | 12.36   | 0.001   |
| [Fe II] (mM)*[Fe II] (mM)         | 1  | 0.62043 | 0.62043 | 10.79   | 0.002   |
| Pulp density (%)*Pulp density (%) | 1  | 3.84499 | 3.84499 | 66.87   | 0.000   |
| 2-Way Interaction                 | 3  | 0.01265 | 0.00422 | 0.07    | 0.974   |
| pH*[Fe II] (mM)                   | 1  | 0.00563 | 0.00563 | 0.10    | 0.756   |
| pH*Pulp density (%)               | 1  | 0.00001 | 0.00001 | 0.00    | 0.990   |
| [Fe II] (mM)*Pulp density (%)     | 1  | 0.00701 | 0.00701 | 0.12    | 0.729   |
| Error                             | 35 | 2.01234 | 0.05750 |         |         |
| Lack-of-Fit                       | 3  | 0.63327 | 0.21109 | 4.90    | 0.007   |
| Pure Error                        | 32 | 1.37907 | 0.04310 |         |         |
| Total                             | 44 | 6.71884 |         |         |         |

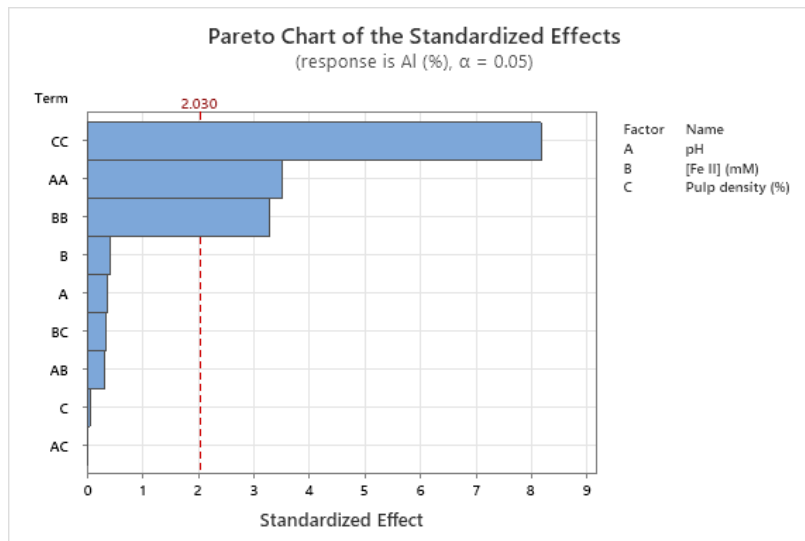
#### Regression Equation in Uncoded Units

Al (%) = 12.65 - 4.03 pH - 0.0268 [Fe II] (mM) - 3.570 Pulp density (%) + 1.013 pH\*pH  
 + 0.000379 [Fe II] (mM)\*[Fe II] (mM) + 0.5892 Pulp density (%)\*Pulp density (%)  
 - 0.00173 pH\*[Fe II] (mM) + 0.002 pH\*Pulp density (%)  
 + 0.00097 [Fe II] (mM)\*Pulp density (%)

#### Fits and Diagnostics for Unusual Observations

| Obs | Al (%) | Fit    | Resid   | Std Resid |   |
|-----|--------|--------|---------|-----------|---|
| 4   | 2.6700 | 3.1733 | -0.5033 | -2.42     | R |
| 38  | 4.1000 | 3.5712 | 0.5288  | 2.55      | R |

R Large residual



*Response Surface Regression: As (%) versus pH, [Fe II] (mM), Pulp density (%) for CP tests*

**Coded Coefficients**

| Term                              | Coef   | SE Coef | T-Value | P-Value | VIF  |
|-----------------------------------|--------|---------|---------|---------|------|
| Constant                          | 3.54   | 1.34    | 2.65    | 0.012   |      |
| pH                                | -3.935 | 0.818   | -4.81   | 0.000   | 1.00 |
| [Fe II] (mM)                      | 0.513  | 0.818   | 0.63    | 0.535   | 1.00 |
| Pulp density (%)                  | -0.950 | 0.818   | -1.16   | 0.253   | 1.00 |
| pH*pH                             | 2.18   | 1.20    | 1.81    | 0.079   | 1.01 |
| [Fe II] (mM)*[Fe II] (mM)         | 0.08   | 1.20    | 0.07    | 0.946   | 1.01 |
| Pulp density (%)*Pulp density (%) | -3.44  | 1.20    | -2.85   | 0.007   | 1.01 |
| pH*[Fe II] (mM)                   | -1.00  | 1.16    | -0.86   | 0.395   | 1.00 |
| pH*Pulp density (%)               | 1.72   | 1.16    | 1.49    | 0.146   | 1.00 |
| [Fe II] (mM)*Pulp density (%)     | 0.18   | 1.16    | 0.16    | 0.877   | 1.00 |

**Model Summary**

| S       | R-sq   | R-sq(adj) | R-sq(pred) |
|---------|--------|-----------|------------|
| 4.00897 | 53.43% | 41.45%    | 26.75%     |

**Analysis of Variance**

| Source                            | DF | Adj SS  | Adj MS  | F-Value | P-Value |
|-----------------------------------|----|---------|---------|---------|---------|
| Model                             | 9  | 645.33  | 71.703  | 4.46    | 0.001   |
| Linear                            | 3  | 399.61  | 133.205 | 8.29    | 0.000   |
| pH                                | 1  | 371.62  | 371.621 | 23.12   | 0.000   |
| [Fe II] (mM)                      | 1  | 6.31    | 6.314   | 0.39    | 0.535   |
| Pulp density (%)                  | 1  | 21.68   | 21.679  | 1.35    | 0.253   |
| Square                            | 3  | 197.90  | 65.968  | 4.10    | 0.013   |
| pH*pH                             | 1  | 52.70   | 52.702  | 3.28    | 0.079   |
| [Fe II] (mM)*[Fe II] (mM)         | 1  | 0.07    | 0.075   | 0.00    | 0.946   |
| Pulp density (%)*Pulp density (%) | 1  | 130.92  | 130.921 | 8.15    | 0.007   |
| 2-Way Interaction                 | 3  | 47.81   | 15.938  | 0.99    | 0.408   |
| pH*[Fe II] (mM)                   | 1  | 11.92   | 11.920  | 0.74    | 0.395   |
| pH*Pulp density (%)               | 1  | 35.50   | 35.501  | 2.21    | 0.146   |
| [Fe II] (mM)*Pulp density (%)     | 1  | 0.39    | 0.392   | 0.02    | 0.877   |
| Error                             | 35 | 562.51  | 16.072  |         |         |
| Lack-of-Fit                       | 3  | 91.50   | 30.501  | 2.07    | 0.123   |
| Pure Error                        | 32 | 471.01  | 14.719  |         |         |
| Total                             | 44 | 1207.84 |         |         |         |

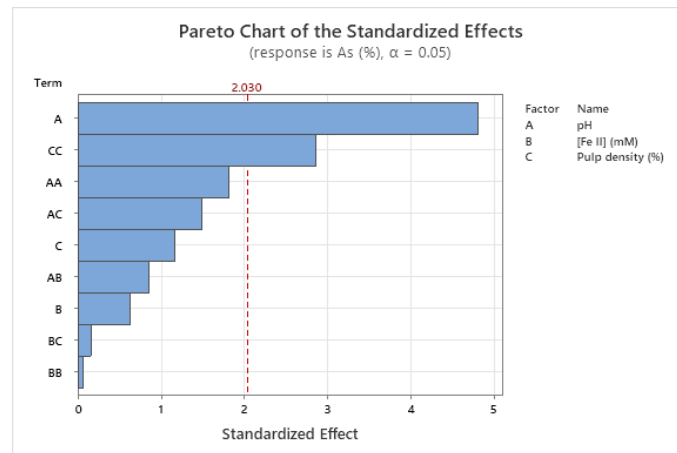
**Regression Equation in Uncoded Units**

As (%) = 41.3 - 50.3 pH + 0.149 [Fe II] (mM) + 12.54 Pulp density (%) + 8.72 pH\*pH  
+ 0.00013 [Fe II] (mM)\*[Fe II] (mM) - 3.44 Pulp density (%)\*Pulp density (%)  
- 0.0797 pH\*[Fe II] (mM) + 3.44 pH\*Pulp density (%)  
+ 0.0072 [Fe II] (mM)\*Pulp density (%)

**Fits and Diagnostics for Unusual Observations**

| Obs | As (%) | Fit  | Resid | Std Resid |   |
|-----|--------|------|-------|-----------|---|
| 31  | 18.94  | 8.23 | 10.71 | 3.09      | R |
| 35  | 0.00   | 8.89 | -8.89 | -2.56     | R |

R Large residual



*Response Surface Regression: Cu (%) versus pH, [Fe II] (mM), Pulp density (%) for CP tests*

#### Coded Coefficients

| Term                              | Coef   | SE Coef | T-Value | P-Value | VIF  |
|-----------------------------------|--------|---------|---------|---------|------|
| Constant                          | 1.117  | 0.454   | 2.46    | 0.019   |      |
| pH                                | -0.123 | 0.278   | -0.44   | 0.662   | 1.00 |
| [Fe II] (mM)                      | -0.036 | 0.278   | -0.13   | 0.898   | 1.00 |
| Pulp density (%)                  | 0.820  | 0.278   | 2.95    | 0.006   | 1.00 |
| pH*pH                             | 0.236  | 0.410   | 0.58    | 0.568   | 1.01 |
| [Fe II] (mM)*[Fe II] (mM)         | 1.234  | 0.410   | 3.01    | 0.005   | 1.01 |
| Pulp density (%)*Pulp density (%) | 2.102  | 0.410   | 5.13    | 0.000   | 1.01 |
| pH*[Fe II] (mM)                   | -0.020 | 0.393   | -0.05   | 0.960   | 1.00 |
| pH*Pulp density (%)               | 0.103  | 0.393   | 0.26    | 0.794   | 1.00 |
| [Fe II] (mM)*Pulp density (%)     | -0.135 | 0.393   | -0.34   | 0.734   | 1.00 |

#### Model Summary

| S       | R-sq   | R-sq(adj) | R-sq(pred) |
|---------|--------|-----------|------------|
| 1.36310 | 54.74% | 43.11%    | 20.47%     |

#### Analysis of Variance

| Source                            | DF | Adj SS  | Adj MS  | F-Value | P-Value |
|-----------------------------------|----|---------|---------|---------|---------|
| Model                             | 9  | 78.661  | 8.7402  | 4.70    | 0.000   |
| Linear                            | 3  | 16.529  | 5.5095  | 2.97    | 0.045   |
| pH                                | 1  | 0.360   | 0.3601  | 0.19    | 0.662   |
| [Fe II] (mM)                      | 1  | 0.031   | 0.0308  | 0.02    | 0.898   |
| Pulp density (%)                  | 1  | 16.138  | 16.1376 | 8.69    | 0.006   |
| Square                            | 3  | 61.781  | 20.5938 | 11.08   | 0.000   |
| pH*pH                             | 1  | 0.616   | 0.6161  | 0.33    | 0.568   |
| [Fe II] (mM)*[Fe II] (mM)         | 1  | 16.872  | 16.8720 | 9.08    | 0.005   |
| Pulp density (%)*Pulp density (%) | 1  | 48.966  | 48.9656 | 26.35   | 0.000   |
| 2-Way Interaction                 | 3  | 0.352   | 0.1172  | 0.06    | 0.979   |
| pH*[Fe II] (mM)                   | 1  | 0.005   | 0.0048  | 0.00    | 0.960   |
| pH*Pulp density (%)               | 1  | 0.128   | 0.1281  | 0.07    | 0.794   |
| [Fe II] (mM)*Pulp density (%)     | 1  | 0.219   | 0.2187  | 0.12    | 0.734   |
| Error                             | 35 | 65.032  | 1.8581  |         |         |
| Lack-of-Fit                       | 3  | 24.450  | 8.1501  | 6.43    | 0.002   |
| Pure Error                        | 32 | 40.581  | 1.2682  |         |         |
| Total                             | 44 | 143.693 |         |         |         |

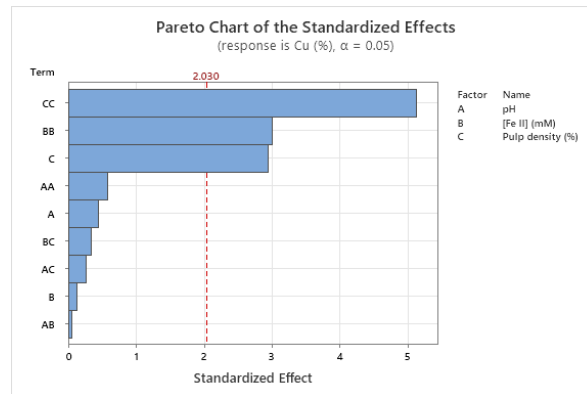
#### Regression Equation in Uncoded Units

$$\begin{aligned}\text{Cu (\%)} = & 24.87 - 4.58 \text{ pH} - 0.1203 [\text{Fe II}] (\text{mM}) - 12.02 \text{ Pulp density (\%)} + 0.94 \text{ pH} \cdot \text{pH} \\ & + 0.001975 [\text{Fe II}] (\text{mM}) \cdot [\text{Fe II}] (\text{mM}) + 2.102 \text{ Pulp density (\%)} \cdot \text{Pulp density (\%)} \\ & - 0.0016 \text{ pH} \cdot [\text{Fe II}] (\text{mM}) + 0.207 \text{ pH} \cdot \text{Pulp density (\%)} \\ & - 0.0054 [\text{Fe II}] (\text{mM}) \cdot \text{Pulp density (\%)}\end{aligned}$$

#### Fits and Diagnostics for Unusual Observations

| Obs | Cu (%) | Fit   | Resid  | Std Resid |   |
|-----|--------|-------|--------|-----------|---|
| 4   | 0.000  | 2.408 | -2.408 | -2.04     | R |
| 9   | 0.970  | 3.534 | -2.564 | -2.17     | R |
| 11  | 2.720  | 5.444 | -2.724 | -2.31     | R |

R Large residual



Response Surface Regression: Mn (%) versus pH, [Fe II] (mM), Pulp density (%) for CP tests

#### Coded Coefficients

| Term                              | Coef   | SE Coef | T-Value | P-Value | VIF  |
|-----------------------------------|--------|---------|---------|---------|------|
| Constant                          | 48.07  | 6.80    | 7.07    | 0.000   |      |
| pH                                | -2.24  | 4.16    | -0.54   | 0.595   | 1.00 |
| [Fe II] (mM)                      | 21.36  | 4.16    | 5.13    | 0.000   | 1.00 |
| Pulp density (%)                  | -17.76 | 4.16    | -4.26   | 0.000   | 1.00 |
| pH*pH                             | -5.07  | 6.13    | -0.83   | 0.414   | 1.01 |
| [Fe II] (mM)*[Fe II] (mM)         | 5.95   | 6.13    | 0.97    | 0.338   | 1.01 |
| Pulp density (%)*Pulp density (%) | 17.07  | 6.13    | 2.78    | 0.009   | 1.01 |
| pH*[Fe II] (mM)                   | -0.27  | 5.89    | -0.04   | 0.964   | 1.00 |
| pH*Pulp density (%)               | 3.30   | 5.89    | 0.56    | 0.578   | 1.00 |
| [Fe II] (mM)*Pulp density (%)     | 5.58   | 5.89    | 0.95    | 0.350   | 1.00 |

#### Model Summary

| S       | R-sq   | R-sq(adj) | R-sq(pred) |
|---------|--------|-----------|------------|
| 20.4034 | 61.34% | 51.40%    | 31.33%     |

#### Analysis of Variance

| Source                            | DF | Adj SS  | Adj MS  | F-Value | P-Value |
|-----------------------------------|----|---------|---------|---------|---------|
| Model                             | 9  | 23117.8 | 2568.6  | 6.17    | 0.000   |
| Linear                            | 3  | 18641.5 | 6213.8  | 14.93   | 0.000   |
| pH                                | 1  | 120.2   | 120.2   | 0.29    | 0.595   |
| [Fe II] (mM)                      | 1  | 10953.8 | 10953.8 | 26.31   | 0.000   |
| Pulp density (%)                  | 1  | 7567.5  | 7567.5  | 18.18   | 0.000   |
| Square                            | 3  | 3971.3  | 1323.8  | 3.18    | 0.036   |
| pH*pH                             | 1  | 284.3   | 284.3   | 0.68    | 0.414   |
| [Fe II] (mM)*[Fe II] (mM)         | 1  | 392.8   | 392.8   | 0.94    | 0.338   |
| Pulp density (%)*Pulp density (%) | 1  | 3226.9  | 3226.9  | 7.75    | 0.009   |
| 2-Way Interaction                 | 3  | 505.0   | 168.3   | 0.40    | 0.751   |

|                               |    |         |       |      |       |
|-------------------------------|----|---------|-------|------|-------|
| pH*[Fe II] (mM)               | 1  | 0.8     | 0.8   | 0.00 | 0.964 |
| pH*Pulp density (%)           | 1  | 131.1   | 131.1 | 0.31 | 0.578 |
| [Fe II] (mM)*Pulp density (%) | 1  | 373.1   | 373.1 | 0.90 | 0.350 |
| Error                         | 35 | 14570.5 | 416.3 |      |       |
| Lack-of-Fit                   | 3  | 1176.1  | 392.0 | 0.94 | 0.434 |
| Pure Error                    | 32 | 13394.4 | 418.6 |      |       |
| Total                         | 44 | 37688.3 |       |      |       |

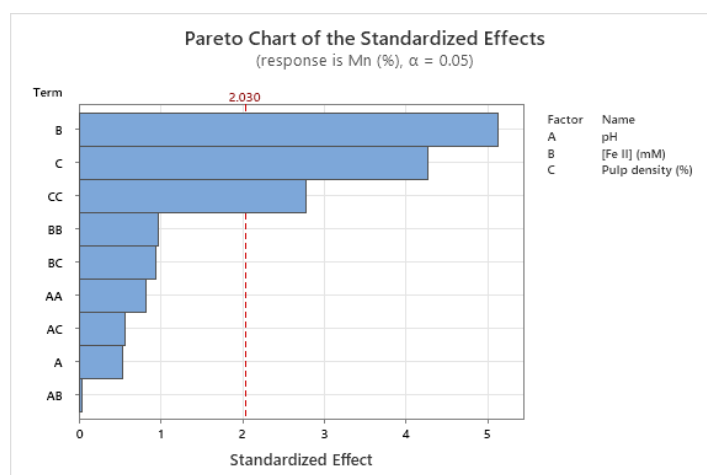
#### Regression Equation in Uncoded Units

$$\begin{aligned} \text{Mn (\%)} = & 226 + 57 \text{ pH} - 0.44 [\text{Fe II}] (\text{mM}) - 141.2 \text{ Pulp density (\%)} - 20.3 \text{ pH} \cdot \text{pH} \\ & + 0.00953 [\text{Fe II}] (\text{mM}) \cdot [\text{Fe II}] (\text{mM}) + 17.07 \text{ Pulp density (\%)} \cdot \text{Pulp density (\%)} \\ & - 0.021 \text{ pH} \cdot [\text{Fe II}] (\text{mM}) + 6.6 \text{ pH} \cdot \text{Pulp density (\%)} \\ & + 0.223 [\text{Fe II}] (\text{mM}) \cdot \text{Pulp density (\%)} \end{aligned}$$

#### Fits and Diagnostics for Unusual Observations

| Obs | Mn (%) | Fit    | Resid  | Std Resid |   |
|-----|--------|--------|--------|-----------|---|
| 9   | 32.01  | 73.06  | -41.05 | -2.32     | R |
| 10  | 42.43  | 104.63 | -62.20 | -3.52     | R |
| 25  | 141.12 | 104.63 | 36.49  | 2.06      | R |
| 39  | 140.66 | 73.06  | 67.60  | 3.83      | R |
| 40  | 143.89 | 104.63 | 39.26  | 2.22      | R |

R Large residual



*Response Surface Regression: Sb (%) versus pH, [Fe II] (mM), Pulp density (%) for CP tests*

#### Coded Coefficients

| Term                              | Coef    | SE Coef | T-Value | P-Value | VIF  |
|-----------------------------------|---------|---------|---------|---------|------|
| Constant                          | 1.8500  | 0.0817  | 22.63   | 0.000   |      |
| pH                                | -0.4483 | 0.0501  | -8.96   | 0.000   | 1.00 |
| [Fe II] (mM)                      | 0.1033  | 0.0501  | 2.06    | 0.046   | 1.00 |
| Pulp density (%)                  | -0.4200 | 0.0501  | -8.39   | 0.000   | 1.00 |
| pH*pH                             | 0.1229  | 0.0737  | 1.67    | 0.104   | 1.01 |
| [Fe II] (mM)*[Fe II] (mM)         | -0.2371 | 0.0737  | -3.22   | 0.003   | 1.01 |
| Pulp density (%)*Pulp density (%) | -0.0587 | 0.0737  | -0.80   | 0.431   | 1.01 |
| pH*[Fe II] (mM)                   | 0.0508  | 0.0708  | 0.72    | 0.478   | 1.00 |
| pH*Pulp density (%)               | 0.0208  | 0.0708  | 0.29    | 0.770   | 1.00 |
| [Fe II] (mM)*Pulp density (%)     | 0.0242  | 0.0708  | 0.34    | 0.735   | 1.00 |

#### Model Summary

| S | R-sq | R-sq(adj) | R-sq(pred) |
|---|------|-----------|------------|
|---|------|-----------|------------|

0.245247 82.93% 78.55% 74.33%

### Analysis of Variance

| Source                            | DF | Adj SS  | Adj MS  | F-Value | P-Value |
|-----------------------------------|----|---------|---------|---------|---------|
| Model                             | 9  | 10.2305 | 1.13672 | 18.90   | 0.000   |
| Linear                            | 3  | 9.3139  | 3.10464 | 51.62   | 0.000   |
| pH                                | 1  | 4.8241  | 4.82407 | 80.21   | 0.000   |
| [Fe II] (mM)                      | 1  | 0.2563  | 0.25627 | 4.26    | 0.046   |
| Pulp density (%)                  | 1  | 4.2336  | 4.23360 | 70.39   | 0.000   |
| Square                            | 3  | 0.8733  | 0.29110 | 4.84    | 0.006   |
| pH*pH                             | 1  | 0.1674  | 0.16736 | 2.78    | 0.104   |
| [Fe II] (mM)*[Fe II] (mM)         | 1  | 0.6226  | 0.62262 | 10.35   | 0.003   |
| Pulp density (%)*Pulp density (%) | 1  | 0.0382  | 0.03823 | 0.64    | 0.431   |
| 2-Way Interaction                 | 3  | 0.0432  | 0.01441 | 0.24    | 0.868   |
| pH*[Fe II] (mM)                   | 1  | 0.0310  | 0.03101 | 0.52    | 0.478   |
| pH*Pulp density (%)               | 1  | 0.0052  | 0.00521 | 0.09    | 0.770   |
| [Fe II] (mM)*Pulp density (%)     | 1  | 0.0070  | 0.00701 | 0.12    | 0.735   |
| Error                             | 35 | 2.1051  | 0.06015 |         |         |
| Lack-of-Fit                       | 3  | 0.1618  | 0.05393 | 0.89    | 0.458   |
| Pure Error                        | 32 | 1.9433  | 0.06073 |         |         |
| Total                             | 44 | 12.3356 |         |         |         |

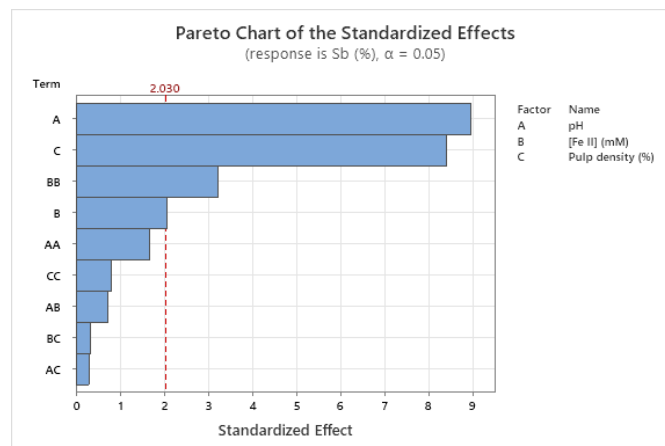
### Regression Equation in Uncoded Units

Sb (%) = 6.37 - 3.13 pH + 0.0197 [Fe II] (mM) - 0.185 Pulp density (%) + 0.492 pH\*pH  
 - 0.000379 [Fe II] (mM)\*[Fe II] (mM) - 0.0587 Pulp density (%)\*Pulp density (%)  
 + 0.00407 pH\*[Fe II] (mM) + 0.042 pH\*Pulp density (%)  
 + 0.00097 [Fe II] (mM)\*Pulp density (%)

### Fits and Diagnostics for Unusual Observations

| Obs | Sb (%) | Fit   | Resid | Std Resid |   |
|-----|--------|-------|-------|-----------|---|
| 13  | 2.350  | 1.850 | 0.500 | 2.16      | R |
| 14  | 2.350  | 1.850 | 0.500 | 2.16      | R |
| 15  | 2.350  | 1.850 | 0.500 | 2.16      | R |

R Large residual



Response Surface Regression: Zn (%) versus pH, [Fe II] (mM), Pulp density (%) for CP tests

### Coded Coefficients

| Term | Coef | SE Coef | T-Value | P-Value | VIF |
|------|------|---------|---------|---------|-----|
|------|------|---------|---------|---------|-----|

|                                   |        |       |       |       |      |
|-----------------------------------|--------|-------|-------|-------|------|
| Constant                          | 21.56  | 1.55  | 13.91 | 0.000 |      |
| pH                                | -0.102 | 0.949 | -0.11 | 0.915 | 1.00 |
| [Fe II] (mM)                      | -0.382 | 0.949 | -0.40 | 0.689 | 1.00 |
| Pulp density (%)                  | -3.751 | 0.949 | -3.95 | 0.000 | 1.00 |
| pH*pH                             | -1.52  | 1.40  | -1.08 | 0.285 | 1.01 |
| [Fe II] (mM)*[Fe II] (mM)         | -3.98  | 1.40  | -2.85 | 0.007 | 1.01 |
| Pulp density (%)*Pulp density (%) | -3.81  | 1.40  | -2.73 | 0.010 | 1.01 |
| pH*[Fe II] (mM)                   | -1.50  | 1.34  | -1.12 | 0.271 | 1.00 |
| pH*Pulp density (%)               | -0.27  | 1.34  | -0.20 | 0.839 | 1.00 |
| [Fe II] (mM)*Pulp density (%)     | 0.43   | 1.34  | 0.32  | 0.752 | 1.00 |

**Model Summary**

| S | R-sq | R-sq(adj) | R-sq(pred) |
|---|------|-----------|------------|
|---|------|-----------|------------|

|         |        |        |        |
|---------|--------|--------|--------|
| 4.65062 | 47.84% | 34.43% | 21.31% |
|---------|--------|--------|--------|

**Analysis of Variance**

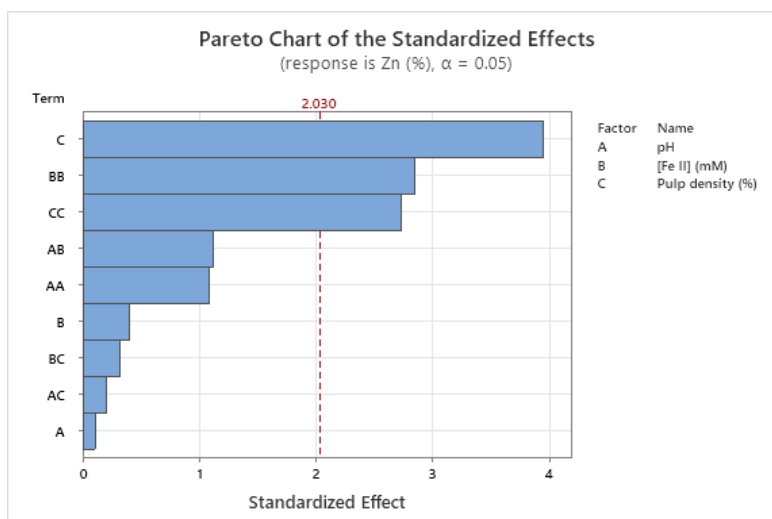
| Source                            | DF | Adj SS  | Adj MS  | F-Value | P-Value |
|-----------------------------------|----|---------|---------|---------|---------|
| Model                             | 9  | 694.41  | 77.157  | 3.57    | 0.003   |
| Linear                            | 3  | 341.41  | 113.803 | 5.26    | 0.004   |
| pH                                | 1  | 0.25    | 0.248   | 0.01    | 0.915   |
| [Fe II] (mM)                      | 1  | 3.51    | 3.511   | 0.16    | 0.689   |
| Pulp density (%)                  | 1  | 337.65  | 337.650 | 15.61   | 0.000   |
| Square                            | 3  | 322.83  | 107.610 | 4.98    | 0.006   |
| pH*pH                             | 1  | 25.45   | 25.452  | 1.18    | 0.285   |
| [Fe II] (mM)*[Fe II] (mM)         | 1  | 175.54  | 175.536 | 8.12    | 0.007   |
| Pulp density (%)*Pulp density (%) | 1  | 161.00  | 161.005 | 7.44    | 0.010   |
| 2-Way Interaction                 | 3  | 30.17   | 10.056  | 0.46    | 0.709   |
| pH*[Fe II] (mM)                   | 1  | 27.06   | 27.060  | 1.25    | 0.271   |
| pH*Pulp density (%)               | 1  | 0.91    | 0.907   | 0.04    | 0.839   |
| [Fe II] (mM)*Pulp density (%)     | 1  | 2.20    | 2.202   | 0.10    | 0.752   |
| Error                             | 35 | 756.99  | 21.628  |         |         |
| Lack-of-Fit                       | 3  | 87.09   | 29.028  | 1.39    | 0.265   |
| Pure Error                        | 32 | 669.90  | 20.935  |         |         |
| Total                             | 44 | 1451.40 |         |         |         |

**Regression Equation in Uncoded Units**

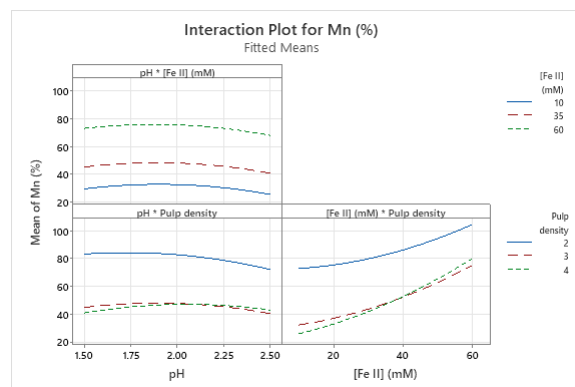
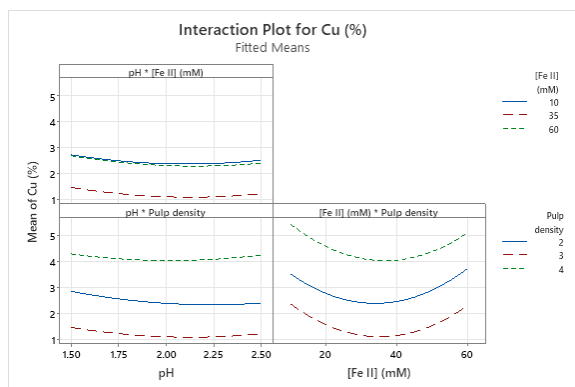
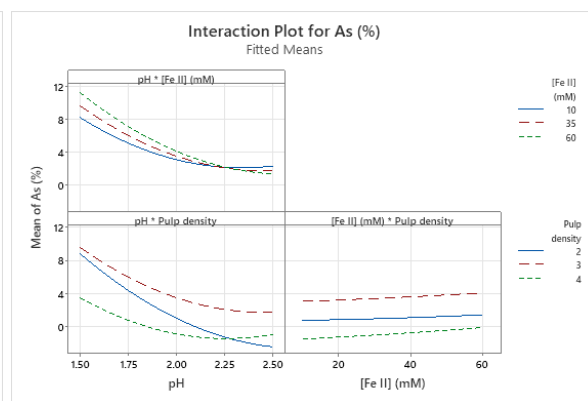
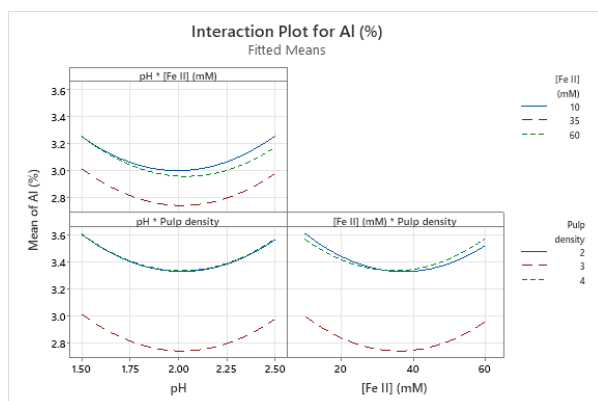
$$\begin{aligned} \text{Zn (\%)} = & -42.5 + 29.9 \text{ pH} + 0.619 [\text{Fe II}] (\text{mM}) + 19.6 \text{ Pulp density (\%)} - 6.06 \text{ pH}^2 \\ & - 0.00637 [\text{Fe II}] (\text{mM})^2 - 3.81 \text{ Pulp density (\%)}^2 \\ & - 0.120 \text{ pH}^2 [\text{Fe II}] (\text{mM}) - 0.55 \text{ pH}^2 \text{ Pulp density (\%)} \\ & + 0.0171 [\text{Fe II}] (\text{mM}) \text{ Pulp density (\%)} \end{aligned}$$
**Fits and Diagnostics for Unusual Observations**

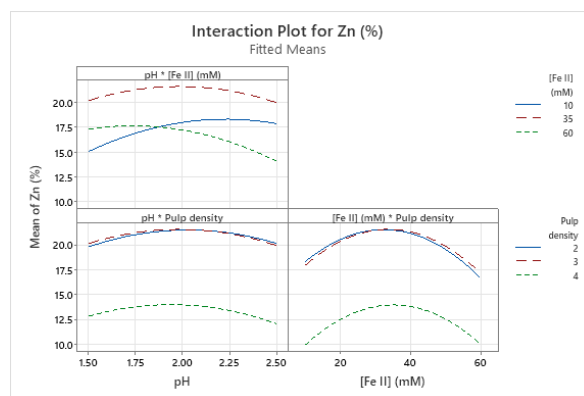
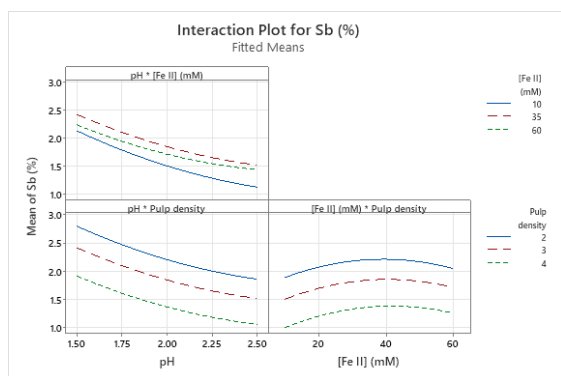
| Obs | Zn (%) | Fit   | Resid | Std Resid |   |
|-----|--------|-------|-------|-----------|---|
| 13  | 30.96  | 21.56 | 9.40  | 2.14      | R |
| 14  | 30.96  | 21.56 | 9.40  | 2.14      | R |
| 15  | 30.96  | 21.56 | 9.40  | 2.14      | R |

R Large residual



### Interaction plots for CP





## Annex – A4

*Response Optimization for CP: Al (%), Zn (%), Sb (%), Cu (%), As (%), Mn (%)*

### Parameters

| Response | Goal    | Lower Target | Upper Target | Weight | Importance |
|----------|---------|--------------|--------------|--------|------------|
| Al (%)   | Maximum | 2.53         | 4.10         | 1      | 1          |
| Zn (%)   | Maximum | 8.62         | 30.96        | 1      | 1          |
| Sb (%)   | Maximum | 0.84         | 2.89         | 1      | 1          |
| Cu (%)   | Maximum | 0.00         | 5.64         | 1      | 1          |
| As (%)   | Minimum | 0.00         | 18.94        | 1      | 1          |
| Mn (%)   | Target  | 17.82        | 100.00       | 143.89 | 1          |

### Solution

| Solution | pH      | [Fe II] (mM) | Pulp density |         |         |         |         |
|----------|---------|--------------|--------------|---------|---------|---------|---------|
|          |         |              | (%)          | Fit     | Fit     | Fit     | Fit     |
| 1        | 1.66162 | 53.9394      | 2            | 3.57188 | 18.5577 | 2.48291 | 3.45371 |

### Mn (%) Composite

| Solution | Fit     | Desirability |
|----------|---------|--------------|
| 1        | 99.8361 | 0.673766     |

### Multiple Response Prediction

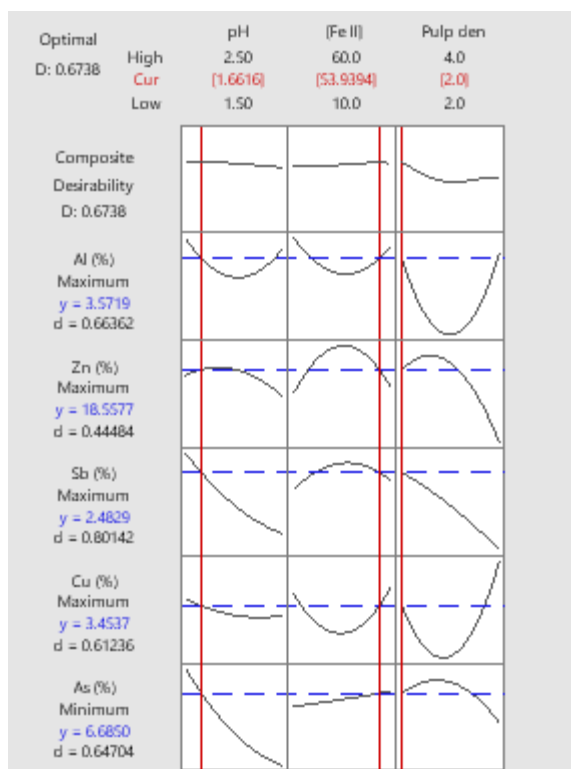
| Variable | Setting |
|----------|---------|
|----------|---------|

pH 1.66162

[Fe II] (mM) 53.9394

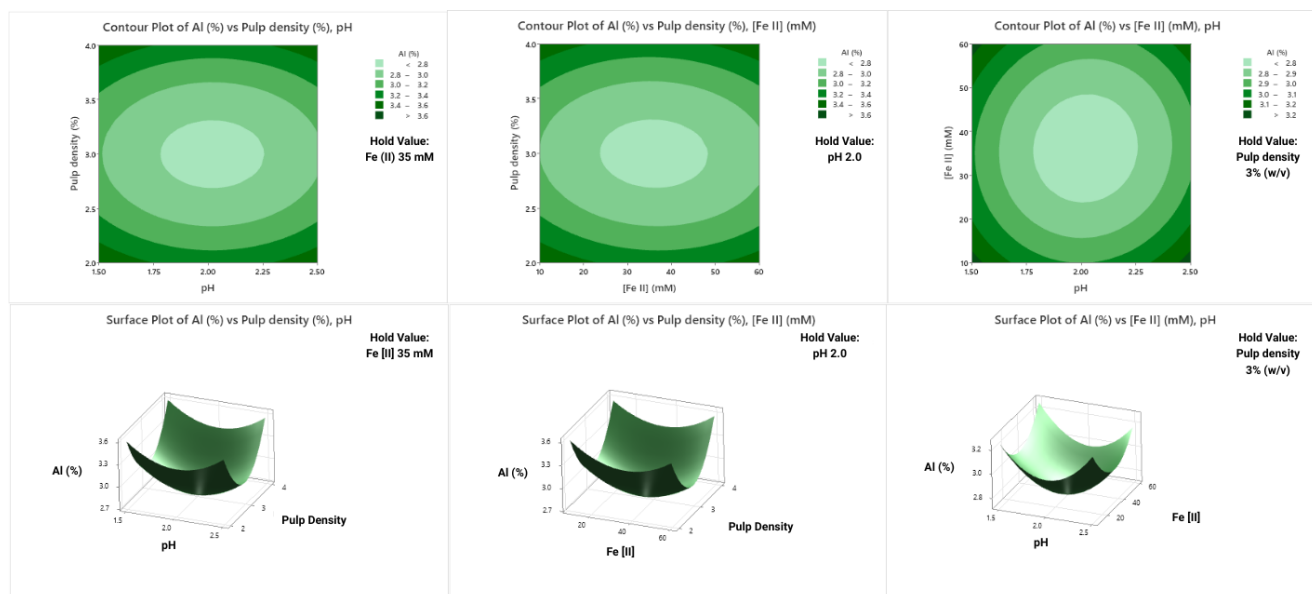
Pulp density (%) 2

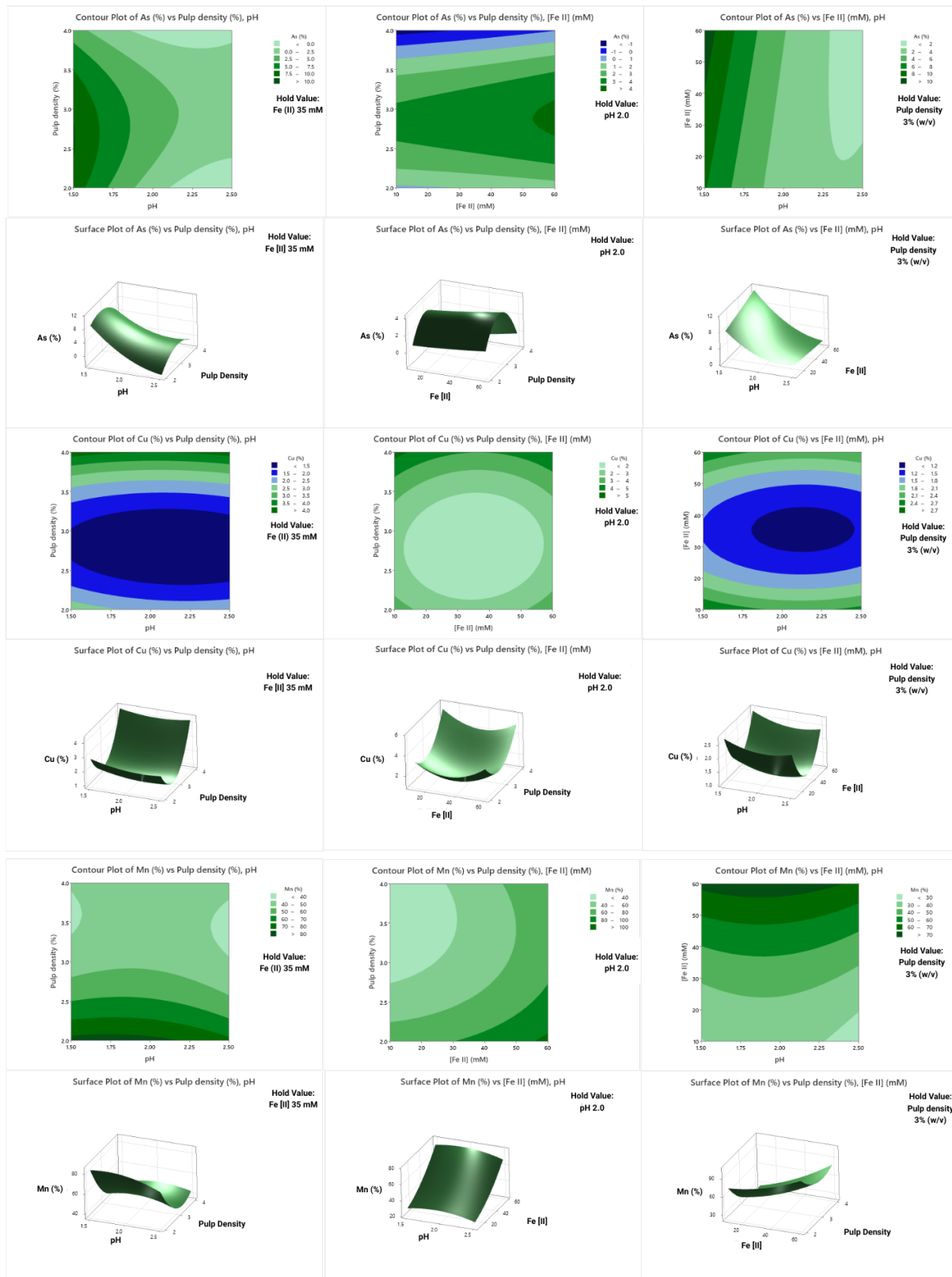
| Response | Fit   | SE Fit | 95% CI          | 95% PI          |
|----------|-------|--------|-----------------|-----------------|
| Al (%)   | 3.572 | 0.116  | (3.336, 3.808)  | (3.031, 4.113)  |
| Zn (%)   | 18.56 | 2.25   | (13.98, 23.13)  | (8.07, 29.05)   |
| Sb (%)   | 2.483 | 0.119  | (2.242, 2.724)  | (1.930, 3.036)  |
| Cu (%)   | 3.454 | 0.661  | (2.113, 4.795)  | (0.379, 6.529)  |
| As (%)   | 6.69  | 1.94   | (2.74, 10.63)   | (-2.36, 15.73)  |
| Mn (%)   | 99.84 | 9.89   | (79.76, 119.91) | (53.81, 145.86) |

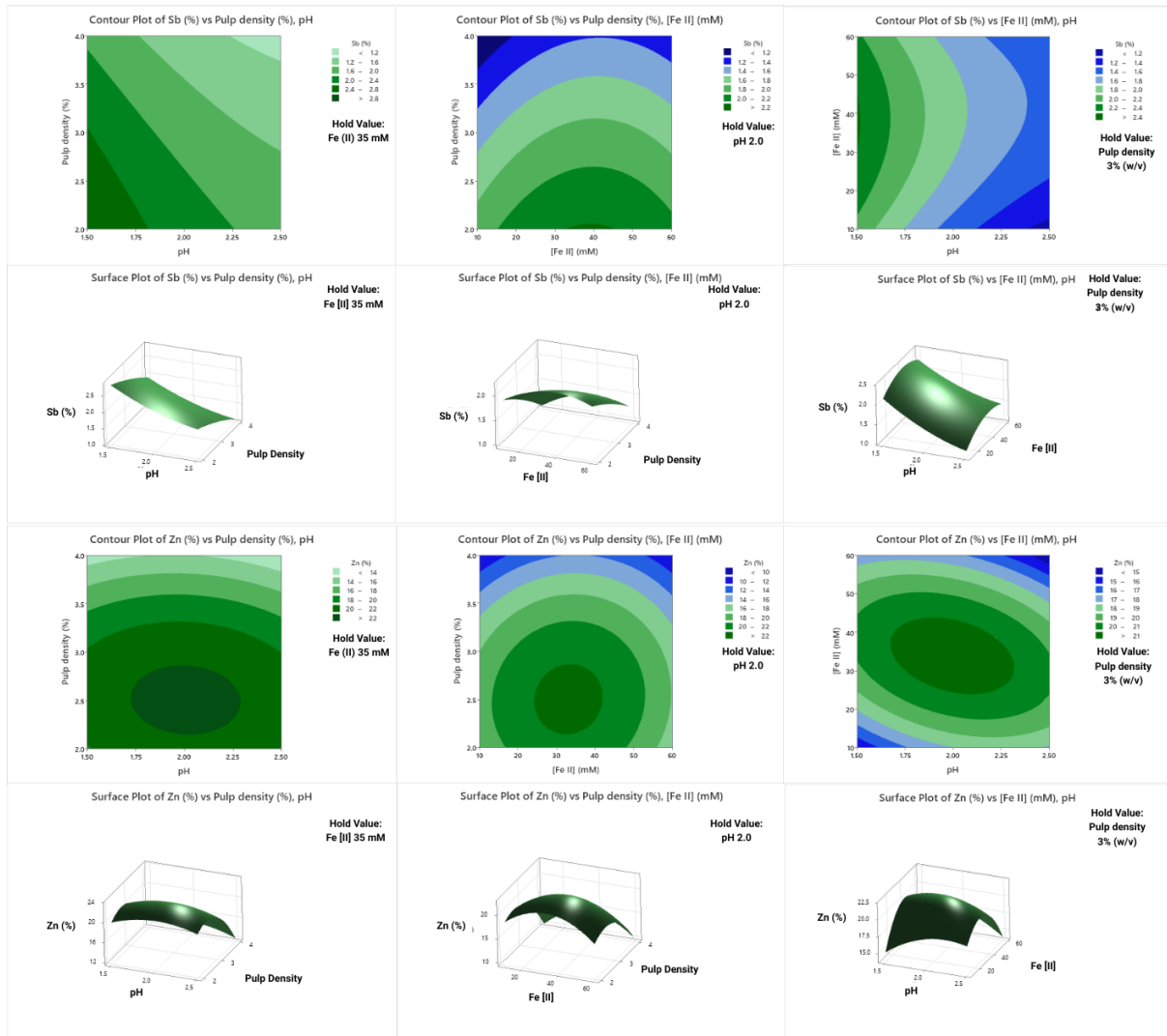


## Annex – A5

### Contour plots and 3D surface response curves for CP

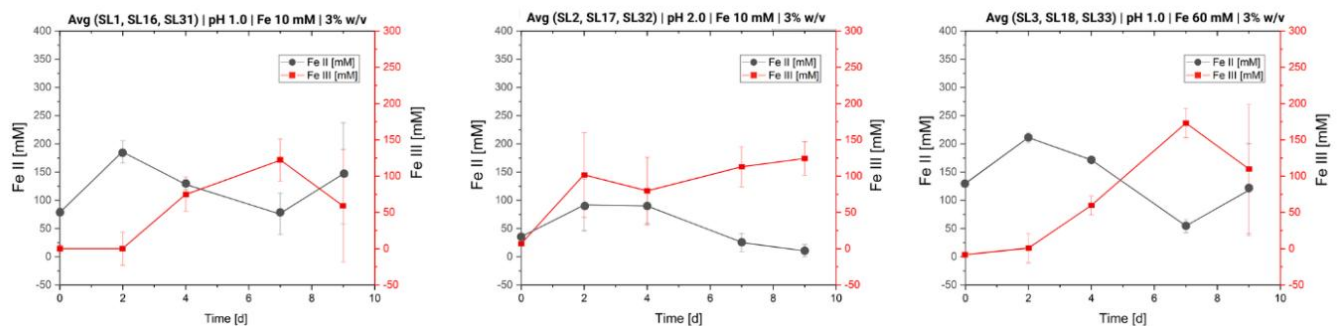


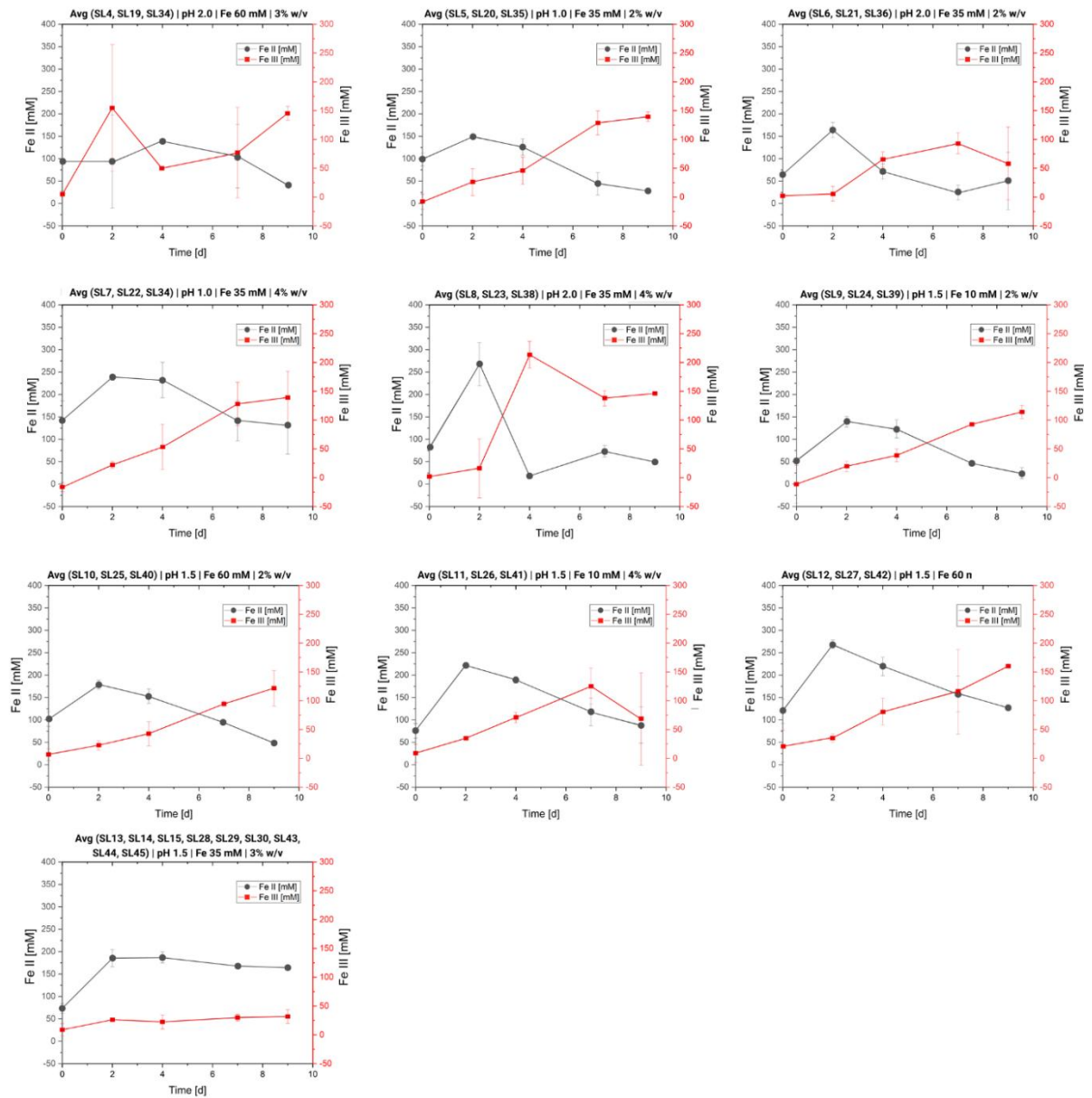




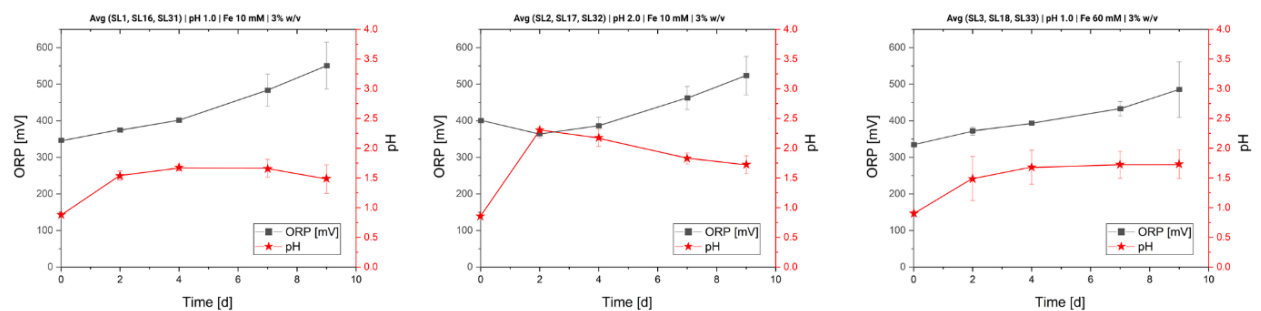
## Annex – A6

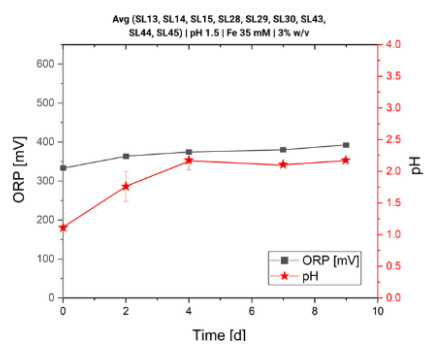
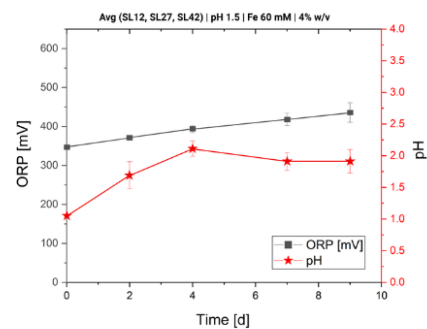
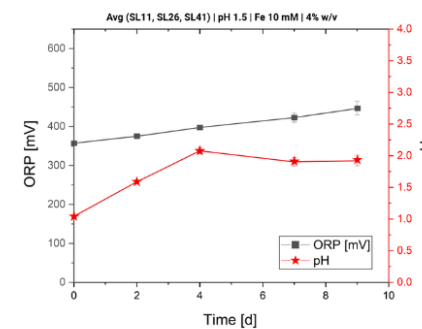
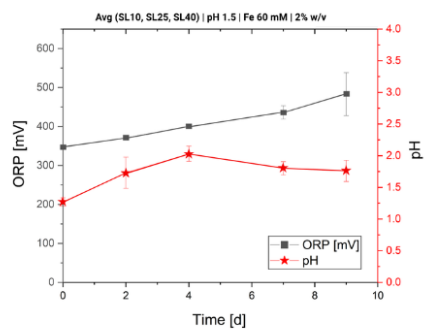
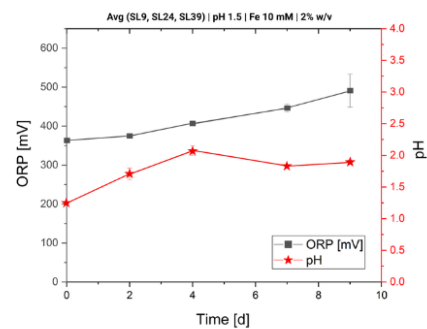
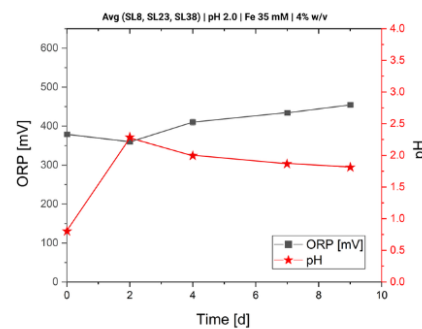
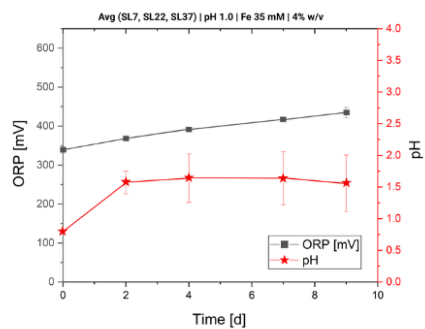
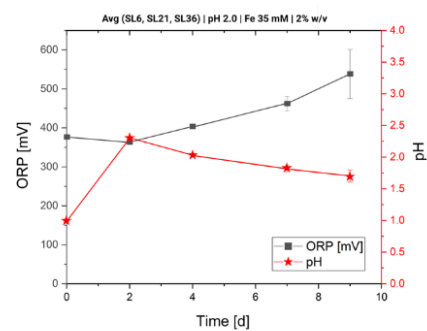
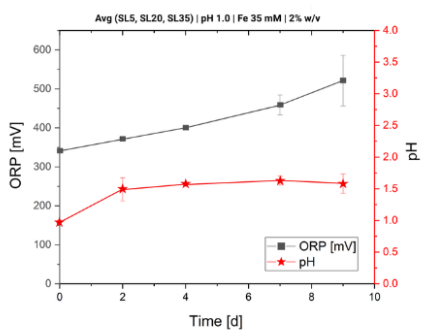
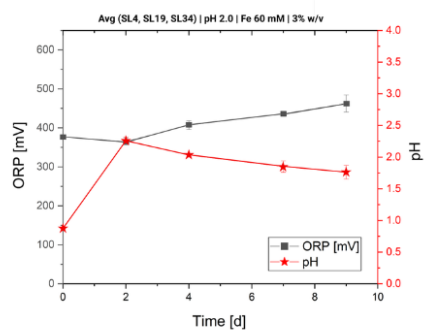
### *Ferrous versus Ferric Iron for SL tests*





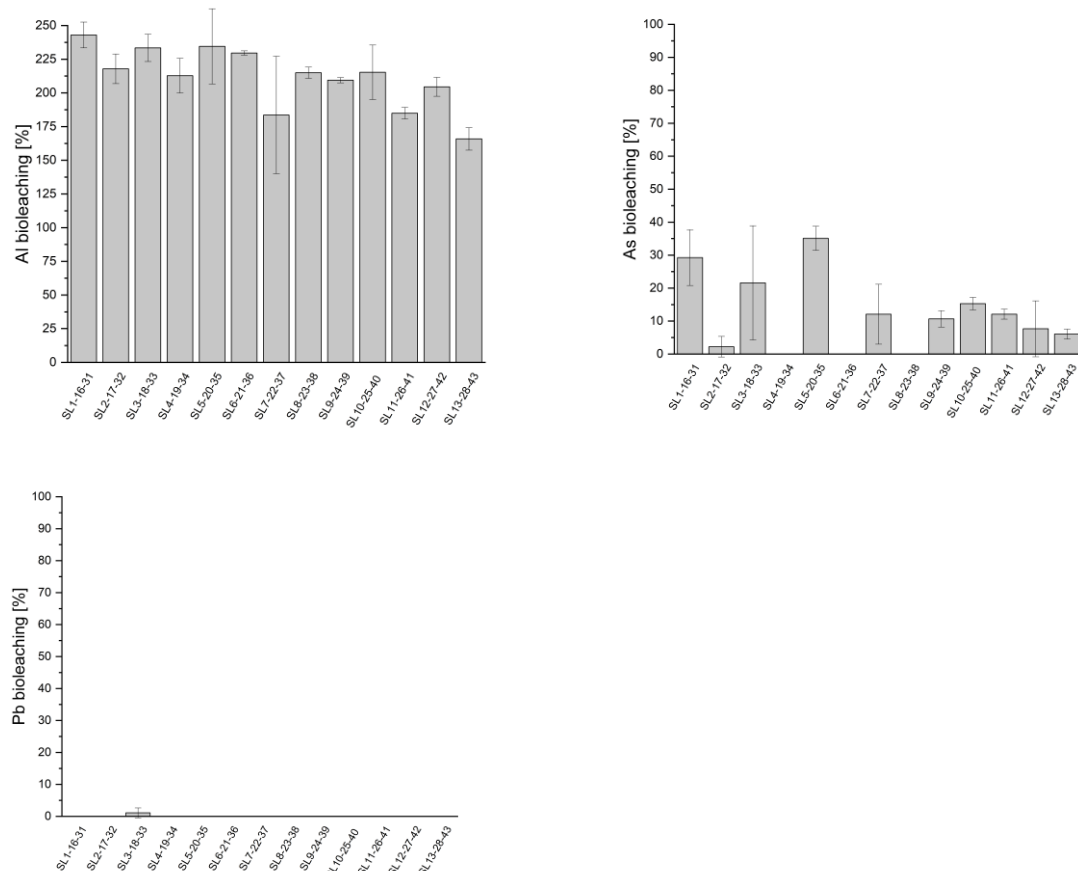
*ORP versus pH for SL tests*





## Annex – A7

### *Bioleaching Efficiency of metals (%) – day 9 for SL tests*



## Annex – A8

### *Response Surface Regression: Al% versus pH, Fe [II], Pulp Density for SL tests*

#### Coded Coefficients

| Term                      | Coef   | SE Coef | T-Value | P-Value | VIF  |
|---------------------------|--------|---------|---------|---------|------|
| Constant                  | 165.84 | 6.78    | 24.45   | 0.000   |      |
| pH                        | -2.41  | 4.15    | -0.58   | 0.566   | 1.00 |
| Fe [II]                   | 1.35   | 4.15    | 0.33    | 0.747   | 1.00 |
| Pulp Density              | -12.61 | 4.15    | -3.04   | 0.005   | 1.00 |
| pH*pH                     | 36.50  | 6.11    | 5.97    | 0.000   | 1.01 |
| Fe [II]*Fe [II]           | 24.38  | 6.11    | 3.99    | 0.000   | 1.01 |
| Pulp Density*Pulp Density | 13.29  | 6.11    | 2.17    | 0.037   | 1.01 |
| pH*Fe [II]                | 1.12   | 5.87    | 0.19    | 0.850   | 1.00 |
| pH*Pulp Density           | 9.07   | 5.87    | 1.54    | 0.131   | 1.00 |
| Fe [II]*Pulp Density      | 3.41   | 5.87    | 0.58    | 0.565   | 1.00 |

#### Model Summary

| S       | R-sq   | R-sq(adj) | R-sq(pred) |
|---------|--------|-----------|------------|
| 20.3508 | 64.19% | 54.99%    | 37.13%     |

#### Analysis of Variance

| Source                    | DF | Adj SS  | Adj MS  | F-Value | P-Value |
|---------------------------|----|---------|---------|---------|---------|
| Model                     | 9  | 25986.4 | 2887.4  | 6.97    | 0.000   |
| Linear                    | 3  | 3999.9  | 1333.3  | 3.22    | 0.034   |
| pH                        | 1  | 139.2   | 139.2   | 0.34    | 0.566   |
| Fe [II]                   | 1  | 43.9    | 43.9    | 0.11    | 0.747   |
| Pulp Density              | 1  | 3816.8  | 3816.8  | 9.22    | 0.005   |
| Square                    | 3  | 20843.4 | 6947.8  | 16.78   | 0.000   |
| pH*pH                     | 1  | 14759.6 | 14759.6 | 35.64   | 0.000   |
| Fe [II]*Fe [II]           | 1  | 6586.4  | 6586.4  | 15.90   | 0.000   |
| Pulp Density*Pulp Density | 1  | 1957.8  | 1957.8  | 4.73    | 0.037   |
| 2-Way Interaction         | 3  | 1143.0  | 381.0   | 0.92    | 0.441   |
| pH*Fe [II]                | 1  | 15.1    | 15.1    | 0.04    | 0.850   |
| pH*Pulp Density           | 1  | 988.1   | 988.1   | 2.39    | 0.131   |
| Fe [II]*Pulp Density      | 1  | 139.9   | 139.9   | 0.34    | 0.565   |
| Error                     | 35 | 14495.4 | 414.2   |         |         |
| Lack-of-Fit               | 3  | 2900.1  | 966.7   | 2.67    | 0.064   |
| Pure Error                | 32 | 11595.3 | 362.4   |         |         |
| Total                     | 44 | 40481.8 |         |         |         |

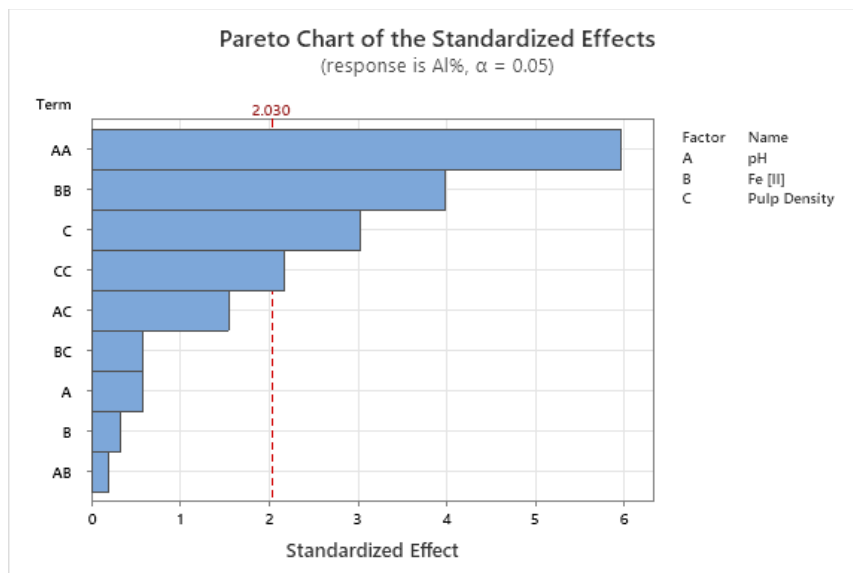
#### Regression Equation in Uncoded Units

$Al\% = 806 - 500.4 \text{ pH} - 3.22 \text{ Fe [II]} - 124.4 \text{ Pulp Density} + 146.0 \text{ pH}^2$   
 $+ 0.03902 \text{ Fe [II]}^2 + 13.29 \text{ Pulp Density}^2 + 0.090 \text{ pH} \cdot \text{Fe [II]}$   
 $+ 18.1 \text{ pH} \cdot \text{Pulp Density} + 0.137 \text{ Fe [II]} \cdot \text{Pulp Density}$

#### Fits and Diagnostics for Unusual Observations

| Obs | Al%    | Fit    | Resid  | Std Resid |   |
|-----|--------|--------|--------|-----------|---|
| 7   | 122.17 | 196.36 | -74.19 | -4.21     | R |
| 35  | 195.62 | 239.73 | -44.11 | -2.50     | R |

R Large residual



*Response Surface Regression: As% versus pH, Fe [II], Pulp Density for SL tests*

#### Coded Coefficients

| Term     | Coef   | SE Coef | T-Value | P-Value | VIF  |
|----------|--------|---------|---------|---------|------|
| Constant | 6.04   | 2.42    | 2.49    | 0.018   |      |
| pH       | -11.97 | 1.48    | -8.07   | 0.000   | 1.00 |

|                           |       |      |       |       |      |
|---------------------------|-------|------|-------|-------|------|
| Fe [II]                   | -1.21 | 1.48 | -0.82 | 0.420 | 1.00 |
| Pulp Density              | -3.65 | 1.48 | -2.46 | 0.019 | 1.00 |
| pH*pH                     | 3.80  | 2.18 | 1.74  | 0.091 | 1.01 |
| Fe [II]*Fe [II]           | 3.43  | 2.18 | 1.57  | 0.126 | 1.01 |
| Pulp Density*Pulp Density | 1.95  | 2.18 | 0.89  | 0.377 | 1.01 |
| pH*Fe [II]                | 1.36  | 2.10 | 0.65  | 0.521 | 1.00 |
| pH*Pulp Density           | 5.76  | 2.10 | 2.74  | 0.010 | 1.00 |
| Fe [II]*Pulp Density      | -2.26 | 2.10 | -1.08 | 0.289 | 1.00 |

**Model Summary**

| S       | R-sq   | R-sq(adj) | R-sq(pred) |
|---------|--------|-----------|------------|
| 7.26979 | 71.19% | 63.78%    | 48.94%     |

**Analysis of Variance**

| Source                    | DF | Adj SS  | Adj MS  | F-Value | P-Value |
|---------------------------|----|---------|---------|---------|---------|
| Model                     | 9  | 4570.34 | 507.82  | 9.61    | 0.000   |
| Linear                    | 3  | 3796.43 | 1265.48 | 23.94   | 0.000   |
| pH                        | 1  | 3440.90 | 3440.90 | 65.11   | 0.000   |
| Fe [II]                   | 1  | 35.14   | 35.14   | 0.66    | 0.420   |
| Pulp Density              | 1  | 320.40  | 320.40  | 6.06    | 0.019   |
| Square                    | 3  | 292.72  | 97.57   | 1.85    | 0.157   |
| pH*pH                     | 1  | 160.06  | 160.06  | 3.03    | 0.091   |
| Fe [II]*Fe [II]           | 1  | 130.10  | 130.10  | 2.46    | 0.126   |
| Pulp Density*Pulp Density | 1  | 42.32   | 42.32   | 0.80    | 0.377   |
| 2-Way Interaction         | 3  | 481.19  | 160.40  | 3.03    | 0.042   |
| pH*Fe [II]                | 1  | 22.25   | 22.25   | 0.42    | 0.521   |
| pH*Pulp Density           | 1  | 397.56  | 397.56  | 7.52    | 0.010   |
| Fe [II]*Pulp Density      | 1  | 61.38   | 61.38   | 1.16    | 0.289   |
| Error                     | 35 | 1849.75 | 52.85   |         |         |
| Lack-of-Fit               | 3  | 144.84  | 48.28   | 0.91    | 0.449   |
| Pure Error                | 32 | 1704.90 | 53.28   |         |         |
| Total                     | 44 | 6420.08 |         |         |         |

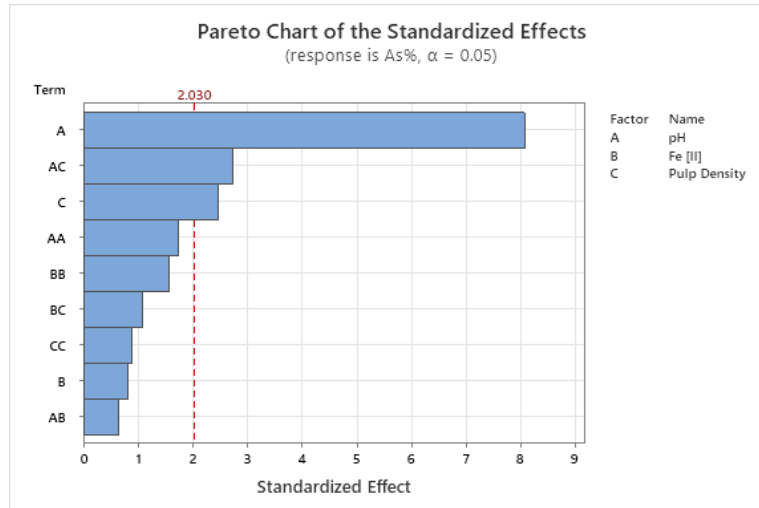
**Regression Equation in Uncoded Units**

As% = 161.2 - 107.9 pH - 0.324 Fe [II] - 29.5 Pulp Density + 15.21 pH\*pH  
+ 0.00548 Fe [II]\*Fe [II] + 1.95 Pulp Density\*Pulp Density + 0.109 pH\*Fe [II]  
+ 11.51 pH\*Pulp Density - 0.0905 Fe [II]\*Pulp Density

**Fits and Diagnostics for Unusual Observations**

| Obs | As%   | Fit   | Resid  | Std Resid |   |
|-----|-------|-------|--------|-----------|---|
| 3   | 0.00  | 22.67 | -22.67 | -3.60     | R |
| 7   | 0.00  | 14.36 | -14.36 | -2.28     | R |
| 18  | 42.44 | 22.67 | 19.77  | 3.14      | R |
| 42  | 19.47 | 4.29  | 15.18  | 2.41      | R |

R Large residual



*Response Surface Regression: Co% versus pH, Fe [II], Pulp Density for SL tests*

#### Coded Coefficients

| Term                      | Coef  | SE Coef | T-Value | P-Value | VIF  |
|---------------------------|-------|---------|---------|---------|------|
| Constant                  | 57.06 | 2.71    | 21.05   | 0.000   |      |
| pH                        | 3.64  | 1.66    | 2.19    | 0.035   | 1.00 |
| Fe [II]                   | 1.11  | 1.66    | 0.67    | 0.507   | 1.00 |
| Pulp Density              | -4.65 | 1.66    | -2.80   | 0.008   | 1.00 |
| pH*pH                     | 4.47  | 2.44    | 1.83    | 0.076   | 1.01 |
| Fe [II]*Fe [II]           | 6.45  | 2.44    | 2.64    | 0.012   | 1.01 |
| Pulp Density*Pulp Density | 1.73  | 2.44    | 0.71    | 0.484   | 1.01 |
| pH*Fe [II]                | -0.09 | 2.35    | -0.04   | 0.969   | 1.00 |
| pH*Pulp Density           | 2.86  | 2.35    | 1.22    | 0.231   | 1.00 |
| Fe [II]*Pulp Density      | 0.90  | 2.35    | 0.38    | 0.705   | 1.00 |

#### Model Summary

| S       | R-sq   | R-sq(adj) | R-sq(pred) |
|---------|--------|-----------|------------|
| 8.13202 | 41.21% | 26.10%    | 0.00%      |

#### Analysis of Variance

| Source                    | DF | Adj SS  | Adj MS  | F-Value | P-Value |
|---------------------------|----|---------|---------|---------|---------|
| Model                     | 9  | 1622.60 | 180.289 | 2.73    | 0.016   |
| Linear                    | 3  | 868.00  | 289.333 | 4.38    | 0.010   |
| pH                        | 1  | 318.41  | 318.405 | 4.81    | 0.035   |
| Fe [II]                   | 1  | 29.66   | 29.659  | 0.45    | 0.507   |
| Pulp Density              | 1  | 519.94  | 519.936 | 7.86    | 0.008   |
| Square                    | 3  | 646.59  | 215.530 | 3.26    | 0.033   |
| pH*pH                     | 1  | 221.01  | 221.005 | 3.34    | 0.076   |
| Fe [II]*Fe [II]           | 1  | 460.85  | 460.852 | 6.97    | 0.012   |
| Pulp Density*Pulp Density | 1  | 33.07   | 33.066  | 0.50    | 0.484   |
| 2-Way Interaction         | 3  | 108.01  | 36.005  | 0.54    | 0.655   |
| pH*Fe [II]                | 1  | 0.10    | 0.098   | 0.00    | 0.969   |
| pH*Pulp Density           | 1  | 98.28   | 98.281  | 1.49    | 0.231   |
| Fe [II]*Pulp Density      | 1  | 9.64    | 9.636   | 0.15    | 0.705   |
| Error                     | 35 | 2314.54 | 66.130  |         |         |
| Lack-of-Fit               | 3  | 430.64  | 143.548 | 2.44    | 0.083   |
| Pure Error                | 32 | 1883.90 | 58.872  |         |         |

Total 44 3937.14

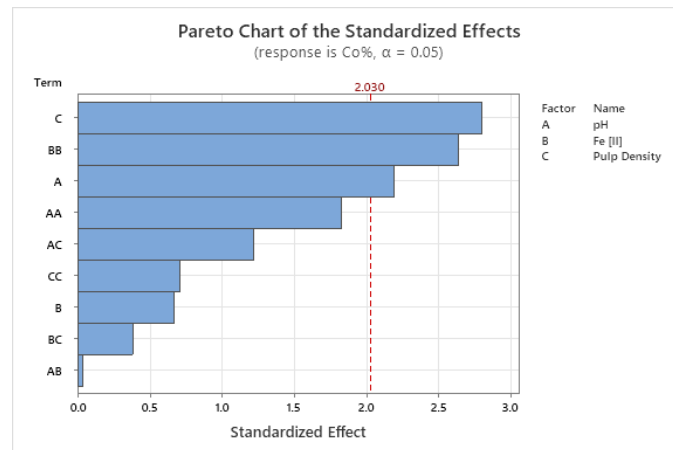
### Regression Equation in Uncoded Units

Co% = 156.1 - 63.2 pH - 0.775 Fe [II] - 24.9 Pulp Density + 17.87 pH\*pH  
 + 0.01032 Fe [II]\*Fe [II] + 1.73 Pulp Density\*Pulp Density - 0.007 pH\*Fe [II]  
 + 5.72 pH\*Pulp Density + 0.0358 Fe [II]\*Pulp Density

### Fits and Diagnostics for Unusual Observations

| Obs | Co%   | Fit   | Resid  | Std Resid |
|-----|-------|-------|--------|-----------|
| 7   | 17.33 | 52.09 | -34.76 | -4.94     |

R Large residual



*Response Surface Regression: Cu% versus pH, Fe [II], Pulp Density for SL tests*

### Coded Coefficients

| Term                      | Coef  | SE Coef | T-Value | P-Value | VIF  |
|---------------------------|-------|---------|---------|---------|------|
| Constant                  | 41.27 | 2.94    | 14.02   | 0.000   |      |
| pH                        | 1.35  | 1.80    | 0.75    | 0.459   | 1.00 |
| Fe [II]                   | -0.23 | 1.80    | -0.13   | 0.899   | 1.00 |
| Pulp Density              | -5.19 | 1.80    | -2.88   | 0.007   | 1.00 |
| pH*pH                     | 11.24 | 2.65    | 4.24    | 0.000   | 1.01 |
| Fe [II]*Fe [II]           | 6.24  | 2.65    | 2.35    | 0.024   | 1.01 |
| Pulp Density*Pulp Density | 1.50  | 2.65    | 0.57    | 0.575   | 1.01 |
| pH*Fe [II]                | -0.40 | 2.55    | -0.16   | 0.877   | 1.00 |
| pH*Pulp Density           | 4.22  | 2.55    | 1.66    | 0.107   | 1.00 |
| Fe [II]*Pulp Density      | 1.48  | 2.55    | 0.58    | 0.566   | 1.00 |

### Model Summary

| S       | R-sq   | R-sq(adj) | R-sq(pred) |
|---------|--------|-----------|------------|
| 8.83129 | 49.33% | 36.30%    | 11.68%     |

### Analysis of Variance

| Source                    | DF | Adj SS  | Adj MS  | F-Value | P-Value |
|---------------------------|----|---------|---------|---------|---------|
| Model                     | 9  | 2657.09 | 295.23  | 3.79    | 0.002   |
| Linear                    | 3  | 692.09  | 230.70  | 2.96    | 0.046   |
| pH                        | 1  | 43.63   | 43.63   | 0.56    | 0.459   |
| Fe [II]                   | 1  | 1.27    | 1.27    | 0.02    | 0.899   |
| Pulp Density              | 1  | 647.19  | 647.19  | 8.30    | 0.007   |
| Square                    | 3  | 1722.93 | 574.31  | 7.36    | 0.001   |
| pH*pH                     | 1  | 1400.05 | 1400.05 | 17.95   | 0.000   |
| Fe [II]*Fe [II]           | 1  | 431.54  | 431.54  | 5.53    | 0.024   |
| Pulp Density*Pulp Density | 1  | 25.03   | 25.03   | 0.32    | 0.575   |

|                      |    |         |        |      |       |
|----------------------|----|---------|--------|------|-------|
| 2-Way Interaction    | 3  | 242.08  | 80.69  | 1.03 | 0.389 |
| pH*Fe [II]           | 1  | 1.90    | 1.90   | 0.02 | 0.877 |
| pH*Pulp Density      | 1  | 213.95  | 213.95 | 2.74 | 0.107 |
| Fe [II]*Pulp Density | 1  | 26.23   | 26.23  | 0.34 | 0.566 |
| Error                | 35 | 2729.71 | 77.99  |      |       |
| Lack-of-Fit          | 3  | 360.77  | 120.26 | 1.62 | 0.203 |
| Pure Error           | 32 | 2368.93 | 74.03  |      |       |
| Total                | 44 | 5386.80 |        |      |       |

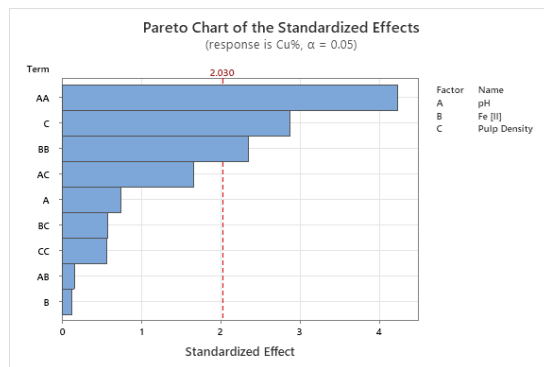
#### Regression Equation in Uncoded Units

$$\begin{aligned} \text{Cu\%} = & 222.6 - 156.4 \text{ pH} - 0.838 \text{ Fe [II]} - 29.0 \text{ Pulp Density} + 45.0 \text{ pH*pH} \\ & + 0.00999 \text{ Fe [II]*Fe [II]} + 1.50 \text{ Pulp Density*Pulp Density} - 0.032 \text{ pH*Fe [II]} \\ & + 8.44 \text{ pH*Pulp Density} + 0.059 \text{ Fe [II]*Pulp Density} \end{aligned}$$

#### Fits and Diagnostics for Unusual Observations

| Obs | Cu% Fit | Resid | Std Resid |
|-----|---------|-------|-----------|
| 7   | 5.56    | 43.25 | -37.69    |

R Large residual



*Response Surface Regression: Mn% versus pH, Fe [II], Pulp Density for SL tests*

#### Coded Coefficients

| Term                      | Coef  | SE Coef | T-Value | P-Value | VIF  |
|---------------------------|-------|---------|---------|---------|------|
| Constant                  | 62.11 | 1.80    | 34.45   | 0.000   |      |
| pH                        | 2.37  | 1.10    | 2.15    | 0.039   | 1.00 |
| Fe [II]                   | 6.29  | 1.10    | 5.70    | 0.000   | 1.00 |
| Pulp Density              | -5.72 | 1.10    | -5.18   | 0.000   | 1.00 |
| pH*pH                     | 4.16  | 1.62    | 2.56    | 0.015   | 1.01 |
| Fe [II]*Fe [II]           | 5.41  | 1.62    | 3.33    | 0.002   | 1.01 |
| Pulp Density*Pulp Density | 4.16  | 1.62    | 2.56    | 0.015   | 1.01 |
| pH*Fe [II]                | -0.39 | 1.56    | -0.25   | 0.803   | 1.00 |
| pH*Pulp Density           | 0.21  | 1.56    | 0.13    | 0.896   | 1.00 |
| Fe [II]*Pulp Density      | -1.14 | 1.56    | -0.73   | 0.470   | 1.00 |

#### Model Summary

| S       | R-sq   | R-sq(adj) | R-sq(pred) |
|---------|--------|-----------|------------|
| 5.40815 | 70.99% | 63.52%    | 48.47%     |

#### Analysis of Variance

| Source  | DF | Adj SS  | Adj MS  | F-Value | P-Value |
|---------|----|---------|---------|---------|---------|
| Model   | 9  | 2504.46 | 278.273 | 9.51    | 0.000   |
| Linear  | 3  | 1869.94 | 623.314 | 21.31   | 0.000   |
| pH      | 1  | 134.90  | 134.900 | 4.61    | 0.039   |
| Fe [II] | 1  | 949.92  | 949.916 | 32.48   | 0.000   |

|                           |    |         |         |       |       |
|---------------------------|----|---------|---------|-------|-------|
| Pulp Density              | 1  | 785.13  | 785.127 | 26.84 | 0.000 |
| Square                    | 3  | 616.54  | 205.512 | 7.03  | 0.001 |
| pH*pH                     | 1  | 192.12  | 192.115 | 6.57  | 0.015 |
| Fe [II]*Fe [II]           | 1  | 324.65  | 324.650 | 11.10 | 0.002 |
| Pulp Density*Pulp Density | 1  | 191.73  | 191.731 | 6.56  | 0.015 |
| 2-Way Interaction         | 3  | 17.98   | 5.993   | 0.20  | 0.892 |
| pH*Fe [II]                | 1  | 1.86    | 1.857   | 0.06  | 0.803 |
| pH*Pulp Density           | 1  | 0.50    | 0.504   | 0.02  | 0.896 |
| Fe [II]*Pulp Density      | 1  | 15.62   | 15.618  | 0.53  | 0.470 |
| Error                     | 35 | 1023.68 | 29.248  |       |       |
| Lack-of-Fit               | 3  | 211.09  | 70.365  | 2.77  | 0.058 |
| Pure Error                | 32 | 812.59  | 25.393  |       |       |
| Total                     | 44 | 3528.14 |         |       |       |

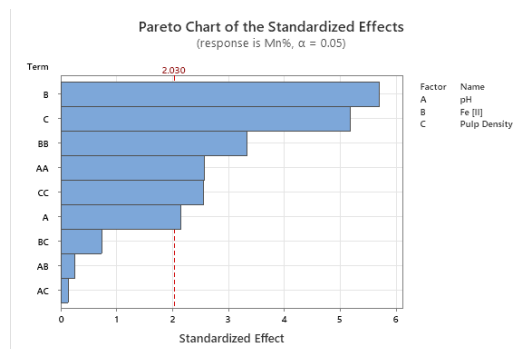
#### Regression Equation in Uncoded Units

$Mn\% = 144.3 - 45.4 \text{ pH} - 0.171 \text{ Fe [II]} - 29.7 \text{ Pulp Density} + 16.66 \text{ pH}^2$   
 $+ 0.00866 \text{ Fe [II]}^2 + 4.16 \text{ Pulp Density}^2 - 0.031 \text{ pH} \cdot \text{Fe [II]}$   
 $+ 0.41 \text{ pH} \cdot \text{Pulp Density} - 0.0456 \text{ Fe [II]} \cdot \text{Pulp Density}$

#### Fits and Diagnostics for Unusual Observations

| Obs | Mn%   | Fit   | Resid  | Std Resid |   |
|-----|-------|-------|--------|-----------|---|
| 3   | 88.71 | 76.00 | 12.71  | 2.71      | R |
| 7   | 50.52 | 62.14 | -11.62 | -2.48     | R |
| 12  | 81.62 | 71.11 | 10.51  | 2.24      | R |
| 35  | 58.97 | 73.99 | -15.02 | -3.21     | R |

R Large residual



*Response Surface Regression: Pb% versus pH, Fe [II], Pulp Density for SL tests*

#### Coded Coefficients

| Term                      | Coef    | SE Coef | T-Value | P-Value | VIF  |
|---------------------------|---------|---------|---------|---------|------|
| Constant                  | -0.000  | 0.160   | -0.00   | 1.000   |      |
| pH                        | -0.1367 | 0.0980  | -1.39   | 0.172   | 1.00 |
| Fe [II]                   | 0.1367  | 0.0980  | 1.39    | 0.172   | 1.00 |
| Pulp Density              | -0.0000 | 0.0980  | -0.00   | 1.000   | 1.00 |
| pH*pH                     | 0.137   | 0.144   | 0.95    | 0.350   | 1.01 |
| Fe [II]*Fe [II]           | 0.137   | 0.144   | 0.95    | 0.350   | 1.01 |
| Pulp Density*Pulp Density | -0.137  | 0.144   | -0.95   | 0.350   | 1.01 |
| pH*Fe [II]                | -0.273  | 0.139   | -1.97   | 0.057   | 1.00 |
| pH*Pulp Density           | -0.000  | 0.139   | -0.00   | 1.000   | 1.00 |
| Fe [II]*Pulp Density      | -0.000  | 0.139   | -0.00   | 1.000   | 1.00 |

#### Model Summary

**S**      **R-sq**   **R-sq(adj)** **R-sq(pred)**  
 0.480143 23.30% 3.57%   0.00%

#### Analysis of Variance

| Source                    | DF | Adj SS  | Adj MS   | F-Value | P-Value |
|---------------------------|----|---------|----------|---------|---------|
| Model                     | 9  | 2.4505  | 0.272280 | 1.18    | 0.337   |
| Linear                    | 3  | 0.8965  | 0.298844 | 1.30    | 0.291   |
| pH                        | 1  | 0.4483  | 0.448267 | 1.94    | 0.172   |
| Fe [II]                   | 1  | 0.4483  | 0.448267 | 1.94    | 0.172   |
| Pulp Density              | 1  | 0.0000  | 0.000000 | 0.00    | 1.000   |
| Square                    | 3  | 0.6575  | 0.219153 | 0.95    | 0.427   |
| pH*pH                     | 1  | 0.2069  | 0.206892 | 0.90    | 0.350   |
| Fe [II]*Fe [II]           | 1  | 0.2069  | 0.206892 | 0.90    | 0.350   |
| Pulp Density*Pulp Density | 1  | 0.2069  | 0.206892 | 0.90    | 0.350   |
| 2-Way Interaction         | 3  | 0.8965  | 0.298844 | 1.30    | 0.291   |
| pH*Fe [II]                | 1  | 0.8965  | 0.896533 | 3.89    | 0.057   |
| pH*Pulp Density           | 1  | 0.0000  | 0.000000 | 0.00    | 1.000   |
| Fe [II]*Pulp Density      | 1  | 0.0000  | 0.000000 | 0.00    | 1.000   |
| Error                     | 35 | 8.0688  | 0.230537 |         |         |
| Lack-of-Fit               | 3  | 0.8965  | 0.298844 | 1.33    | 0.281   |
| Pure Error                | 32 | 7.1723  | 0.224133 |         |         |
| Total                     | 44 | 10.5193 |          |         |         |

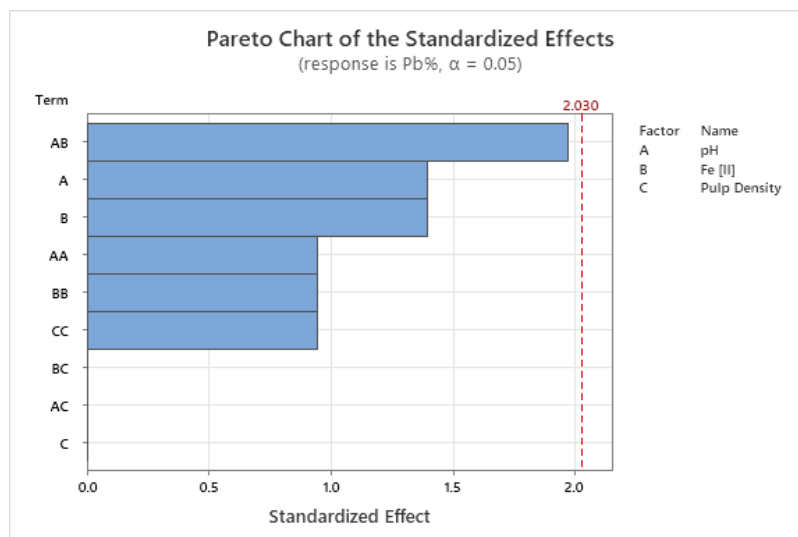
#### Regression Equation in Uncoded Units

Pb% = -0.66 - 1.15 pH + 0.0230 Fe [II] + 0.820 Pulp Density + 0.547 pH\*pH  
 + 0.000219 Fe [II]\*Fe [II] - 0.137 Pulp Density\*Pulp Density - 0.0219 pH\*Fe [II]  
 + 0.000 pH\*Pulp Density + 0.00000 Fe [II]\*Pulp Density

#### Fits and Diagnostics for Unusual Observations

| Obs | Pb%   | Fit   | Resid | Std Resid |
|-----|-------|-------|-------|-----------|
| 18  | 3.280 | 0.820 | 2.460 | 5.92      |

R Large residual



*Response Surface Regression: Zn% versus pH, Fe [II], Pulp Density for SL tests*

#### Coded Coefficients

| Term     | Coef  | SE Coef | T-Value | P-Value | VIF |
|----------|-------|---------|---------|---------|-----|
| Constant | 64.04 | 2.77    | 23.08   | 0.000   |     |

|                           |       |      |       |       |      |
|---------------------------|-------|------|-------|-------|------|
| pH                        | 1.88  | 1.70 | 1.11  | 0.276 | 1.00 |
| Fe [II]                   | 2.23  | 1.70 | 1.31  | 0.197 | 1.00 |
| Pulp Density              | -2.36 | 1.70 | -1.39 | 0.174 | 1.00 |
| pH*pH                     | 6.58  | 2.50 | 2.63  | 0.013 | 1.01 |
| Fe [II]*Fe [II]           | 7.61  | 2.50 | 3.04  | 0.004 | 1.01 |
| Pulp Density*Pulp Density | 3.33  | 2.50 | 1.33  | 0.192 | 1.01 |
| pH*Fe [II]                | -1.62 | 2.40 | -0.67 | 0.505 | 1.00 |
| pH*Pulp Density           | 0.77  | 2.40 | 0.32  | 0.751 | 1.00 |
| Fe [II]*Pulp Density      | 2.38  | 2.40 | 0.99  | 0.330 | 1.00 |

**Model Summary**

| S       | R-sq   | R-sq(adj) | R-sq(pred) |
|---------|--------|-----------|------------|
| 8.32464 | 38.93% | 23.23%    | 0.00%      |

**Analysis of Variance**

| Source                    | DF | Adj SS  | Adj MS  | F-Value | P-Value |
|---------------------------|----|---------|---------|---------|---------|
| Model                     | 9  | 1546.47 | 171.830 | 2.48    | 0.026   |
| Linear                    | 3  | 337.92  | 112.641 | 1.63    | 0.201   |
| pH                        | 1  | 84.79   | 84.788  | 1.22    | 0.276   |
| Fe [II]                   | 1  | 119.80  | 119.796 | 1.73    | 0.197   |
| Pulp Density              | 1  | 133.34  | 133.340 | 1.92    | 0.174   |
| Square                    | 3  | 1102.33 | 367.443 | 5.30    | 0.004   |
| pH*pH                     | 1  | 479.23  | 479.227 | 6.92    | 0.013   |
| Fe [II]*Fe [II]           | 1  | 642.33  | 642.331 | 9.27    | 0.004   |
| Pulp Density*Pulp Density | 1  | 122.65  | 122.647 | 1.77    | 0.192   |
| 2-Way Interaction         | 3  | 106.21  | 35.405  | 0.51    | 0.677   |
| pH*Fe [II]                | 1  | 31.40   | 31.396  | 0.45    | 0.505   |
| pH*Pulp Density           | 1  | 7.08    | 7.084   | 0.10    | 0.751   |
| Fe [II]*Pulp Density      | 1  | 67.74   | 67.735  | 0.98    | 0.330   |
| Error                     | 35 | 2425.49 | 69.300  |         |         |
| Lack-of-Fit               | 3  | 379.03  | 126.343 | 1.98    | 0.137   |
| Pure Error                | 32 | 2046.46 | 63.952  |         |         |
| Total                     | 44 | 3971.95 |         |         |         |

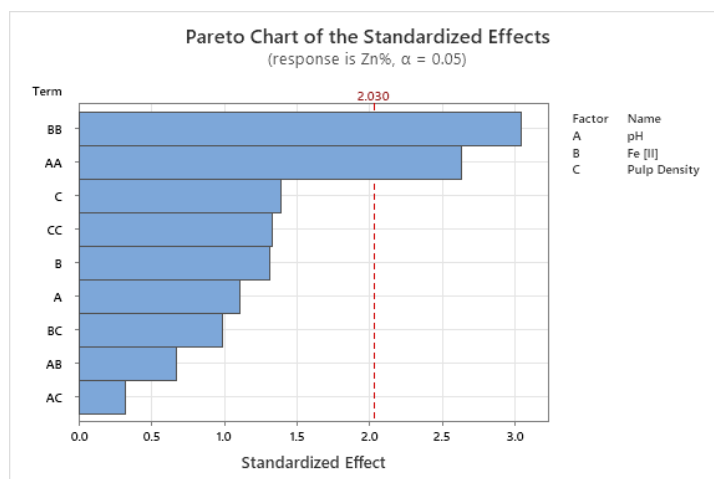
**Regression Equation in Uncoded Units**

$$\begin{aligned} \text{Zn\%} = & 176.5 - 75.3 \text{ pH} - 0.855 \text{ Fe [II]} - 28.0 \text{ Pulp Density} + 26.3 \text{ pH*pH} \\ & + 0.01218 \text{ Fe [II]*Fe [II]} + 3.33 \text{ Pulp Density*Pulp Density} - 0.129 \text{ pH*Fe [II]} \\ & + 1.54 \text{ pH*Pulp Density} + 0.0950 \text{ Fe [II]*Pulp Density} \end{aligned}$$

**Fits and Diagnostics for Unusual Observations**

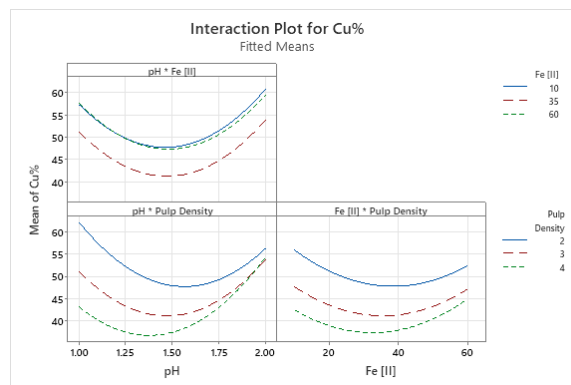
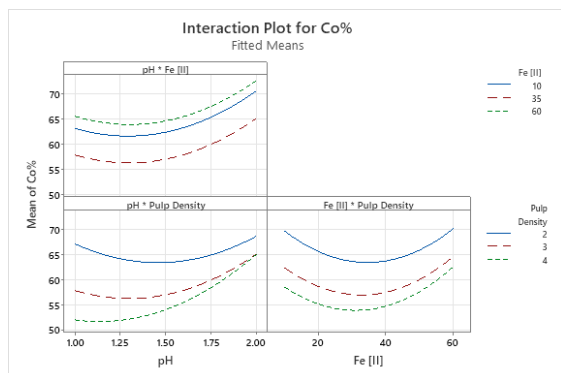
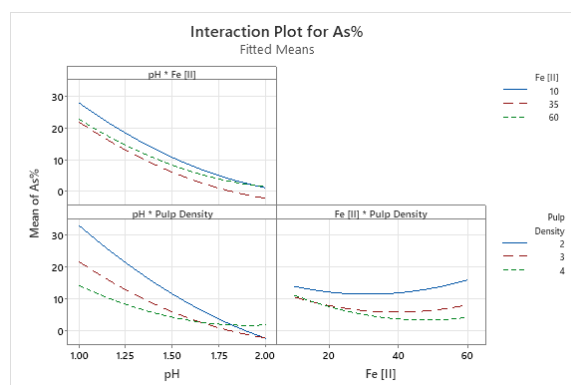
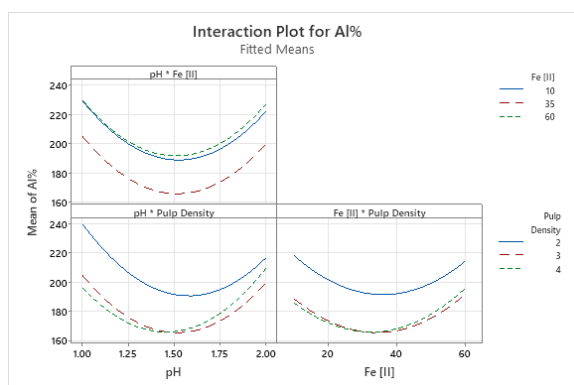
| Obs | Zn%    | Fit   | Resid  | Std Resid |   |
|-----|--------|-------|--------|-----------|---|
| 3   | 106.64 | 80.20 | 26.44  | 3.67      | R |
| 7   | 52.24  | 68.94 | -16.70 | -2.32     | R |
| 12  | 99.24  | 77.24 | 22.00  | 3.05      | R |
| 35  | 60.28  | 75.19 | -14.91 | -2.07     | R |

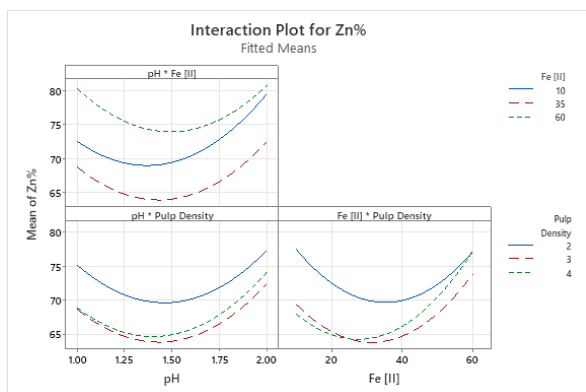
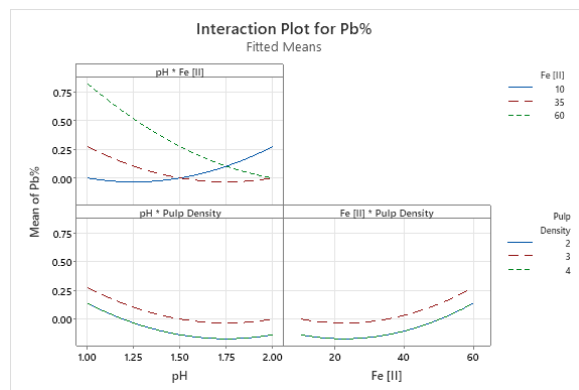
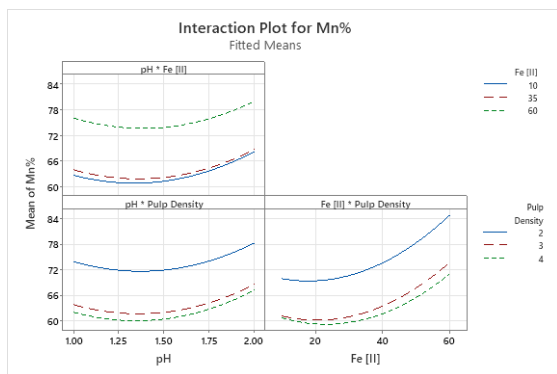
*R Large residual*



## Annex – A9

### Interaction plots for SL





## Annex – A10

### Optimization of SL

### Response Optimization: Zn%, Mn%, Cu%, Co%, As%, Al%

#### Parameters

| Response | Goal    | Lower  | Target | Upper  | Weight | Importance |
|----------|---------|--------|--------|--------|--------|------------|
| Zn%      | Target  | 52.240 | 100    | 106.64 | 1      | 1          |
| Mn%      | Target  | 50.520 | 100    | 110.00 | 1      | 1          |
| Cu%      | Target  | 5.560  | 100    | 110.00 | 1      | 1          |
| Co%      | Target  | 17.327 | 100    | 110.00 | 1      | 1          |
| As%      | Minimum | 0      | 42.44  | 1      | 1      | 1          |
| Al%      | Target  | 90.000 | 100    | 259.84 | 1      | 1          |

#### Solution

| Solution | pH      | Fe [II] | Pulp Density | Fit     | Zn%     | Mn%     | Cu%     | Co%     | As% |
|----------|---------|---------|--------------|---------|---------|---------|---------|---------|-----|
| 1        | 1.89899 | 60      | 2.32323      | 79.3831 | 84.4826 | 56.1185 | 72.0849 | 3.99622 |     |

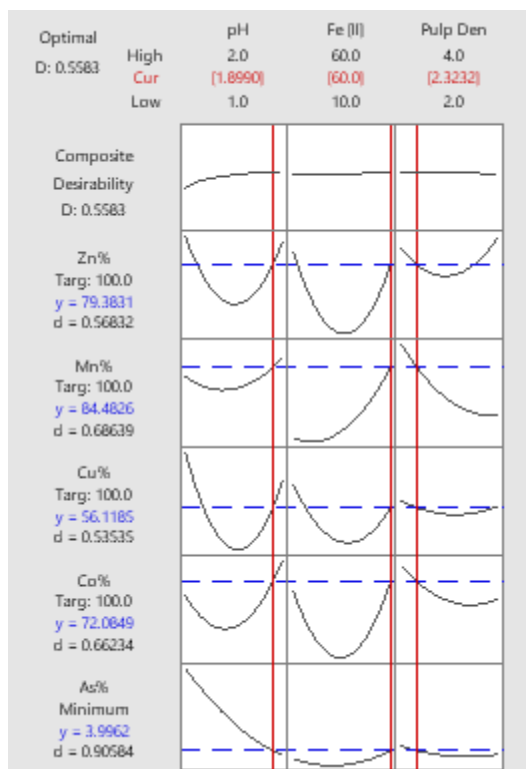
| Al%          | Composite    |
|--------------|--------------|
| Solution Fit | Desirability |

1 221.210 0.558295

#### Multiple Response Prediction

| Variable     | Setting |
|--------------|---------|
| pH           | 1.89899 |
| Fe [II]      | 60      |
| Pulp Density | 2.32323 |

| Response | Fit   | SE Fit | 95% CI         | 95% PI          |
|----------|-------|--------|----------------|-----------------|
| Zn%      | 79.38 | 4.13   | (71.00, 87.77) | (60.52, 98.25)  |
| Mn%      | 84.48 | 2.68   | (79.03, 89.93) | (72.23, 96.74)  |
| Cu%      | 56.12 | 4.38   | (47.22, 65.02) | (36.10, 76.13)  |
| Co%      | 72.08 | 4.04   | (63.89, 80.28) | (53.66, 90.51)  |
| As%      | 4.00  | 3.61   | (-3.33, 11.32) | (-12.48, 20.47) |
| Al%      | 221.2 | 10.1   | (200.7, 241.7) | (175.1, 267.3)  |



## Annex – A11

