Microbial-electrochemical systems for metal recovery

Suanny Sophia Mosquera Romero
Student number: 01600774
Mentor: Dr. ir. Jeet Varia

Promotor: Prof. dr. Korneel Rabaey, Dr. ir. Jeet Varia

Master's Dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Master of Science in Environmental Sanitation

Academic year: 2017 – 2018
Copyright

"The author and the promoter(s) give permission to make this master dissertation available for consultation and to copy parts of this master dissertation for personal use. In the case of any other use, the copyright terms have to be respected, in particular with regard to the obligation to state expressly the source when quoting results from this master dissertation."

Ghent University, July 27th 2018

Promoter(s)                        The author

Jeet Varia                        Suanny Sophia Mosquera Romero
Jeet.varia@Ugent.be               Suannysophia.mosqueraromero@Ugent.be

Korneel Rabaey
Korneel.rabaey@Ugent.be
Preface

This thesis is carried out as a completion of the master education in Environmental Sanitation. This manuscript is a compilation of several efforts geared towards common objectives.

First of all, I want to dedicate this work to the almighty God. Everything I had accomplished has been enlightened by our merciful creator.

Especially, I thank my husband Byron Galarza for all the hours dedicated encouraging me to follow my dreams. Without any doubt thanks to my parents who gave me all the principles and initial impulse in this life. Gratitude to my brother for exemplifying a model to follow, to my sister and niece who taught how to be strong in life, and my little sister for always been there to listen to us despite being the youngest. Thanks to my friends to support me, without them the path would be harder. Especially, to Bruno and Joyce for taking their time to read this manuscript and give valuable advice. All their collaboration has borne fruit, thanks.

Following, I would like to thank my promotor and mentor Jeet Varia for his valuable input and support throughout this period in CMET. I appreciate my mentor advice to force me to learn beyond the expectations. In the same way, I want to thank my professor Korneel Rabaey for sharing his knowledge on this topic. I also must express that without the invaluable pioneer work of Nico Boon this thesis would not be materialised. I extend my great gratitude to Juan Anaya for the cooperative work and tuition in electrochemical practicalities.

In addition, it is vital to mention that this work would not be possible without the valuable opportunity of VLIR-UOS and the support of the Walter Valdano Raflo program from my Ecuadorian university ESPOL, throughout my master period.

In the same sense, this work in cooperation with Jeet Varia was thanks to MetGrow+ project. Additionally, I would like to acknowledge other scientists and technicians that contributed in the execution of this thesis as Van Dorpe, Lisa Claeys, Ran Rumes, and Xochitl Dominguez for their cooperation.

“To be worthy does not mean to be perfect. Perfection is not attainable. Everything is about effort for chasing our goals. Do not deviate from getting there without disregarding those you love”

Gong, Lombardi
Table of contents

COPYRIGHT .................................................................................................................. I
PREFACE ..................................................................................................................... II
TABLE OF CONTENTS ............................................................................................... III
LIST OF FIGURES ..................................................................................................... V
LIST OF TABLES ........................................................................................................ VI
LIST OF ABBREVIATIONS ......................................................................................... VII
ABSTRACT ................................................................................................................. VIII

I LITERATURE REVIEW .............................................................................................. 1
1. METAL RECOVERY AND BEYOND: INTER/MULTIDISCIPLINARY OVERVIEW .... 1
   1.1. BIOHYDROMETALLURGY ............................................................................. 2
2. MICROBIAL - METAL INTERACTIONS .................................................................. 2
   2.1. PARADIGM SHIFTS FROM REMEDIATION TO RECOVERY TO VALORIZATION 2
   2.2. MECHANISMS OF MICROBIAL METAL RECOVERY SORPTION AND
       PRECIPITATION/CRYSTALLISATION BY USING VIABLE CELLS ...................... 3
       2.2.1. Microbial metal ion sorption as passive mechanisms .............................. 4
       2.2.2. Active microbial metal ion reduction and crystallisation ....................... 5
       2.2.2.1. Dissimilatory respiration and assimilative pathway for metal reduction
                and crystallisation .............................................................................. 5
       2.2.2.2. Resistance mechanisms for metal reduction and crystallisation .......... 6
       2.2.3. Passive microbial metal ion reduction and precipitation ....................... 6
   2.3. MICROBIAL MEDIATED CRYSTALLIZATION: GREEN APPROACH, APPLICATION, IDENTIFICATION, AND PERSPECTIVES .......................... 7
3. METALS FOR METAL RECOVERY ......................................................................... 9
   3.1. MECHANISMS AND METHODOLOGIES FOR MICROBIAL – ELECTROCHEMICAL METAL RECOVERY ................................................................. 9
   3.2. CURRENT STATE OF THE ART OF MES: BIO-CATHODES FOR METAL RECOVERY ........................................................... 12
4. METALS TARGETED, AND BACTERIAL STRAINS APPLIED IN THIS STUDY .... 14
   4.1. METALS TARGETED ..................................................................................... 14
   4.1.1. Electrochemical equilibria of metal solutions ............................................ 14
   4.2. BACTERIAL STRAINS APPLIED .................................................................. 17
5. DESIGN OF INVESTIGATION ................................................................................. 19
   5.1. LOWER RESOLUTION FRACTIONAL FACTORIAL DOES ................................. 19
       5.1.1. Matrix construction ............................................................................. 20
OBJECTIVES AND EXPERIMENTAL OVERVIEW ................................................. 21
II MATERIALS AND METHODS ................................................................................. 23
1. DESIGN OF EXPERIMENTS ................................................................................. 24
2. BACTERIAL STRAINS AND GROWTH CONDITIONS ......................................... 25
3. CHEMICALS ......................................................................................................... 26
4. MICROBIAL METAL RECOVERY ....................................................................... 26
5. MICROBIAL-ELECTROCHEMICAL FOR METAL RECOVERY ........................... 26
6. ANALYTICAL METHODS ....................................................................................... 27
   BACTERIAL CONCENTRATION AND VIABILITY MEASUREMENTS .................. 27
   METAL REMOVAL AND CrVI REDUCTION MEASUREMENTS ............................. 28
   HEADSPACE ANALYSIS .................................................................................... 28
   LACTATE REMOVAL .......................................................................................... 28
   METAL NANOPARTICLES CHARACTERIZATION .............................................. 29
7. ANALYSIS .............................................................................................................. 29
8. STATISTICAL ANALYSIS ...................................................................................... 30
III RESULTS AND DISCUSSION .............................................................................. 31
1. BACTERIA CELLS IDENTIFICATION AND CELL WEIGHT CALCULATION OF
   SHEWANELLA ONEIDENSI S MR-1 AND CUPRIAVIDUS METALLIDURANS CH34 .. 32
2. MICROBIAL STRATEGIES FOR METAL RECOVERY ........................................ 33
   2.1. Ni²⁺, Cu²⁺, Au³⁺, AND CrVI REMOVAL BY S. ONEIDENSI S MR-1 AND C. METALLIDURANS CH34 .............................................................. 33
       2.1.1. Ni²⁺, Cu²⁺ experiments with S. oneidensis MR-1 and C. metallidurans CH34 ...... 35
       2.1.2. Au³⁺ and CrVI experiments with S. Oneidensis MR-1 and C. metallidurans CH34 CH3438
   2.2. Cu²⁺ REMOVAL WITH PSEUDOMONAS PUTIDA AND SERRATIA MARCESCENS ....... 46
   2.3. SUMMARY OF DOE METAL RESULTS ......................................................... 51
3. RESULTS VALIDATION OF THE BEST RESPONSE FROM DOE GOLD AND CHROMIUM

3.1. Gold triplicates of best run with pH comparison

3.1. Chromium duplicates of best run with higher concentration comparison

4. PART B: MICROBIAL ELECTROCHEMICAL SYSTEMS FOR AU$^{3+}$ AND CR$^{6+}$ RECOVERY

4.1. ELECTROCHEMICAL ANALYSIS OF AU$^{3+}$ AND CR$^{6+}$ SOLUTIONS

4.2. MES FOR METAL RECOVERY

4.2.1. Gold experiments, effect of step potential

4.2.2. Chromium experiments, effect of set potential

5. GENERAL DISCUSSION AND PERSPECTIVES, OUTLOOK

V CONCLUSION AND FURTHER RESEARCH

1. CONCLUSION

2. FUTURE RESEARCH

VI REFERENCES
Figure 3- 18 Chromium removal with microbial - electrochemical reactors. ........................................65
Figure 3- 19 Chromium reduction by MES at initial 15 mg/L of Cr^{6+} with step potentials of -0.795 V vs. SHE .................................................................66

List of tables
Table 1- 1 Reported minimum inhibitory concentration (MIC) of Shewanella oneidensis MR-1 and Cupriavidus metallidurans CH34 for the metals of interest in this thesis.............................................6
Table 1- 2 UV-Vis wavelength characteristics for reported metallic nanoparticles (NPs) ..........9
Table 1- 3 Summary of METs technologies system for metal recovery by biocathodes ..........14
Table 1- 4 Gold and chromium corrected reduction potential (E°) with Nernst equation at 0.2 mM of metal concentration and pH 7 for chromium. .............................................................17
Table 1- 5 Characteristics and behaviours of Gram-negative (Gram-ve) studied bacteria in microbial mediated nanoparticles and in MES. Inspired from (Kracke et al., 2015) ..............18
Table 1- 6 Analysis of application according to the type and resolution of the factorial designs in DOE..................................................................................................................20
Table 1- 7 Orthogonal matrix used to exemplify the DOE for a fractional factorial with seven parameters .................................................................20
Table 1- 8 Metal set of experiments (DOE Metal) for gold, copper, nickel, and chromium with the tested bacteria strains. .................................................................................................21
Table 2- 1 Summary of various factors investigated for bioprecipitation/biosorption experiments in design of experiments (DOE) .....................................................................................24
Table 2- 2 Design matrix applied for experiments: green box – RES(III) Ni, Cu, Au, and Cr experiments and red box – RES(III) Cu DOE 2 experiments..........................................................24
Table 2- 3 Summary of factors and levels (-1, +1) applied in fractional DOE matrix (a) Ni2+, Cu2+, and Au3+ experiments with S. oneidensis and C. metallidurans, (b) Cr6+ experiments with S. oneidensis and C. metallidurans and (c) Cu2+ experiments with S. marcescens and P. putida 25
Table 2- 4 Initial conditions of reactors at the levels high and low for the tested metal: nickel, copper, gold and chromium with S. oneidensis and C. metallidurans. Copper DOE 2 with S. Marcescens and P. Putida. .................................................................25
Table 3- 1 Results of DOE factors applied are detailed in materials and methods (Table 2- 3). Factors (A,B,C,D,E,F,G) and their levels (-1/+1) for Ni2+, Cu2+ and Au3+, A: OD (1 or 0.5/2 or 1), B: temperature (28/37 °C), C: anoxic/oxic (N2/air), D: pH (1or 2/5), E: metal concentration (0.2/2 mM), F: e-donor (H2/lactate), G: cells (MR-1/CH34). For Cr6+ A: medium (NaCl/M9), B: metal concentration (0.2/1 mM), C: anoxic/oxic (N2/air), D: pH (1or 2/5), E: OD (1/2), F: e-donor (H2/lactate) G: cells (MR-1/CH34) ............................................................34
Table 3- 2 : Summary of the factors of gold removal; and chromium removal and reduction...42
Table 3- 3 Summary of best results of DOE: nickel, copper, gold, and chromium with S. oneidensis MR-1 and C. metallidurans CH34; and *copper with Serratia marcescens and Pseudomonas putida with the studied factors. [Cs] bacteria concentration in cells mL\(^{-1}\) and [Cs] metal concentration mg mL\(^{-1}\).................................................52
Table 3- 4 Gold description of the validation sets with initially viable cells at pH 2 and 5, and the control with dead cells ........................................................................................................53
Table 3- 5 Chromium validation description with initially viable cells at 0.2 mM and 1 mM, and their controls (dead cells and abiotic) ..................................................................................................56
Table 3- 6 Initial conditions of the electrolytes, catholyte mimicking conditions of penicillin reactor 5 from DOE Au^{3+} and Cr^{6+}........................................................................59
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEM</td>
<td>Anion exchange membrane</td>
<td>METs</td>
<td>Microbial electrochemical technologies</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>AuNPS</td>
<td>Gold nanoparticles</td>
<td>NB</td>
<td>Nutrient Broth</td>
</tr>
<tr>
<td>CA</td>
<td>Chronoamperometry</td>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>Cb</td>
<td>Initial bacteria concentration, cells.mL⁻¹</td>
<td>OCP</td>
<td>Open circuit potential</td>
</tr>
<tr>
<td>CEM</td>
<td>Cation exchange membrane</td>
<td>OD₆₁₀</td>
<td>Optical density at 610 nm</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
<td>PSE</td>
<td>Pseudostandard error</td>
</tr>
<tr>
<td>C₀</td>
<td>Initial metal concentration, mg.L⁻¹</td>
<td>PVD</td>
<td>Physical vapour deposition</td>
</tr>
<tr>
<td>CVD</td>
<td>Chemical vapor deposition</td>
<td>OVAT</td>
<td>One variable at the time</td>
</tr>
<tr>
<td>CV</td>
<td>Cyclic Voltammetry</td>
<td>Ref</td>
<td>Reference electrode</td>
</tr>
<tr>
<td>CW</td>
<td>Cell wall</td>
<td>RPM</td>
<td>Rotational speed</td>
</tr>
<tr>
<td>DMRB</td>
<td>Dissimilatory Metal-Reducing Bacteria</td>
<td>RCF</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>DOE</td>
<td>Design of experiments</td>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>E₀</td>
<td>Standard potential</td>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>Eₑ</td>
<td>Nernst equilibrium potential</td>
<td>SPR</td>
<td>Surface Plasmon resonance</td>
</tr>
<tr>
<td>Eᵣ</td>
<td>Reversible potential (CVs)</td>
<td>SG</td>
<td>SYBR Green</td>
</tr>
<tr>
<td>EDX</td>
<td>X-ray spectroscopy</td>
<td>SGPI</td>
<td>SYBR Green and propidium iodide</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular polymeric substance</td>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
<td>w</td>
<td>width of bacterial cells</td>
</tr>
<tr>
<td>FC</td>
<td>Flow cytometry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram-ve</td>
<td>Gram negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂</td>
<td>Hydrogen gas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICP-OES</td>
<td>Inductive coupled plasma - optical emission spectroscopy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Current (mA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>j</td>
<td>Current density (mA/cm²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>l</td>
<td>length of bacterial cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M9</td>
<td>Minimum medium M9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBE</td>
<td>Molecular beam epitaxy</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Abstract

This thesis focused on the study of microbial and microbial - electrochemical systems (MES) for the recovery of metals via precipitation from simulated aqueous waste waters leading to the synthesis of micro to nanoscale precipitates or nanoparticles (NPs). Microbial mediated NPs have potential valorisation as functional materials.

Microbial strategies were first tested using a design of experiment (DOE) methodology, with screening of various factors reported to influence metal recovery from dilute solutions (< 2 mM) by using a fractional factorial design matrix (7 levels, 8 reactors). DOEs targeted metals as Au$^{3+}$, Cr$^{6+}$, Cu$^{2+}$, and Ni$^{2+}$. Metal removal of 92.8%, 53.5%, 64.2%, 28.4% was obtained for each system, respectively. Metal precipitates were detected for experiments with copper, gold, and chromium by transmission electron microscopy (TEM). Optimal condition for metal recovery from gold and chromium were validated with further testing and selected for further studies. Factors such as pH and initial metal concentration were significant ($\alpha = 0.05$) to describe gold metal removal, optimized at pH 5 in 0.2 mM Au$^{3+}$ solutions. Whereas, anaerobic conditions in M9 medium at neutral pH combined with *Shewanella oneidensis* MR-1 and H$_2$ proved to enhance the Cr$^{6+}$ reduction and removal significantly. The comparison of these set of experiments showed that initially viable cells, with the precise conditions, interpreted from DOE screening, perform higher removal/reduction of metals in comparison with the controls, variable pH and concentration.

Following on, a proof of principal in microbial-electrochemical system (MES) was developed for metal recovery and NP synthesis of Au$^{3+}$ and Cr$^{6+}$, with the perspective application of cathodes as sustainable electron donors for microbial respiration. Since the microbial reduction of metal ions, leading to NP synthesis, is typically mediated by an electron donor such as H$_2$. Conditions selected from the DOE experiment, were mimicked in the MES. Three recirculating reactors, set in parallel, were used for these experiments. For two reactors an applied potential to the working electrode (cathodes), where H$_2$ production was evidenced, was set based on previous electrochemical analysis of metals in three electrode set-ups. One of these reactors served as an abiotic control (no cells added). A second control experiment was designated as biotic (with addition of bacterial cells) in open circuit (OCV). *Shewanella oneidensis* MR-1 in presence of a step potential at - 0.5 V vs. Ag/AgCl removed 91% of gold from a synthetic solution in 24 h, whereas 83% Cr$^{6+}$ was reduced after 24h at -1 V vs. Ag/AgCl. ICP-OES and standard colorimetric methods were used to follow the metal concentration and reduction within time. Bacteria viability during the experiments was followed by flow cytometry, where live/dead staining (SGPI) on bacterial cells displayed an increasing red fluorescence emission when membrane damage increases. UV-Vis and TEM analysis confirmed the presence of zero valent gold NPs (AuNPs) and Cr hydroxide/oxide NPs (CrNPs). These results suggest that the microbial – electrochemical system could enhance the removal/reduction of metals when using initially viable cells, providing routes for further optimisation.
I Literature Review
1. Metal recovery and beyond: inter-multidisciplinary overview

In the modern era, metals have become indispensable for the society and industry in almost every extent. Starting from copper for electricity conduction, gold for production of jewels and catalysts, nickel and chromium for corrosion resistance, to integrated production of devices (e.g. computers, mobiles) used in daily-life. The multiple applications in a growing society demand continuous metal extraction, usually undertaken by primary mining of metals. There, twofold challenge can be highlighted. First, depletion of strategic metals since reserves are finite (Diederen, 2010), and followed by excessive environmental burden (“ecological rucksack”) (Sarsby et al., 2001).

Metals are essential for European economy, which depends mainly (80%) on imports (European Commission, 2010). There, the risk of supply shortage and their impacts on their economy (“Critical Raw [Materials - European Commission”) rise an alert to look for innovative alternatives to cover their needs. On the other hand, contaminated sites in Europe (35% caused by heavy metals pollution) catapulted the remediation urgency (Van Liedekerke et al., 2014). Against these backdrops, EU launched several projects (BioelectroMET, 2016; GetAMet, 2016; MetGrow, 2018) pursuing a circular economy to recover metals from both primary and secondary sources in a gun-sight for sustainable development (“Critical Raw [Materials - European Commission”). Metal recovery from secondary sources is fundamental to foster the sustainability of raw materials for low carbon energy technologies and tackle the metal disturbance in the environment.

Metal-laden waste streams from one side pose a threat for the environment, but from the other side, metal wastes could symbolize potential sources for recovery. Industrial wastewaters and leachates often contain valuable metal candidates for recovery (Nancharraiah et al., 2015). Spent leachates from superfluous materials such as landfilled sludges (1 million ton/year), fayalitic slags (2.95 million ton/year) could represent rich sources of Cu, Ni, and Cr (MetGrow, 2018). Gold can be found from jewelry leachates, eluates from activated carbon, and spent liqueurs in concentrations ranging from 1 - 2000 mg.L⁻¹ (Flores et al., 1995). Chromium can be also present in effluents from electroplating, pigment, leather tanning, vanadium mining and processing, lumber and wood product processing (Huang, Chai, et al., 2011; Myers et al., 2000), its concentrations range from 0.004 to 400 mg.L⁻¹ (H. Wang et al., 2014). All of these exemplify metal secondary sources. Considering that these streams bear market valuable metals such as gold, copper, nickel; and a critical metal such as chromium, economic incentives for the technology development are emerging.

As metal concentration in these sources can be relatively low (from ug.L⁻¹ to mg.L⁻¹), technologies should be developed in a more meaningful way for metal recover efficiency (H. Wang et al., 2014). Classical methods have been applied for metal removal from polluted environments, such as chemical (precipitation, electroextraction, membrane electrolysis) and physical (membrane filtration, ion exchange, carbon adsorption) strategies (He et al., 2015; C. Y. Yin et al., 2007). Membranes can remove metals selectively, but their challenges include high operational cost due to energy consumption and membrane fouling (H. Wang et al., 2014). Ion exchange is limited by their resins saturation and scarce selectivity (Gupta et al., 2015). Chemical precipitation is the most traditional method; however, it produces a large amount of toxic sludge. In overall, these methods generally use and/or produce considerable amounts of toxic chemicals (He et al., 2015), be energy intensive, or require expensive equipment (Dozie-Nwachukwu et al., 2017). More sustainable techniques, as microbial assisted mining by retrieving metals from diluted aqueous system have attracted much attention, which in addition can synthesize valuable products as metallic nanoprecipitates (Durán et al., 2012). For instance, gold from acidic leachate of a jewelry waste (115 mg/L Au³⁺) was completely recovered as gold NPs by using microbial strategies (Deplanche et al., 2008). Which opens a window for a possible viable application in biohydrometallurgy.
1.1. Biohydrometallurgy

Hydrometallurgy implies the extraction of the metal via aqueous solution. Conventional strategies can be expensive and energy intensive (Courtney, 2011). Therefore, bio-hydrometallurgy (application of microbes for the extraction and processing of metals) rise as a viable alternative to reclaim metals in a cost-effective, energy-saving, and environmentally friendly manner (Rawlings, 2002). However, bio-metallurgy (metal – microbial interactions) is not sufficiently explored for metal recovery and recycling, as only few biotechnologies have been applied at industrial scale for metal recovery from effluents (Hennebel et al., 2015). One example is the metal precipitation via biogenic H₂S (Paques, 2014), which consists of the introduction of chemicals in the first stage (elemental sulphur and fertilizer) for the bioproduction of H₂S; following by the chemical precipitation in a second stage. Drawbacks of this two-stage process is clearly elucidated at the dependency of toxic chemicals and the production of a non-highly marketable sludge, which is the main goal when recovery is targeted.

A smarter bio-application has risen by considering that microbes can influence the redox reactions of both insoluble minerals and dissolved metal(loid)s (Hennebel et al., 2015). Generally, these techniques can involve two distinct steps: (a) selective dissolution (leaching) of the metal from the ore and (b) selective recovery of the metal from solution (Dominguez-Benetton et al., 2015). In this concept, bioleaching consists on the release of the desired elements in an aqueous medium (Rawlings, 2002), meanwhile biosorption/bioprecipitation can be applied for retrieving and transforming the selected metal from these leachate for a recovering cycle.

Bacteria have been recognized to extract and concentrate metals from leachate solutions, industrial streams and wastewaters (Gupta et al., 2015). In addition, their microbial precipitation is attractive for the recovery and synthesis of NPs (Durán et al., 2012). Therefore, by applying this biohydrometallurgy strategy, the direct microorganism treatment allows the conversion of waste stream to a product with added value in one step (Hennebel et al., 2009), and suppresses the addition of toxic chemicals. This thesis will be focused on the metal recovery via microbial mediated sorption, reduction and/or precipitation, through microbial-metal interactions that could induce or enhance NPs production leading to valorisation.

2. Microbial - metal interactions

Much of our understanding of microbial-metal interactions roots from fundamental studies of the role of microbes in the geochemical cycling of metals (Banfield et al., 1997; G. Gadd, 2010). Investigations by geochemists reveal that microorganisms participate actively in the natural metal cycling and mineral formation, promoting changes in metal speciation, including mineral formation, dissolution or deterioration (G. Gadd, 2010). Properties of interest for in-vitro application in metal recovery.

2.1. Paradigm shifts from remediation to recovery to valorisation

Based on previous work in-vivo, in-vitro application initiated principally for the remediation of metals since the 1970’s (Voilesky, 1987). There applied for immobilizing or detoxifying heavy metals such as Cu, Zn, Pb, Ni, Cd from mining industries (G. M. Gadd et al., 1993). However, over the last 30 years, the application shifts from remediation to metal recovery perspectives (Dominguez-Benetton et al., 2018). Recovery of metals became the paramount objective in light of the challenges related to risk of supply shortages (“Critical Raw Materials - European Commission”). Moreover, metals are ideal materials for fulfilling the European circular economy initiative by metal recycling without losing their properties (Hagelüken et al., 2016). At the very state of the art, the focus of research has been further shifted to recover metals present as by-products in diluted environments by synthesizing valuable products (Deplanche et al., 2008; Y. Konishi et al., 2007). There, the possibility of synthesizing nanoscale precipitates by their microbial – metal transformations found myriad application in nanotechnology (Murray et al.,
Accordingly, a paradigm shift is evidenced from remediation to recovery, and then to valorisation at present and in the future.

The bacteria concomitantly can bio-recover metals by synthesising NPs from metal diluted environments. Thus, it provides a way to integrate remediation, recovery, and valorisation (L E Macaskie et al., 2010). The system could satisfy the recovery of targeted metals in biohydrometallurgy processes; however, the understanding of the holistic microbial mechanism has not yet been well defined. The specificity and diversity of microbial metabolism complicates the recognition of the general active elements, specially, in the nucleation and growth of the metal NPs (Castro et al., 2014). In the following section general mechanisms that have been speculated for their passive and active interaction will be discussed.

2.2. Mechanisms of microbial metal recovery sorption and precipitation/crystallisation by using viable cells

Gadd (2010) illustrated the microbial mechanisms in presence of metals. This comprise redox transformations, production of metal binding peptides and proteins (e.g. metallothioneins, phytochelatins), organic and inorganic precipitation, active transport, efflux and intracellular compartmentalization, metal-binding abilities in cell walls (CWs) and other structural components. These mechanisms summarized in Figure 1-1, can lead to the metal biosorption, bioreduction and formation of precipitates.

![Figure 1-1 Microbial mechanisms involved in the detoxification and transformation of metals. A variety of specific or non-specific mechanisms may also result in redox transformations, intracellular chelation and intracellular precipitation. Biomineral formation (biomineralization) may be biologically induced, i.e. caused by physicochemical environmental changes mediated by the microbes, or biologically controlled. Adapted from (G. Gadd, 2010).](image)

Once the metal reduction takes place, many reduced atoms start to agglomerate in colloids due to higher binding energy between the atoms than the atom-solvent energy (Southam et al., 1994). Thus, many metal atoms diffuse to the nucleation site, while encountering progressively into the growing crystals by a cascade of coalescence processes (Pandian et al., 2015; Southam et al., 1994). This phenomenon can be called crystallisation, when nanoscale solids/precipitates (NPs) are synthesised.

Microbial synthesis of metal NPs depends upon the localization of the reductive components of the cell. Extracellular NPs could be explained by the release of reductases secreted during their specific microbe defence mechanisms (Bao et al., 2010; Dahl et al., 2007). When the CW reductive enzymes or soluble secreted enzymes are involved in the reductive process of metal ions, it is more likely to find more extracellular NPs than in another location (Castro et al., 2014).

A wide range of microorganisms have been used for these NPs synthesis (bacteria, yeasts, fungi, and algae). However, bacteria have received a substantial attention for its potential synthesis of
Literature review

NPs (Sastry et al., 2003). This is especially because bacteria can be cultured easily, producing NPs mainly extracellular, requires mild conditions (circumneutral pH, ambient temperature) (Parikh et al., 2008), with fast reaction rate for NPs synthesis (< 24 hours) (Deplanche et al., 2008; Y. Konishi et al., 2007). In addition, the wide range of molecules in their structure can mimic the properties of chemical agents involved in metal reduction, stabilization and synthesis of the NPs. These natural molecules will prevent the consumption of expensive and toxic chemicals, making this method more attractive to synthesize NPs in a green chemistry approach. To exemplify, the energy requirements for metallic reduction are met by oxido/reductases enzymes located on the bacteria membrane structure (Saif Hasan et al., 2008). Moreover, the amphipathic nature of lipid structures make them a promising capping agent for stability of biogenic NPs, which are produced in higher amounts under oxidative stress in the stationary phase (Saif Hasan et al., 2008). In this sense, bacteria serves as reducing/capping agent and also as a carrier for NPs (De Corte et al., 2011). Furthermore, initially viable bacteria have shown advantageous metal removal and NPs synthesis in comparison with initially deactivated cells (Chubar et al., 2008; De Corte et al., 2011; Malik, 2004; J. Varia et al., 2016). Therefore, this thesis will first focus on the strategies of initially viable bacteria for metal recovery.

Figure 1-2 schematize the different interactions that microbes can have with metal ions leading to their recovery. Such interactions can occur in parallel or series (G. Gadd, 2010; Pasula et al., 2017), involving passive (sorption, physicochemical reduction) or active bacterial metabolism by their dissimilative, assimilative, and resistance mechanisms of initially viable cells (Pasula et al., 2017). Whether NPs are formed, they could be located extracellular, intracellular, or on the CW (Castro et al., 2014).

<table>
<thead>
<tr>
<th>Passive</th>
<th>Dissimilative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active</td>
<td>Assimilative</td>
</tr>
<tr>
<td>Resistance</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Location</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular</td>
<td></td>
</tr>
<tr>
<td>On Cell Wall(CW)</td>
<td></td>
</tr>
<tr>
<td>Intracellular</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 1-2** Categorization of mechanisms for NPs synthesis, aiming to be applicable for other microbial–metal interactions. Inspired from (Pasula et al., 2017; J. Varia, 2018).

### 2.2.1. Microbial metal ion sorption as passive mechanisms

The term biosorption is commonly used to cluster several physicochemical interactions with bacterial cells, such as adsorption, absorption, ion exchange, entrapment, reduction-oxidation and methylation (G. Gadd, 2010; G. M. Gadd et al., 1993). Adsorption immobilize the metal ions onto the CW, exopolymers (EPS), other structural components, and/or derive/excreted products. Functional groups of the cell membrane (i.e. carboxyl, phosphate, and amine) confer a net negative charge to some microbial surface (e.g. on *Bacillus subtilis*) allowing them to uptakes
substantial amounts of metal cations (Mg$^{2+}$, Fe$^{3+}$, Cu$^{2+}$, Na$^+$, K$^+$, Mn$^{2+}$, Zn$^{2+}$, Ca$^{2+}$, and Ni$^{2+}$) (Beveridge et al., 1976).

In this context, physicochemical parameters play an important role in the biological pathways for capturing metals from a solution, with special attention to pH (Abbas et al., 2016). The pH can affect the solubility and speciation of metal ions, as well as protonation and deprotonation of the CW by increasing the acidity or basicity of the aqueous medium (Gupta et al., 2015). A study showed that at low pH, higher protonation of the CW ligands gave a surface more positively charged, which increases the electrostatically driven passive sorption of the negatively charged ions, such as AuCl$_4^-$ species (Nakajima, 2003). This was also reported by Varia et al. (2014), at pH 1 – 3 in 300 mg L$^{-1}$ AuCl$_4^-$ solutions. Also, Cr$^{6+}$ species form chromate CrO$_4^{2-}$ anions (pH 8 – 2.5) have been reported to have accumulation on bacterial biomass at lower pH (< 2.5) (Srinath et al., 2002). Higher pH (6 – 7) were determined as optimum for Cu$^{2+}$ biosorption (Fan et al., 2014), and for Ni$^{2+}$ by reducing the competition for sorption sites with H$^+$ (Gupta et al., 2015).

Biosorption is generally referenced as a passive uptake mode by pre-treated/deactivated, and non-living microbial biomass (Volesky, 1987). However, it is also a property in living biomass. Higher metal removal has been reported by using growing metal-resistant cells (e.g. extra capacity of 46.5 mg Ni/g cell compared to pre-treated cells) through a combination of biosorption, and continuous metabolic uptake of metals after physical adsorption (Malik, 2004). Accordingly, a biosorption study by J. Varia et al. (2014) showed an efficient removal capacity of initially viable *Shewanella putrefaciens* (1343 mg AuCl$_4$/g cell), in contrast with lower removal with initially dead cells (749 mg AuCl$_4$/g cell) at pH 3. This mechanism has been proved as a low cost, greener, and promising technology depending on the physicochemical interactions with the cellular compounds of the species applied (Abbas et al., 2016).

### 2.2.2. Active microbial metal ion reduction and crystallisation

After biosorption, precipitation may occur as a result of active metabolism: energy conservation; intracellular deposition and sequestration by enzymatic components; or metabolite release (sulphite, oxalate) or reductive agents (G. Gadd, 2010). In microbial energy conservation, metals can act as indirect or direct electron donors/acceptors for oxido-reduction reactions (Courtney, 2011). This process can be considered as a respiring mechanism. In addition, the enzymatic processes or the reducing groups on the CW, can later on produce metallic precipitates (De Gusseme, 2010). Metabolites or other reducing agents can be secreted by bacteria when suffer greater stress, therefore it has been suggested that the crystallisation of NPs takes place in those conditions (Saif Hasan et al., 2008). The aforementioned interactions are considered as a resistance mechanism. Moreover, the microbial mediate NPs from industrial leachates has been categorised as cheap and scalable, with the ability to recover metal with satisfactory purity (Lynne E. Macaskie et al., 2008). Opening the gates for innovative valorisation and viable commercial application.

Enzyme activity and biochemical pathways of the organisms are the most relevant intrinsic properties to analyse for selecting the suitable candidates for metal biosynthesis. Moreover, optimal conditions for cell viability, enzyme activity, and reaction development are recommended to be optimized by temperature, pH, mixing speed, yield and production rate (Castro et al., 2014). The deposition phenomena with the oxy/red redox mediators, protein enzymes on the bacterial cell, or released by the bacterial cell is of interest to enhance the metal ion nucleation in engineered application for metal recovery (J. Varia et al., 2013).

#### 2.2.2.1. Dissimilatory respiration and assimilative pathway for metal reduction and crystallisation

Active bacterial mechanisms for metal reduction or accumulation can be broadly categorised in dissimilatory metal reduction and/or assimilate metals into cellular materials (H. Wang et al.,
Dissimilatory metal reduction is a respiration process in which bacteria use metals ions as electron acceptors for respiration under anoxic conditions (Lovley, 1993). Dissimilatory metal reducing bacteria (DMRB) follow this pathway outside the cell membrane for cellular energetics (H. Wang et al., 2014). To illustrate, *Pseudomonas sp.* and *Shewanella sp.* coupled the oxidation of lactate or hydrogen gas (H₂) to the reduction of Fe³⁺ via dissimilatory metal reduction, providing energy (chemolithothrophic) to support bacterial growth (Lovley, 1993). Moreover, this reduction mechanism mediated by H₂ and lactate in combination with *Shewanella* genus has been studied for enhancing metallic NPs formation (Kane et al., 2016; Yasuhiro Konishi et al., 2006).

On the other hand, there has been metal reduction in aerobic microorganisms involved in the assimilation of metals into cellular components (Lovley, 1993). For instance, chromate anion (Cr₂O₇⁻) in solutions present structural similarities to biologically important inorganic anions, such as sulphate (SO₄⁻) and phosphate (PO₄³⁻), conferring the ability to Cr₂O₇⁻ to transverse cell membranes, via the sulphate transport system, and be incorporated into the cell (Cervantes et al., 2001; Codd et al., 2001).

### 2.2.2. Resistance mechanisms for metal reduction and crystallisation

It has been revealed that bacteria can actively reduce some metal ions as a mechanism to reduce their toxicity. Some microbes can grow and even flourish in metal stress conditions (minimum inhibitory concentration, MIC, as indicator Table 1-1), because they are capable of evolving survival mechanisms that contribute to metal toxic resistance (G. Gadd, 2010). This can involve transformation of the toxic metal ions into their corresponding metal or metal oxides/sulphide (Nies, 1999), thus, modifying the metal speciation and mobility (G. Gadd, 2010). Additionally, efflux mechanisms or enzymatic detoxification (i.e. reduction) can be involved (G. Gadd, 2010; Silver et al., 2009) which in some cases lead to NPs formation.

**Table 1-1** Reported minimum inhibitory concentration (MIC) of *Shewanella oneidensis* MR-1 and *Cupriavidus metallidurans* CH34 for the metals of interest in this thesis.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Ni²⁺ (mM)</th>
<th>Cu²⁺ (mM)</th>
<th>Au³⁺ (mM)</th>
<th>Cr⁶⁺ (mM)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Shewanella oneidensis</em> MR-1</td>
<td>0.05</td>
<td>0.05</td>
<td>0.06</td>
<td>2</td>
<td>(Brown et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Singapore, 2015)</td>
</tr>
<tr>
<td><em>Cupriavidus metallidurans</em></td>
<td>0.6</td>
<td>3</td>
<td>0.0008</td>
<td>0.4</td>
<td>(Monsieurs et al., 2011)</td>
</tr>
<tr>
<td>CH34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Additionally, some bacterial plasmids have metal and metalloids resistance genes (for instance, Cu²⁺, CrO₄⁻ and Ni²⁺), sequenced in previous studies (Osman et al., 2008; Silver et al., 2009). Upregulation of the stress response genes occur from the moment that the toxic substance is inside the cells (Nies, 1999). To exemplify, DMRB may contain a gene encoding for an enzyme that can precipitate soluble ionic forms of gold to insoluble metallic nanoprecipitates (Kashefi et al., 2001). In addition, Cr⁶⁺ reductase in the cytoplasmic membrane of *S. oneidensis* reduced Cr⁶⁺ in solution under anaerobic conditions (Myers et al., 2000), and formed Cr³⁺ precipitates when chromate was the sole terminal electron acceptor (Daulton et al., 2007). In these ways, abating the toxic load.

### 2.2.3. Passive microbial metal ion reduction and precipitation

After biosorption, reduction and precipitation may also occur via physicochemical influence of an external reducing agent, such as hydrogen. H₂ is not capable to reduce nor synthesize metallic NPs by standing alone (De Corte et al., 2011; Deplanche et al., 2008). However, in combination with a support matrix, for instance bacterial cells, reduction could be favoured in the sorption site,
leading to nanoprecipitates formation by nucleation. This assumption can be supported by previous experiments using heat killed cells of the Shewanella genus in presence of H₂ as electron donor. AuNPs were synthesised without the involvement of any active bacterial metabolic pathway (De Corte et al., 2011; J. Varia et al., 2014). Thus, it has been hypothesized that Au³⁺ is reduced to a Au¹⁺-S complex (bacteria), but pure physicochemical mechanism could further reduced the Au⁰ (De Corte et al., 2011). In that sense, Au³⁺ that is bounded to or complexed by the bacteria cell component, can utilized H₂ as a reductant to form metallic AuNPs (De Corte et al., 2011). Figure 1-3 illustrate a passive microbial precipitation.

2.3. Microbial mediated crystallization: green approach, application, identification, and perspectives

Previously it was explained how bacteria can both mediate and/or support NPs from their microbial - metal interactions. NPs are of keen interest as their properties differ from their bulk materials (Gericke et al., 2006). Their large surface area to volume ratio enhance catalytic reactivity, optical properties, and chemical steadiness (Agarwal et al., 2017; Gericke et al., 2006). There is an increasing interest of the microbial mediated NPs due to their unique properties with potential wide-ranging applications (Horeyalla et al., 2017; Murray et al., 2000). The as-synthesized NPs are found in many fields such as energy, environmental remediation, disinfection, etc (G. Ingale, 2013). With these principles, metal recovery by bacterial strategies from waste streams can easily achieve added marketable products (Hennebel et al., 2009).

NPs are usually produced by conventional methods on a top-down approach or bottom-up approach. Top-down approach involves process of milling or etching from macroscopic materials to nanoscale level through plastic deformation; these processes demand energy and can be costly (Agarwal et al., 2017). On a bottom-up approach, assembly of NPs from atomic/molecular components can save costs but are complicated (Shamaila et al., 2016). Both approaches need an additional capping and stabilizing agent (Agarwal et al., 2017). Thus, conventional NPs production (physical and chemical) involve an inherent use of chemicals, many of which are toxic to the environment (Shamaila et al., 2016). In contrast, microbial strategies used self-produced reducing, stabilizing and capping agents in mild-conditions, fulfilling the concept of green chemistry (Shantkriti et al., 2014). Figure 1-4 represents the current methods for NPs synthesis.

The microbial mediated NPs has been highlighted for potential commercial application (J.-W. Wu et al., 2017). More commercial applications in various fields can be found for extracellular production of metallic NPs (Castro et al., 2014). But also, metallic NPs in bacterial carrier matrix are interesting for degradation catalysis of persistent organic pollutants, as was reported for Bio-Pd and Bio-Ag (De Gusseme, 2010; Windt et al., 2005). As polydispersity is the major concern, optimization of conditions (e.g. pH, temperature, time) become important for achieving monodispersed particles (Castro et al., 2014).
Gold NPs (AuNPs) play a prominent role today in nanotechnology (Hutchings et al., 2008). AuNPs and their compounds have been applied in biosensors, medical diagnostic and intracellular delivery, especially due to their biocompatibility and low inherent toxicity (Castro et al., 2014). AuNPs morphology could define their application. For instance, nanotriangles could be used in photonics, optoelectronics, and optical sensing (Castro et al., 2014), whereas nanoflowers can be an effective drug delivery for cancer cells (Song et al., 2018). In addition, gold represent the best catalyst for a numerous of reactions (Hutchings et al., 2008). Deplanche et al. (2008) showed that similar catalytic activities were achieved by AuNPs bio-recovered from waste leachates, than those produced chemically (Carrettin et al., 2004). This research reinforce the potential to unify recovery of metal and conversion of valuable products in a one-pot process (Lynne E. Macaskie et al., 2008).

Copper NPs (CuNPs) have been studied profoundly for applications in healthcare and industry (Rajesh et al., 2016). In contrast with the extended studied silver NPs (AgNPs), copper is a cheaper essential metal suitable for medical applications, and present supreme antibacterial/antifungal activity by growth inhibition (Rubilar et al., 2013). Therefore, their usage can be applied in hospital sterilization (Mikolay et al., 2010). In this last decade, CuNPs as catalyst have been studied due to their low-cost and remarkable catalytic efficiency (Rubilar et al., 2013). Another application in the industry are in gas sensors, high temperature superconductors, solar cells, and catalytic processes (Majeed et al., 2015).

Nickel NPs (NiNPs) from green synthesis were studied for removal of dye and pollutant adsorption from aqueous solution (Pandian et al., 2015). This study suggested NiNPs to treat textile and tannery effluents. Another study explored other applications in antimicrobial, anti-inflammatory and anti-proliferative activities (Horeyalla et al., 2017).

Cr$_2$O$_3$ NPs is of interest due to its reported use as green pigments, antibacterial properties, high temperature and corrosive resistance (Al-Saadi, 2015; Jaswal et al., 2014). Green synthesis of Cr$_2$O$_3$ NPs were reported by using Leaf extract with tested antibacterial activity against Escherichia coli (Ramesh et al., 2012). Bacterial synthesis of chromium NPs as useful materials have not been a focus of research, although some studies reported chromium precipitates by bacterial as Shewanella putrefaciens and oneidensis MR-1 (Daulton et al., 2006; Myers et al., 2000).

Sizes of particles play a key role in the optical properties of the NPs (Kreibig et al., 1995). UV–Vis spectroscopy is widely being used to initially identify the formation and stability of NPs in aqueous solution (Dozie-Nwachukwu et al., 2017). The surface plasmon resonance (SPR) of NPs
will exhibit a clear peak of absorption in a certain wavelength (Mulvaney, 1996). The reported wavelength in literature can be observed in Table 1-2. Of note, AuNPs SPR could shift due to differences in their size, agglomeration, and conformational protein (Deplanche et al., 2008).

<table>
<thead>
<tr>
<th>Metallic NPs</th>
<th>Range of NPs</th>
<th>Description Specific wavelength / characteristic or conditions/ Colour change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nickel</td>
<td>370 – 630 nm</td>
<td>395 nm / colour change from yellow to light brown</td>
<td>(Pandian et al., 2015)</td>
</tr>
<tr>
<td>Copper</td>
<td>570 – 630 nm</td>
<td>590 to 630 nm / Serratia m. exposed to CuSO4 570 nm / Colour change to yellow</td>
<td>(Horeyalla et al., 2017)</td>
</tr>
<tr>
<td>Gold</td>
<td>520 – 580 nm</td>
<td>520 nm / pH 2 / Pale yellow to bright red 550 nm / pH 6 / Pale yellow to purple 580 nm / pH 9 / Pale yellow to dark blue</td>
<td>(Rajesh et al., 2016; Sair Hasan et al., 2008)</td>
</tr>
<tr>
<td>Chromium</td>
<td>460 nm</td>
<td>460 nm / Chromium NPs oxides(Cr2O3) / 20 to 70 nm of hexagonal structure /</td>
<td>(Varshney et al., 2010; Deplanche et al., 2008; Dozie-Nwachukwu et al., 2017; J. Vartia et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>430 nm</td>
<td>430 nm / leaf extract synthesis</td>
<td></td>
</tr>
</tbody>
</table>

As discussed in the previous section microbial reduction of metal ions leading to NP synthesis can typically be enhanced and/or mediated by an electron donor, such as H₂. Giving that and the need for more environmental friendly solution for remediation and recovery, clean energy supply to microorganisms without chemicals surge as an alternative. In that sense, a landmark study reported the application of biocathodes for metal recovery via NPs precipitation (Gregory et al., 2005). Based on that, further investigation is certainly required on the bases of this investigation within the broader field of microbial electrochemical technologies (METs).

3. METs for metal recovery

METs incorporate an interdisciplinary synergy between electrochemistry and microbiology (Rabaey et al., 2010). This field is based on the fact that electrochemically active bacteria submerged in one or both compartment of the electrochemical-cell can switch from the natural soluble electron donor or acceptor to an insoluble electrode (Rabaey et al., 2005). The knowledge that the electrical current applied to the electrochemical configurations can also drive the microbial metabolism has led to a vast of applications from bioremediation to the production of valuable products (Rabaey et al., 2010).

Research in METs was initially implemented to generate electric current (bio-galvanic) and clean organic pollutants by using bioanodes in the so-called microbial fuel cells (MFCs) (Rabaey et al., 2005). Later on, the application shifted and expanded by applying electricity to harvest valuable products at the cathode such as H₂ in microbial electrolysis cells (MECs) (Gildemyn et al., 2017). In this past decade, the focus has been to obtain other high valuable chemicals (e.g. hydrogen peroxide, organic synthesis) in microbial electrosynthesis reviewed by Rabaey (2010) and Logan et al. (2013). At the very state of the art, the application for metal recovery is very much in its infancy (Catal et al., 2009; Luo et al., 2015; Shen et al., 2015; H. Wang et al., 2014).

3.1. Mechanisms and methodologies for microbial – electrochemical metal recovery

METs offers an innovative approach for efficient metal recovery, in light of the flexible platform for both oxidation and reduction reaction oriented processes (Logan et al., 2013; H. Wang et al., 2014). Recently, METs have demonstrated excellent performances in removal and recovery of metals from different wastewaters, by using electrodes as electron suppliers (H. Wang et al., 2014). The main advantages of METs for metal recovery is that one can couple both remediation
of complex wastewaters and metal recovery; reduce process energy demand by comparison with traditional extractions; and finally generate metallic products with lower greenhouse emissions (Dominguez-Benetton et al., 2018).

The use of bioanodes has been reported extensively for recovery of metals on the reduced abiotic compartment, observed in the Figure 1-5 A for the microbial-electrochemical system (MES). The system runs on the bases that electrons are generated from biological oxidation (exoelectrogens) of organic substrate in the anode, which travel through resistances (or aided by extra power source) to an abiotic cathode where metal ions are reduced. The metal can be recovered from the deposition in the electrode and precipitation in the electrolyte (Dominguez-Benetton et al., 2018; H. Wang et al., 2014). MES with bioanodes have received considerable attention for recovering metals from metallurgical wastewater in absence or limited energy input, by EU projects as “BioelectroMET” (BioelectroMET, 2016).

However, less research has been carried for the application of biocathodes for metal recovery (Figure 1-5 B). Here, bacteria could use solid electrodes as electron donors for respiration from the cathode (Rabaey et al., 2010) and couple this to metal sorption and or precipitation (Dominguez-Benetton et al., 2018). Indeed, such mechanisms involving biotic cathodes could boost favourable conditions for metal reduction and transformation of metal diluted effluents (Kim et al., 2015; J. C. Varia et al., 2014).

In the biocathodes, two strategies for metal recovery are highlighted. First, electrochemical reactions in the cathode can be bio-catalysed (J. C. Varia et al., 2014). Second, stimulation of the microbial metabolisms can be perceived in the cathodic compartment either by direct electron transfers through membrane enzymes or indirectly via redox mediators and/or hydrogen (Figure 1-6) (Thrash et al., 2008a). In biocathodes, electrotrophs are used as catalysts to accept cathodic electrons, and enhance metal reduction (Huang, Regan, et al., 2011) or for the production of value-added products (Huang, Chai, et al., 2011). Further advantages of biocathodes, are that electrotrophs can decrease overpotentials and remove recalcitrant waste particularly a neutral pH (Shen et al., 2015).
Of note, the membrane enzyme Cytochrome-c exhibits a standard potential of +0.26 V vs. SHE (at neutral pH), whose function can be crucial for metal respiration (Scott Mathews, 1985). Accordingly, the Cytochrome-c potential can approximately set a thermodynamic limit for respiratory microbial metal reduction (Dominguez-Benetton et al., 2018). This could lead to the statement that metal ions with higher standard potential than the Cytochrome-c are the greatest for demonstrating the promising application of recovery on biocathodes (Dominguez-Benetton et al., 2018).

The electrochemically active microorganisms can be attached to the cathode as biofilms or suspended in the medium (planktonic microorganisms). Biofilms exhibit enhanced metals tolerance compared with planktonic microorganisms; and also separate easily the treated liquid from the biomass (Nancharaiah et al., 2010). However, dense biofilms (>40 µm) can hinder the active sites for recovery of metals (J.-W. Wu et al., 2017), and pose a challenge to maintain the activity of the biofilm (H. Wang et al., 2014). Another limitation is consistency, the biomineralization of active biofilm in MES exhibited a non-uniform metallic precipitates, in which pre-treatment by inactivation was proposed as a solution (Courtney, 2011). On the other hand, planktonic cells can provide a greater mass transfer due to a large surface fraction of the bacteria which favours the metal contact and uniform precipitation. Planktonic cells mainly interact with electrodes via electrons shuttles or H2 (Guo et al., 2015). Another advantage would be that the biotic catholyte can be constantly refreshed with initially viable cells, and sequentially the valuable products can be harvested.

Microorganism-electrode interactions can influence the reactions by the biocompatibility, surface topography, and conductivity of the electrode (Guo et al., 2015). Thrash et al. (2008a) indicate that stainless steel can be used effectively as electrodes in contact with bacteria, although most research has been done with carbon. Carbon graphite electrodes are preferable for this system as they are cheaper, good for bacterial adhesion, chemically stable, good conductor; and well documented to transfer electrons directly and indirectly to bacterial cells for anaerobic respiration (Gregory et al., 2004). While metallic electrodes such as stainless steel have good conductivity, mechanical strength, competitive-cost against carbon; their biocompatibility can be limited (Guo et al., 2015). The unbeatable advantage of stainless steel is the enhanced H2 generation, capacity to transfer of electrons to bacteria (Dumas et al., 2008; Selembo et al., 2009), with a supportive report of biofilm formation (Sharma et al., 2014). Both materials can be fabricated as plates, rods, meshes and felts; in which planar electrodes are preferable for fundamental research (Guo et al., 2015).

MES configurations can be constructed as a single, or 2 or more chambers. Separation of the chambers is achieved with selective permeable membranes, depending on the added value.
functions in each electrolyte (Sleutels et al., 2009). Two-chamber MES have been typically applied for metal recovery to separate the reduction process of metals in the catholyte from the anolyte (Shen et al., 2015). Were the aim is to maintain the potential of the working electrode (e.g. cathode) to ensure precise redox potential, three-electrode systems can poise the cathode potential versus a constant potential reference electrode (Thrash et al., 2008a).

The majority of MES reported the installation of cation exchange membranes (CEM) (Sleutels et al., 2009). Most metal species coexist as cations in the catholyte compartment. Therefore, just by considering the concentration gradient, metals can be transported from cathode to anode through the CEM (Dominguez-Benetton et al., 2018). Anions such as \( \text{AuCl}_4^- \) and \( \text{Cr}_2\text{O}_7^{2-} \) could initially not interfere in CEM membranes (Figure 1- 7 A). However, their developed intermediate reduced metal cations could transverse the membrane unless a greater attraction force retains them in the catholyte as reported by Tandukar et al. (2009). Therefore, to present a scalable design, an analysis of CEM selectivity, resistance, and transport across the membrane needs to be studied (Sleutels et al., 2009).

To avoid metal cation migration, anion exchange membranes (AEM) can be used in metal recovery, as mainly allows the transportation of anions from the cathodic to the anodic compartment (Dominguez-Benetton et al., 2018). Moreover, pH variation lead the movement of ions between compartments, thus, for both cases CEM and AEM, pH truly plays and important role. However, the situation can be overcome with pH correction for each compartment respectively. Bipolar membrane (comprises a CEM and AEM ensemble together) can also be a solution, as it can enhance proton/hydroxide selectivity despite some possible energy cost implications (Dominguez-Benetton et al., 2018).

In a more interesting perspective, electrodialysis processes consist of a smart configuration in which the ionic movement is facilitated/impeded by the CEM and AEM membranes along the applied electrical field (Figure 1- 7 B). This has been extensively applied for desalination, demineralization, and to a lesser extent for acid-alkali production (Sleutels et al., 2009) or metal recovery. There, the energy content of the wastewater power the separation process of anions (bio- anode chamber separated by an AEM) and cations (cathode/CEM) (Sleutels et al., 2009).

![Figure 1- 7](image-url)

*Figure 1- 7* Configuration of electrochemical cells with ion exchange membranes. (A): Schematic overview of continuous flow of a biocathodes system with cation exchange membranes (CEM) allowing proton movement in microbial-electrochemical systems (MES) (B): Bio-electrodialysis cells (AEM and CEM configuration) (Varcoe et al., 2014).

### 3.2. Current state of the art of MES: bio-cathodes for metal recovery

The following table summarizes so far, the research carried out for metal recovery using biocathodes (category B Figure 1- 5). The first application of biocathodes was demonstrated by Gregory and Lovely for the reduction of hexavalent uranium (\( \text{U}^{6+} \)) to their insoluble tetravalent form (\( \text{UO}_2 \)) with *Geobacter sulfurreducens*. The cathode served as an electron donor when poised at -500 mV vs. Ag/AgCl (Gregory et al., 2005). Uranium was shown to be absorbed onto the
surface of the electrode when the potential was applied, however, metal reduction was only achieved when bacterial cells were present as electroactive biofilms on the electrode surface. Similarly, reduction was not observed when no potential step was applied in the biotic cathode. A total of 87% (89 µmol) was removed from the system with biotic cathodes. With these results, it can be said that the electrode not only allowed the electron transfer, but also could drive electrostatic adsorption and metal ion transport in the electric double layer. Furthermore, it was proved that the microbial-electrochemical mechanism leads to transformation of metal precipitates (UO₂), which was not achieved in the absence of microbes and the applied potential (Dominguez-Beneton et al., 2018).

Bioanodes and biocathodes can be coupled together to decrease the energy applied in the process. This is the case of the experiments done for selenium recovery (Catal et al., 2009), and the two-chamber system for simultaneous copper and cobalt recovery (Shen et al., 2015). In a single aerobic chamber MFC, elemental selenite was found as deposits on the electrode surface and as colloids in the catholyte. There, carbon felts were installed as electrodes in both compartments. Domestic wastewater was used for the enrichment of the electrodes, with addition of acetate and glucose to serve as anodic electron donors. Selenite removal of 99% were achieved by using glucose, and 88% by using acetate (Catal et al., 2009). Furthermore, Shen et al. (2015) reported the recovery of cobalt in the biocathodes of a MEC, drove by a MFC with bioanodes. Copper was simultaneously recovered in the abiotic cathode of the MFC. In such a system with a mixed culture, the *proteobacteria* was dominant (67.9%) that comprised of α- (33.7%), β- (23.8%), γ- (9.0%), and δ- (1.2%) classes. Removal rates of both metals were enhanced up to 1.7 times and 3.3 times for copper and cobalt respectively, by comparing with those with one-system of abiotic cathodes. Additionally, H₂ was generated without any external energy consumption (Shen et al., 2015), which could also influence the reduction and removal of the mentioned metals.

The first report of biological Cr⁶⁺ reduction in a biocathode (as biofilm) of a MFC was published by Tandukar et al. (2009). A mixed culture from an anaerobic digester obtained a maximum Cr⁶⁺ reduction rate of 0.46 mg Cr⁶⁺/g VSS.h. On the running experiment, an abiotic control exhibited a minimal Cr⁶⁺ reduction and biomass decay, which can lead to a conclusion that in the treatment removal was aided by the microbial metabolisms. Within 5 hours, initially hexavalent chromium concentration of 80 mg/L were totally absent in the filtrate solution. There, it was considered as Cr(OH)₃ precipitated that could be adsorbed on the cathode or on the bacteria. In a similar system, Huang et al. (2011) achieved higher reduction and removal of Cr⁶⁺ by setting a cathode potential. The latter shows that the fastest MFC start-up time and highest Cr⁶⁺ reduction was achieved by setting the potential at -0.30 V vs. SHE. As similar reduction was also achieved at a potential of -0.15 V vs. SHE, optimization can be suggested (Dominguez-Beneton et al., 2018). Of note, these experiments were capable to produce nanoscale precipitates (Cr(OH)₃) on the bacterial biomass with potential for recovery, which were not feasible by abiotic configurations.

By connecting a research of biocathodes for gold recovery with a subsequent research that studied the analysis of AuCl₃ sorption leading to gold nanoparticle synthesis by *Shewanella putrefaciens* (J. Varia et al., 2016) the viable idea of production of NPs in METs has been highlighted. On cathodes bacteria can make this technology possible for recovery and/or metallic NPs synthesis. The cells can be supported by application of negative potential at the electrode, interacting with bacteria for example, by direct electron donors to bacterial cells or the in-situ production of electron donors such as H₂. Moreover, capacitive deionization can improve the diffusion of metal ions to bacterial cells immolated on electrodes (Gregory et al., 2005). Finally, CW enzymes or acid functional groups implicated in metal sorption and reduction could be influenced by applied potential. This can set the foundation, for a selective sorption and subsequent metal reduction in these systems (J. Varia et al., 2016).
However, these systems have their limitations. First, there is an inherent necessity of a power source. Second, there should be a necessity of a pre-treatment before entering the biological chamber, considering the toxicity towards microbes. Further limitations, is that low amount of metal recovered can make market logistics difficult (Dominguez-Beneton et al., 2018). As discussed, MES offers potential for metals recovery. However, the restricted experience and gaps in knowledge to understand the interactions of metals ions, bacteria and electrodes is required for further application assessment (H. Wang et al., 2014).

4. Metals targeted, and bacterial strains applied in this study

4.1. Metals targeted

In this study, metals as nickel, copper, gold, and chromium will be discussed, based on their representative importance. Gold exhibited the greatest interest due to their environmental implications of mining and processing (Graedel et al., 2015), in addition to its economic value and long history in nanotechnology (Hutchings et al., 2008). On the other hand, manufacturing metals with economically importance as copper exhibited many advantages in their efficient microbial NPs production (Rubilar et al., 2013). Nickel hold high economic importance, and is found in a wide range of secondary resources (MetGrow, 2018). Finally, chromium exhibits high attention as a critical raw material (CRM) (“Critical Raw Materials - European Commission”) due to supply risk and environmental implications (Graedel et al., 2015).

4.1.1. Electrochemical equilibria of metal solutions

Analysis of the limits of thermodynamically equilibria of metal ions in aqueous solution is required to understand the microbial interactions in the hydrometallurgy strategy. By this effect, Pourbaix diagrams provide a panoramic map of the equilibrium state for the variety of species of a metal in function of pH (abscissa) and potential (ordinate, reported vs. SHE) (Pourbaix, 1966). Particularly, multielement Pourbaix can be constructed to introduce additional elements (i.e. chloride) for characterizing regions where targeted redox conversion occurs (Thompson et al., 2011). Moreover, microbial metal binding agent can modified these graphs, but can be negligible if an appropriate membrane is implemented in MES with biotic anodes (Figure 1- 5 A) (Dominguez-Beneton et al., 2018). However, metal thermodynamic equilibria could shift when microbes are present in the catholyte (Figure 1- 5 B) (Dominguez-Beneton et al., 2018; J. Varia, 2012).

Gold form very stable complexes in aqueous solution at +1 and +3 oxidation state, but also gold can be present in other oxidation states (0,+2, and variety of polynuclear clusters) (Hutchings et al., 2008). Au^{3+} is a strong oxidant (Bard et al., 1985). Figure 1- 8 A, shows the speciation of gold
in chloride solutions. Tetrachloraurate (AuCl₄⁻) is predominantly present in solutions at pH < 6; thus, AuCl₄⁻ reduction reaction to metallic Au⁰ can occur in acidic electrolytes close to neutral pH. AuCl₄⁻ experiences stepwise hydrolysis whose products are represented as AuCl₃·x(OH)ₓ⁻ in the alkaline going direction (S. Wang et al., 2009). Gold biosorption and bioprecipitation have been reported from pH 1 to 7 (Suresh et al., 2011; J. Varia et al., 2013). Nonetheless, important consideration is given to pH, as it could also impact the size, shape, and number of particles produced by microbial systems (Dozie-Nwachukwu et al., 2017).

Copper +1 and +2 are the most stables form in aqueous media (Bard et al., 1985). Copper metal species and their equilibrium are illustrated in the Pourbaix Figure 1-8 B. Cu²⁺ can be present as copper oxides (pH > 3) and as zero valent copper (E < 0.2 V vs. SHE) (Thompson et al., 2011). Ni²⁺ ion can be oxidized to Ni³⁺ in the form of chelates or reduced to Ni⁺ or Ni⁰ in the presence of a suitable complexing ions (Bard et al., 1985). However, in aqueous solution it is mainly represented by Ni²⁺ (Beverskog et al., 1997), which remains in solution as a single cation in a wide range of pH. The uncharged complex Ni(OH)₂ predominates only at very alkaline conditions (Figure 1-8 C). Microbial biosorption have been mainly reported for nickel (Abbas et al., 2016; Chubar et al., 2008). Moreover, microbial precipitation of copper leading to CuNPs have been reported in neutral to acidic pH in few reports (Rajesh et al., 2016; Saif Hasan et al., 2008; Shantkriti et al., 2014; Taran et al., 2017).

In the majority of aqueous environments, the redox active metal of chromium is mainly present in two oxidation states: Cr³⁺ and Cr⁶⁺ (Kimbrough et al., 1999). Cr²⁺ can also be present (Daulton et al., 2006), but is easily oxidized to Cr⁵⁺ by oxygen. Cr⁵⁺ is the most stable and can form many inert complexes. Unstable Cr⁴⁺ and Cr⁶⁺ species are intermediates in redox reactions (Bard et al., 1985). The valence of Cr largely controls the biogeochemical properties of Cr complexes including solubility, adsorption affinity, chemical reactivity, and toxicity (Kimbrough et al., 1999). For instance, Cr⁶⁺ species are strong oxidants (especially in acidic solutions) which act as carcinogens, mutagens, and teratogens in biological systems (Daulton et al., 2006). The reduction of the mobile and toxic Cr⁶⁺ to innocuous and immobile Cr³⁺ is of technological and biological importance (Daulton et al., 2007). The Pourbaix diagram in Figure 1-8 E allows to understand the equilibrium in aqueous solution to see this through. Cr⁶⁺ will reduce to soluble Cr³⁺ at low pH but Cr precipitates as Cr-oxide species will be observed at pH higher than 5.

In a general view, it is possible to represent the standard reduction potential (E°) of the main reaction in the selected pH of study in a redox tower (Figure 1-9). From there, the position of Cytochrome-c complex can be observed as it functions as an electron transport chain in reduction - oxidation reactions pathways by bacteria 0.26 V vs. SHE (pH 7) (Scott Mathews, 1985). This representation aid to predict the movement of electrodes in the system. Metal ions with lower redox than the (a)biotic anode potential cannot receive electrons spontaneously, but when enough potential is applied, theoretically, all the metal ions can be reduced (H. Wang et al., 2014).
Figure 1-8 Pourbaix diagrams of gold, copper, nickel, chromium. **A**: Au Pourbaix diagram at 298 K (25 °C) in chloride solution at 1M concentration Cl⁻. Replicated from (Thompson et al., 2011). **B**: Cu Pourbaix diagram at 298 K (25 °C) (Thompson et al., 2011). **C**: Ni Pourbaix (Takeno, 2005) **D**: Cr Pourbaix (Lindsay et al., 2012).

![Pourbaix diagrams](image)

**Figure 1-9** Redox tower of the metal ion standard reduction potentials in solutions of the studied metals, and cytochrome-c (MtrC) (Scott Mathews, 1985) reported vs. SHE at 25 °C. Adapted from (Dominguez-Benetton et al., 2018).

<table>
<thead>
<tr>
<th>E vs. SHE/V</th>
<th>Reduction reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4</td>
<td>$\text{HCrO}_4^{-} + 3\text{e}^- + 7\text{H}^+ \rightarrow \text{Cr}^{3+} + 4\text{H}_2\text{O}$</td>
</tr>
<tr>
<td>1.2</td>
<td>$\text{Cr}_2\text{O}_7^{2-} + 6\text{e}^- + 14\text{H}^+ \rightarrow 2\text{Cr}^{3+} + 7\text{H}_2\text{O}$</td>
</tr>
<tr>
<td>1.0</td>
<td>$\text{AuCl}_4^{-} + 3\text{e}^- \rightarrow \text{Au} + 4\text{Cl}^-$</td>
</tr>
<tr>
<td>0.8</td>
<td>$\text{AuCl}_2 + 2\text{e}^- \rightarrow \text{AuCl}_2^{-} + 2\text{Cl}^-$</td>
</tr>
<tr>
<td>0.6</td>
<td>$\text{Cu}^+ + \text{e}^- \rightarrow \text{Cu}$</td>
</tr>
<tr>
<td>0.4</td>
<td>$\text{Cu}^{2+} + 2\text{e}^- \rightarrow \text{Cu}$</td>
</tr>
<tr>
<td>0.2</td>
<td>Cytochrome c (Fe³⁺)+ e⁻ → Cytochrome c (Fe²⁺) (pH 7)</td>
</tr>
<tr>
<td>0.1</td>
<td>$\text{CrO}_4^{2-} + 3\text{e}^- + 4\text{H}_2\text{O} \rightarrow \text{Cr(OH)}_3(\text{aq}) + 5\text{OH}^-$(basic)</td>
</tr>
<tr>
<td>-0.2</td>
<td>$\text{Ni}^{2+} + 2\text{e}^- \rightarrow \text{Ni}$</td>
</tr>
<tr>
<td>-0.4</td>
<td></td>
</tr>
</tbody>
</table>

A qualitative indication of which species are being reduced in MES, in regard to applied potential, can be obtained from the comparison of the theoretical reversible potentials ($E_r$) from slow scan cyclic voltammetry (CV) with the calculated Nernst ($E_{eq}$) potentials, that consider the standard equilibrium potentials $E^0$ (Table 1-4) (Bard et al., 1985). Overpotential and ohmic drops in the MES can reflect some differences between the thermodynamic and the practical case. Furthermore, microorganisms can successfully achieve metal redox transformations to added-
value NPs sometimes by influencing favourable thermodynamic or kinetically conditions in the cathode (Dominguez-Benetton et al., 2018; J. Varia, 2012). To illustrate theoretical corrected reduction (Equation 1), gold and chromium reactions at 0.2 mM are described in Table 1-4. The electrochemistry of chromium is complex due to all its oxidation states, besides, at pH 7 two species are almost equally present: HCrO$_4^-$ and CrO$_4^{2-}$ (Bard et al., 1985).

\[
E_v = E^0 + \frac{RT}{nF} \ln \left[ \frac{C_u}{C_R} \right] \equiv E_r
\]

**Equation 1**

<table>
<thead>
<tr>
<th>Half reaction</th>
<th>Electrons</th>
<th>(E_v) / V vs. SHE</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AuCl$_4^-$/Au$^0$</td>
<td>3</td>
<td>0.934</td>
<td>(Bard et al., 1985)</td>
</tr>
<tr>
<td>AuCl$_4^-$/AuCl$_2^-$</td>
<td>2</td>
<td>0.824</td>
<td></td>
</tr>
<tr>
<td>AuCl$_2^-$/Au$^0$</td>
<td>1</td>
<td>1.154</td>
<td></td>
</tr>
<tr>
<td>CrO$_4^{2-}$/Cr(OH)$_3$</td>
<td>3</td>
<td>1.246</td>
<td></td>
</tr>
<tr>
<td>HCrO$_4^-$/Cr(OH)$_3$</td>
<td>3</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>HCrO$_4^-$/Cr$^{3+}$</td>
<td>3</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Cr$^{3+}$/Cr$^{2+}$</td>
<td>1</td>
<td>-0.47</td>
<td></td>
</tr>
<tr>
<td>Cr(OH)$_3$/Cr$^{2+}$</td>
<td>1</td>
<td>-0.95</td>
<td></td>
</tr>
<tr>
<td>Cr$^{2+}$/Cr</td>
<td>1</td>
<td>-1.128</td>
<td></td>
</tr>
</tbody>
</table>

4.2. Bacterial strains applied

*Shewanella oneidensis* MR-1 has the ability to respire with a wide range metal (Fredrickson et al., 2008) and is considered as an archetypal DMRB, capable of enhance reduction by the presence of electron donors as H$_2$ (through hydrogenase) (Myers et al., 2000) and lactate (through Mtr pathway) (Kane et al., 2016). In addition, *S. oneidensis* have been studied for its ability to use solid electrodes for electron transport, with special attention along bacterial nanowires (El-Naggar et al., 2010), redox mediators and direct electron shuttling (Kotloski et al., 2013), of interest for microbial - electrochemical systems (MES). *Cupriavidus metallidurans* CH34 showed notable metal resistance (Max Mergeay et al., 2003) which involved resistance metabolisms and metal reduction mediated by H$_2$ in the periplasmic and cytosolic hydrogenase (M Mergeay et al., 1985). Limited reports of copper NPs by bacteria were available, emphasizing the work of Hasan and colleagues (2008) with *Serratia marcescens* and Shantkriti & Rani (2014) with *Pseudomonas* genus. This last one exhibits an extended list of application in MES, but no reports were found for metal mediated reduction and crystallisation. Figure 1- 10 illustrates some of the NPs synthesis by the studied bacteria and simplified the most recognized Mtr pathway on *S. oneidensis* MR-1. Table 1- 5 summarizes the relevant literature review of the bacteria strains applied in this thesis.
Figure 1-10 Mechanisms and pathways represented for metal-microbial interactions for synthesis of metallic NPs: A: *S. oneidensis* MR-1 Mtr pathway for synthesis of AuNPs. B: EDX and SEM of AuNPs mediated by *C. metallidurans* CH34. C and D: Copper oxide NPs on cells-free *S. marcescens* medium. E: CuO NPs in *Pseudomonas* genus (Avendaño et al., 2016; Reith et al., 2009; Saif Hasan et al., 2008; J.-W. Wu et al., 2017).

Table 1-5 Characteristics and behaviours of Gram-negative (Gram-ve) studied bacteria in microbial mediated nanoparticles and in MES. Inspired from (Kracke et al., 2015).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Characteristics, Key components</th>
<th>Application for NP synthesis</th>
<th>Application in MES</th>
<th>Relevant reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Shewanella oneidensis</em> MR-1</td>
<td>Gram-ve, facultative anaerobic DMRB. Mtr pathway: proton gradient created by cytochromes (c-type), soluble electron carriers and membrane bound NADH-hydrogenase. Anaerobic oxidation of lactate. Bacterial nanowires and self-produced mediators</td>
<td>Au(^+) from AuCl(_4)-, Te(^6) from TeO(_3)-, Pd(^0) from Pd(^2+), uranium oxide NPs from aqueous UO(_2)-, and Cr(^{6+}) to divalent deposits</td>
<td>Anode: model organisms for direct and self-mediated electron transfer</td>
<td>(El-Naggar et al., 2010; Kotloski et al., 2013; Suresh et al., 2011; C. Wu et al., 2013; Xafenias et al., 2013)</td>
</tr>
<tr>
<td><em>Cupriavidus metallidurans</em> CH34</td>
<td>Gram-ve, facultative aerobic chemolithoautotrophic, H(_2) oxidation by periplasmic bound and cytosolic hydrogenase. Cation/ proton chemiosmositic efflux system. Detoxification gene clusters in the two mega plasmids pMOL28 and pMOL30</td>
<td>Metal precipitates of Cu, Cd, Zn, Co, Ni, Pb, Pd, Y, Ge. AuNPs as two stage reduction</td>
<td>No report</td>
<td>(Diels et al., 1995; M Mergeay et al., 1985; Max Mergeay et al., 2003; Monchy et al., 2006; Reith et al., 2009; Rozycki et al., 2009)</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>Gram-ve, cells at stationary phase experience oxidative stress and release reductive biomolecules due to resistance mechanisms</td>
<td>Copper oxides NPs AuNPs, AgNPs, BiNPs</td>
<td>Cathode: reduction as part of microbial consortium in MFCs</td>
<td>(Dozie-Nwachukwu et al., 2017; Nazari et al., 2012; Saif Hasan et al., 2008; Taran et al., 2017; Xue et al., 2017)</td>
</tr>
<tr>
<td><em>Pseudomonas putida, fluorescence, aeruginosa</em></td>
<td>Gram-ve, electroactive used for their diverse respirational capacity.</td>
<td>P. putida AgNPs from Ag(^+), Nano-Se from selenite. P. aeruginosa Anode: current production by</td>
<td>(Avendaño et al., 2016; Friman et al., 2012; Gopinath et al., 2015)</td>
<td></td>
</tr>
</tbody>
</table>
5. Design of investigation

Based on previous discussed literature for this research topic, many parameters or factors may influence the outcome that is expected. By simplicity, design of experiments of one variable at the time (OVAT) tends to be extensively applied due to the practical perception of the effect of each factor. However, this technique impedes to visualize if the effect of one factor varies over the levels of other factors. Thus, it is necessary to include something different into classical experimental designs to increase research efficiency (Lazic, 2004).

Design of experiments (DOE) by factorial experiments is a smart way to conduct experiments. By this sense, it is possible to collect maximum amount of pertinent information with minimum time and resources expenditures (Lazic, 2004). DOE is a powerful optimization technique that investigates multiple (two or more) independent variables and one (or more) dependent variables (response). This technique allows visualization of the effect of any factor (independent variable) for any situation regarding the other factors (Kempthorne, 1960).

By assigning two levels for each studied factor (k), low (-1) and high (1), a complete $2^k$ experimental matrix can be constructed, termed full fractional factorial; where all possible combinations of level and factors are studied (Lazic, 2004). For large number of k, fractional factorials are preferable. They can be derived from the full factorial matrix by dividing into number of parts by two (2,4,8,16). The obtained results are named $\frac{1}{2}$ factorial ($2^{k-1}$), $\frac{1}{4}$ factorial ($2^{k-2}$), and continue systematically following the model of $2^{k-p}$, with p= extend of fractionation (Lazic, 2004).

### 5.1. Lower resolution fractional factorial DOES

In fractional factorial DOES, the number of runs (reactors or experimental points-trial) are selected in a statistical distribution obeying the sparsity-of-effects principle to obtain the necessary important information with a minimal number of runs (Lazic, 2004). Fractional factorial designs imply less experiments, costs, and time. From there, valuable outcomes for screening and optimization can be obtained.

The strength of DOE by fractional factorials is measured by resolution which determines the application of the selected methodology, represented as Roman number (III, IV, V) in Table 1-6. Resolution will give an idea of the level of aliasing or confounding (meaning that same element can have more than one representation) of the main factors with their interaction in the design Resolution III are DOE designs suitable for screening approaches to determine the significance of the main factors, with minimum number of experiments. Resolution IV implies that there is a
confounding between main factors and higher interactions, but in practice, higher level of interactions is rather inexistent. Higher resolution fractional factorials overcome confounding and ascertains the results but bears in mind the higher costs. Furthermore, higher resolution allows the creation of empirical models which predict responses for different parameters investigated and provides routes for fast track optimization.

Table 1-6 Analysis of application according to the type and resolution of the factorial designs in DOE.

<table>
<thead>
<tr>
<th>Resolution</th>
<th>Application</th>
<th>Confounding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Res III</td>
<td>Initial screening</td>
<td>Main effects confounded with 2 factor interactions</td>
</tr>
<tr>
<td>Res IV</td>
<td>Characterization of a system</td>
<td>Main effects confounded with 3 factor interactions or two factor interactions are confounded with each other</td>
</tr>
<tr>
<td>Res V</td>
<td>Used for robust modelling, optimization of a process, understanding complex effects, but expensive</td>
<td>Main effects confounded with 4 factor interactions or 2 factors confounded with 3 factor interactions.</td>
</tr>
<tr>
<td>Full factorial</td>
<td></td>
<td>No confounding</td>
</tr>
</tbody>
</table>

5.1.1. Matrix construction

The orthogonal matrix represents the different combinations of factors (k) and levels (low: -1, high: 1); columns will correspond to the individual factors (A, B, C, etc) and rows to different experimental runs (Lazic, 2004).

The first three columns are selected from a full fractional (independent vectors) with a length equal to the number of trials (Box et al., 1978). Depending on the factorial design, generators are used for the construction of the complete matrix. This means that by taking the first three factors as unequal vectors, the product between them will generate unsurpassed vectors corresponding to the remaining factors (Lazic, 2004). The product between factors also represents interactions, i.e. D=AB, which leads to the term aliased or confound (Box et al., 1978). Generators mathematically obtained are represented in Table 1-7 leading to the construction of the matrix. Finally, the experiments run in a completely randomized design used in this thesis as reactor numbers.

Table 1-7 Orthogonal matrix used to exemplify the DOE for a fractional factorial with seven parameters.

<table>
<thead>
<tr>
<th>Standard Order</th>
<th>Random Run Order (reactor)</th>
<th>Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>-1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>-1</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>-1</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>
Objectives and experimental overview

This study was divided into two main parts. First microbial strategies targeted Cu$^{2+}$, Ni$^{2+}$, Au$^{3+}$, and Cr$^{6+}$ recovery from diluted (< 2 mM or < 350 mg/L) synthetic solutions. As discussed in the introduction various factors are reported to influence metal recovery from diluted solutions. Furthermore, mechanisms can vary depending on bacterial species applied. Therefore, microbial strategies were tested using a design of experiment (DOE) methodology with the application of a fractional factorial DOE (Lazic, 2004) to identify optimal conditions. A resolution III (Res III) fractional factorial orthogonal matrix for 7-6 factors with 8 experimental runs was applied Table 1-7. Although such highly fractionated factorial experiments are not ideal for robust modelling and determination of interactions due to confounding; they are ideal for fast track screening and a basis for further optimisation (Box et al., 1978). Subsequently to DOE screening, a validation for optimal metal recovery was also performed for some experiments. The response analysed was metal removal, metal reduction, and/or metal NPs synthesis. Specific strains were selected based on extensive literature survey summarized in the introduction of thesis and detailed in Table 1-8.

Table 1-8 Metal set of experiments (DOE Metal) for gold, copper, nickel, and chromium with the tested bacteria strains.

<table>
<thead>
<tr>
<th>Metal set of experiments (DOE Metal)</th>
<th>Bacteria strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOE Gold, DOE Copper, DOE Nickel, DOE Chromium</td>
<td>Shewanella oneidensis MR-1</td>
</tr>
<tr>
<td>DOE 2 Copper</td>
<td>Cupriavidus metallidurans CH34</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas putida UWC3 (LMG2322), Serratia marcescens (LMG 2792)</td>
</tr>
</tbody>
</table>

In Part B, the best validated conditions for removal, reduction and valorisation from the DOE experimental set were simulated in electrochemical reactors in the cathodic chamber. In this sense, a proof of concept was sort in MES by the application of cathodes for enhanced metal recovery by supporting/enhancing highlighted microbial routes.
Part A
Screening Microbial strategies
Design of experiments (DOE)

Part B
Microbial Electrochemical systems (MES)
Effect of step potential

Results validation to best response
Repetition
Controls
Cells viability

Figure 1-11 Schematic overview of the methodology applied in this study.
II Materials and Methods
1. Design of experiments

Microbial mediated recovery was evaluated first using 1/8 fractional (Resolution III, $2^{7-4}$) or (Resolution III, $2^{6-3}$) factorial design matrix for screening of various physicochemical factors highlighted to influence microbial mediated metal recovery (Table 2-1). Each set of experiments consisted of 8 experimental reactors with high and low levels specified in the Table 2-3. Considering the factors and levels low (-1) and high (+1) an orthogonal matrix is constructed (Table 2-2). Before each experiment, the run order was randomised to ensure that each trial was independently distributed and for elimination of any bias in the experimental units and/or treatment of combinations-trials (Lazic, 2004). The run order was considered as the identification of the reactors described through this thesis. Initial conditions of the individual reactors at each level is described in Table 2-4.

Table 2-1 Summary of various factors investigated for bioprecipitation/biosorption experiments in design of experiments (DOE).

<table>
<thead>
<tr>
<th>Factors investigated</th>
<th>Categorical/numerical values</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial concentration</td>
<td>(OD)$_{600}$</td>
<td>Indicator of bacterial removal capacity (g mg metal removal / g bacteria)</td>
</tr>
<tr>
<td>Temperature</td>
<td>28 °C - 37 °C</td>
<td>Incubation temperature is a key influence on bacterial growth</td>
</tr>
<tr>
<td>Medium</td>
<td>Nutrient medium/M9 medium/0.9% NaCl</td>
<td>Incubation medium is a key influence on bacterial maintenance</td>
</tr>
<tr>
<td>N$_2$ / air</td>
<td>anoxic/oxic</td>
<td>Some microbial mechanisms reported to require anoxic conditions for bioprecipitation</td>
</tr>
<tr>
<td>pH</td>
<td>pH 1 - pH 7</td>
<td>The pH influences metal ion sorption, bacterial cell viability and metal speciation</td>
</tr>
<tr>
<td>Metal concentration</td>
<td>$\approx$ 10 - 350 mg/L</td>
<td>Considering metal toxicity to bacterial cells</td>
</tr>
<tr>
<td>Electron donor</td>
<td>H$_2$, Na-lactate</td>
<td>Electron donors commonly shown to mediate bioprecipitation</td>
</tr>
<tr>
<td>Bacteria species</td>
<td>Various</td>
<td>As highlighted in literature review</td>
</tr>
</tbody>
</table>

Table 2-2 Design matrix applied for experiments: green box – RES(III) Ni$^{2+}$, Cu$^{2+}$, Au$^{3+}$, and Cr$^{6+}$ experiments and red box – RES(III) Cu DOE 2 experiments.

<table>
<thead>
<tr>
<th>Standard Order</th>
<th>Random Run Order (reactor)</th>
<th>Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7 1 -1 -1 -1 1 1 1 -1</td>
<td>A B C D E F G</td>
</tr>
<tr>
<td>2</td>
<td>2 1 -1 -1 -1 1 1 1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1 1 1 -1 1 -1 -1 -1</td>
<td>F G</td>
</tr>
<tr>
<td>4</td>
<td>5 1 1 -1 1 -1 -1 1</td>
<td>E F G</td>
</tr>
<tr>
<td>5</td>
<td>8 -1 -1 1 1 -1 -1 -1</td>
<td>D E F G</td>
</tr>
<tr>
<td>6</td>
<td>4 1 -1 1 -1 1 -1 1</td>
<td>C D E F G</td>
</tr>
<tr>
<td>7</td>
<td>3 -1 1 1 -1 -1 1 -1</td>
<td>B C D E F G</td>
</tr>
<tr>
<td>8</td>
<td>6 1 1 1 1 1 1 1 1</td>
<td>A B C D E F G</td>
</tr>
</tbody>
</table>
Table 2-3 Summary of factors and levels (-1, +1) applied in fractional DOE matrix (a) Ni^{2+}, Cu^{2+}, and Au^{3+} experiments with *S. oneidensis* and *C. metallidurans*, (b) Cr^{6+} experiments with *S. oneidensis* and *C. metallidurans* and (c) DOE 2: Cu^{2+} experiments with *S. marcescens* and *P. putida*.

<table>
<thead>
<tr>
<th>a</th>
<th>A: OD_{610}</th>
<th>B: Temperatur e</th>
<th>C: anoxic/oxic</th>
<th>D: pH</th>
<th>E: [Metal] 0.2 - 2 mM</th>
<th>F: Electron donor</th>
<th>G: cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni^{2+}, Cu^{2+}, Au^{3+} RES(III)</td>
<td>(-1) 1 Ni and Cu, 0.5 Au and (+1) 2 Ni and Cu, 1 Au</td>
<td>(-1) 28 °C ↔ (+1) 37 °C</td>
<td>(-1) N_{2} ↔ (+1) air</td>
<td>(-1) Ni, Cu pH 2, Au pH 1 ↔ (+1) pH 5</td>
<td>(-1) (Cu 12 mg/L), (Ni 10 mg/L), (Au 35 mg/L) ↔ (+1) (Cu 125 mg/L), (Ni 100 mg/L), (Au 350 mg/L)</td>
<td>(-1) H_{2} ↔ (+1) CH34</td>
<td>(-1) MR-1 ↔ (+1) CH34</td>
</tr>
<tr>
<td>b</td>
<td>A: Medium</td>
<td>B: [Metal] 0.2 - 1 mM</td>
<td>C: anoxic/oxic</td>
<td>D: pH</td>
<td>E: OD_{610}</td>
<td>F: Electron donor</td>
<td>G: cells</td>
</tr>
<tr>
<td>Cr^{4+} RES(III)</td>
<td>(-1) 0.9% NaCl ↔ (+1) M9 medium</td>
<td>(Cr 15 mg/L) ↔ (+1) (Cr 75 mg/L)</td>
<td>(-1) N_{2} ↔ (+1) air</td>
<td>(-1) pH 2 ↔ (+1) pH 7</td>
<td>(-1) 1 ↔ (+1) 2.0</td>
<td>(-1) H_{2} ↔ (+1) Lac</td>
<td>(-1) MR-1 ↔ (+1) CH34</td>
</tr>
<tr>
<td>c</td>
<td>A: Medium</td>
<td>B: Bacterial viability</td>
<td>C: Temperature</td>
<td>D: [Metal] 5 - 50 mM</td>
<td>E: Electron donor</td>
<td>F: cells</td>
<td></td>
</tr>
<tr>
<td>Cu^{2+} RES(III)</td>
<td>(-1) Nutrient Broth ↔ (+1) 0.9% NaCl</td>
<td>(-1) non-viable heat treated ↔ (+1) viable</td>
<td>(-1) 28 °C ↔ (+1) 37 °C</td>
<td>(-1) 5 ↔ (+1) 50 mM</td>
<td>(-1) None ↔ (+1) Lac</td>
<td>(-1) <em>S. marcescens</em> ↔ (+1) <em>P. putida</em></td>
<td></td>
</tr>
</tbody>
</table>

Table 2-4 Initial conditions of reactors at the levels high and low for the tested metal: Ni^{2+}, Cu^{2+}, Au^{3+}, and Cr^{6+} with *S. oneidensis* and *C. metallidurans*. Cu^{2+} DOE 2 with *S. marcescens* and *P. putida*.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Level</th>
<th>OD_{610} (Cu\textsubscript{eq} cells.mL\textsuperscript{-1})</th>
<th>pH</th>
<th>[Metal]\textsubscript{eq} mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nickel, Ni\textsuperscript{2+}</td>
<td>High</td>
<td>2 (=3x10\textsuperscript{8})</td>
<td>5.27 ± 0.30</td>
<td>120.94 ± 3.19</td>
</tr>
<tr>
<td>Low</td>
<td>1 (=1.5x10\textsuperscript{8})</td>
<td>2.10 ± 0.01</td>
<td>11.31 ± 0.40</td>
<td></td>
</tr>
<tr>
<td>Copper, Cu\textsuperscript{2+}</td>
<td>High</td>
<td>2 (=5x10\textsuperscript{9})</td>
<td>5.17 ± 0.03</td>
<td>125.34 ± 5.56</td>
</tr>
<tr>
<td>Low</td>
<td>1 (=2.5x10\textsuperscript{9})</td>
<td>2.14 ± 0.03</td>
<td>12.64 ± 1.71</td>
<td></td>
</tr>
<tr>
<td>Gold, Au\textsuperscript{3+}</td>
<td>High</td>
<td>1 (=6x10\textsuperscript{4})</td>
<td>5.01 ± 0.06</td>
<td>352.19 ± 67.13</td>
</tr>
<tr>
<td>Low</td>
<td>0.5 (=3x10\textsuperscript{3})</td>
<td>1.02 ± 0.02</td>
<td>44.48 ± 7.22</td>
<td></td>
</tr>
<tr>
<td>Chromium, Cr\textsuperscript{6+}</td>
<td>High</td>
<td>2 (=9x10\textsuperscript{4})</td>
<td>6.95 ± 0.05</td>
<td>77.17 ± 3.88</td>
</tr>
<tr>
<td>Low</td>
<td>1 (=3x10\textsuperscript{5})</td>
<td>2.02 ± 0.05</td>
<td>15.50 ± 0.79</td>
<td></td>
</tr>
<tr>
<td>Copper, Cu\textsuperscript{2+} DOE 2</td>
<td>Low</td>
<td>2 (=2x10\textsuperscript{8})</td>
<td>Medium 5.39</td>
<td>326.83 ± 91.98</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td><em>P. putida</em></td>
<td>NaCl 3.48</td>
<td>3013.13 ± 261.11</td>
<td></td>
</tr>
</tbody>
</table>

2. Bacterial strains and growth conditions

Frozen cells (frozen in 20 % glycerol at −80 °C) were grown under aerobic conditions on trypticase soy agar (Oxoid, England) for *Shewanella oneidensis* MR-1 (LMG 19005) and *Cupriavidus metallidurans* CH34 (SCK•CEN (Mol, Belgium)), and on nutrient agar (Oxoid, England) for *Pseudomonas putida* (LMG 2322) and *Serratia marcescens* (LMG 2792).
Materials and Methods

Following, a single colony of cells where transferred to 500 mL of sterile trypticase soy broth (TSB) (Carl Roth, Germany) for *S. oneidensis* MR-1 and *C. metallidurans* CH34, and nutrient broth (NB) (Carl Roth, Germany) for *P. putida* and *S. marcescens*. Cells were grown to a stationary phase (72-96-hours of incubation, OD$_{610}$ = 1) and harvested by centrifugation (Sorval RC 6+, Thermo Scientific, USA), washed twice and concentrated with sterile 0.9% w/v NaCl to OD$_{610}$ = 10. The concentrated bacteria cells were made anoxic by degassing for 21 cycles of vacuum and filling with N$_2$ gas (Linde, Germany).

3. Chemicals

All chemical reagents used were of analytical grade and supplied by Carl Roth (Germany), VWR International (USA), or Sigma-Aldrich (UK). Metal stocks were prepared from respective salts using distilled water (Merck Millipore, Burlington, MA). I.e. Ni$^{2+}$ (5.87 g/L) from nickel (II) sulphate hexahydrate ($M_r = 262.75$ g/mol), Cu$^{2+}$ (6.35 g/L) from copper (II) sulphate pentahydrate ($M_r = 249.69$ g/mol), AuCl$_4^-$ (5.00 g/L) from gold (III) chloride hydrate ($M_r = 339.79$ g/mol) and Cr$_2$O$_7^{2-}$ (54.00 g/L) from potassium dichromate (VI) ($M_r = 294.185$ g/mol). Metal solutions were prepared in a medium consisting of 0.9 w/v % NaCl from a decuple concentrate stock of NaCl salt ($M_r = 58.4$ g/mol) unless otherwise stated (NB or M9 medium). M9 medium, prepared also from a decuple concentrated stock, consist of 6 g/L Na$_2$HPO$_4$, 3 g/L KH$_2$PO$_4$, 0.5 g/L NaCl, 1 g/L NH$_4$Cl.

4. Microbial metal recovery

Metal solutions (50 mL) were prepared from respective stock solutions in 120 mL penicillin bottles. With pH adjustment using 0.9 M NaOH or 0.9 M H$_2$SO$_4$ for Ni$^{2+}$, Cu$^{2+}$; or with 0.9 M NaOH and 0.9 M HCl for Au$^{3+}$ and Cr$^{6+}$ solutions. After adding the test solution, each reactor was plugged with thick butyl rubber stoppers and sealed with an aluminium crimp. For anoxic experiments, reactors where degassed with N$_2$ (21 cycles, vacuum and pumping N$_2$, Linde, Germany) and autoclaved at 121 °C for 21 min. After this, H$_2$ gas (Linde, Germany) was added to the reactors that specified this as electron donor by purging on the reactor for 10 minutes. Or whether lactate as electron donor was specified, the corresponding aliquot of the 0.1 M sodium lactate (Sigma-Aldrich, USA) was added to obtain ≈1-10 mM on each reactor. As described by J. Varia et al. (2014). For a typical experiment, 5 or 10 mL of washed viable bacterial cells (OD$_{610}$ ≈ 10, 1 × 10$^{10}$ cells/mL) were added to the metal solution. The reactors were gently shaken (120 rpm) in an incubator (KS 400i, IKA, Germany) at the temperatures specified on the matrix.

5. Microbial-electrochemical for metal recovery

Based on the results from microbial mediated metal recovery, a MES was further developed. All experiments were conducted in temperature-controlled room (28 °C). Electrochemical analysis and polarisations were carried out using a BioLogic potentiostat VSP 1008 multi-channel with processing software EC-Lab (VSP, Bio Logic, Science Instruments, 2014b). An Ag/AgCl reference electrode (- 0.204 vs. SHE / V, 3 M KCl, Bio-Logic, France) was placed in the cathode compartment. All potentials are reported vs. SHE unless otherwise stated. Two types of electrochemical reactors were applied for these experiments as illustrated in Figure 2-1 A. The first (Figure 2-1 A), a standard three electrode system in a cylindrical reactor was applied for preliminary electrochemical characterisation of metal electrolyte solutions. This reactor had a working volume of 150 mL in the cathodic compartment, with an inner concentric anode compartment of 20 mL. Two reactors were operated in parallel with a constant stirring at 350 rpm. A second plate reactor (Figure 2-1 B) was applied for actual assessment of microbial-electrochemical recovery. The second constructed with Plexi glass frames, providing an equally size bi-chamber of a 150 mL working volume. Three reactors were operated in parallel during the experiments with a continuous flow of 0.7 mL/s, to assure adequate mixing.
For both configurations, the anodic and cathodic compartment were separated by a cation exchange membrane (CEM) (CEM, Fumasep FKB, FumaTech GmbH Germany). For anoxic conditions, the electrolytes were degassed by flushing with N₂ (Linde, Germany) for 15 minutes before starting up of the electrochemical experiments. A solution of 0.15 M Na₂SO₄, at the selected pH, was used as the anolyte to maintain solute equilibrium between cathodic and anodic chamber. The working electrode was made with graphite plates (Müller & Rössner GmbH & Co., Troisdorf, Germany). Graphite electrode and connections were isolated with epoxy glue and rubber, to allow only a one-dimensional face (A = 3 cm²) in contact with the aqueous medium. Counter electrodes were cylindrical carbon for the cylindrical stirring reactors, and stain steel mesh (Magneto Special Anodes Bv, The Netherlands) for the three bi-chamber recirculating reactors. After performing the experiments and before starting a new one, the reactors and auxiliaries were soaked in disinfectant, and 0.1 M HNO₃. Then rinsed with copious amount of deionized water (J. Varia, 2012). Samples were typically taken every hour during the first 3 hours of experiment and then after a day.

6. Analytical methods

Bacterial concentration and viability measurements

Flow cytometric (FC) is emerging as a promising and powerful method to analyse bacterial community (Buysschaert et al., 2018). Concentrated bacterial solutions and samples were analysed by FC techniques to obtain information about the number of cells, and their viability during the exposure to metals. Two different stains were applied propidium iodide (PI) (20 mM in dimethyl sulfoxide (DMSO), Invitrogen, Carlsbad, CA) and SYBR Green I (SG) (10,0003 concentrate in DMSO, Invitrogen, Carlsbad, CA). Measurements were obtained by the Accuri C6.
Materials and Methods

A flow cytometer with autosampler (BD Biosciences, Erembodegem, Belgium). The performance of the instrument was monitored daily, and the instrument was calibrated with the CS&T calibration beads (BD Biosciences, Belgium). The occurrence of cells was determined by analysing the number of events per volume on green vs red fluorescence plot in the BD CSampler Plus software (version 1.0.264.21, BD Biosciences, Belgium) on gating defined by the expertise of the operator on FC density plots.

The total cell counting was performed by staining a dilute sample (<10⁶ cells/mL) with SG. Subsequently the stained samples were placed in the incubator (37 °C) for 13 minutes. As a viability indicator, cells were stained with a combination of SG and PI (SGPI) following the same procedure in triplicates assays. Using SGPI, intact cells from damaged cells were differentiated, PI only enters cells with a damaged or permeabilized membrane, thus, differentiating intact versus putative dead or damaged cells (Buysschaert et al., 2018). There, an increasing red fluorescence emission is displaying when membrane damage increases. A viability ratio was determined based on the events count with the software, according to Equation 2:

\[
\text{viability ratio} = \frac{\# \text{ cells intact}}{\# \text{ cells intact}}
\]  

Metal removal and Cr⁶⁺ reduction measurements

The removal concentration of metal from experimental assays were measured with inductively coupled plasma optical emission spectrometry (ICP-OES) (Varian Vista-MPX CCD simultaneous, Australia). All samples were filtered through 0.2 µm disposable syringe filters (Macherey-Nagel, Germany) and diluted with 1% v/v HNO₃ prior to analysis. The analysis of chromium reduction was performed following the hexavalent colorimetric Method 7196 (EPA, 1992). Briefly, 0.2 mL of supernatant form the sample was diluted in 10 mL of milli-Q water in each studied time. Then, 0.4 mL of indicator, diphenyl carbazide, were added, followed by pH adjustment with H₂SO₄ to achieve values of pH between 2 – 2.5. The prepared samples by the presence of Cr⁶⁺ exhibited an intense violet coloration, which resulted in a peak at 540 nm. The Cr⁶⁺ concentration in the system was calculated vs. a linear calibration (R² > 96.9%) with chromium standards (0.6 – 2.0 mg/L Cr⁶⁺).

Headspace analysis

During validation of treatments with H₂ as the electron donor, H₂ removal was determined. First, the pressure of air tight sealed reactor was measured with a tensiometer. Subsequently, 1.5 mL of headspace was sampled before and after the experiment and analysed with a Compact gas chromatograph (GC) (Global Analyzer Solutions, Breda, The Netherlands). H₂ are separated by a pre-column (Molsieve 5A) and then by a central column (PorabondQ), then gas concentration was analysed by means of a thermal conductivity detector. Before a sample analysis, an air sample was injected to the GC as a control. Finally, the concentration of aqueous H₂, before and after treatment, was calculated using Henry’s constant (Hcp: defined as concentration in the aqueous phase and partial pressure) of 7.7 × 10⁻⁷ mol/m³Pa in H₂O (298.15 K, 1 atm) with the Equation 3 (Sander, 2015).

\[
C_{H_2} = P_{H_2} \times H^{cp}
\]  

Lactate removal

Chromium reactors were sampled before and after the experiment, to determine a removal of lactate by bacterial respiration. Lactate removal was carried out using ion chromatography 930
Dual channel Compact IC Flex with 944 Professional UV/Vis detector Vario (Metrohm, Switzerland).

**Metal nanoparticles characterization**

Spectroscopic and optical techniques were used to identify and characterize NPs formed extracellular or at the outer membrane. UV-Vis spectrophotometry, transmission electron microscopy (TEM), and scanning electron microscopy (SEM) were performed at the end of the experiments for selected reactors.

The measurements for UV-vis scans were carried on the light wave spectrophotometer (Biochrom WPA1100nm II, Germany) to identify characteristic peaks which would indicate any nano to micro scale precipitate synthesis. A multi-well plate reader at a single wavelength of 540 nm (Tecan Infinite M200 Pro, UK) were used to read 96-well plates every hour for the follow-up of AuNPs formation and for the colorimetric chromium determination during the validation of results section.

For TEM analysis a 1.5 mL sample was centrifuged (Eppendorf, 5430) for 5 minutes at room temperature at 5000 rcf. The supernatant was discarded, and the pellet was fixed with 1 mL of fixing solution (4% paraformaldehyde, 5% glutaraldehyde in 0.1 M cacodylate buffer (VWR, Merck, USA)). The samples are centrifuged once again and stained with OsO4. After this, the sample pass by dehydration steps with alcohol. The pellets were then embedded at room temperature in Epon medium. Semithin sections of 1 μm sections were stained with toluidine blue to better observation of the cells. Ultrathin sections (≈ 60 nm) were cut with a diamond knife and contrasted with uranyl acetate and lead citrate. Imagery of the sections was carried out with a Zeiss TEM 900 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) at 50kv. The dimensions (width and length) of the bacteria cells and particles were determined using the ImageJ software (National Institutes of Health, 2016). For SEM analysis filtered samples of 200 μL were placed on a sample holder on carbon tape. The analysis was carried out with a Quanta-450F field emission gun (FEG) scanning electron microscope (Thermo Scientific, USA). In addition, energy dispersive X-ray spectroscopy (EDX) was used to determine the elemental analysis of the samples, using the same equipment and the same sample point.

7. **Analysis**

Metal removal efficiency ($\eta$) was determined using Equation 4. For chromium reduction, the Equation 5 was applied using the concentration from the colorimetric analysis. Removal yield ($\gamma$) mg/l was taken as the change in initial ($C_0$) and concentration at specific time ($C_t$) (Equation 6). While removal capacity ($q$) mgmetal removed/gcell was determined from Equation 7, where $V$ is the volume of the solution and $M_C$ is the mass of cells applied. The last term also considers the initial bacterial cell concentration ($C_b$) on the reactors.

\[
\text{Removal efficiency (\(\eta\))} \quad \eta = \frac{C_0 - C_t}{C_0} \times 100 \quad \text{Equation 4}
\]

\[
\text{Reduction efficiency (Cr}^{6+} \eta_r) \quad \eta_r = \frac{C_0 - C_t}{C_0} \times 100 \quad \text{Equation 5}
\]

\[
\text{Removal yield (\(\gamma\))} \quad \gamma = C_0 - C_t \quad \text{Equation 6}
\]

\[
\text{Removal capacity (\(q\))} \quad q = (C_0 - C_t) \frac{V}{M_C} \quad \text{Equation 7}
\]
8. Statistical analysis

The Pareto plots indicate the magnitude of the effects of the factors of the studied response variable $\eta$ during each DOE analysis. From there, the Lenth’s pseudo standard error, based on the concept of sparse effects, determines which effects are higher than a random error (Lenth, 1989). Then, the analysis of variance (ANOVA) was used to determine the level of significance indicated by the $p$-value $< 0.05$. 
III Results and Discussion
Results and Discussion

1. Bacteria cells identification and cell weight calculation of *Shewanella oneidensis* MR-1 and *Cupriavidus metallidurans* CH34

The bacteria cells, *Shewanella oneidensis* MR-1 and *Cupriavidus metallidurans* CH34, were analysed before exposure to metal ions by TEM as illustrated by Figure 3- 1 i a-b. Cells were shown as typically oblate-shaped rods. *C. metallidurans* CH34 showed minor inclusion bodies (white structures) corresponding to polyhydroxybutyrate granules (PHB) (Beeby et al., 2012), that were acknowledged as cellular metal pools (Avoscan et al., 2009; Herzberg et al., 2014). In addition, flow cytometry (FC) analysis were performed for cells counting and viability differentiation. SGPI were used as a viability indicator that differentiates between cells with intact and damaged cytoplasmic membranes (De Roy et al., 2012). The Figure 3- 1ii a-b illustrates a typical FC figure print of both cultures concentrated in 0.9% NaCl with control gates. The gates are drawn in the software to focus on a certain group of cells according to their cells distribution (De Roy et al., 2012). Figure 3- 1 ii c illustrates a FC figure print of cells after heat treatment (121 °C, 15 min). As observed, heat treatment shifts the cells distribution to the upper left (killed gate), indicating a total loss of cell viability.

Calculating biomass weight is crucial for quantitative comparisons of metal ion recovery (J. Varia et al., 2013). The total bacterial mass in the reactors was calculated using the allometric relationship between bacterial volume and mass (Equation 8). First developed by Norland et al. (1987) and further optimised by Loferer-Krößbacher et al. (1998), with application for Au³⁺ removal using viable cells of *S. putrefaciens* (J. Varia et al., 2013). The average volume of bacterial cells was calculated from measurements of 25 bacterial cells from TEM analysis. This by using Equation 9, where $V_b$ corresponds to the bacteria volume, calculated from the measurements of the width (w) and the length (l) (figure not shown), of bacterial cells unexposed to metal ions. The constant 435 represents the conversion factor between weight and volume, and 0.86 is a scaling factor (Loferer-Krößbacher et al., 1998). Calculations runs under the assumption that the bacterial
Results and Discussion

original projection on the supporting grid is conserved (J. Varia et al., 2013). The total mass of bacteria in solution \( (M_c) \), further calculated using Equation 10, where \( C_b \) is the concentration of cells (cells/mL) determined from FC, \( V_c \) is the volume of cells injected into the reactors in mL (typically 5 or 10 mL).

\[
M_b = 435V_b^{0.866} \quad \text{Equation 8}
\]

\[
V_b = \left[ \left( w^2 \times \frac{\pi}{4} \right) (l - w) + \left( \pi \times w^3 / 6 \right) \right] \quad \text{Equation 9}
\]

\[
M_c = C_b \times V_c \times M_b \quad \text{Equation 10}
\]

Bacterial dimensions of \( S. \) oneidensis MR-1 (W= 0.62 ± 0.08 and L= 1.34 ± 0.64 µm) and \( C. \) metallidurans CH34 (W= 0.55 ± 0.08 µm and L= 1.14 ± 0.35 µm) were comparable to a previous characterization of the \textit{Shewanella algae} (Yasuhiro Konishi et al., 2006) and the \textit{C. metallidurans} (Goris et al., 2001). For this thesis, the calculated average of the mass of single cell was of \( 1.73 \times 10^{-13} \) g/cell and \( 1.23 \times 10^{-13} \) g/cell for \( S. \) oneidensis MR-1 and \( C. \) metallidurans CH34 respectively. Which is comparable with previous reports for \( S. \) putrefaciens \( (1.28 \times 10^{-13} \) g/cell) (J. Varia et al., 2013).

2. Microbial strategies for metal recovery

Microbial strategies (Part A) aim to evaluate optimal metal recovery by exposing initially viable bacteria cells to metal solutions under the factors described in each DOE. Response variables of metal removal/reduction and/or metal NPs synthesis for the four metals (\( \text{Ni}^{2+}, \text{Cu}^{2+}, \text{Au}^{3+}, \) and \( \text{Cr}^{6+} \)) were considered.

2.1. \( \text{Ni}^{2+}, \text{Cu}^{2+}, \text{Au}^{3+}, \) and \( \text{Cr}^{6+} \) removal by \( S. \) oneidensis MR-1 and \( C. \) metallidurans CH34

Table 3-1 summarises results of metal removal efficiency (\( \eta \)), capacity (\( q \)), and yield (\( \gamma \)) from RES(III) fractional factorial experiments with \( S. \) oneidensis MR-1 and \( C. \) metallidurans CH34 with metal solutions of \( \text{Ni}^{2+}, \text{Cu}^{2+}, \text{Au}^{3+}, \) and \( \text{Cr}^{6+} \). In a typical experiment, the results are recorded after three days of equilibrium, as there have been usually reported as thermodynamic equilibrium (Chubar et al., 2008; Y. Konishi et al., 2007; Yasuhiro Konishi et al., 2007).
Table 3-1 Results of DOE factors applied are detailed in materials and methods (Table 2-3). Factors (A,B,C,D,E,F,G) and their levels (-1/+1) for Ni^{2+}, Cu^{2+} and Au^{3+}, A: OD (1 or 0.5/2 or 1), B: temperature (28/37 °C), C: anoxic/oxic (N\textsubscript{2}/air), D: pH (1or 2/5), E: metal concentration (0.2/2 mM), F: e-donor (H\textsubscript{2}/lactate), G: cells (MR-1/CH34). For Cr\textsuperscript{6+} A: medium (NaCl/M9), B: metal concentration (0.2/1 mM), C: anoxic/oxic (N\textsubscript{2}/air), D: pH (1or 2/5), E: OD (1 /2), F: e-donor (H\textsubscript{2}/lactate) G: cells (MR-1/CH34).

| Standard order | Run order | Factor | Ni \textsuperscript{2+} | | Cu \textsuperscript{2+} | | Au \textsuperscript{3+} | | Cr \textsuperscript{6+} |
|----------------|-----------|--------|-----------------|-----|-----------------|-----|-----------------|-----|
| Reactor       | A         | B      | C    | D    | E    | F    | G    | \(\eta\) % | q mg/g | \(\gamma\) mg/L | \(\eta\) % | q mg/g | \(\gamma\) mg/L | \(\eta\) % | q mg/g | \(\gamma\) mg/L |
| 3              | 1         | -1     | 1    | -1   | 1    | -1   | 1    | 13.4    | 730.0   | 13.7 | 4.6 | 20.5 | 4.8 | 9.7 | 14928 | 28.2 | 28.95 | 168   | 3.8 |
| 2              | 2         | 1      | -1   | -1   | -1   | 1    | 1    | 1.6     | 4.2    | 0.16 | 18.0 | 4.9 | 2.3 | 58.8 | 5584 | 21.1 | 6.7   | 379   | 4.3 |
| 7              | 3         | -1     | 1    | -1   | 1    | -1   | 1    | 7.8     | 2.7    | 0.72 | 15.3 | 12.9 | 1.6 | 42.1 | 4023 | 15.6 | 10.2  | 16.9  | 1.4 |
| 6              | 4         | 1      | -1   | 1    | -1   | 1    | -1   | 14.5    | 27.7   | 14.8 | 4.5 | 18.7 | 4.6 | 7.9 | 2744 | 21.2 | 20.2  | 83.8  | 14.3 |
| 4              | 5         | 1      | 1    | -1   | 1    | -1   | -1   | 18.8    | 3.2    | 1.7  | 64.2 | 25.3 | 6.2 | 88.5 | 3420 | 23.1 | 53.5  | 88.7  | 7.6 |
| 8              | 6         | 1      | 1    | 1    | 1    | 1    | 1    | 18.0    | 45.1   | 1.7  | 15.8 | 37.4 | 17.5 | 39.9 | 4046 | 42.1 | 20.5  | 134.4 | 3.1 |
| 1              | 7         | -1     | -1   | 1    | 1    | 1    | -1   | 13.3    | 48.9   | 13.0 | 12.0 | 99.5 | 12.2 | 50.1 | 29352 | 98.9 | 43.8  | 187.6 | 32.0 |
| 5              | 8         | -1     | -1   | -1   | 1    | -1   | -1   | 28.4    | 146.6  | 2.8  | 40.2 | 16.5 | 3.9 | 92.8 | 4785 | 24.9 | 20.0  | 1251.0 | 14.3 |
Results and Discussion

2.1.1. \( \text{Ni}^{2+}, \text{Cu}^{2+} \) experiments with \textit{S. oneidensis MR-1} and \textit{C. metallidurans CH34}

Figure 3-2 shows the (i) \( \text{Ni}^{2+} \) (a) and \( \text{Cu}^{2+} \) (b) metal removal \( \eta \) (%), \( y \) (mg/L) and \( q \) (mg/g) in all reactors, (ii) Pareto plots of main effects, and (iii) factorial plots of main effects for metal removal \( \eta \).

For \( \text{Ni}^{2+} \) the highest \( \eta \) achieved was 28.39% (reactor 8), with \textit{C. metallidurans} CH34 (\( [C_{\text{b}}]_{\text{low}} = 1.52 \times 10^8 \text{ cells/mL} \)). With a \( y \) of 2.75 mg/L (\( [C_{\text{o}}]_{\text{low}} = 9.67 \text{ mg/L} \)) and \( q \) of 146.6 mg/g. The highest \( q \) is exhibited by reactor 1, which had high initial metal concentration and a low \textit{C. metallidurans} CH34 concentration. This would suggest high metal removal of \textit{C. metallidurans} CH34 for \( \text{Ni}^{2+} \). Higher \( y \) was achieved with reactor 4 of 14.78 mg/L when the initial metal concentration is higher (\( C_{\text{c}} = 102.16 \text{ mg/L} \)), but with a lower \( \eta \) (14.47%). Reactor 5, with \textit{S. oneidensis} MR-1 (\( [C_{\text{b}}]_{\text{high}} = 3.05 \times 10^9 \text{ cells/mL} \)), achieved the second highest \( \eta \) of 18.78 % at low metal concentration (\( q = 3.2 \text{ mg/g}, y = 1.7 \text{ mg/L} \)). Reactor 2 and 3 with low metal concentration achieved the lowest \( \eta \) (< 8 %), \( y \) (< 0.7 mg/l), and \( q \) (< 5 mg/g). For these experiments, the combination of low metal concentration at low pH was not favourable for metal removal. This can be explained, as \( \text{Ni}^{2+} \) remains as a cation in the studied range of pH (Figure 1-8), highly protonated CW lessen the metal cation adsorption process by decreasing electrostatic attraction, which is worse when are dispersed (less concentrated) (Abbas et al., 2016; Fan et al., 2014). Likewise, removal efficiency (\( \eta \)) seems to be a better estimator to characterize the standardized removal process independently from the input variable (metal concentration). Thus, it is used herein as main response.

For copper experiments reactor 5, with \textit{S. oneidensis} MR-1 (\( [C_{\text{b}}]_{\text{high}} = 1.4 \times 10^9 \text{ cells/mL} \)), exhibited the highest \( \eta \) 64.2 % (\( q = 25.33 \text{ mg/g}, y = 6.19 \text{ mg/L} \)) (Figure 3-2). Although the highest \( y \) was given by reactor 6 (17.51 mg/L), at \( [C_{\text{o}}]_{\text{high}} \) of 111.13 mg/L which only corresponded to a \( \eta \) 15.8% (\( q = 38.095 \text{ mg/g} \)). Reactor 8, with \textit{C. metallidurans} CH34 (\( [C_{\text{b}}]_{\text{low}} = 1.9 \times 10^9 \text{ cells/mL} \)), exhibited the second highest metal removal with 40.2% (\( q = 14.35 \text{ mg/g} \)) retrieved from the initial \( \text{Cu}^{2+} \) solution. For reactors 5 and 8 a general trend in conditions of \( [C_{\text{o}}]_{\text{low}}, [e-\text{donor}]_\text{H}_2, \) and [\( \text{pH} \)]_\text{high} where shown to favour higher removal. Of note, the removal of \( \text{Cu}^{2+} \) is predominantly higher than that of \( \text{Ni}^{2+} \), this could suggest higher affinity for copper, despite both cations have similar atomic structure (Bard et al., 1985; Chubar et al., 2008).

Figure 3-2 B illustrates Pareto plots of main effects for \( \text{Cu}^{2+} \) and \( \text{Ni}^{2+} \) removal. These charts reveal the magnitude of effect of various factors on the response variable, here taken as \( \eta \). From there, ANOVA can be used to determine their significant effect at CI = 95 % in regards of the Lenth’s pseudo standard error (PSE), which is calculated based on the median effects of the factors (Lenth, 1989). ANOVA found anoxic/oxic (\( p = 0.04 \)), pH (\( p = 0.003 \)), and electron donor (\( p = 0.005 \)) to be significant for \( \text{Ni}^{2+} \) removal. While for \( \text{Cu}^{2+} \) removal pH (\( p = 0.04 \)) and initial metal concentration (\( p = 0.03 \)) were significant. Factorial plots for \( \text{Ni}^{2+} \) (Figure 3-2iiia) show that oxic conditions, \( \text{H}_2 \) as electron donor, and high pH were favourable for \( \text{Ni}^{2+} \) removal. These conditions are met in the reactor 8 that exhibited the highest \( \eta \), among the other reactors. For \( \text{Cu}^{2+} \) solutions at high pH 5 and low \( \text{Cu}^{2+} \) concentration give the highest \( \text{Cu}^{2+} \) removal. pH at the higher level was found as a common significant factor for both studied metals, \( \text{Ni}^{2+} \) and \( \text{Cu}^{2+} \).
Results and Discussion

Figure 3- 2 DOE results from Ni\(^{2+}\) and Cu\(^{2+}\) (i) Response plots for (a) Ni\(^{2+}\) and (b) Cu\(^{2+}\) experiments inoculated with S. Oneidensis MR-1 (black bars) and C. metallidurans CH34 (blue bars) for (A) removal efficiency $\eta$, (B) removal yield $y$, and (C) removal capacity $q$, (ii) Pareto plots of the factors studied for (a) Ni\(^{2+}\) and (b) Cu\(^{2+}\) experiments, and (iii) factorial plots of main factors for (a) Ni\(^{2+}\) and (b) Cu\(^{2+}\) experiments.
Higher pH is favourable for sorption of metals cations, as less competition occurs between metal cations and hydronium ions ($H_3O^+$) at the CW interface (Abbas et al., 2016; Fan et al., 2014). Furthermore, these results correlate well with previous reports, in which pH between 5 and 6 were optimal for copper removal with C. metallidurans CH34 (15% of initial 100 mg/L $Cu^{2+}$) (Fan et al., 2014) and Shewanella genus (q = 45 mg/g $Cu^{2+}$) (Chubar et al., 2008). Also of note, removal of both $Cu^{2+}$ and $Ni^{2+}$ by the studied bacteria strains are > 2.5 times higher than those reported using deactivate bio-substrates, such as barley straw ash (q = 6.61 mg/g $Ni^{2+}$ and q = 6.04 $Cu^{2+}$ at pH 6.5) (Arshadi et al., 2014); and comparable with fruit peels, hull, bark, and seed (15.95 – 158 mg/g $Ni^{2+}$ and 3.81 - 74 mg/g $Cu^{2+}$ ) challenged with similar initial metal ion concentration (Gupta et al., 2015). Both studied strains had higher $\eta$ than other reports using bacteria for remediation of nickel and copper (staphylococcus saprophyticus, pseudomonas aeruginosa, bacillus licheniformis, actinomycete) at low metal concentration ($< 1000$ mg/L, maximum removal $Cu^{2+}: 32\% ; Ni^{2+}: 37\%$) (Abbas et al., 2016). By using initially viable cells, bacteria strains as Bacillus thuringiensis was able to remove 34% of $Cu^{2+}$ after two days of contact time, and Pseudomonas spp. exhibited a $q= 74.9$.mg/g reviewed in the work of Malik (2004). Both results were lower to the greatest results in this thesis with the studied strains ($Cu^{2+}: \eta = 64.2$ mg/l, $Ni^{2+}: q= 146.6$).

Reinforcing the idea of obtaining optimised conditions represented by reactor 5 and 8. In addition, initially viable cells have been reported to remove twice that of autoclaved bacteria ($q= 25$ mg/g), for the Shewanella genus in solutions of pH 6 (Chubar et al., 2008). Thus, rising the hypothesis that initially viable cells and higher pH influences the metal removal process. Higher pH is favourable for physicochemical sorption to the CW, whereas low pH imposes proton competition and toxicity to the cells. Based on the results of this thesis and the work of Chubal and colleagues (2008), the pH significantly influences the metal cation removal by initially viable bacteria cells in solution obtaining higher responses at pH > 5.

Reactors for experiments of $Ni^{2+}$ and $Cu^{2+}$, exhibited neither a colour change nor an indicative absorption representative for NP synthesis by UV-Vis analysis (Table 1-2). TEM analysis was carried out to determine whether any intracellular metal precipitation was present, and for inference of the effect of metal ions on bacterial condition via physiological analysis. For Ni, a selection of two reactors with the highest amount of metal removed from solution, were analysed (Figure 3-3). C. metallidurans CH34, in reactor 1 Figure 3- 3 a, exhibited CW with undulating appearance and larger inclusion bodies. These metal pools seem to be enlarged when the metal cations accumulated inside the cells. This might be attributed to a stress resistance mechanism (Herzberg et al., 2014). S. oneidensis MR-1, in reactor 4 Figure 3- 3 b, exhibited irregularly shaped cells and external cytoplasmic content release. This was previously reported as a protective barrier delaying the harmful effects of the accumulation of cations in the cell (Krapf et al., 2016). Copper reactors inoculated with C. metallidurans CH34 were compared at both level of pH using TEM (Figure 3- 3 c, d). Reactor 2 pH 2 was contrasted with reactor 6 with the highest absolute amount of copper removed at pH 5. Regardless of the pH, both reactors showed enlargement of the CW with exteriorisation of cytoplasmic content. This effect could be related to the efflux mechanisms of metal detoxification (Max Mergeay et al., 2003). After analysing the selected micrographs, neither nickel nor copper precipitates were clearly distinguished.
Results and Discussion

2.1.2. \( \text{Au}^{3+} \) and \( \text{Cr}^{6+} \) experiments with \textit{S. Oneidensis} MR-1 and \textit{C. metallidurans} CH34

\( \text{Au}^{3+} \) removal efficiency (\( \eta \)) for all reactors were analysed as a response variable after 3 days of incubation. In the case of \( \text{Cr}^{6+} \), both reduction efficiency (\( \eta_r \)) and removal efficiency (\( \eta \)) were studied after 4 days of incubation. Considering that equilibrium is reached at that point.

Of note, \( \text{Cr}^{6+} \) would be mainly present as \( \text{HCrO}_4^- \) and less as \( \text{Cr}_2\text{O}_7^{2-} \) at pH 2; and will be distributed evenly between \( \text{HCrO}_4^- \) and \( \text{CrO}_4^{2-} \) at pH 7. \( \text{Au}^{3+} \) in chloride solutions would be mainly as \( \text{AuCl}_4^- \) at pH 1, but at pH 5 the presence of some \( \text{AuCl}_3(\text{OH})^- \) can be observed. As can be noticed, both metals and their speciation are presents as anions (Figure 1-8), thus physico-chemical sorption processes at the CW will be different compared to cations such as \( \text{Cu}^{2+} \) and \( \text{Ni}^{2+} \).

Figure 3-4 (i-a) illustrates that higher gold \( \eta \) occurred within the first hour of the experiment. This can be attributed to rapid biosorption process reported previously in a range of pH between 3 and 7 (De Corte et al., 2011; J. Varia et al., 2014). However, not only electrostatic attraction can be attributed to this process, as for reactors at higher pH is believed that the CW is less positively charged, that could repel the initial \( \text{AuCl}_4^- \) anions. This for the case of reactor 8 which exhibited the highest \( \eta \) (\( \eta = 92.84 \% \), \( q_e = 4785 \text{ mg/g}_{\text{cell}} \text{Au}^{3+} \)) for \textit{C. metallidurans} CH34 (pH 5, [e-donor]_{e2}) since the first hours of the experiment; then it reached rapidly a plateau indicating maximum sorption capacity. Similarly, the second highest \( \eta \) is observed in reactor 5, where \textit{S. oneidensis} MR-1 (pH 5, [e-donor]_{e2}) achieved 88.49 \% of removal (\( q_e = 3420 \text{ mg/g}_{\text{cell}} \text{Au}^{3+} \)). In a previous
study, up to 1 mM AuCl₄⁻ (339.79 mg/L) reduction was H₂ dependent suggesting the involvement of hydrogenases in Shewanella genus (Yasuhiro Konishi et al., 2006). Although hydrogenase enzymes on bacteria CW play a significant role in gold reduction, it was proved to be not the only mechanism (Deplanche et al., 2008), others such as physicochemical reduction of adsorbed metals and further metal nucleation can increase the removal efficiency. Moreover, the highest metal removal capacity (q=29352 mg/gcell), and yield (γ=98.93 mg/L Au³⁺) was achieved by reactor 7 inoculated with S. oneidensis MR-1. Higher initial metal concentration ([C₀]₀ of 197.38 mg/L) showed higher γ, and low cells concentration ([C₀]₀ 2.4 x10⁷ cells/mL) would convey a relative higher q (Figure 3-4 i-b and i-c). But also, reactor 7 exhibited a significant η of 50.1%. Once more, reactor 1, inoculated with low concentration of C. metallidurans CH34 ([C₀]₀ = 3.3 x10⁷ cells/mL) exhibited one of the highest q (14928 mg/gcell Au³⁺), but removal is limited to a η 9.7%. This would suggest high affinity of S. oneidensis MR-1 for retrieving gold from solution regardless of the concentration of cells.

For Cr⁶⁺, S. oneidensis MR-1 exposed to low initial Cr⁶⁺ concentration ([C₀]₀=14.14 mg/l, pH=7, anaerobic conditions, [e-donor]₀) showed the highest Cr⁶⁺ reduction (η) (Figure 3-5, reactor 5). H₂ consumption (>5% of the headspace) were noticed for reactor 5. Similarly, in reactor 7, S. oneidensis MR-1 at high initial Cr⁶⁺ concentration ([C₀]₀=73.12 mg/l, pH=7, anaerobic conditions, [e-donor] lactate), exhibited the second highest Cr⁶⁺/η. Lactate was present in the designated reactors stoichiometrically in excess. There, reactor 7 achieved the highest consumption (33% of initial lactate) which coincide with the highest metal reduction. Reactor 5 exhibited also the highest removal (η= 53.53%) followed by reactor 7 (η=43.76%) as shown in Figure 3-5. According to these results, S. oneidensis MR-1 is superior to C. metallidurans CH34 for Cr⁶⁺ reduction and removal. It is relevant to notice that η for Cr⁶⁺ were < 60% as in comparison with high removals of Au³⁺ (up to 93%). This could suggest different microbial mechanisms for metal removal regarding the physicochemical conditions of the anions in solutions.

Figure 3-5 ii. illustrates the Pareto plots of main effects for Au³⁺ and Cr⁶⁺ removal and reduction, taken as η and ηᵢ. ANOVA, CI = 95%, determined pH (p = 0.002) and initial gold concentration (p = 0.001) to be significant for Au³⁺ removal (η). For Cr⁶⁺ reduction (ηᵢ) anoxic/oxic conditions (p=0.048) were significant while for Cr⁶⁺ removal (ηᵢ) both anoxic/oxic conditions and pH were significant. The factorial plots (Figure 3-5 iiia) show that pH 5 at low initial concentration of gold (2 mM or 39 mg/L Au³⁺) provide the optimal response, agreeing with a recent study of gold reduction by Shewanella genus (J.-W. Wu et al., 2017). Previously, it was reported that Au³⁺ removal by Shewanella putrefaciens at pH 3 achieved the maximum η of 59% (q=1243 mg/gcell) (J. Varia et al., 2014). Here, pH 5 allowed higher removals concomitantly with less bacteria biomass causing 3 to 3.5 times higher removal capacity. This highlights the significant role of pH for metal removal when using initially viable cells.

Optimal conditions for Cr⁶⁺ reduction and removal, interpreted from the DOE, are M9 medium at pH 7 in anoxic conditions (Figure 3-5 iii b and c). Higher pH allows precipitation of Cr(OH)₃ according to Pourbaix diagram (Figure 1-8), and anoxic conditions avoid the re-oxidation of the Cr³⁺. Oxygen is an acknowledged oxidant, thus in their absence, reduction of Cr⁶⁺ prevails. Thus, Cr³⁺ should be stable at these reducing conditions. In addition, it was previously reported that pH close to 7 allows higher Cr⁶⁺ reduction by S. oneidensis MR-1 (98% of 0.1 mM Cr⁶⁺) within 6 hours of experiment, but lower reduction was observed at pH 6 (23% 0.1 mM) indicating the implications of higher Cr³⁺ solubility at lower pH (Parker et al., 2011). These authors suggested that soluble Cr³⁺ exhibited a more lethal effect for the bacteria than Cr⁶⁺, thus higher pH was preferable for precipitating Cr³⁺ to allow higher removal and reduction of initially Cr⁶⁺. Furthermore, bacteria actively uptake Cr⁶⁺, whereas for Cr³⁺ removal was only attributed to physicochemical effects (Parker et al., 2011).
Results and Discussion

Figure 3-4 DOE experiments for gold and chromium removal/reduction during the study time. Au\textsuperscript{3+} removal i.a: efficiency (\(\eta\)); i.b: yield (\(\gamma\)); and i.c: capacity (\(q\)). Cr\textsuperscript{6+} ii.a: reduction efficiency \(\eta_r\) (%) and ii.b: removal efficiency (\(\eta\)).
Results and Discussion

Figure 3-5 i.A: Final \( \text{Au}^{3+} \) removal after 3 days of incubation (a: removal capacity \( q \), b: removal efficiency \( \eta \), c: removal yield \( y \)). i.B: Results of \( \text{Cr}^{6+} \) after 4 days of incubation (a: reduction and b: removal) with \( S. \) oneidensis MR-1 (black bars) and \( C. \) metallidurans CH34 (blue bars). Pareto plots ii.a: \( \text{Au}^{3+} \) \( \eta \); ii.b: \( \text{Cr}^{6+} \) reduction; and ii.c: \( \text{Cr}^{6+} \) removal. Factorial plots of relevant factors iii.a: \( \text{Au}^{3+} \) \( \eta \); iii.b: \( \text{Cr}^{6+} \) reduction; and iii.c: \( \text{Cr}^{6+} \) removal.

Statistical information of significant factors for \( \text{Au}^{3+} \) removal is detailed in Table 3-2. For \( \text{Cr}^{6+} \) reduction, medium (\( p = 0.135 \)), and pH (\( p = 0.216 \)) were also considered as relevant factors as it exhibited a significant magnitude in the pareto plots. Similarly, for \( \text{Cr}^{6+} \) removal factors as type of cells (\( p = 0.070 \)) and e-donor (\( p = 0.112 \)) were also considered in the factorial plots and are represented in the Table 3-2.
Results and Discussion

Table 3-2: Summary of the factors of gold removal; and chromium removal and reduction.

<table>
<thead>
<tr>
<th>Model</th>
<th>Term</th>
<th>Effect</th>
<th>Coefficient</th>
<th>T-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Au$^{3+}$ removal</td>
<td>Constant</td>
<td></td>
<td>48.74</td>
<td>21.27</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td></td>
<td>38.20</td>
<td>19.10</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>[Au]</td>
<td></td>
<td>-43.63</td>
<td>-21.82</td>
<td>0.001</td>
</tr>
<tr>
<td>% Cr$^{6+}$ reduction</td>
<td>Constant</td>
<td></td>
<td>34.78</td>
<td>5.23</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td></td>
<td>25.83</td>
<td>12.92</td>
<td>1.94</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td></td>
<td>-39.79</td>
<td>-19.89</td>
<td>2.99</td>
</tr>
<tr>
<td></td>
<td>edonor</td>
<td></td>
<td>19.98</td>
<td>9.99</td>
<td>1.50</td>
</tr>
<tr>
<td>% Cr removal</td>
<td>Constant</td>
<td></td>
<td>-15.49</td>
<td>-7.75</td>
<td>-3.32</td>
</tr>
<tr>
<td></td>
<td>edonor</td>
<td></td>
<td>-17.92</td>
<td>8.96</td>
<td>3.84</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td></td>
<td>-10.38</td>
<td>-5.19</td>
<td>-2.23</td>
</tr>
<tr>
<td></td>
<td>cells</td>
<td></td>
<td>-12.91</td>
<td>6.46</td>
<td>-2.77</td>
</tr>
</tbody>
</table>

For gold assays, after two days of experiment, a colour change from slightly yellow to a vivid purple colour was visualized in reactors 2, 5, and 8 of DOE gold (Figure 3-6). At the end of the experiments, the absorbance at 550 nm from the UV-Vis spectra of the unfiltered concentrated solution were obtained. Peaks at 550 nm, which is indicative of AuNPs synthesis (Amendola et al., 2017; Deplanche et al., 2008), were found not only in the reactors with visible colour change but also in reactors 3, 4, and 6 (Figure 3-6). TEM, SEM and EDX were performed for all reactors to corroborate the formation of NPs.

![Figure 3-6](image-url)

Figure 3-6 Gold solutions of the 8 penicillin reactors from DOE gold, before microbial exposure (i) and after 2 days of microbial treatment (ii). Absorbance values at 550 nm from the Uv-Vis spectra for the 8 reactors (iii).

TEM and SEM revealed that AuNPs were synthesized in the eight reactors with different size, distribution, and location (Figure 3-7 and Figure 3-8). AuNPs mainly intracellular, were smaller as in reactor 3 (10.5 ± 2.1 nm) in comparison to those in the periplasmic space as reactor 1 (15.6 ± 2.9 nm) and in a combination of both as in reactor 4 (18.3 ± 3.7 nm) and 6 (19.1 ± 3.3 nm). Larger NPs were observed extracellular or on CW in the reactors 2 (103 ± 43 nm), 5 (43 ± 13 nm), and 8 (31 ± 7 nm); whose solutions exhibited a colour change (Figure 3-6). Likewise, these reactors also exhibited the highest metal removal efficiency ($\eta$). The location of these NPs is suggested to be easier to recover (Castro et al., 2014). Additionally, Au$^0$ NPs from 20 – 50 nm...
are the most appropriate for certain catalytic applications (Carrettin et al., 2004), being this the case of reactor 5 and 8.

Filtrated solutions of all reactors were analysed by SEM and EDX. After filtration, reactors 5 and 8 retained the vivid purple colour over the filter and no NPs were determined from the filtrate solution. These colourless gold depleted filtrates, suggested that the AuNPs were bound to the biomass. Conversely, reactors 2, 6, and 7 (Figure 3-8) exhibited Au\(^0\) particles in the remaining filtrate gold solution. Mainly spherical Au\(^0\) particles were observed in reactor 2 and 6, whereas in reactor 7, gold micro particles with a flower like morphology is observed. A possible synthesis mechanism can be proposed: (i) Au-reduction took place during the experimental time (46\% removal Figure 3-5) forming Au\(^0\) nuclei. (ii) After removing the biomass (cells), the particles start to form branched structures on the Au\(^0\) nuclei, forming this interesting morphology. Only few recent reports have successfully synthesized 3D flower like Au-structures by using chemical reducing agents and stabilizers (Chen et al., 2016; Sai-Anand et al., 2013; Song et al., 2018). Application of these particles were studied as efficient spots for surface enhanced Raman for in-vitro imaging, and as effective companion of drug delivery for cancer cells (Song et al., 2018).

For chromium reactors UV-Vis spectra did not corroborate the presence of NPs (absence of peaks at 430 or 470 nm) (Jaswal et al., 2014; Rakesh et al., 2013). However, TEM analysis was carried out to determine whether any metal precipitates where formed. Considering that previous studies have reported chromium precipitates to be found intracellular (Belchik et al., 2011; Middleton et al., 2003; Ravindranath et al., 2011) and on the CW (Myers et al., 2000). The micrographs for reactor 5 and 7 (Figure 3-9), shows some black electron-dense precipitates. In reactor 5, CrNPs were found in the cytoplasmic membrane and extending into the cytoplasm of S. oneidensis, suggesting that the microbial pathway for Cr\(^{6+}\) reduction is intracellularly localized (Myers et al., 2000). Moreover, the reactor 7 showed nanocomposites, mainly around the cells with unmarked edges but could be speculative of chromium precipitates on comparison with the control (Figure 3-1). After filtrating the solution at the end of the experiments, reactor 5 was analysed by using SEM, no NPs were found in the filtrate solution which confirms that CrNPs were associated with the bacterial CW or precipitated intracellular.

C. metallidurans CH34 (reactor 6 Figure 3-9 c, and reactor 8 Figure 3-9 d) exhibited greater stress than S. oneidensis MR-1 (reactor 5 Figure 3-9 a and reactor 7 Figure 3-9 b), as evident in TEM micrographs. Reactor 6 micrograph shows ultrastructural damage in bacterial cells, induced by the toxic presence of Cr\(^{6+}\), as an atypical stress response. By exposing these cells to higher concentration (1 mM) above the MIC (0.4 mM), profound CW damage and disintegration attributed to the stress condition is observed (reactor 8). Suggestions for this behaviour are that (i) CH34 was not able to use Cr\(^{6+}\) as energy source when oxygen was present, (ii) the reduction of toxic Cr\(^{6+}\) to their reduced form were not favoured, imposing a constant toxic effect. Uncomplexed Cr\(^{3+}\) present in the cytoplasm was hypothesized to exert a toxic effect by interfering with bacterial gene transcription (Belchik et al., 2011). These conditions were overcome by the reactors 5 and 7, in which S. oneidensis MR-1 was capable to reduce Cr\(^{6+}\) (> 48 \%) and complexed/ or bioaccumulate (> 43 \%) chromium precipitates, thus lessening the toxicity effect.
Results and Discussion

Figure 3-7 TEM results of DOE Gold for reactors at pH 1: Reactor 1 and reactor 2 with *S. oneidensis* MR-1, reactor 3 and 4 with *C. metallidurans* CH34; and at pH 5: Reactor 5 and reactor 7 with *S. oneidensis* MR-1, reactor 6 and 8 with *C. metallidurans* CH34. Red circles indicate some of the Nanoparticles (NPs).
Results and Discussion

Figure 3-8 SEM and EDX results of filtrate solutions of the reactors of DOE gold at the end of the experiments. 

i.a: SEM and i.b: EDX of reactor 2 inoculated with *C. metallidurans* CH34. 

ii.a: SEM and ii.b: EDX of reactor 6 inoculated with *C. metallidurans* CH34. 

iii.a: SEM and iii.b EDX of reactor 7 inoculated with *S. oneidensis* MR-1, which reveals micro flowers structures.
Results and Discussion

Figure 3- 9 TEM analysis of DOE chromium reactors at pH 5 after experiments , S. oneidensis MR-1 is observed in a: reactor 5 and b: reactor 7, whereas, C. metallidurans in c: reactor 6 and d: reactor 8.

2.2. Cu\textsuperscript{2+} removal with *Pseudomonas putida* and *Serratia marcescens*

Copper recovery was tested after 3 days of incubation by using the bacterial cells *P. putida* and *S. marcescens*, based on previous reports of microbial mediated CuNPs (Saif Hasan et al., 2008; Taran et al., 2017). Reactor 3 achieved the highest removal efficiency ($\eta$) with 24.90 % ($q=118.83$ mg/g cell Cu\textsuperscript{2+}) (Figure 3- 10 i). Reactors 5 and 6 with high initial Cu\textsuperscript{2+} achieved the highest $q$ and $\eta$. There was a marginal removal ($\eta$, $y$, and $q$) when using initially death cells. Pareto plot (Figure 3- 10 ii) indicates that the factor of dead/alive ($p=0.038$) was the most significant factor to describe copper $\eta$ with a 95% CI. Factorial plot (Figure 3- 10) corroborates that the categorical value of alive cells allowed higher $\eta$.

The medium was not a significant factor for metal removal, although only reactors in nutrient broth (NB) (reactors 1, 3, 7, and 8) showed a colour change from yellow to dark green (Figure 3- 10 iv) suggesting the formation of metal precipitates (Taran et al., 2017). Filtered samples were analysed by UV-Vis. Clear peaks at 621 nm were observed for reactors 1 and 3 (Figure 3- 10 v.a), which can be an indicative of nanoparticle synthesis (Hassan et al 2008). The absorbance at 621 nm was monitored for 4 days, showing stability after three days of the experimental time (Figure 3- 10 v.b).
Results and Discussion

Figure 3-10 i. Final Cu^{2+} removal after 3 days of incubation with *Pseudomonas Putida* (black bars) and *Serratia marcescens* (blue bars) (a: removal capacity \( q \), b: removal efficiency \( \eta \), c: removal yield \( y \)). ii. Pareto plot indicating dead/alive factor as significant. iii. Factorial plots indicating higher removals with alive cells. iv. Colour change of reactors 1, 3, 7, and 8 in nutrient broth after adding Cu^{2+}. va: UV-Vis absorbance from 300 – 900 nm of reactors 1 and 3 compared with an abiotic copper solution; and vb: Monitoring the absorbance at 621 nm indicative of copper precipitation of reactors 1 and 3.
Results and Discussion

TEM, SEM, and EDX analysis were performed for the reactors with initially viable cells that also exhibited the highest metal removal. Suggested copper particles can be observed in Figure 3-11. Reactor 1 (Figure 3-11 i), with initially viable *S. marcescens* in NB medium, showed spherical copper oxide particles (4.0 ± 0.9 µm) in filtrate solution by SEM, but precipitates around the cell were not clearly verified on TEM micrograph. EDX confirmed that the concentration of the particles was 49.92% Cu and 18.47% O which would suggest the formation of CuO precipitates. Initially viable *S. marcescens* exposed to higher copper concentration (50 mM) in NaCl, exhibited spherical particles (0.6 ± 0.1 µm) (reactor 5, Figure 3-11 iii). EDX analysis revealed Cu concentration higher than 50% in the studied sample, but as a crystal-layer was covering the spherical particles, it cannot be conclusive if it corresponds to the spherical structures. In addition, TEM micrographs do not reveal any particle formation on the bacteria cell, but this does not discard that the particles were produced extracellularly. These results suggest that the synthesis is mediated by substances produced extracellularly by bacteria cells, which was reported previously in the biosynthesis of CuO NPs with the microbial supernatant as reducing agent (Taran et al., 2017).

Suggested copper precipitates can be observed in the TEM micrographs of reactor 3, with viable *P. putida* in NB medium (Figure 3-11 ii.b). However, SEM and EDX analysis of the filtrate solution, do not detect any copper particle at that magnification (1200 x). This could suggest that most of the metallic precipitates were attached to the bacterial matrix and not in solution. Following on, reactor 6 with initially alive *P. putida* in NaCl exhibited polydisperse spherical particles in the SEM micrographs (Figure 3-11 iv.a and iv.d). Larger spherical particles had diameters ranging from 7 - 34 µm in magnification 600x (Figure 3-11 iv.d). At higher magnification 6000x (Figure 3-11 iv.a), smaller spherical particles were found ranging from 0.37 to 0.66 µm with a composition of 50.82 % Cu, 25.37% O. Similarly, TEM analysis (Figure 3-11 iv b) showed abundantly electron-dense particles around the bacteria cells, suggested to be Cu₂O precipitates.

It was reported that cells at stationary phase experience oxidative stress because of the accumulation of waste and energy sources depletion, then the stress is increased after exposure to the copper (II) sulphate (Saif Hasan et al., 2008). The release of enzymatic reductases, secreted during their microbe defence mechanisms (Bao et al., 2010; Dahl et al., 2007), can lead to the reduction of copper and then to the precipitation of nano-composites (Saif Hasan et al., 2008). By filtrating the solutions (through 0.2 µm syringe filters) biomass was removed, but reductive extracellular molecules would remain in solution; which could induce the formation of copper as microscale precipitates. Moreover, *S. marcescens*, in stationary phase, was reported to synthesize copper oxides NPs (10 - 30 nm copper) in 48 hours of experiment (Saif Hasan et al., 2008). Suggesting that bigger composites were formed, in our results, as nucleation time was increased up to 3 days.

Alive cells were a significant factor for Cu²⁺ removal (η) from solution, of which reactor 3 exhibited the optimal response. For initially viable tests, copper oxides particles were identified by UV-Vis (reactor 1 and 3), suggested by TEM micrographs (reactor 3 and 6), and/or corroborated by EDX and SEM micrographs (reactor 1, 5 and 6). Therefore, it can be said that particles can be microbial mediated for both bacteria strains at the studied Cu²⁺ initial concentration (5 mM, 50 mM). However, optimization studies with alive cells are suggested at similar conditions of pH and concentration in typical copper waste streams.

DOE with *S. marcescens* and *P. putida* achieved lower removal (up to 25% after 3 days) but extracellular particles were synthesized. As removal was minor, these experiments were limited to these results.
Results and Discussion

**Mag**

<table>
<thead>
<tr>
<th>1 200 x</th>
<th>15.00 kV</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µm</td>
<td>10 µm</td>
</tr>
</tbody>
</table>

**Crystal-layer**

**Mag**

<table>
<thead>
<tr>
<th>6 000 x</th>
<th>15.00 kV</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 nm</td>
<td>500 nm</td>
</tr>
</tbody>
</table>

**i.a**

**i.b**

**i.c**

**ii.a**

**ii.b**

**ii.c**

**iii.a**

**iii.b**

**iii.c**

**i.a**

**i.b**

**i.c**

**ii.a**

**ii.b**

**ii.c**

**iii.a**

**iii.b**

**iii.c**
Figure 3-11 Comparative micrographs between (a) SEM of filtrate solutions, (b) TEM of bacteria pellets, and (c) EDX for the reactors of DOE copper with initially viable *S. marcescens* and *P. putida*. i. Reactor 1, ii. Reactor 3, iii. Reactor 5, iv. Reactor 6 and iv.d SEM of same sample at magnification 600x, iv.e. EDX of micro rounded precipitated.
2.3. Summary of DOE metal results

Reactor 5 with *S. oneidensis* MR-1 was found as one of the best responses for metal removal ($\eta$) in the DOE nickel, copper, gold, and chromium. Similarly, reactor 8 with *C. metallidurans* CH34 exhibited the second highest $\eta$ for DOE nickel, copper, and gold. In both reactors, the conditions were low metal concentration (0.2 mM), H$_2$ as electron donor, and pH > 5. Specially pH at the high level, was found as a significant factor for all the DOEs. The results of the reactors that achieved the highest $\eta$ from each DOE are summarized in Table 3. DOE nickel achieved a maximum $\eta$ of 28.4%. DOE copper exhibited considerable removal (65% by reactor 5), but no copper NPs were identified. DOE 2 copper showed that both *S. marcescens* and *P. putida* were capable to synthesise copper oxides particles, whereas the removal was limited (25% by reactor 3).

In the case of gold experiments, reactor 5 and 8 exhibited the highest metal removal from solution, and gold NPs were synthesised extracellularly or on CW. Similarly, reactor 5 from chromium experiments exhibited the best response for both removal and reduction of Cr$^{6+}$. Chromium precipitates were observed on reactor 5 and 7, both with initially viable *S. oneidensis* MR-1. Between those reactors, reactor 5 with the recognized electroactive *S. oneidensis* MR-1 in combination with H$_2$ as electron donor, enabling its application in microbial – electrochemical systems (MES) for in-situ production of H$_2$ as an electron donor. Therefore, further validation of these conditions (reactor 5) for both metals (gold and chromium) were performed and detailed in the next section.
Table 3-3 Summary of best results of DOE: nickel, copper, gold, and chromium with *S. oneidensis* MR-1 and *C. metallidurans* CH34; and *copper* with *Serratia marcescens* and *Pseudomonas putida* with the studied factors. [Co] bacteria concentration in cells mL\(^{-1}\) and [Co] metal concentration mg mL\(^{-1}\)

<table>
<thead>
<tr>
<th>Metal</th>
<th>Reactor (bacteria)</th>
<th>Factors</th>
<th>η (%)</th>
<th>(q_{\text{max}}) mg g(^{-1}) cell</th>
<th>Nanoparticles identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni(^{2+})</td>
<td>5 (MR-1)</td>
<td>([\text{Co}]_{\text{high}}) 3.1 (\times) 10(^7) (\text{mg mL}^{-1}) (\text{cell}^{-1})</td>
<td>Anoxic</td>
<td>pH 2</td>
<td>([\text{Co}]_{\text{low}}) 9.05 (\text{mg mL}^{-1})</td>
</tr>
<tr>
<td>Ni(^{2+})</td>
<td>8 (CH34)</td>
<td>([\text{Co}]_{\text{high}}) 1.5 (\times) 10(^8) (\text{mg mL}^{-1}) (\text{cell}^{-1})</td>
<td>Oxic</td>
<td>pH 2</td>
<td>([\text{Co}]_{\text{low}}) 9.68</td>
</tr>
<tr>
<td>Cu(^{2+})</td>
<td>5 (MR-1)</td>
<td>([\text{Co}]_{\text{high}}) 1.4 (\times) 10(^9) (\text{mg mL}^{-1}) (\text{cell}^{-1})</td>
<td>Anoxic</td>
<td>pH 1</td>
<td>([\text{Cu}]_{\text{low}}) 9.63 (\text{mg mL}^{-1})</td>
</tr>
<tr>
<td>Cu(^{2+})</td>
<td>8 (CH34)</td>
<td>([\text{Co}]_{\text{low}}) 1.9 (\times) 10(^9) (\text{mg mL}^{-1}) (\text{cell}^{-1})</td>
<td>Oxic</td>
<td>pH 5</td>
<td>([\text{Cu}]_{\text{low}}) 9.63</td>
</tr>
<tr>
<td>Au(^{3+})</td>
<td>5 (MR-1)</td>
<td>([\text{Co}]_{\text{high}}) 4.9 (\times) 10(^7) (\text{mg mL}^{-1}) (\text{cell}^{-1})</td>
<td>Anoxic</td>
<td>pH 1</td>
<td>([\text{Co}]_{\text{low}}) 26.05</td>
</tr>
<tr>
<td>Au(^{3+})</td>
<td>8 (CH34)</td>
<td>([\text{Co}]_{\text{low}}) 2.4 (\times) 10(^7) (\text{mg mL}^{-1}) (\text{cell}^{-1})</td>
<td>Oxic</td>
<td>pH 5</td>
<td>([\text{Co}]_{\text{low}}) 26.78</td>
</tr>
<tr>
<td>Cr(^{6+})</td>
<td>5 (MR-1)</td>
<td>NaCl</td>
<td>([\text{Co}]_{\text{low}}) 14.14 (\text{mg mL}^{-1}) (\text{cell}^{-1})</td>
<td>Anoxic</td>
<td>pH 7</td>
</tr>
<tr>
<td>Cr(^{6+})</td>
<td>7 (MR-1)</td>
<td>NaCl</td>
<td>([\text{Co}]_{\text{high}}) 7.14 (\text{mg mL}^{-1}) (\text{cell}^{-1})</td>
<td>Anoxic</td>
<td>pH 7</td>
</tr>
<tr>
<td>*Cu(^{2+})</td>
<td>NB</td>
<td>Alive, ([\text{Co}]_{\text{low}}) 2.30 (\times) 10(^7)</td>
<td>28 °C</td>
<td>Lactate</td>
<td>(\text{S. marcescens})</td>
</tr>
<tr>
<td>*Cu(^{2+})</td>
<td>NB</td>
<td>Alive, ([\text{Co}]_{\text{low}}) 1.80 (\times) 10(^7)</td>
<td>37 °C</td>
<td>None</td>
<td>(\text{P. putida})</td>
</tr>
</tbody>
</table>
Results and Discussion

3. Results validation of the best response from DOE gold and chromium

3.1. Gold triplicates of best run with pH comparison

ANOVA analysis of the DOE gold indicate the significant factors to enhance metal removal (η). These conditions were fulfilled for gold reactor 5 which exhibited one of the best responses for metal removal and NPs synthesis from DOE gold. Therefore, conditions of this reactor were validated in triplicates (Table 3-4). Moreover, to compare the metal removal efficiency (η): (i) a second validation at pH 2 with the same other factors, and (ii) a control with autoclaved (heat treated) cells were conducted.

Table 3-4 Gold description of the validation sets with initially viable cells at pH 2 and 5, and the control with dead cells

<table>
<thead>
<tr>
<th>Au³⁺ reactor</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment, pH 2</td>
<td>Initially viable S. oneidensis MR-1 OD 1, with initial [Au]= 0.2 mM, anoxic and ( \text{H}_2 ) headspace, at 38 °C</td>
</tr>
<tr>
<td>Treatment, pH 5 (R5)</td>
<td>Initially viable S. oneidensis MR-1 OD 1, with initial [Au]= 0.2 mM, anoxic and ( \text{H}_2 ) headspace, at 38 °C</td>
</tr>
<tr>
<td>Control, dead cells</td>
<td>Heat killed S. oneidensis MR-1 OD 1, with initial [Au]= 0.2 mM, anoxic and ( \text{H}_2 ) headspace, at 38 °C</td>
</tr>
</tbody>
</table>

Figure 3-12 shows that treatments, with initially viable cells are favourable for the removal of metals from solution. The η of the treatment at pH 5 was 88.19 ± 3.52 %, comparable with the previous results obtained from the DOE. Whereas it cannot be conclusive whether there is a significant difference between the initially viable cells treatment between pH 5 and pH 2, initially (0 - 3 hours) higher η is observed at pH 5. Also, after 2 days (when NPs synthesis is observed by purple coloration of the solution), the gold removal at pH 5 is evidently higher than at pH 2.

Moreover, a control experiment with initially heat-treated cells achieved up to 68% removal after 4 days of incubation. This could be suggested due to the external exposure of reducing biomolecules (e.g. aminoacids) (Nies, 1999), and other sorption sites due to cell lysates occurring after the heating process (De Corte et al., 2011). The treatment at pH5 and the control with dead cells, were found to consume similar amount of \( \text{H}_2 \) (≈ 20 % from the headspace) during the experimental time. \( \text{H}_2 \) could serve as an electron donor to reduce the metals that were attached to the cells (viable or death), suggesting that the reduction is related to passive physicochemical mechanisms in agreement with De Corte et al. (2011). Based on previous reports, \( \text{H}_2 \) alone will not induce precipitation from initially AuCl₄⁻ solutions in an abiotic test (Deplanche et al., 2008). Thus, bacterial cells were needed to catalyse the reduction of gold leading to the nanoprecipitates.
Results and Discussion

Figure 3-12 Validation experiments for Au\(^{3+}\) microbial strategies. a: pH 5, and b: pH 2 reported mean ± standard deviation, n=3, c: control of heat-treated cells, and DOE: results of reactor 5.

The synthesis of gold NPs was monitored over 96 hours by measuring the UV-Vis absorbance at 540 nm. Figure 3-13 i illustrates the UV-Vis spectra from 300 nm to 900 nm. It can be observed a clear peak at 540-550 nm with higher absorbance (quantitative indicator of higher AuNPs yield) for the treatment with initially viable cell (Amendola et al., 2017; Deplanche et al., 2008). The control with initially dead S. oneidensis MR-1 shows a flatten peak at the same wavelength, while the abiotic test shows no absorbance peak nor colour change (Figure 3-13 i and ii). This agrees with previous reports in which dead cells produce fewer NPs in comparison with initially viable cells (De Corte et al., 2011; J. Varia et al., 2014). In addition, after 24 hours of treatment at pH 5 a steady rise in the absorbance (Figure 3-13 ii) is observed which coincides with the colour change of the reactors from slightly yellow to purple.

FC was performed in addition to monitor the viability of the cells. Figure 3-13 iii illustrates that the cells were intact during the first hour of the experiment, then the number of damage cells started to increase t >2 hours. After 24 hours of incubation, only 6 % of the initial cells remained intact (2.8 x 10^5 cells/mL)
Results and Discussion

Figure 3-13 Uv-Vis and Flow cytometry (FC) analysis of Au$^{3+}$ validation experiments. (i.) Uv-Vis spectra from 300 to 900 nm. (ii.) Absorbance at 540 nm during each study time for 48 hours of experiment. (iii.) Monitoring cells viability during 24 hours of gold experiments; percentage of intact cells in the alive gate. (iv.a.) FC of initial cells (iv.b.) at the end of the experiment.

Overall, the treatment with initially viable cells exhibited higher removal and UV-vis (Abs)$_{540}$ than heat-killed cells. Nevertheless, AuNPs synthesis was accomplished with both viable and non-viable cells. Gold reduction with the heat-killed cells seems to be a passive physicochemical treatment, formerly reported as non-enzymatic process by De Corte et al. (2011). Enhanced gold removal and NPs yield by initially active cells would imply that gold reduction and NPs synthesis could be via active (dissimilatory respiration, resistance mechanisms) (Kane et al., 2016; Yasuhiro Konishi et al., 2006; Lovley, 1993) and passive mechanism (H$_2$ as reductant in the sorption site) (De Corte et al., 2011; Pasula et al., 2017; J. Varia et al., 2014). Further coalescence of colloids to form bigger nanoparticles can be attributed to physicochemical mechanisms, which occurs after biocatalysts of the initially viable bacterial cells.
3.1. Chromium duplicates of best run with higher concentration comparison

Pareto and factorial plots of the DOE chromium suggested that the conditions of reactor 5 enhanced the Cr\textsuperscript{6+} removal ($\eta$) and reduction ($\eta_r$). For validation purposes, two reactors with same conditions as reactor 5 (R5) from DOE chromium were compared with higher concentration of the metal (1 mM) and two controls. Control 1 was established with same conditions as in R5 from the DOE matrix, but with heat-treated cells. Control 2 was a Cr\textsuperscript{6+} solution set with same conditions as R5 but without adding any bacterial cells. This is summarized in Table 3-5.

Table 3-5 Chromium validation description with initially viable cells at 0.2 mM and 1 mM, and their controls (dead cells and abiotic).

<table>
<thead>
<tr>
<th>Cr\textsuperscript{6+} reactors</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment, 1 mM</td>
<td>Initially viable S. oneidensis MR-1 OD 1 in M9 medium, pH 7, anoxic and H\textsubscript{2} headspace</td>
</tr>
<tr>
<td>Treatment, 0.2mM (R5)</td>
<td>Heat killed S. oneidensis MR-1 OD 1 in M9 medium, pH 7, anoxic and H\textsubscript{2} headspace</td>
</tr>
<tr>
<td>Control, dead cells</td>
<td>Heat killed S. oneidensis MR-1 OD 1 in M9 medium, pH 7, anoxic and H\textsubscript{2} headspace</td>
</tr>
<tr>
<td>Control, abiotic</td>
<td>No cells, M9 medium, pH 7, anoxic and H\textsubscript{2} headspace</td>
</tr>
</tbody>
</table>

Cr\textsuperscript{6+} reduction and removal were monitored during 96 hours of experiments (4 days). Figure 3-14 shows that the treatment at 0.2 mM Cr\textsuperscript{6+} achieved reduction up to 44 ± 1 % and total removal of 26 ± 2 %. By modifying one factor as initial Cr\textsuperscript{6+} concentration to 1 mM, both response variables were negatively affected, reduction (up to 12 %) and removal (up to 20 %). This can be attributed to toxic effects towards the bacteria. Myers et al., 2000 reported that toxicity could cause a decrease in Cr\textsuperscript{6+} reduction rate at concentrations > 0.1 mM of CrO\textsubscript{4}\textsuperscript{2-}. However, it can be clearly seen that the optimized treatment (0.2 mM Cr\textsuperscript{6+}) was capable to reduce Cr\textsuperscript{6+} more than twice with initially viable cells than with dead cells (15%) with similar H\textsubscript{2} consumption (≈ 33 % from the headspace). In addition, abiotic control (without cell) insignificantly reduced (up to 7%) and removed (1%) Cr\textsuperscript{6+}. This could suggest that H\textsubscript{2} alone was not the main reducing agent, attributing the significant reduction and removal of Cr\textsuperscript{6+} as microbial mediated mechanisms. Limited reduction (15%) and removal (<10%) was observed using heat-killed cells, confirming an active part played by the initially viable cells. Thus, it can be proposed an active mechanism for the production of nano-deposits after reduction and bioaccumulation into the cells, which is in good agreement with the suggestion of Daulton et al. (2007).

Of note, Figure 3-14 ii shows the previous DOE results of the reactor 5 with a total reduction within 24 h of experiments. Similarly, Figure 3-14 iii illustrates higher chromium removal (54%) of DOE than in the validation results. Possible reasons to explain the variations remain elusive but could be attributed to analytical variances in the preparation of the reactors during DOE, e.g. excess of electron donor provision (H\textsubscript{2} pressure at saturation or mistakenly lactate addition) could enhance the reduction rates. The duplicates presented in this section exhibited a low standard deviation (<2%) which can be reliable to represent this practice. Furthermore, it was corroborated H\textsubscript{2} concentration as the single electron donor supplied. Moreover, removal is limited when reduction is also limited. Process as adsorption of chromate anions (CrO\textsubscript{4}\textsuperscript{2-}) does not occur due to the nature of the cell wall charge at that pH (Middleton et al., 2003). Therefore, it can be said that first reduction occurs, followed by bioaccumulation inside the cells.

Moreover, cells viability was monitored during the first 24 hours of experiment. Figure 3-15 shows that most of the cells suffer instantaneous damage after exposed to chromium solution. However, some bacterial cells (6 x 10\textsuperscript{7} cells/mL) were shown to be viable up to 24 hours of incubation.
Results and Discussion

i. Validation experiments for Cr$^{6+}$ in microbial strategies. (i.) Penicillin bottles for chromium validation, (ii.) Cr$^{6+}$ reduction ($\eta_r$), and (iii.) Chromium removal ($\eta$) of a: two reactors at same conditions as Reactor 5 [DOE Chromium], b: at higher concentration (1mM), c1: control with initially dead cells, and c2: control 2 without cells. DOE reactor 5 of Chromium.

Figure 3-14 Validation experiments for Cr$^{6+}$ in microbial strategies. (i.) Penicillin bottles for chromium validation, (ii.) Cr$^{6+}$ reduction ($\eta_r$), and (iii.) Chromium removal ($\eta$) of a: two reactors at same conditions as Reactor 5 [DOE Chromium], b: at higher concentration (1mM), c1: control with initially dead cells, and c2: control 2 without cells. DOE reactor 5 of Chromium.
Results and Discussion

Figure 3-15 Viability analysis of *S. oneidensis* MR-1 exposed to Cr$^{6+}$ (i) Intact cells analysis for the first 24 hours of chromium experiment. Fingerprint of flow cytometry analysis at (ii.a) the start and (ii.b) after 24 hours.
4. Part B: Microbial electrochemical systems for \( \text{Au}^{3+} \) and \( \text{Cr}^{6+} \) recovery

The experiments described above (DOE metals and validation experiments) revealed the highest microbial reduction and removal for gold and chromium, with initially viable \( S.\ oneidensis \) MR-1 for conditions summarised in Table 3-6. These conditions were obtained based on the ANOVA, Pareto, and factorial plots of the significant factors which allows the higher response. Therefore, those conditions were mimicked for microbial electrochemical systems (MES). In the cathodic compartment, the working electrode is placed to study the bioelectrochemical phenomena. There, preceding electrochemical evaluations of the solutions were performed before the experiments for metal removal and reduction. These set of experiments aimed to further validate the application of cathodes as source of electrons for the microbial bio-catalysed reduction and the removal process of the selected metal electrolyte: \( \text{Au}^{3+} \) and \( \text{Cr}^{6+} \).

<table>
<thead>
<tr>
<th>Table 3-6 Initial conditions of the electrolytes, catholyte mimicking conditions of penicillin reactor 5 from DOE ( \text{Au}^{3+} ) and ( \text{Cr}^{6+} ).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metal</strong></td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Gold</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Chromium</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

4.1. Electrochemical analysis of \( \text{Au}^{3+} \) and \( \text{Cr}^{6+} \) solutions

The reversible potential \((E_r)\) and reductive peaks from steady state cyclic voltammetry (CV) were compared with the theoretical reduction potentials \((E_\text{th})\) (Table 1-4) by considering the conditions of the system (Table 3-6). Figure 3-16 i. illustrates a CV for gold electrolytes (scan rate = 20 mV/s) and reveals two reductive peaks \((\text{IP}_1 = 0.984 \text{ V vs. SHE}, \text{IP}_2 = 0.804 \text{ V vs SHE})\). Which can be related to the gold reduction reactions \((\text{AuCl}_4^-/\text{Au}^0, \text{AuCl}_4^-/\text{AuCl}_2^-)\) as detailed in Table 1-4.

For \( \text{Cr}^{6+} \) in 0.9% NaCl (Figure 3-16 iii and iv b), an inflexion point \((\text{IP}_b)\) was noticed at 0.5 V vs. SHE. This would be attributed to the redox couples \( \text{HCrO}_4^-/\text{Cr}^{3+} \) and \( \text{Cr}_2\text{O}_7^{2-}/\text{Cr(OH)}_3 \) (Table 1-4).

For \( \text{Cr}^{6+} \) in M9 medium (Figure 3-16 iii and iv a) the first point \((\text{IP}_1)\), already described in NaCl medium, was seen at 0.2 V vs. SHE, but another reaction is evident \((\text{IP}_2)\) at 0 V vs. SHE. This last reaction could be related to one of the components of the M9 medium (e.g. reduction of \( \text{PO}_4/\text{PO}_3 \) \( E^{0} = -0.2 \text{ V vs. SHE} \) (Haynes, 2012)).

As discussed, \( \text{H}_2 \) evolution using the cathode is the key strategy for microbial-electrochemical mediated metal recovery. Therefore, a set potential at -0.295 V vs. SHE was selected for gold experiments, and \( \leq -0.795 \text{ V vs. SHE} \) for chromium, where \( \text{H}_2 \) evolution was determined as illustrated by Figure 3-16 i and iii. It is worth to mention that the faradic current related to \( \text{H}_2 \) evolution for chromium electrochemistry is very low compared to the standard reduction potential in neutral pH \((-0.4 \text{ V vs. SHE})\), which implies that the overpotential of the reaction is very high on the graphite electrode, reported previously for chromium reduction using graphite (Q. Yin et al., 2000).
Results and Discussion

Figure 3-16 Electrochemical analysis of Au$^{3+}$ and Cr$^{6+}$ solutions. (i) Cyclic voltammograms (CVs) of Au$^{3+}$ (scan rate = 0.020 V s$^{-1}$) recorded on a graphite electrode in 0.9% NaCl a: with 35 mg/L AuCl$_4^-$ at pH=5 and blank in the absence of the metal. (ii) CVs of Au$^{3+}$ (scan rate = 0.050 V s$^{-1}$) on a graphite electrode at 28 °C from 35 mg/L Au$^{3+}$ in 0.9% NaCl pH = 2 a: without bacterial cells, b: with S. oneidensis MR-1 (≈ 0.5x10$^8$ cells/mL). (iii) CVs recorded on a graphite electrode in a: M9 medium, b: 0.9% NaCl with 1 mM Cr$^{6+}$ at pH7, and blank in the absence of the metal (scan rate = 0.020 V s$^{-1}$) with a (iv) focus on the reduction zone. (v) Linear sweep voltammetry (LSV) of graphite vs stainless steel of 1 mM Cr$^{6+}$ at pH7 in M9 medium, and vi. LSV a stainless steel at different Cr$^{6+}$ concentration

Preliminary CVs experiments (Figure 3-16 ii) were also carried out for analysing the electrochemistry of gold at slow scan rate (0.050 V s$^{-1}$ on a graphite electrode at 28 °C from 35 mg/L Au$^{3+}$ in 0.9% NaCl pH = 2), to show the influence of bacteria on the electrochemical reactions. Despite the standard reduction potential of the electrolytes (E$_r$) seems to be the same for both cases, in the kinetics zone (lower overpotential < 100 mV) (Bockris et al., 1973) a slightly
Results and Discussion

A positive shift is observed in the biotic experiment in comparison with the abiotic one. Similar results were previously reported by Varia et al., (2014), suggesting a reduction in energy requirements for the biotic experiments. Conversely, by observing the region at the limiting current (steady state) (Bard et al., 2001), lower current density is observed in the biotic experiments than for abiotic. Suggesting some influence of the bacteria in the mass transfer region.

For Cr$^{6+}$ electrolytes, as sluggish kinetics for $\text{H}_2$ evolution was observed using graphite electrodes. Further analysis with stainless steel was also conducted. Figure 3-16 illustrates $\text{H}_2$ evolution using graphite vs stainless steel. As observed from the LSV, a lower overpotential is required for the evolution of $\text{H}_2$ by using stainless steel (-0.6 V vs SHE). In addition, as Figure 3-16 vi illustrates that the higher the concentration of Cr$^{6+}$ in solution the higher the overpotential for $\text{H}_2$ evolution on stainless steel. This phenomenon can be attributed to a reported oxyhydroxides monolayers of Cr$^{3+}$ on the surface of the electrode after reduction, leading to electrode fouling (Xafenas et al., 2013).

4.2. MES for metal recovery

In metal removal experiments, the frame recirculating reactors in parallel were used, as describe in the Figure 2-1B. One reactor was used as an abiotic control (without adding any bacteria cells) operated at a step cathode potential where $\text{H}_2$ evolution could be observed, by a chronoamperometry technique (CA). Another reactor was designated as the biotic experiment, in which bacterial cells were injected into the cathode, at the same step cathode potential as in the abiotic control during the study time. A biotic control without external addition of electron donor was also studied, being this a case of an open circuit potential (OCP) in MES. In a usual experiment, 3 mL of aqueous samples were collected from the side bottom of the reactors for metal removal and reduction analysis. The research presented in this master thesis investigate the proof of concept in MES configurations to support metal recovery.

4.2.1. Gold experiments, effect of step potential

Electrodeposition (Figure 3-17 ia) occurs on the abiotic cathode, with a maximum of 57 % Au$^{3+}$ removal. When an step cathodic potential of -0.295 V vs. SHE with planktonic bacteria (biotic experiment) in the cathode was applied, 91 ± 1 % gold removal was achieved after 24 h (Figure 3-17 ii). There, without any apparent electrodeposition (Figure 3-17 ib). In the biotic experiment electrodeposition could not occur presumably because (1) bacteria appears to hinder diffusion phenomena of gold ions onto the electrified carbon surface that could be attached for defensive and/or dissimilatory respiration (Gregory et al., 2004; J. Varia et al., 2013), and (2) rapid gold sorption to the bacterial cells could also decrease the concentration of gold ions on solution (De Corte et al., 2011; Y. Konishi et al., 2007; J.-W. Wu et al., 2017). Thus, the reduction and precipitation of Au$^{3+}$ would be occurring in the surrounding of the bacteria. Mediated via electron transfer to $\text{S. oneidensis}$ MR-1 via electron shuttling (Kotloski et al., 2013) or with the indirect electron transfer reduction via $\text{H}_2$ (Thrash et al., 2008b), that promotes higher microbial reduction of the gold ions and further nucleation leading to NPs. In addition, $\text{H}_2$ could play an important role by a physicochemical reduction of the adsorbed ions of gold attached to the cells (Courtney, 2011; Lynne E. Macaskie et al., 2008).

Figure 3-17 iii illustrates changes in current density during the experiment for the abiotic control and biotic experiment. At that step potential more than one reduction reactions could occur, which include the $\text{H}_2$ evolution and the reduction of AuCl$_4^-$. A lower current density is observed in the biotic experiment than in the abiotic control, which was also previously observed in the mass transfer region of Figure 3-16 ii. This could be due to inhibition of electrochemical reactions due to interference of the bacteria (J. C. Varia et al., 2014), or decrease of gold ions in solution which were rapidly adsorbed by the bacterial cells (De Corte et al., 2011; Y. Konishi et al., 2007; J.-W. Wu et al., 2017).
Valorisation of metals can be evidenced by the AuNPs synthesis in the catholyte of the reactors, which is initially illustrated by the colour change. In the biotic experiment, a change from slightly yellow to purple was first observed (Figure 3- 17 iv), indicating the formation of colloidal gold (Amendola et al., 2017; Deplanche et al., 2008). Further analysis of the biotic experiment during an UV-Vis spectra (Figure 3- 17 v) depicts the symbolic peak at 550 nm, indicative of higher AuNPs yield (Amendola et al., 2017; Deplanche et al., 2008). Finally, there was no evidence of NPs synthesis in the abiotic electrochemical system. This is not unusual, as the abiotic solution lacked capping agents and nucleation sites needed for NPs synthesis (Shantkriti et al., 2014).

TEM corroborated AuNPs synthesis in the biotic experiment (Figure 3- 17 vi). In microbial strategies, the particles were observed to be produced extracellularly or on the CW at sizes ranging from 30 nm to 64 nm (Figure 3- 7, reactor 5). While in the MES, NPs sizes were found at sizes between 15 and 22 nm mainly on CW, which are smaller and less disperse. Suggested reasons of this variation presumably be: (1) as the time was shorten in MES, less contact time to coalescence to bigger particles was provided (Liz-Marzan 2004); (2) H₂ in penicillin reactor were set at saturation, probably at higher concentrations than that produced H₂ in MES. Thus, higher agglomeration of Au⁰ leading to larger AuNPs has been reported, at excess of electron donor (Deplanche et al., 2008); and/or (3) penicillin reactors are a closed system, in which conservation of mass is assured, whereas in continuous flow system, the NPs or solid deposits could be trapped in the compartment and appliances. Thus, possible extracellular NPs were not captured and validated in TEM.

To summarise these results showed that MES has the ability to reduce aqueous Au³⁺ ions to metallic Au⁰, and after colloidal agglomeration, also produce AuNPs. Therefore, it can be suggested that MES could couple NPs synthesis to removal of gold-bearing wastes.
Figure 3-17 Gold removal with microbial-electrochemical reactors at set cathode potential (-0.295 V vs. SHE) with *S. oneidensis* MR-1 in comparison with an abiotic control at same set cathode potential ([AuCl₄] = 35 mg/L). (i) Carbon electrodes used in the a: abiotic control and b: biotic experiment. (ii) Removal efficiency (ƞ) during 24 h for the biotic experiment (average and SD, n=2) and abiotic control. (iii) Current density evolution during application of step potential, (iv) Catholytes after experiments, and (v) UV-Vis spectra after the experiments of the of the a: abiotic control and b: biotic experiment. (vi) TEM micrograph of the catholyte of the biotic experiment showing AuNPs with average size: 18.0 ± 2.7 nm. Min 15.1 nm Max 22.0 nm.
Results and Discussion

4.2.2. Chromium experiments, effect of set potential

First a set of experiments were performed with graphite cathodes in stirring reactors as described in Figure 2-1 A, at same initial conditions shown in Table 3-6. With step potential of -0.895 V vs. SHE for H₂ evolution, the maximum Cr⁶+ reduction and removal were limited to 21% and 2%, respectively. Further experiments varying the initial Cr⁶+ concentration (0.2 mM and 1 mM) and step potential (-0.895 V and -0.295 V vs. SHE) were tested. However, the results were not promising. To illustrate, in pure electrochemical conditions (abiotic test) at a set potential -0.295 V vs SHE with initially 1 mM Cr⁶+ solution, 16% of Cr⁶+ was reduced and 14% removed after 24 hours. In same conditions, the biotic electrochemical test (S. oneidensis MR-1 in the catholyte) was only able to reduce 1% with a maximum removal of 5% of Cr⁶+. Cr⁶+ removal by MES was three times lower than the previous experiments of purely microbial strategies at same conditions in Table 3-6, but with H₂ headspace. Also, the maximum reduction of 44% after 48 h of incubation in microbial strategies was scarcely observed with MES. A possible restriction in H₂ provision was analysed to further understand the results.

As stated before, reduction of Cr⁶+ by MES in graphite electrodes showed that Cr³⁺ oxyhydroxides can form monolayers on the electrodes interfering with the electrochemistry of the reaction (electrode fouling) (Xafenias et al., 2013). This also can explain the low efficiency in abiotic test, and consequently their interference in biotic test with the graphite electrodes. Therefore, modifications in the configuration were made. The set-ups were defined in the frame recirculating reactors (Figure 2-1 B) showing less ohmic resistance, with stainless steel cathodes that are well-recognize for H₂ evolution and possess the ability to transfer electrons with microbes (Dumas et al., 2008; Selembo et al., 2009). Stainless steel at set cathode potential of -0.795 V vs SHE (H₂ evolution) were tested for 24 hours at conditions described in Table 3-6. Two controls were demarcated. Control 1, the cathode potential was identically as in the biotic experiment but without biomass (abiotic). Control 2 was enriched with the pure culture without setting a potential (OCP).

After 24 hours, in the biotic experiment where stainless steel electrode was poised for H₂ evolution, 61% of initial Cr⁶+ was removed (Figure 3-18 i.) and 83% reduced (Figure 3-18 ii.). Meanwhile, for the biotic experiment where electrodes where at OCP, 5% of initial Cr⁶+ was removed without any apparent Cr⁶+ reduction. Moreover, in the absence of cells (abiotic test) Cr⁶+ removal was not evidenced, but maximum Cr⁶+ reduction of 31% were observed by the first three hours. Following which oxidation of Cr³⁺ was observed. ICP analysis of the anolyte excluded the possibility of reduced chromium migration and/or diffusion to the anode in the biotic conditions. This is in good agreement with the results of Tandukar et al. (2009), were Cr⁶⁺ removal were mainly attributed to their interaction with the bacteria and the cathode, excluding the migration to the other chamber. Thus, one could propose that bacteria play an important role for reduction of Cr⁶⁺, leading to chromium removal when electrons from the cathode and H₂ were provided.

Figure 3-18 iii displays the current density evolution when the step potentials of -0.795 V vs SHE was applied to the working electrode. Once again, a lower current density is observed in the biotic experiment than in the abiotic control. Removal could occur only when reduction was evidenced after the first 3 hours in the biotic experiment and not when electrode where held at OCP. Thus, less current density would not be attributed to sorption, but could be related to the interference of bacterial cells in solution. During OCP the potential rapidly shifts positively during the first hour, presumably due to stabilization after the routinely CVs. After which, it maintained a marginally steady increase from ≈ 0 to 0.15 V vs. SHE without any evident relation with the Cr⁶⁺ redox reactions or removal as observed in Figure 3-18 Chromium removal with microbial - electrochemical reactors.

i and ii.
Results and Discussion

Figure 3-18 Chromium removal with microbial-electrochemical reactors. (i.) Total chromium removal ($\eta$) and (ii.) Cr$^{6+}$ reduction ($\eta_r$) of a: an abiotic control at step potential (-0.795 V vs SHE), b: biotic experiment with *S. oneidensis* MR-1 at same set cathode potential, and c: a biotic control in open circuit (OCP) during 24 hours of electrochemical tests. (iii.) Current density evolution during application of step potential of the a: abiotic and b: biotic test. (iv.) Measured potential in OCP conditions of the biotic control.

At the end of the experiments, Uv-vis spectra was performed on the catholyte of the abiotic control and the biotic experiment. Although both catholytes had same initial Cr$^{6+}$ concentration, it can be observed in Figure 3-19 i. that the abiotic control displays a clear peak at 370 nm, which represent the remaining Cr$^{6+}$ in solution, whereas the biotic experiment shows a flatten line at that wavelength. Furthermore, during the colorimetric method for determination of Cr$^{6+}$ with diphenyl carbazide, it was evident that the colourless test tube in the biotic experiment (Figure 3-19 ii b) would indicate that almost all Cr$^{6+}$ was reduced from the solution. However, some background absorbance of the bacterial cells in the biotic experiment (Figure 3-19 i) could influence an underestimation of the total Cr$^{6+}$ reduction described in Figure 3-18 ii.

Additionally, neither the biotic experiment nor the controls exhibited a SPR at 430 or 460 nm reported for chromium NPs (Jaswal et al., 2014; Rakesh et al., 2013). However, nanoprecipitates were detected by TEM (Figure 3-19 iii a and b). The micrographs illustrate that the cells are decorated with the chromium deposits of sizes from 19.4 to 36.7 nm. In addition, the oblate morphology of *S. oneidensis* MR-1 had been transfigured, indicating serious damage to the cells after bioaccumulation of the reduced chromium. Similarly, in a previous microbial electrochemical studies using biocathodes (Huang, Chai, et al., 2011) the chromium precipitates were also found mainly outside the cells and not on the bare electrodes, suggesting that the Cr$^{6+}$ reduction was mainly occurring on bacterial surfaces.
Results and Discussion

Figure 3-19 Chromium reduction by MES at initial 15 mg/L of Cr$^{6+}$ with step potentials of -0.795 V vs. SHE. (i) UV-vis spectra for Cr$^{6+}$ in the catholyte at the end of the experiment. (ii) Using the diphenyl carbazide reagent, indicating Cr$^{6+}$ present by colour change to purple a. abiotic control and b. biotic experiment. (iii. a and b) TEM results from catholyte showing NPs with between 19.4 - 36.7 nm, mainly on CW (periplasmic space) and intracellularly.

Of note, the pure electrochemical system (abiotic test) showed that the reduction of Cr$^{6+}$ is not stable in the system (re-oxidation occurs), meanwhile the MES achieved a steady increase in reduction of soluble Cr$^{6+}$. Therefore, a distinctive advantage is apparent for the bacterial interaction with the cathode, which in the absence of the bacterial cells, Cr$^{6+}$ removal was not observed. In the biotic experiment, the Cr$^{6+}$ was reduced and assimilated by the bacteria to form nano-precipitates by a presumed combination of active metabolism (Daulton et al., 2006; Tandukar et al., 2009) mediated by H$_2$. This suggestion can be reinforced with the results of the biotic control where the cathode was maintained at OCP, with minimal removal of chromium from solution and no Cr$^{6+}$ reduction. The Cr$^{6+}$ reduction in the biotic experiment occurred at faster rates (7.85 mg/g cell.h) than removal (5.99 mg/g cell.h). These reduction and removal rates are higher than the reported in previous studies in MES with a more positively set potential (> -0.30 vs SHE achieved up to 0.82 mg/L.h (Huang, Chai, et al., 2011) and 0.46 mg Cr$^{6+}$/g VSS.h (Tandukar et al., 2009). Thus, presumably the method of Cr$^{6+}$ reduction and removal was microbial–mediated as reported in a previous study (Huang, Chai, et al., 2011) and bio-catalysed in interaction with H$_2$. There, one can stipulate that higher reduction could be attributed to the indirect transfer of electrons through H$_2$ evolution.

In our approach the aim was to maximize reduction and removal of the chromium by applying recognized electroactive bacteria, such as S. oneidensis MR-1. This was studied as biocatalysts for the reduction reaction with H$_2$. Despite the working mechanism has not been entirely understood, previous studies speculated that H$_2$ played an important role for Cr$^{6+}$ reduction (Huang, Chai, et al., 2011), and that microorganisms interact with the electrons for enhancing the
Results and Discussion

process (Tandukar et al., 2009). Further research in the genetic domain is suggested to prove the implication of the direct or indirect electron transfer routes, and further boost the speculated mechanisms in MES. However, from engineering perspectives, the MES was able to reduce and recover Cr faster and with higher yields. Retrieving Cr\(^{6+}\) from solution via precipitation as Cr-oxide NPs that could be further valorised.

5. General discussion and perspectives, outlook

The main outcome of this study was the exploration of initially viable cells for metal recovery with further studies in MES as a proof of concept for the enhanced microbial mediated reduction and removal of metals. Two main parts were studied: microbial strategies (Part A) and microbial electrochemical systems (Part B).

In Part A of the experiments, optimized conditions for metal removal were interpreted form the DOE. Factors as pH (> 5) was determined as significant for all the studied DOEs (nickel, copper, gold, and chromium), which was studied due to their electrostatic interaction, metal speciation, and possible influence in the viability of the cells (Chubar et al., 2008; Daulton et al., 2006; Fan et al., 2014; J.-W. Wu et al., 2017). Green synthesis of NPs was achieved by microbial strategies in conditions with optimal metal removal from gold and chromium solution. In general, S. oneidensis MR-1 exhibited one of the best responses when H\(_2\) as an electron donor was provided. This was reported previously due to presence of hydrogenases enzymes catalysing their activation as electron donor for nucleation and precipitation of metals (Daulton et al., 2006; Yasuhiro Konishi et al., 2006; J. C. Varia et al., 2014). Additionally, here in a novel interpretation of physicochemical interaction of bacteria cells as biocatalyst for the metal reduction (De Corte et al., 2011).

In Part B, MES with the conditions interpreted from DOE proved to be capable to remove efficiently gold and chromium within 24 hours. At a faster (< 24 hours) and higher yields (Au\(^{3+}\) \(\eta = 91% \pm 1\%\); Cr\(^{6+}\) \(\eta = 61\% \pm 1\%\); Cr\(^{6+}\) \(\eta = 26 \pm 2\%\); Cr\(^{6+}\) \(\eta = 44 \pm 1\%\)) than in microbial strategies (validation of results after 3 days: Au\(^{3+}\) \(\eta = 88.2 \pm 3.5\%\); Cr\(^{6+}\) \(\eta = 26 \pm 2\%\); Cr\(^{6+}\) \(\eta = 44 \pm 1\%\)). In addition, when planktonic bacteria and H\(_2\) was provided in the cathode of MES, higher removal and NPs yield were observed in comparison with the abiotic or OCP control experiments. With this, it can be said that the biotic experiment in MES clearly outperformed the controls (abiotic electrochemistry, biotic OCP).

Sensitivity of living cells to extremes conditions (pH or high metal concentration) are some of the major constraints to implement technologies with initially viable cells (Malik, 2004). However, by applying initially viable bacterial cells in their stationary phase, one can overcome the concern of the growth effects and redirect the focus in the bacteria as biocatalyst for removal, reduction, and precipitation of metal in the cathode of MES. Moreover, concentrating the bacterial cells needed for inoculation in higher reactor volumes of MES was presented as a viable alternative for this technology. Less requirement for the maintenance of the biomass in the reactor is glimpsed, thus a cost-saving analysis could bring another advantage.

Also, for true valorisation, two strategies can be outlined. For intercellular metal precipitation, the biological matrix can act as a carrier. For instance, the remediation of organic and biological pollutants (e.g. PCBs, diclofenac and viruses) has been enhanced in advanced water treatment with metal NPs decorating the CW of bacterial cells, as reported by Windt et al. (2005), De Gusseme (2010), and Hennebel et al. (2009). Using bioPd NPs embedded in the bacterial matrix of Desulfovibro desulfuricans, the production of H\(_2\) was catalysed (Courtney, 2011), suggested as an alternative way to economically enhance MFC (L E Macaskie et al., 2010). For external extraction of NP, osmotic lyses by sonication can liberate AuNPs in the solution (J. Varia et al., 2014), there separation by microfiltration can be analysed. With this approach, up to 75% recovery of metal NPs located in the intracellular matrix was obtained by using sodium dodecyl sulphate (SDS) detergent (Krishnamurthy et al., 2013). Another alternative would be centrifugation to
obtain the metal pellets, then a drying process at 70 °C after a RO water wash can be useful to recover the metals (B. N. Singh et al., 2014).

Therefore, this system has the potential to recycle metal resources from waste streams, and concomitantly reducing cost and potential chemical wastes for the synthesis of metallic NPs. In this sense, NPs can be biosynthesized in a green chemistry approach. Unifying two concepts of metal recovery from aqueous system and valorisation by the produced metallic NPs.
V Conclusion and further research
1. Conclusion
This research studied A) optimized conditions for metal recovery and NPs yield mediated by initially viable bacteria and B) a proof of concept to apply microbial – electrochemical system for metal recovery and valorisation, considering the lucrative microbial mediated metal NPs.

DOEs metals target were Au^{3+}, Cr^{6+}, Cu^{2+}, and Ni^{2+} in the microbial strategies. Their metal removals in an incubation time > 3 days were 92.8%, 53.5%, 64.2%, 28.4% for each system, respectively. Metal NPs were detected for experiments with copper, gold, and chromium by transmission electron microscopy (TEM). In general, the combination of *S. oneidensis* MR-1 with H_{2} as electron donor at low concentration of the metal (0.2 mM) and higher pH (5 or 7) in anoxic conditions, exhibited one of the enhanced responses for removal and reduction of metals. These results suggested a combination of mechanisms such as passive biosorption and physicochemical reduction of metal attached in the cellular matrix, accompanying with presumably active anaerobic respiration with preference to H_{2} as an electron donor.

The electroactive microorganism was suggested as biocatalysts in MES, which seems to be able to use the protons and possibly electrons from the cathode for metal reduction and precipitation. When H_{2} evolution was set, the biological experiment in MES with a pure culture of *S. oneidensis* MR-1 performed significantly better than the abiotic control. The concept was successfully applied to recover 91 ± 1 % of the Au^{3+} from synthetic solutions; and a considerable reduction (83%) and removal (61%) of Cr^{6+} within 24 hours. For both cases, NPs were identified with potential for valorization.

These results suggest that the microbial – electrochemical system was able to enhance the removal/reduction of metals when using initially viable cells. In addition, there is a potential to applied MES for both recovery and valorisation of metals. In this sense, a novel application of MES is suggested towards a green chemistry for production of metallic NPs while recovering metals from diluted environments. This research is in the early stages but can be a key of interesting mechanisms for recovery and integrated synthesis.

2. Future research
Based on these results from a screening approach, the significant factors can be further studied with higher resolution in DOE, to better characterize the system, propose empirical models, and provide clear routes for optimization.

Nickel, copper, and gold in reactor 8 from their respective DOE, exhibited also higher removal by *Cupriavidus metallidurans* CH34 at the presence of H_{2}. *C. metallidurans* CH34 are less studied in MES, but the optimized conditions provided in this thesis can set a base for future tests. Chromium reactor 7 from the DOE, exhibited precipitates and the second highest removal. Microbial-electrochemical test at the precise conditions of this reactor with *S. oneidensis* MR-1, could be further studied for effluents with higher chromium concentration. There, the cells were submerged in 0.9% NaCl, providing a greener approach against the preparation of chemical intensive M9 medium as the one studied in this thesis.

Further work at the molecular level is suggested to better describe the presumable mechanisms of the bacteria for the removal and synthesis of metallic NPs. This in the sense, to apply genetic engineering of the recognized biomolecular mechanisms to enhance the metal recovery. In addition, there is a need to elucidate the behaviour for metal removal under non-ideal conditions from real streams environments. This can include studies of scalability, liability, and system lifetime. Competition and inhibition of a mixture of metals will also need to be considered for future viable application.
References


References


