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Faculteit Industriële ingenieurswetenschappen  
master in de industriële wetenschappen: biochemie

## Masterthesis

The influence of copper and silver ions on *Cupriavidus metallidurans* biofilm formation and development

PROMOTOR :

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Michelle Billen

Scriptie ingediend tot het behalen van de graad van master in de industriële wetenschappen: biochemie

Gezamenlijke opleiding UHasselt en KU Leuven



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**KU LEUVEN**



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## LIST OF ABBREVIATIONS

284	Tris-buffered mineral
Ag	Silver
AgNO <sub>3</sub>	Silver nitrate
ATP	Adenosine triphosphate
CFU	Colony forming units
Cu	Copper
CuSO <sub>4</sub>	Copper sulfate
CV	Crystal violet
DEPC	Diethyl pyrocarbonate
DNA	Deoxy ribonucleic acid
EPS	Extracellular polymeric substances
ETC	Electron transport chain
HME	Heavy metal efflux
ISS	International Space Station
LB	Lysogeny broth
MIC	Minimum inhibitory concentration
OD	Optical density
RDA	RNA Dilution buffer
RLA	RNA Lysis buffer
RNA	Ribonucleic acid
RND	Resistance, nodulation, cell division
ROS	Reactive oxygen species
SCK•CEN	Studiecentrum voor Kernenergie; Centre d'Étude de l'énergie Nucléaire
SEM	Scanning electron microscopy
TE	Tris-EDTA
TiO <sub>2</sub>	Titanium dioxide



# ABSTRACT

Bacterial biofilm formation is a widely known problem for water storage and distribution systems since it often leads to recurring contamination and water spoilage. *Cupriavidus metallidurans* strains, such as NA4, have been discovered aboard the International Space Station (ISS). Their presence is notable, especially in water supplies, because of the absence of nutrients and the presence of water decontaminants such as silver. *C. metallidurans*, and in particular strain CH34, is mostly studied for its metal resistance. Both *C. metallidurans* CH34 and NA4 are able to form biofilms.

The aim of this thesis was to study *C. metallidurans* biofilms in the presence of various concentrations of copper ( $\text{Cu}^{2+}$ ) and silver ( $\text{Ag}^+$ ), since these metal ions are frequently used as water disinfectants. Biofilm formation and development were studied in Lysogeny broth (LB) medium and Tris-buffered mineral (284) medium.

Optical density (OD) measurements, crystal violet (CV) quantification, RNA extraction, and scanning electron microscopy (SEM) were carried out in order to determine the effect of silver and copper ions on biofilm formation.

In 284 medium, an inhibiting effect on biofilm growth was found for 5  $\mu\text{M}$   $\text{Ag}^+$  and 5 mM  $\text{Cu}^{2+}$ . RNA extraction of biofilms was performed, but optimization of the extraction protocol is needed. Furthermore, SEM visualised interesting structural differences in CH34 and NA4 biofilm formation, revealing a more developed extracellular polymeric substance (EPS) structure of CH34 biofilm and a higher density of NA4 biofilms.



## NEDERLANDS ABSTRACT

De vorming van biofilms in water opslag- en distributiesystemen kan aanleiding geven tot terugkerende contaminatie en een verslechtering van de waterkwaliteit. *Cupriavidus metallidurans* stammen, waaronder NA4, werden reeds aangetroffen in het International Space Station (ISS). Hun aanwezigheid in watersystemen is opmerkelijk gezien de afwezigheid van nutriënten en de aanwezigheid van zilver als desinfectans. *C. metallidurans*, en in het bijzonder stam CH34, zijn vooral gekend voor hun metaalresistentie. Zowel *C. metallidurans* CH34 als NA4 kunnen biofilms vormen.

Het effect van koper ( $\text{Cu}^{2+}$ ) en zilver ( $\text{Ag}^+$ ) op de ontwikkeling van deze *C. metallidurans* biofilms werd nagegaan, aangezien deze metaalionen frequent gebruikt worden om drinkwater te desinfecteren. Biofilms werden bestudeerd in Lysogeny broth (LB) medium en Tris-buffered mineral (284) medium.

De evaluatie van biofilms gebeurde door middel van verschillende experimenten waaronder optische densiteit (OD) en crystal violet (CV) metingen, RNA extractie, en rasterelektronenmicroscopie (SEM) in aanwezigheid van verschillende concentraties aan koper en zilver.

In 284 medium werd er een inhiberend effect op biofilm groei waargenomen voor  $5 \mu\text{M Ag}^+$  and  $5 \text{mM Cu}^{2+}$ . RNA extractie van biofilms werd uitgevoerd, maar dit protocol moet geoptimaliseerd worden voor biofilms. SEM visualiseerde de structurele verschillen tussen biofilms, waaronder een meer ontwikkelde *extracellular polymeric substance* (EPS) structuur voor CH34 biofilms en een meer dense structuur voor NA4 biofilms.





# 1. INTRODUCTION

SCK•CEN (Studiecentrum voor Kernenergie; Centre d'Étude de l'énergie Nucléaire) is active in numerous areas of research in the nuclear sector and spaceflight. One of the research lines focuses on microbial contamination during spaceflight and its possible impact on human health.

## 1.1 BACKGROUND

The International Space Station (ISS) is a habitable satellite in low Earth orbit. The crew members face extreme circumstances such as high pressure, limited hygienic practices, radiation, and microgravity. According to previous studies, these conditions affect the astronauts' immune systems and may increase the risk of infections. Remarkably, microorganisms have been discovered in air, food, and water supplies aboard the ISS. Their presence is notable, especially in water supplies, because of the absence of nutrients and the presence of water decontaminants such as silver. Bacterial contamination of water sources in the ISS is prevented by adding silver ( $\text{AgNO}_3$ ) ions (0.5 mg/L) to these sources. The microorganisms that were detected mainly originate from the astronauts themselves, but also environmental microorganisms are discovered. The compromised immune system might lead to infections by opportunistic pathogens. Also, microorganisms might interfere with water quality. Therefore, stringent microbial contamination monitoring is required to guarantee their health [1].

To obtain specific maximal levels of contamination, international microbiological quality standards have been defined and implemented by space agencies [2]. Maximal concentrations of bacterial contamination in water are internationally defined and documented in the ISS Medical Operations Requirements Document. These threshold levels are dependent on the acceptable risk and achievable levels according to current technologies [1, 2].

## 1.2 MICROBIAL CONTAMINATION CONTROL OF DRINKING WATER

Contamination control of drinking water is necessary to prevent health risks and water quality impairment, as well as microbially mediated corrosion. Microorganisms are able to develop into biofilms when present in drinking water storage and distribution systems, which allows them to be more persistent and resistant to disinfectants, such as silver or copper ions [3]. This resistance can be caused by slower penetration of antimicrobials into the biofilm and adaptation of cells or subpopulations in response to external factors, leading to more persistent cells [1].

Potable water for the ISS is provided through various sources. The transportation of water to space is expensive since transport costs might run up to 10.000 Euros per kilogram, and therefore the emphasis lies on the recycling of water in space. Aboard the ISS, humidity condensate originating from the astronauts' breath or sweat, and urine distillate are purified into potable water [1].

*Cupriavidus metallidurans* strains, such as NA4, have been isolated from the cooling and drinking water of the ISS. The persistence of these strains in these strictly controlled and oligotrophic environments is remarkable, and the underlying mechanisms remain unclear [3, 4]. To decontaminate these water systems, such as drinking water storage and distribution systems, silver ions are widely used since they are expected to have a higher and more specific toxicity towards prokaryotic cells in comparison to mammalian cells [5]. However, after analysis of these silver sanitized water samples aboard the ISS, a severe decrease in the dissolved silver concentration was observed. This phenomenon is caused by the deposition of silver onto the surface of the water tanks [3].

The current drinking water distribution and storage system setups aboard the ISS do not guarantee a complete decontamination of the water, even when using silver ions as a disinfectant. After attempts to decontaminate the drinking water, resilient microorganisms have been found which can withstand high silver concentrations [6]. This might not pose an immediate problem, since most of the present bacteria are non pathogenic [4]. However, during long-term spaceflight missions, this might lead to opportunistic pathogens causing infections. An additional prominent problem is the presence of microorganisms in drinking water ( $>100$  colony-forming units (CFU)/100 mL), leading to a decrease in water quality or spoilage [7, 8].

In order to ensure enhanced and prolonged water decontamination, a thorough understanding of these bacterial strains is needed. Therefore, their biofilm formation and development dynamics need to be studied in the presence of copper and silver ions in order to determine the optimal concentrations of copper and silver for the inhibition of bacterial growth and biofilm formation.

## 2. LITERATURE STUDY

### 2.1 CUPRIAVIDUS METALLIDURANS

The *Cupriavidus* genus is defined as a group of Gram-negative, rod-shaped, aerobic bacteria that are motile. They are able to form colonies within 48 h at 30 °C [9-11].

All *Cupriavidus* genomes are characterized by the presence of a large replicon, with a size of around 2-3 Mb, in addition to their chromosome and megaplastids. This replicon carries genes that are essential for cell viability. It is often referred to as a chromid, since it has a plasmid-type replication system, its nucleotide composition is relatively similar to chromosomes, and it carries core genes found on chromosomes in other species [12]. Most *Cupriavidus* strains carry in addition at least one megaplastid with a size of 100 kb or more, which allows them to adapt to environmental changes [13].

#### 2.1.1 STRAIN CH34

*Cupriavidus metallidurans* CH34 was isolated in a heavy metal contaminated decantation basin in a metallurgical plant near Liège [14]. *C. metallidurans* CH34 is a key model organism that has been used to study bacterial resistance mechanisms for over 30 years because it endures relatively high concentrations of over 20 different heavy metal ions, including copper and silver. This resistance is mostly attained by ion efflux, metal complexation and reduction [15]. The interactions between various types of efflux pumps can lead to an increased resistance and detoxification [3].

Its genome is rather rich in genes involved in the adaptation and resistance to relatively high heavy metal concentrations [15, 16]. The corresponding genes are mainly associated with plasmids, transposons and genomic islands [9, 17]. Numerous metal resistance determinants have been associated with its native megaplastids pMOL28 and pMOL30. Nevertheless, genome sequencing showed the intricate structure of its numerous replicons, as well as the presence of unknown metal resistance determinants on several replicons [9, 12].

#### 2.1.2 STRAIN NA4

*Cupriavidus metallidurans* strain NA4 has been found in the International Space Station (ISS) drinking and cooling water [3, 9, 18]. *C. metallidurans* NA4 carries two plasmids which resemble the size of both pMOL28 and pMOL30 of *C. metallidurans* CH34. Additionally, it also carries a smaller plasmid with a size of approximately 95 kb [19].

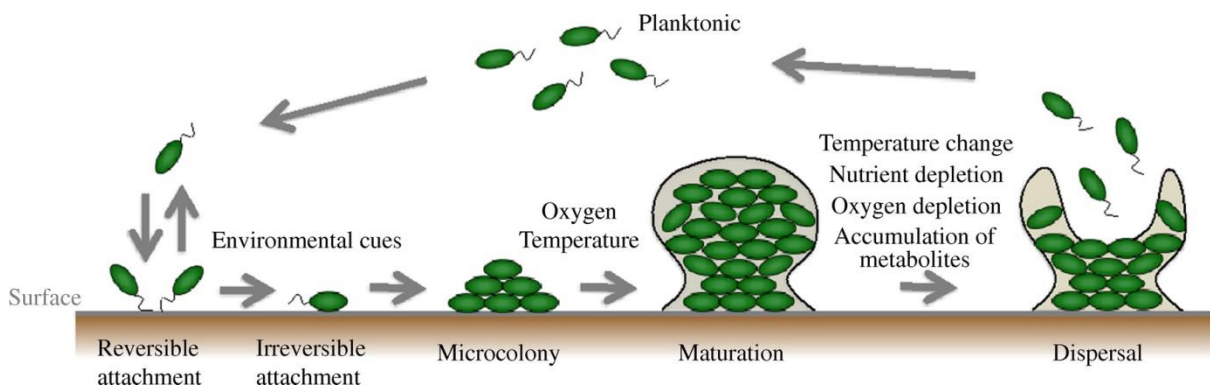
## 2.2 BIOFILM FORMATION

Biofilms are defined as biologically active matrices of cells and extracellular polymeric substances (EPS) that are able to form in association with a solid surface [20]. These EPS are mostly polysaccharides, proteins and extracellular DNA, which are produced by the cells within the biofilm itself [21]. The microbial cells present in a biofilm are sessile and physiologically different from planktonic cells, which are the non adherent separate cells, of the same organism. Biofilms are formed on numerous surfaces in response to different factors, such as stress due to the presence of metal ions or temperature changes. The microorganisms that are present are able to share nutrients and are partly shielded from disinfectants due to the gradient of nutrients and stressors that establishes itself along the biofilm [22].

### 2.2.1 BIOFILM DEVELOPMENT AND MATURATION

Biofilm growth is directed by various of chemical, environmental and biological processes, and the mechanisms by which gene expression directs biofilm formation has been the topic of extensive research. Several environmental factors determine whether cells form or detach from a biofilm. Also, environmental factors influence the structure of the biofilm, indicating the importance of biofilms to adapt to local conditions. Additionally, biofilm forming cells usually exhibit a different gene expression pattern compared to their planktonic counterparts [23]. There are several types of attachment of a biofilm to a solid surface, such as the attachment of a cell to a substrate, referred to as adhesion, and cell-to-cell attachment, referred to as cohesion. The formation of a biofilm onto a surface is described in three stages: adsorption, attachment, and colonisation. Adsorption is the attachment of an organism on a certain surface, which functions as a substrate. This is followed by the attachment between the microorganism and a surface, which is often attained by the formation of polymer bridges. After attachment, the microorganisms form colonies, grow and divide on the present surface, which is the initial phase of biofilm maturation [20, 24].

Figure 1 shows that the actual process of maturation is much more complex, also including the formation of a primary conditioning layer (the adsorption of (macro)molecules onto the substrate), adhesion of microorganisms, and the detachment of microorganisms from a mature biofilm [20, 23]. This figure highlights the different steps in biofilm development. First, the bacterial cells attach reversibly onto the surface. Afterwards, the cells attach irreversibly and lose their motility. Later, the maturation phase occurs, which is the premature development of the actual biofilm structure. During the dispersion stage, single cells become motile and are able to separate themselves from the microcolonies [23, 25].



**Figure 1: Environmental factors that influence biofilm formations [23]**

After attachment, the bacterial cells of the biofilm develop into micro-colonies by assembling with previously attached cells as shown in Figure 2. These micro colonies undergo further cell division and proliferation, which produces EPS. The EPS comprises over 90% of the dry mass in the majority of

mature biofilms and is often responsible for the adhesion properties of the biofilms to surfaces. Moreover, the EPS protects the bacterial biofilm cells against stressors such as decontaminants, oxidation, and metal ions. Cell communication is made possible by the presence of metabolic products and extracellular enzymes. Previous studies revealed the complexity of biofilm architecture and dynamic alterations caused by environmental changes [23].

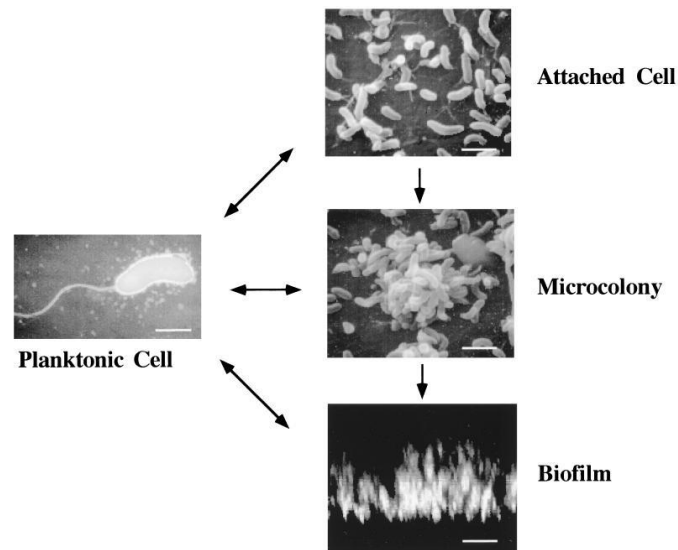


Figure 2: A microscopic study of the steps in biofilm formation [26].

In order to facilitate access to bacteria, the surface can be altered in to stimulate the attachment of the microorganisms. The growth of the microorganisms is enhanced by the provision of anchorage and nutrients. Primarily, planktonic cells are transferred from the bulk liquid to the surface by cell motility. Bacterial adhesion occurs when a segment of the cells that reach the surface adsorb, this is a reversible process and is dependent on multiple factors, such as temperature and pressure. Occasionally, reversible adhesion takes place, causing the bacteria to detach from the surface [20]. The capacity to form biofilms can enhance the persistence of microorganisms, and lead to a higher resistance to antibiotics and other biocides [27].

## 2.2.2 ENVIRONMENTAL FACTORS

As previously mentioned, bacterial biofilm cells can respond to the environment by altering gene expression. Biofilm growth is directed by a various of chemical and biological processes, referred to as environmental factors [20].

### 2.2.2.1 pH, temperature, and adhesive properties of biofilms

Fluctuations in pH can influence bacterial growth. Bacteria have membrane-bound proton pumps to transport protons out of the cytoplasm. The passive influx of protons due to external pH changes might pose a problem for cells attempting to regulate their cytoplasmic pH. Bacteria are only able to respond to limited pH fluctuations by altering the activity and synthesis of certain proteins associated with various cellular processes [20, 28].

A rapid formation of biofilms is observed at the optimum temperature and is associated with an increase in nutrient intake. Nutrient metabolism is dependent on the presence of enzymes, and biofilm formation is controlled by the temperature that controls the reaction rate of enzymes which influence the development of cells [20].

The matrix, which consists of EPS, is able to respond to stress such as the presence of metal ions by exhibiting elastic tension, and alignment of the polymers in the shear direction [20, 29].

#### **2.2.2.2 METAL IONS**

Heavy metals are well-known as for their antimicrobial properties, but recently they also gained interest for their activity against biofilms. It has been well known that biofilm bacteria show an increased tolerance to antimicrobials. Heavy metal resistance of biofilms, however, has not been studied extensively yet [30]. Metal ions, such as copper and silver, might either inhibit or stimulate biofilm formation and development. However, if these concentrations are near the minimum inhibitory concentration (MIC), bacterial growth and biofilm formation will be inhibited [22].

### **2.3 COPPER AND SILVER AS ANTIMICROBIALS**

Heavy metal ions are able to interact with microorganisms. First of all, they can be coupled on the bacterial membrane, which leads to irreversible damage (e.g. loss of membrane integrity) [31, 32]. Also, they can be absorbed in the cytoplasm where they oxidize enzymes or inactivate bacterial organelles [33]. Last but not least, they might interact directly with DNA [34].

#### **2.3.1 COPPER**

Copper (Cu) is a metal that is essential to aerobic forms of life because of its involvement as an electron donor or acceptor in redox-active enzymes, or in the electron transport chain (ETC) [35].

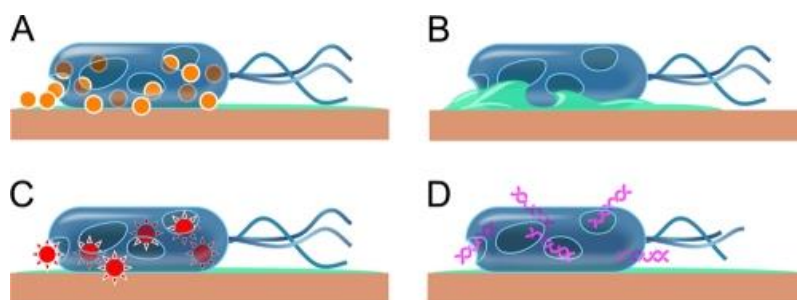
##### **2.3.1.1 COPPER METABOLISM IN HUMANS**

Copper is found in multiple cells and tissues, especially in the liver and brain. It is mainly present in biological systems as cupric form ( $\text{Cu}^{2+}$ ). In the human body, all copper is linked to enzyme prosthetic groups or tightly bound to copper chaperones [36]. These chaperones decrease the concentration of unbound copper and ensure delivery of copper ions to a specific target. The amount of copper ingested in water or food is relatively low, and excess amounts are strictly controlled by either decreased absorption or increased excretion through bile and urine, preventing acute and chronic copper toxicity. Due to the tight regulation of copper homeostasis, excess accumulation in the body is prevented. Cases of acute and chronic copper toxicity are relatively rare and only take place as a result of an exposure to excess copper (>30 mg/L) [37, 38]. The oral consumption of copper that is bound to particulates in water is less likely to cause health related effects in comparison to ionic copper, since the bound forms of copper show reduced bioavailability [39]. The main targets for acute copper toxicity are the gastrointestinal tract, the liver, and the central nervous system [38].

##### **2.3.1.2 COPPER METABOLISM IN BACTERIA**

Copper compounds are used as antimicrobial, algicidal, pesticidal and antifungal agents. Copper has been used extensively for medical applications as astringent, antiseptic, and to decontaminate drinking water [40, 41]. Various studies have shown that copper ensures killing of bacteria [42-44]. Previous studies indicated that 4-6 mM of copper was toxic to *E. coli* [45, 46].

The mechanism of copper as an antimicrobial is shown in Figure 3. First, copper dissolves and is released from the copper surface. Then, copper interferes with the cell membrane and causes rupture, which leads to loss of cytoplasmic content. Afterwards, reactive oxygen species (ROS) generation is induced by redox cycling between the different copper species, this causes cell damage. Ultimately, genomic and plasmid DNA is degraded. It has been suggested that the main damage to cells is due to membrane rupture, followed by DNA damage [42, 44].



**Figure 3: Antimicrobial mechanism of copper: (A) Copper is released from the copper surface, (B) Copper induces rupture of the cell membrane, (C) Reactive oxygen species (ROS) are induced by copper ions, leading to cell damage, (D) Genomic and plasmid DNA are degraded [42]**

## 2.3.2 SILVER

Silver (Ag) plays no valuable role in the human metabolism and is lethal to bacteria. Over the years, silver has been used as an antimicrobial in drinking water containers and is to date extensively used as potable water disinfectant [47].

### 2.3.2.1 SILVER METABOLISM IN HUMANS

In living organisms, silver can be present as metallic silver, silver salts (ionic silver  $\text{Ag}^+$ ), silver complexes or colloidal silver [48]. Silver is generally considered a harmless metal for humans [39]. However, after chronic exposure, silver might cause permanent blue staining of the skin (argyria) and/or eyes (argyrosis). Silver may also affect the kidneys, liver, or respiratory and intestinal tract [48]. Excretion of silver is mediated by removal through bile and urine [49].

The ingested estimated total dose that is needed for chronic toxicity due to silver salts is approximately 1 to 30 g. The estimated fatal dose to induce acute toxicity by ingestion is approximately 10 g of silver nitrate ( $\text{AgNO}_3$ ) [50].

### 2.3.2.2 SILVER METABOLISM IN BACTERIA

The antimicrobial properties of silver have been studied extensively. Previous research showed that micromolar concentrations of silver lead to the disconnection of the respiratory electron transport from oxidative phosphorylation and inhibition of respiratory chain enzymes [51, 52]. Additionally, higher concentrations of silver have been described to interfere with cytoplasmic components and nucleic acids [53, 54].



In bacteria, short term exposure of silver leads to interaction with membrane proteins and weakening of the outer membrane, failure of the plasma membrane potential, blocking of respiration and electron transfer, and exhaustion of intracellular ATP as shown in Figure 4. Additionally, silver ions are able to interact with DNA and proteins, and can induce the production of reactive oxygen species (ROS) [52, 55].

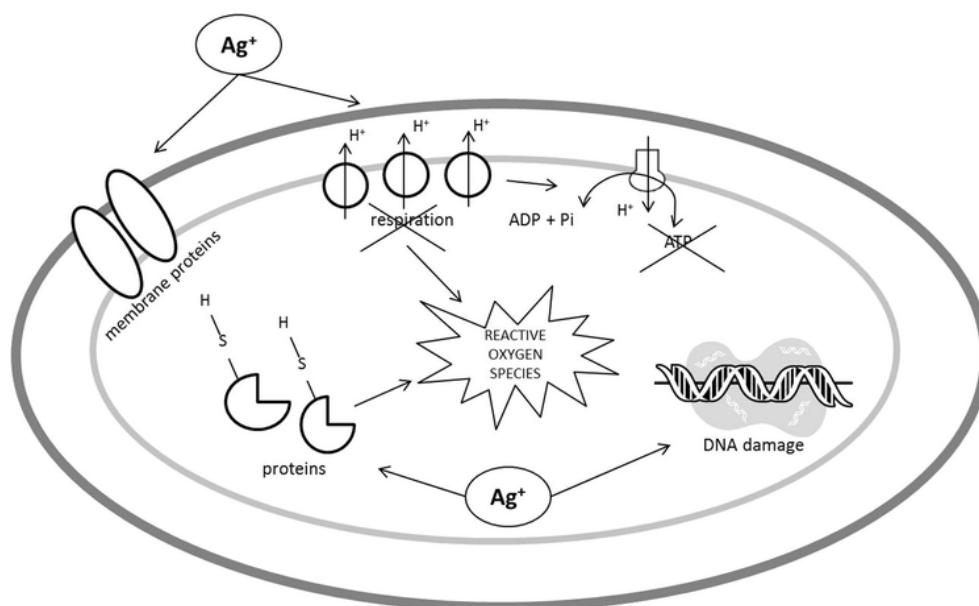


Figure 4: Antimicrobial effects of silver [55]

Silver has been found to be toxic at concentrations of 0.5 to 5 ppm for  $Ag^+$  ions, and 12.5 to 50 ppm for nanoparticles [56]. Currently, the application of silver ions has been used in hospital settings and aboard the ISS [1].

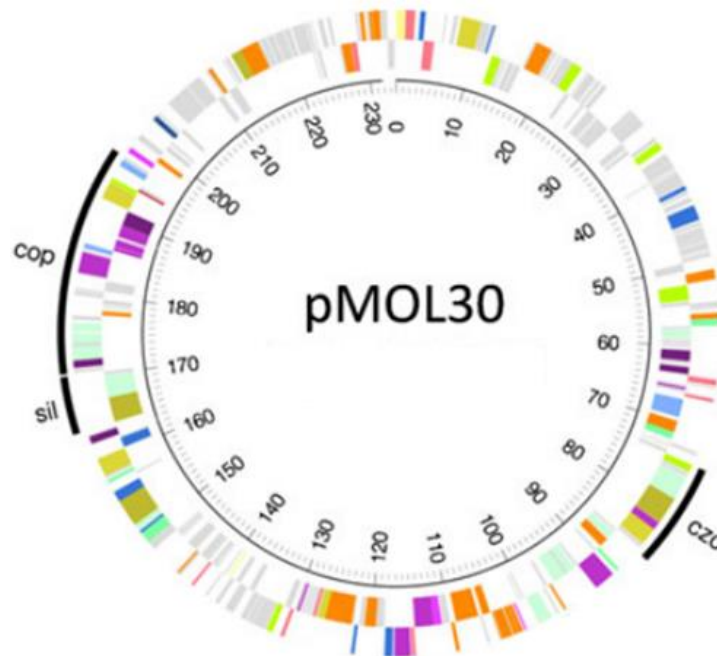
#### 2.4 METAL RESISTANCE IN *CUPRIAVIDUS METALLIDURANS*

For all bacteria, metal homeostasis is an important process to react to scarcity or excess of either essential or toxic metals [57]. Bacteria need to warrant adequate concentrations of essential nutrients, such as iron and copper, in order to guarantee a normal functioning of the cells. Excessive concentrations however, may lead to biochemical deficiencies such as structural damage to the cell membrane, alteration of enzymatic activities, or denaturation of proteins. Therefore, bacteria possess several heavy metal resistance mechanisms such as a variety of efflux pumps, proteins able to change the oxidation state of certain metals [58].

To date, *C. metallidurans* CH34 contains the highest number of heavy metal resistance genes [16]. The *C. metallidurans* CH34 genome consists out of two chromosomes and two megaplasmids and has been sequenced and studied extensively [15]. *C. metallidurans* CH34 carries megaplasmids that confer heavy metal resistance. These megaplasmids range between 180 to 450 kb. Two of these megaplasmids, pMOL28 and pMOL30, are fully sequenced and thoroughly studied at the transcriptomic level in response to metal ions [9, 15]. Resistance to heavy metals in bacteria carried by plasmids or transposons has been extensively described [59, 60]. It is notable that numerous heavy metal resistance determinants are associated with genomic islands and transposons that are present on the four replicons of *C. metallidurans* [17, 58, 61].

### 2.4.1 MEGAPLASMID pMOL30

Megaplasmid pMOL30 (Figure 5), consisting of 234 kb, confers resistance to a number of heavy metals, such as silver and copper [62, 63]. Self-transmission of the megaplasmid does not occur, but it can be transferred for example with the assistance of conjugation genes that are present on other replicons. However, this occurs at a very low frequency [64]. Figure 5 shows a circular map that indicates several metal response clusters and their locations (*cop*, *sil* and *czc*).



**Figure 5: Circular map indicating the location of the different metal response clusters for megaplasmid pMOL30 of *C. metallidurans* CH34 (Distance marks are in kbp) [58]**

*C. metallidurans* CH34 is equipped with several RND (Resistance-Nodulation- cell Division)-driven efflux systems, which are able to actively pump out metal ions such as copper and silver. Along with various efflux systems, RND proteins ensure an effective defence against metal ions. RND proteins are located in the cytoplasmic membrane. These trimeric proteins are involved in efflux reactions of cations driven by a gradient that is established due to the proton-motive force. HME-RND (heavy metal efflux) is a RND family that is extensively found in Gram-negative bacteria [65].

In addition to the previously mentioned RND-driven efflux systems, the chromid also carries a genes involved in copper resistance. The chromid-located gene *copS2R2A2B2C2D2* encodes for homologs of the Cop/Pco system, which regulates the transport of copper [9, 66].

## 2.4.2 COPPER RESISTANCE

Plasmid encoded copper resistance has previously been studied in *C. metallidurans* CH34. Copper detoxification can occur through several mechanisms, such as efflux pumps or chaperones. Copper induces a large number of pMOL30 genes [9].

Also, the presence of copper-induced genes neighbouring to the pMOL30 *cop* genes play an important role. For example, the *silDCBA* cluster that includes *silA* and *silC*, is induced by both  $\text{Ag}^+$  and  $\text{Cu}^{2+}$ , and is involved in the resistance to toxic amounts of copper [17, 67].

Three copper detoxification systems are encoded by pMOL30 copper-induced regions. A certain ATPase (CopF), which is able to form a transport system for metal cations, is responsible for cytoplasmic detoxification. CopF is supported by the periplasmic copper detoxification system CopABCD, and by the HME-RND-driven efflux system SilDCBA. Table 1 summarizes copper resistance proteins in *C. metallidurans* CH34 [9, 68].

**Table 1: *Cupriavidus metallidurans* CH34 copper resistance proteins**

Function	Protein
Periplasmic detoxification of Cu	CopA <sub>1</sub> B <sub>1</sub> C <sub>1</sub> D <sub>1</sub> / CopA <sub>2</sub> B <sub>2</sub> C <sub>2</sub> D <sub>2</sub>
Multicopper oxidase	CopA <sub>1</sub> /CopA <sub>2</sub>
Efflux P <sub>IB1</sub> -type ATPase	CupA/CopF
HME-RND-driven efflux	CusCBA/SilCBA

## 2.4.3 SILVER RESISTANCE

Silver resistance determinants are extensively spread among bacteria [69, 70]. Chemical detoxification of silver in the periplasm can occur by precipitation. Also, active efflux systems are often responsible for bacterial resistance to silver. There are two types of efflux pumps. First of all P-type ATPases are able to transport silver ions ( $\text{Ag}^+$ ) from the cytoplasm to the periplasm. Additionally, three-polypeptide membrane-potential dependent antiporters (HME-RND family) are able to transport silver ions from the periplasm out of the cell. Often, mobile genetic elements carry these resistance determinants, this facilitates their spread [47, 71, 72].

*C. metallidurans* CH34 carries various systems that are responsible for the detoxification of silver. The *silDCBA* and *cusDCBAF* operons, encoding several proteins that are part of the HME-RND transporter family (efflux systems), are located on pMOL30 and the chromid. On the chromosome, the *cupRAC* operon is located which codes for an ATPase [9]. Previous studies have shown the expression of all three operons in the presence of 0.25  $\mu\text{M}$   $\text{Ag}^+$  [15, 17, 67].

SilB, SilC, CusB and CusC proteins were also induced after they were grown in the presence of 1  $\mu\text{M}$   $\text{AgNO}_3$  [16, 73]. Furthermore, it was shown that the C-terminal domain of SilB is able to bind  $\text{Ag}^+$  [62]. Additionally to Sil and Cus, the induction of AgrR after growth of *C. metallidurans* CH34 in the presence of 1  $\mu\text{M}$   $\text{AgNO}_3$  was shown [73]. AgrR is part of the two-component system AgrR-AgrS. This system comprises a histidine kinase AgrS that can act as a sensor and is able to transmit a signal through a phosphorylation cascade to AgrR, which is a cytoplasmic transcriptional response regulator. This induction of AgrR by silver ions may indicate that this efflux pump is involved in the silver resistance of *C. metallidurans* CH34. The presence of this *sil* gene cluster might give them the capability to endure the disinfection process using silver [3].

#### 2.4.4 MIC DETERMINATION FOR COPPER AND SILVER IN *C. METALLIDURANS* CH34

In previous studies [17, 58] the minimum inhibitory concentrations (MIC) of copper and silver on *C. metallidurans* CH34 have been evaluated by 96 well microtiter plate MIC-assays in Tris-buffered mineral (284) medium after 4 days as shown in Table 2.

**Table 2: Overview of minimum inhibitory concentrations (MIC) of copper and silver compounds applied on *C. metallidurans* CH34 [58]**

Metal (ionic form)	Compound	MIC
Ag <sup>+</sup>	AgNO <sub>3</sub>	0.5 μM
Cu <sup>2+</sup>	CuSO <sub>4</sub> ·6H <sub>2</sub> O	3 mM

Another study found a silver MIC of 4 μM for *C. metallidurans* CH34 in 284 medium and 800 μM in LB medium after 7 days. Thus, the 284 medium for the MIC determination reduces the MIC in comparison to a complex rich medium, such as LB medium [3].

Generally, it has been revealed that heavy metal ions can inhibit bacterial growth. Also, this often results in elongated lag times of bacterial growth. Nevertheless, traces of several heavy metals may stimulate growth, since they can be employed by microorganisms as enzyme cofactors. This effect depends on the type and concentrations of the heavy metal ions, and the bacterial strain. Usually, high heavy metal concentrations result in an extended lag time and a reduced growth level [34, 74-76].



## 3. METHODS

The effect of several concentrations of copper and silver on *C. metallidurans* CH34 and NA4 biofilm growth and development were assessed using various experiments. In order to perform these experiments, liquid precultures were prepared and biofilms were grown using 96 well plates or glass slides. Afterwards, biofilms were evaluated using optical density (OD) measurements to quantify bacterial growth, crystal violet (CV) staining to quantify biofilm formation, RNA extraction and scanning electron microscopy (SEM).

### 3.1 BIOFILM (PRE)CULTURE

*C. metallidurans* CH34 and NA4 strains were used to inoculate the media. Both Lysogeny broth medium and Tris-buffered mineral medium were used. All added copper solutions were prepared from a 60 mM or 600 mM CuSO<sub>4</sub> stock solution and all silver solutions were prepared from a 50 mM AgNO<sub>3</sub> stock solution.

#### 3.1.1 MEDIA USED FOR BIOFILM PRECULTURES AND CULTURES

*C. metallidurans* CH34 and NA4 were grown in either Lysogeny broth (LB) medium or Tris-buffered mineral (284) medium.

##### 3.1.1.1 LYSOGENY BROTH (LB) MEDIUM

In order to prepare LB medium, 10 g LB Broth Base (Lennox L Broth Base) Invitrogen™ was weighed and dissolved in 500 mL Milli-Q® (Millipore). After the LB Broth Base dissolved completely, the medium was autoclaved at 120 °C during 20 minutes.

##### 3.1.1.2 TRIS-BUFFERED MINERAL (284) MEDIUM

###### 10X STOCK SOLUTION

One liter of a 10X stock solution was prepared by adding 60.6 g Trizma® hydrochloride (Sigma-Aldrich), 46.8 g NaCl (Sigma-Aldrich), 14.9 g KCl (Sigma-Aldrich), 10.7 g NH<sub>4</sub>Cl (Sigma-Aldrich), 4.3 g Na<sub>2</sub>SO<sub>4</sub> (Sigma-Aldrich), 2.0 g MgCl<sub>2</sub>•6H<sub>2</sub>O (Sigma-Aldrich), and 0.3 g CaCl<sub>2</sub>•2H<sub>2</sub>O (Sigma-Aldrich) to Milli-Q® (Millipore).

###### SI 7 TRACE ELEMENTS

One liter of a SI 7 trace elements solution was prepared by adding 1.30 mL of a 25% HCl solution (Sigma-Aldrich), 144 mg ZnSO<sub>4</sub>•7H<sub>2</sub>O (Sigma-Aldrich), 100 mg MnCl<sub>2</sub>•4H<sub>2</sub>O (Sigma-Aldrich), 62 mg H<sub>3</sub>BO<sub>3</sub> (Sigma-Aldrich), 190 mg CoCl<sub>2</sub>•6H<sub>2</sub>O (Sigma-Aldrich), 17 mg (0.12 mM) CuCl<sub>2</sub>•2H<sub>2</sub>O (Sigma-Aldrich), 24 mg NiCl<sub>2</sub>•6H<sub>2</sub>O (Sigma-Aldrich), and 36 mg Na<sub>2</sub>MoO<sub>4</sub>•2H<sub>2</sub>O (Sigma-Aldrich) to Milli-Q® (Millipore).

###### NA-GLUCONATE

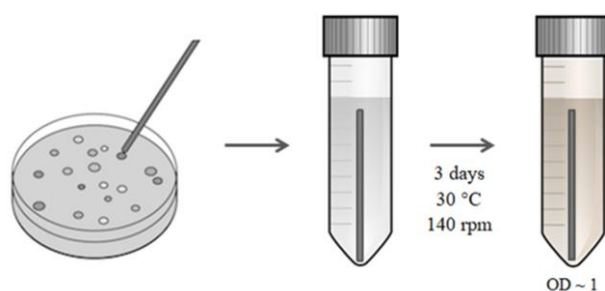
To prepare 50 mL of a 200 g/L Na-gluconate stock solution; 10 g Na-gluconate (Sigma-Aldrich) was dissolved in 50 mL of Milli-Q® (Millipore). Afterwards, the solution was filter sterilized using 0.45 µm Pall Corporation Acrodisc® Syringe Filters.

###### 284 MEDIUM

For the actual preparation of one liter of Tris-buffered mineral (284) medium; 100 mL of 10X stock solution, 4 mL of a 1% Na<sub>2</sub>HPO<sub>4</sub>•2H<sub>2</sub>O stock solution, 10 mL of a 48 mg/100 mL Fe(III)NH<sub>4</sub>.citrate solution, 1 mL of SI 7 trace elements solution, and 10 mL of a Na-gluconate solution (200g/l) were added. Afterwards, HCl was added up to a pH of 7.

### 3.1.2 PRECULTURE METHOD

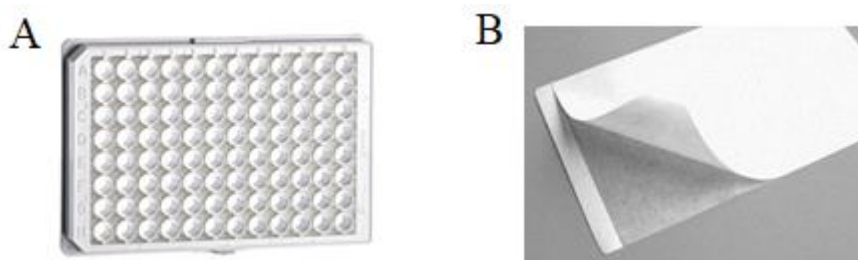
In order to grow *C. metallidurans* biofilms as shown in Figure 6, precultures were prepared by taking a single colony using a sterile toothpick to inoculate 5 mL Tris-buffered mineral (284) medium. This preculture was incubated on an orbital shaker at 140 rpm during 3 days at 30 °C until an OD of approximately 1, indicating that a stationary phase was reached. The caps were loosely screwed on to allow oxygen influx. Afterwards, the bacterial suspension was diluted using 10 mM MgSO<sub>4</sub> in Milli-Q® (Millipore) up until a final OD of 0.1 was reached. Since bacterial cells burst in hypotonic solutions such as water due to difference in osmotic pressure, an isotonic dilution solution (10 mM MgSO<sub>4</sub>) was provided.



**Figure 6: Preculture method for *C. metallidurans* strains**

#### Culture method

Biofilms were cultured and assessed using Greiner Bio-One CELLSTAR® flat bottom 96 well plates (Figure 7A). To inoculate these microtiter plates, 20 µL of a bacterial suspension (OD = 0.1) was used up to a final OD of 0.01 in the microtiter plate. The OD of this bacterial suspension was measured at 600 nm with an Eppendorf BioPhotometer® using Tris-buffered mineral (284) medium as a blank. In brief, these 96 well plates were filled with 170 µL of Lysogeny broth (LB) or Tris-buffered mineral (284) medium, 20 µL of a bacterial suspension of either *Cupriavidus metallidurans* CH34 or NA4 (OD = 0.1), and 10 µL of either copper (CuSO<sub>4</sub>) solutions, silver (AgNO<sub>3</sub>) solutions, or Milli-Q if no metal ions were added. The bacterial suspensions were incubated on an orbital shaker at 140 rpm during 7 days at 30 °C and covered with Sigma-Aldrich AeraSeal™ film (Figure 7B) to prevent evaporation.



**Figure 7: Biofilm culture method: A) Greiner Bio-One CELLSTAR® flat bottom microtiter plat, B) Sigma-Aldrich AeraSeal™ film**

### 3.2 OPTICAL DENSITY (OD) MEASUREMENTS

Optical density measurements of *C. metallidurans* growth were performed by transferring 100 µL of the bacterial suspension that was cultured during 7 days into a new 96 well plate. This microtiter plate was read using a CLARIOstar® BMG LABTEC plate reader at 600 nm.

### 3.3 STAINING AND QUANTIFICATION OF BIOFILMS

#### 3.3.1 CRYSTAL VIOLET (CV) STAINING

Biofilms were visualized using the intercalating dye crystal violet (CV). After biofilm growth and development in the microtiter plates, the 96 well dish was turned over and unattached cells were removed by shaking out the liquid. Then, the plate was gently submerged in a container filled with 750 mL tap water. Afterwards, the tap water was removed again by shaking out the liquid. This rinsing step was repeated twice to lower background staining. To stain the biofilm, 250  $\mu$ L of a 0.1% solution of CV in Milli-Q was added to each well of the 96 well plate as shown in Figure 8A. This CV solution was prepared by weighing 0.5 g Crystal violet (Sigma-Aldrich) and dissolving it into 500 mL of Milli-Q<sup>®</sup> (Millipore). The microtiter plate was then incubated at room temperature during 15 minutes. Subsequently, the plate was rinsed three times using tap water by submerging it, followed by shaking out the liquid and blotting the plate on paper in order to remove all excess cells and dye. The microtiter plate was covered with aluminium foil and dried upside down overnight. An inoculated 96 well plate was also stained and used as a blank. Figure 8 shows an example of a CV staining of inoculated 96 well plates before and after rinsing.



**Figure 8: An example of the biofilm assay quantification: (A) Staining of biofilms using 250  $\mu$ L of a 0.1% CV solution, (B) Microtiterplate after rinsing with tap water one time, (C) Microtiterplate after rinsing with tap water three times**

#### 3.3.2 QUANTIFICATION

##### 3.3.2.1 BIOFILMS FORMED IN 96 WELL PLATES

For quantification of biofilms formed in 96 well plates, 250  $\mu$ L of 30% acetic acid (Suprapur<sup>®</sup> glacial acetic acid) in Milli-Q was added to each well of the microtiter plate to solubilize the CV dye. Afterwards, the microtiter plate was incubated at room temperature for 15 minutes. After incubation, 200  $\mu$ L of the solution was transferred to a new flat bottomed microtiter dish. The absorbance was quantified using the CLARIOstar<sup>®</sup> BMG LABTEC plate reader at 550 nm and 30% acetic acid in Milli-Q<sup>®</sup> (Millipore) as a blank [77].



### 3.4 BIOFILM FORMATION IN THE PRESENCE OF SILVER AND COPPER

The effect of various concentrations of  $\text{CuSO}_4$  or  $\text{AgNO}_3$  on *C. metallidurans* CH34 and NA4 biofilm formation and development was assessed. The minimum inhibitory concentration (MIC) was evaluated in an attempt to determine the lowest concentration of  $\text{CuSO}_4$  or  $\text{AgNO}_3$  that prevents visible growth of *Cupriavidus metallidurans* CH34 or NA4 planktonic bacteria. This was carried out in 96 well plates using various concentrations of  $\text{Cu}^{2+}$  and  $\text{Ag}^+$  with bacterial suspensions of *C. metallidurans* CH34 and NA4 in LB medium (Figure 9) or in 284 medium (Figure 10). The microtiter plates were inoculated and incubated as previously mentioned in section 3.1.2. The OD was measured at 600 nm, and the biofilms were stained using CV and quantified using an absorbance measurement at 550 nm after solubilisation.

#### 3.4.1 EXPERIMENTAL SETUP 1: BIOFILM FORMATION IN LYSOGENY BROTH (LB) MEDIUM

The effect of various concentrations of  $\text{CuSO}_4$  (Figure 9A and B) or  $\text{AgNO}_3$  (Figure 9C and D) on *C. metallidurans* CH34 and NA4 biofilm formation and development was assessed in LB medium. For copper, concentrations of 0.1 mM, 0.2 mM, 0.5 mM, 1 mM, 2 mM and 5 mM  $\text{Cu}^{2+}$  were used. For silver, concentrations of 10  $\mu\text{M}$ , 20  $\mu\text{M}$ , 50  $\mu\text{M}$ , 100  $\mu\text{M}$ , 200  $\mu\text{M}$  and 500  $\mu\text{M}$   $\text{Ag}^+$  were used. The concentrations were based on previously determined minimum inhibitory concentrations in Tris-buffered mineral medium as shown in Table 2. These concentrations were increased to compensate for copper and silver interactions with LB medium.

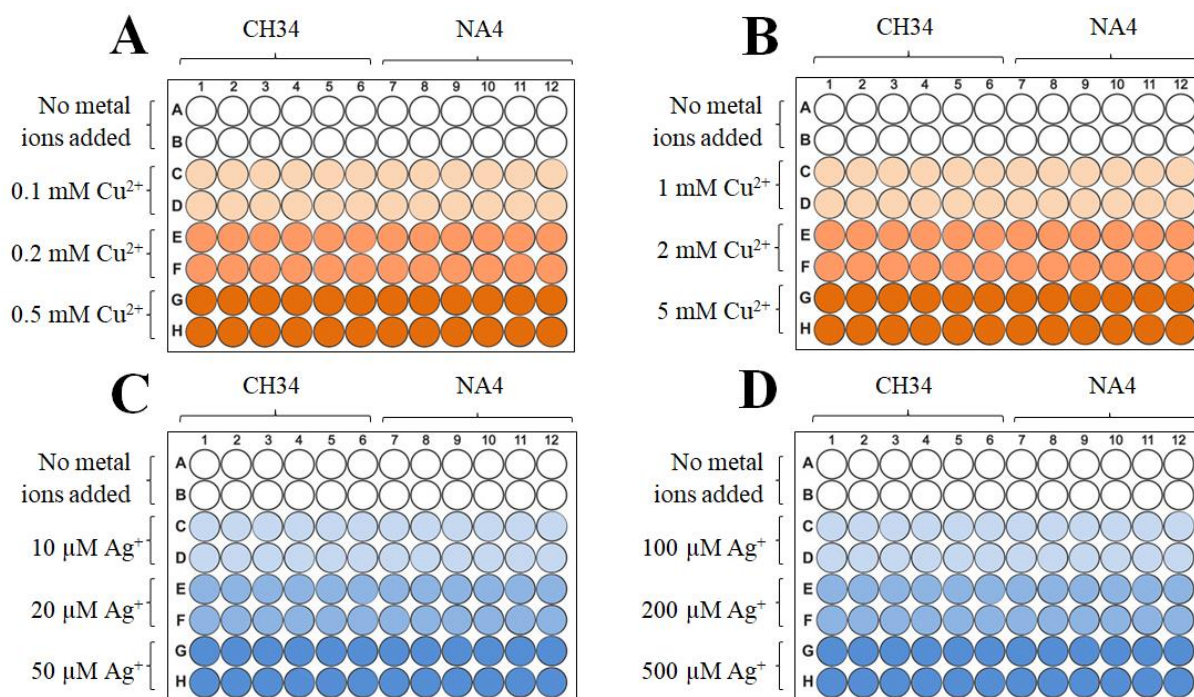


Figure 9: Layout of assays to determine the effect of copper (A and B) and silver (C and D) on *C. metallidurans* CH34 and NA4 biofilm formation in Lysogeny broth (LB) medium

### 3.4.2 EXPERIMENTAL SETUP 2: BIOFILM FORMATION IN TRIS-BUFFERED MINERAL (284) MEDIUM

The effect of various concentrations of copper (Figure 10A and B) or silver (Figure 10C and D) on *C. metallidurans* CH34 and NA4 biofilm formation and development was assessed in 284 medium. For copper, concentrations of 0.1 mM, 0.2 mM, 0.5 mM, 1 mM, 2 mM and 5 mM  $\text{Cu}^{2+}$  were used. This is the same concentrations range that was used in LB medium. For silver, concentrations of 0.1  $\mu\text{M}$ , 0.2  $\mu\text{M}$ , 0.5  $\mu\text{M}$ , 1  $\mu\text{M}$ , 2  $\mu\text{M}$  and 5  $\mu\text{M}$   $\text{Ag}^+$  were used. These concentrations were based on previously determined minimum inhibitory concentrations in Tris-buffered mineral (284) medium as shown in Table 2.

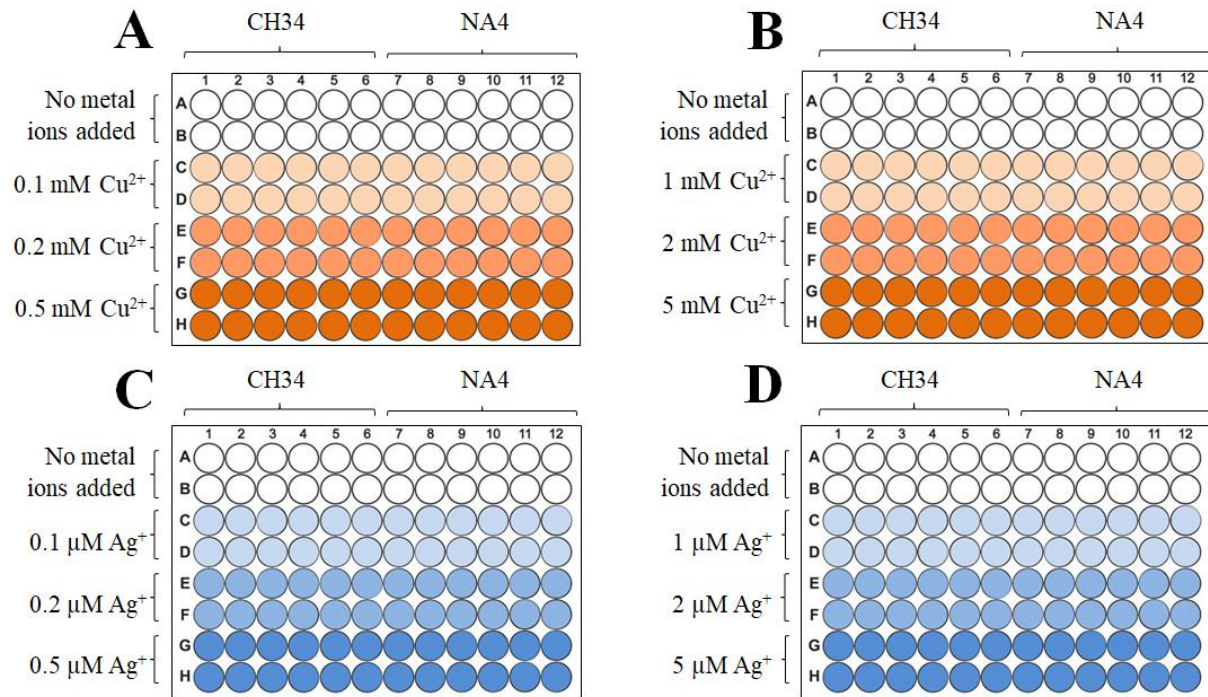


Figure 10: Layout of assays to determine the effect of copper (A and B) and silver (C and D) on *C. metallidurans* CH34 and NA4 biofilm formation in Tris-buffered mineral (284) medium

### 3.5 SCANNING ELECTRON MICROSCOPY

Structural biofilm changes under the influence of copper and silver ions were studied using scanning electron microscopy (SEM). Several parameters were observed, such as clustering, distinct morphology of biofilm cells, the increased or decreased presence of EPS and their relation to copper and silver ion concentration. These measurements were carried out using the Phenom Pro X (Phenom World) with preferably the highest electron acceleration voltage leading to a resolution where cells and biofilms are still clearly visible, ranging from 10 kV - 15 kV.

#### 3.5.1 MBEC™ ASSAY

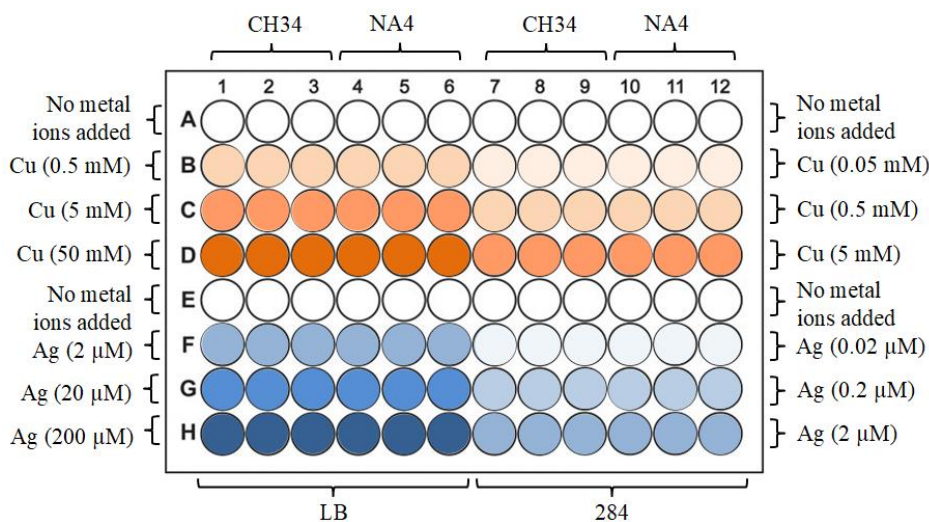
An MBEC™ Assay Biofilm Inoculator (Innovotech) was used as setup for the scanning electron microscopy experiment. This biofilm inoculator consists of a plastic lid with 96 pegs (pins) and a corresponding base, as shown in Figure 11. Two different 96 well plates were used: the MBEC™ Biofilm Inoculator with uncoated pegs and the MBEC™ Biofilm Inoculator with Titanium dioxide (TiO<sub>2</sub>) coated pegs. These different coatings were used to observe differences in biofilm growth or attachment.



**Figure 11: MBEC™ Biofilm Inoculator with 96 well base**  
<https://www.genengnews.com/new-products/biofilm-generation/4091>

This experiment was carried out using various concentrations of CuSO<sub>4</sub> and AgNO<sub>3</sub> in LB and 284 medium with *C. metallidurans* CH34 and NA4 as shown in Figure 12. This setup was used for both the MBEC™ Biofilm Inoculator with uncoated pegs and the MBEC™ Biofilm Inoculator with TiO<sub>2</sub> coated pegs.

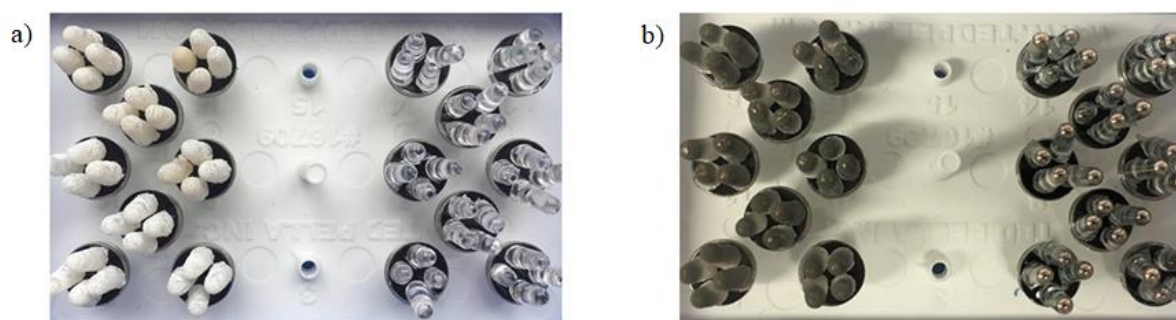
The bacterial suspensions of *Cupriavidus metallidurans* CH34 were added in columns 1-3 and 7-9, and these of *C. metallidurans* NA4 were added in columns 4-6 and 10-12. Metal ion solutions were added in all rows except for A and E. The MBEC™ microtiter plates were inoculated and incubated as previously mentioned in section 3.1. The pegs of rows 1, 4, 7 and 10 were used for SEM imaging. All other pegs were used for CV staining.



**Figure 12: Setup for MBEC analysis**

### 3.5.2 SCANNING ELECTRON MICROSCOPY (SEM) ANALYSIS

The biofilms on the pegs from rows 1, 4, 7 and 10 were fixed by submerging the lid with the pegs in a 96 well plate containing the fixing solutions. First, the pegs were submerged in 3% glutaraldehyde (Sigma-Aldrich) to crosslink proteins and 0.132 M cacodylate (Sigma-Aldrich) to avoid microprecipitation on thin sections that can occur with phosphate buffers. Afterwards, the excess of glutaraldehyde was removed by submerging the pegs again in 0.1 M cacodylate. The samples were dehydrated using a dilution series of ethanol (30%, 50%, 70%, 95%, and 100%). The pegs were submerged during 5 minutes in each of the ethanol dilutions, starting with the least concentrated one. After fixation, the pegs were removed individually using tweezers, and four pegs were placed on one alumina stub (Ted Pella) with the use of double-sided adhesive carbon conductive tape (Ted Pella) as shown in Figure 13a. Afterwards, the samples were sputter coated with gold using the Edwards Scancoat Six. Before sputter coating, the cooling water and Argon valve were opened, while making sure the pressure for oxygen was around 0.6 bar. Next, the power of the Edwards Scancoat Six was turned on and the stubs were mounted onto an alumina holder and loaded into the sputter-coat chamber. After closing the upper lid, the vacuum pump was switched on and when the pressure reached 0.04 mbar, a current of 500 V and 50 mA was administered. At this point, the pressure was stabilized around 6 mbar and the sputter coating process was executed during 200 seconds. The sputter coated peg samples are shown in Figure 13b.



**Figure 13: (a) MBEC™ Biofilm Inoculator with TiO<sub>2</sub> coated pegs (left) and uncoated pegs (right) before sputter coating, (b) MBEC™ Biofilm Inoculator with TiO<sub>2</sub> coated pegs (left) and uncoated pegs (right) after sputter coating**

### 3.5.3 PEGS USED FOR CV STAINING

The coated and uncoated pegs from rows 2, 3, 5, 6, 8, 9, 11 and 12 were removed using tweezers and placed in a new 96 well plate. To stain the pegs, 250  $\mu$ L of a 0.1% solution of CV in Milli-Q<sup>®</sup> (Millipore) was added to each well containing a peg. The microtiter plate was then incubated at room temperature during 15 minutes. Subsequently, the pegs were rinsed individually by submerging them in tap water using tweezers. The microtiter plate was dried overnight. The quantification of biofilms formed on these pegs was carried out using 250  $\mu$ L of 30% acetic acid as described in section 3.3.2.1.

The coated and uncoated MBEC™ Assay's Biofilm Inoculator bottom plates were also stained using CV and quantified as mentioned in section 3.3.1 and 3.3.2.1.

## 3.6 RNA EXTRACTION

RNA extraction of *C. metallidurans* bacterial suspensions and biofilms was performed for RNA quantification and quality assessment.

### 3.6.1 BACTERIAL SUSPENSIONS

RNA extraction of bacterial suspensions of *C. metallidurans* CH34 and NA4 was carried out using the Promega SV Total RNA Isolation System. First, the bacterial culture of either *C. metallidurans* CH34 or NA4 was grown overnight in LB medium at 30 °C. The following day, this culture was diluted 1:10 and grown in fresh LB medium until the OD reached 0.6-1.0. RNA extraction was performed using a RNA isolation protocol that was adjusted for the RNA isolation of *C. metallidurans* biofilms (Appendix 1). The samples were centrifuged at 14,000 x g for one minute and the purified RNA was stored at -80 °C.

### 3.6.2 BIOFILMS

RNA extraction of biofilms formed by *C. metallidurans* CH34 and NA4 in 96 well plates was carried out by adding 20 µL of DEPC treated Tris-EDTA buffer containing 3 mg/mL Lysozyme from chicken egg white (Sigma-Aldrich) to each well and removing the biofilm by scraping using a pipette tip. Next, five wells from the same condition were pooled to obtain a total volume of 100 µL. Afterwards, the same protocol for RNA extraction for bacterial suspensions was used (Appendix 1).

## 3.7 DATA PROCESSING

All data were visualized using GraphPad Prism 5 software. Significance was determined using a parametric One-way ANOVA (one-way analysis of variance) and Tukey as a post test ( $P < 0.05$ ). Only significant differences with regard to the zero (no metal ions added) were displayed to keep a clear overview. The level of significance was indicated using asterisks.

*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$

## 4. RESULTS

### 4.1 BIOFILM FORMATION IN THE PRESENCE OF SILVER AND COPPER

OD measurements were carried out to determine bacterial (planktonic) growth, CV measurements were carried out to determine biofilm formation, and CV/OD ratios were determined to normalize biofilm formation to planktonic cell density.

#### 4.1.1 BIOFILM FORMATION IN LYSOGENY BROTH (LB) MEDIUM

The influence of silver and copper ions on *Cupriavidus metallidurans* CH34 and NA4 biofilms in LB medium was assessed using 96 well plates. Also, the minimum inhibitory concentration (MIC) was assessed to determine the lowest concentration of  $\text{Cu}^{2+}$  or  $\text{Ag}^+$  that prevents visible growth of *C. metallidurans* CH34 or NA4.

##### 4.1.1.1 SILVER

###### OD MEASUREMENTS

The optical density of the bacterial suspensions of *C. metallidurans* CH34 and NA4 in the presence of silver was measured after 7 days of incubation in LB medium at 600 nm as shown in Figure 14. Three repeats of this experiment were carried out.

For both *C. metallidurans* CH34 and NA4, a decrease in OD was observed with increasing concentrations of  $\text{Ag}^+$  for repeats 1 and 2. This might indicate that  $\text{Ag}^+$  has an inhibiting effect on *C. metallidurans* growth. Repeat 3 however, shows a very different pattern with twofold absolute values for 0  $\mu\text{M}$   $\text{Ag}^+$  for *C. metallidurans* NA4. In general, *C. metallidurans* CH34 growth decreases from 100  $\mu\text{M}$   $\text{Ag}^+$  onwards, whereas for NA4 growth decreases from 200  $\mu\text{M}$   $\text{Ag}^+$  onwards. This might indicate that these concentrations are the MIC and that *C. metallidurans* NA4 is slightly more resistant to  $\text{Ag}^+$ .

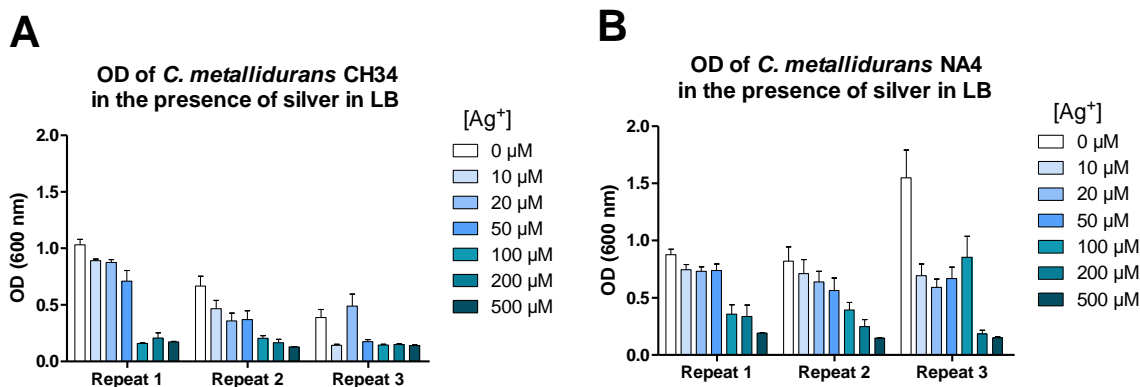


Figure 14: Optical density (OD) measurements for *C. metallidurans* CH34 (A) and NA4 (B) bacterial suspensions grown in LB medium appended with  $\text{AgNO}_3$

### CV MEASUREMENTS

A CV staining was performed to quantify biofilm formation of *C. metallidurans* CH34 and NA4 after 7 days of incubation in LB medium in the presence of silver as shown in Figure 15.

For *C. metallidurans* CH34 (Figure 15C) a decrease in biofilm quantity is observed with increasing concentrations of  $\text{Ag}^+$  for repeat 1. Whereas repeats 2 and 3 showed almost no biofilm growth, even for  $0 \mu\text{M}$   $\text{Ag}^+$ . In these repeats, biofilm formation was prevented independently from the added silver ions. This might be caused by the inaccuracy that is induced by the medium that was used. *C. metallidurans* NA4 (Figure 15D) shows an overall decrease in biofilm growth with increasing concentrations of  $\text{Ag}^+$ . However, large differences in absolute CV values were observed between the different repeats. This emphasizes the unsuitability of using LB medium to grown biofilms. Overall, *C. metallidurans* NA4 formed more biofilm than CH34 and copper seems to have an inhibiting effect on *C. metallidurans* biofilms.

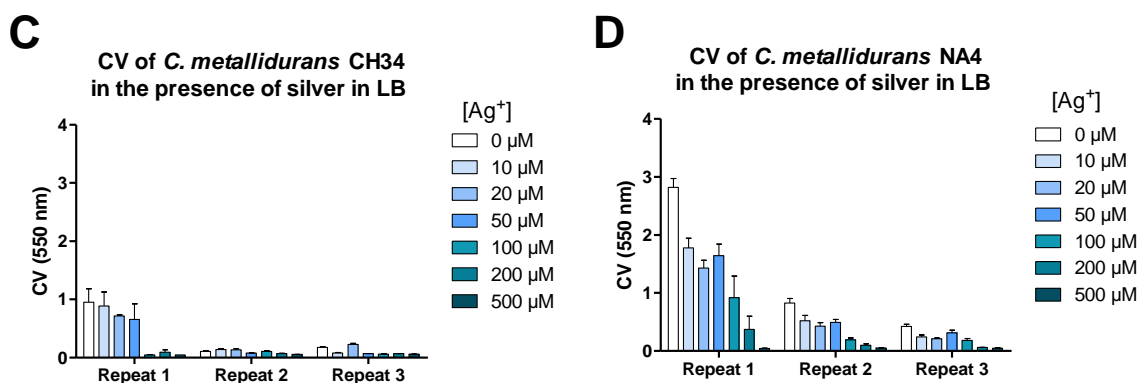
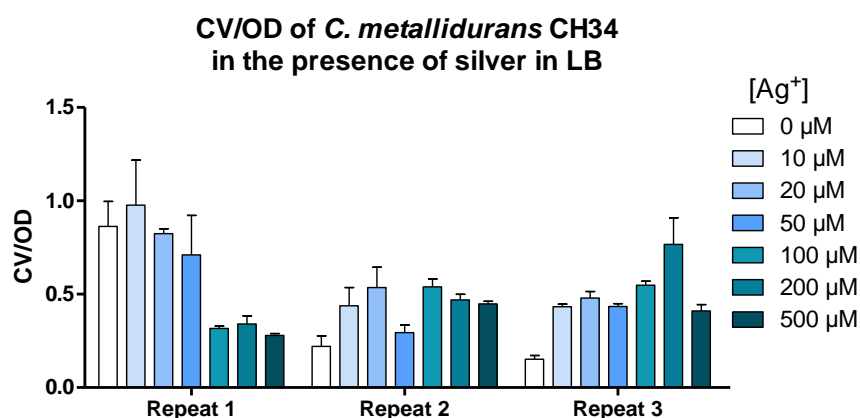


Figure 15: Crystal violet (CV) absorbance measurements for *C. metallidurans* CH34 (C) and NA4 (D) biofilms grown in LB medium appended with  $\text{AgNO}_3$

### CV TO OD RATIO

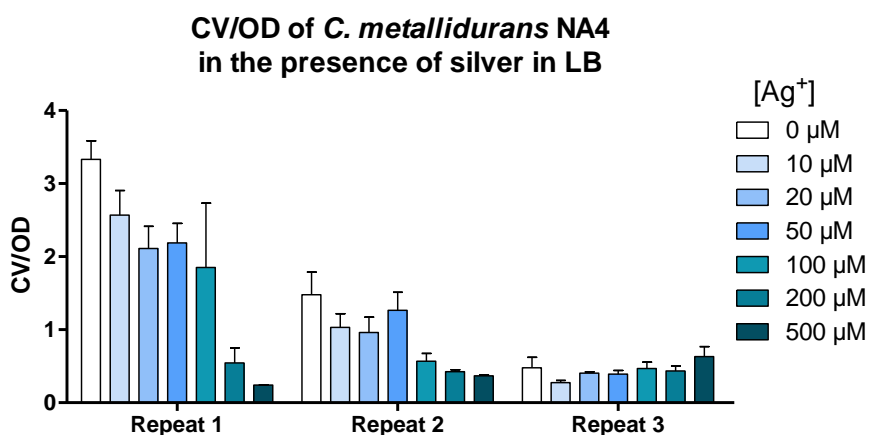
An increased biofilm growth might be caused by either increased bacterial growth or stimulation of biofilm growth due to stressors, such as copper or silver ions. This was verified by determining the CV/OD ratio values. OD measurements were carried out to determine bacterial (planktonic) growth, CV measurements were carried out to determine biofilm formation, and CV/OD ratios were determined to normalize biofilm formation to planktonic cell density. A high CV/OD value indicates increased biofilm growth with regard to bacterial growth.

Figure 16 shows the CV/OD values of *C. metallidurans* CH34 in LB medium in the presence of silver. Repeat 1 shows a decrease in biofilm formation with increasing  $\text{Ag}^+$  concentrations, whereas repeats 2 and 3 show an overall increase. Also, the absolute CV/OD value for repeat 1 for  $0 \mu\text{M}$   $\text{Ag}^+$  is about fourfold the values of repeats 2 and 3. This large variation is probably due to the used medium.



**Figure 16: Ratio of CV absorbance value to optical density (OD) for *C. metallidurans* CH34 bacterial suspensions and biofilms grown in LB medium appended with  $\text{AgNO}_3$**

Figure 17 shows the CV/OD values of *C. metallidurans* NA4 in LB medium in the presence of silver. Repeats 1 and 2 show a decrease in biofilm formation with increasing  $\text{Ag}^+$  concentrations, whereas repeat 3 ratios are practically zero. This might indicate that  $\text{Ag}^+$  exhibits an inhibiting effect on biofilm formation. The absolute values of the CV/OD value of repeat 1 is much higher than the values for repeats 2 and 3, again stressing the variations of biofilm formation in LB medium.



**Figure 17: Ratio of CV absorbance value to optical density (OD) for *C. metallidurans* NA4 bacterial suspensions and biofilms grown in LB medium appended with  $\text{AgNO}_3$**



#### 4.1.1.2 COPPER

##### OD MEASUREMENTS

Figure 18 shows the results of the OD measurements of the bacterial suspensions of *C. metallidurans* CH34 and NA4 in LB medium in the presence of copper. For both *C. metallidurans* CH34 (A) and NA4 (B), an overall increase in OD is observed with increased concentrations of  $\text{Cu}^{2+}$  for repeats 1 and 2. This might indicate that copper concentrations up to 5 mM have a stimulating effect on *C. metallidurans* growth. Repeat 3 however, shows a different pattern. Especially *C. metallidurans* NA4 repeat 3 shows deviant OD values. Also, the overall *C. metallidurans* NA4 OD values are higher compared to CH34 OD values. This might indicate that *C. metallidurans* NA4 is more resistant to copper ions. Since there is still visible growth at 5 mM  $\text{Cu}^{2+}$ , the MIC could not be determined.

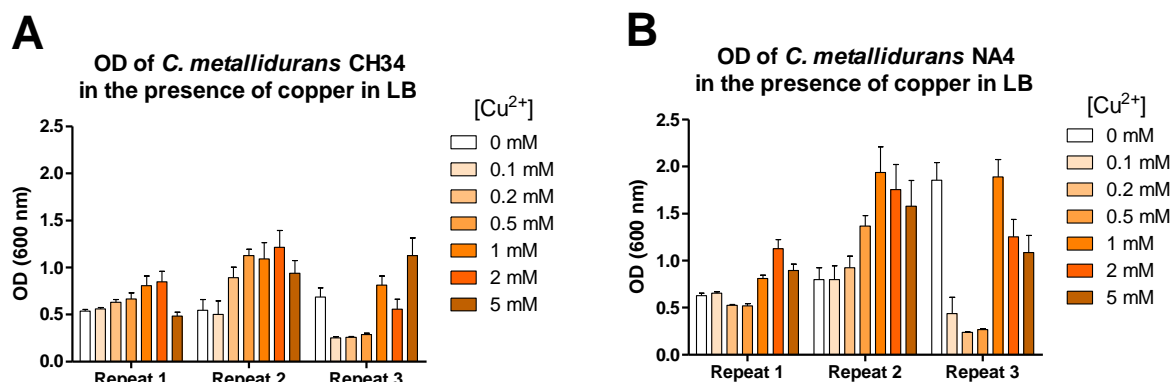


Figure 18: Optical density (OD) measurements for *C. metallidurans* CH34 (A) and NA4 (B) bacterial suspensions grown in LB medium appended with  $\text{CuSO}_4$

##### CV MEASUREMENTS

Figure 19 shows the results of the CV measurements of *C. metallidurans* CH34 and NA4 biofilm formation in LB medium in the presence of copper. For *C. metallidurans* CH34 (Figure 15C) an overall increase in biofilm quantity is observed with increasing concentrations of  $\text{Cu}^{2+}$ . The absolute CV value of repeat 1 however, is about fourfold the values of repeats 2 and 3. For *C. metallidurans* NA4 (D) an overall decrease in biofilm quantity is observed with increasing concentrations of  $\text{Cu}^{2+}$ . Again, the absolute CV value of repeat 1, is about fourfold the values of repeats 2 and 3. Also, the overall *C. metallidurans* NA4 biofilm CV values are higher than for CH34, indicating that NA4 forms more biofilm compared to CH34.

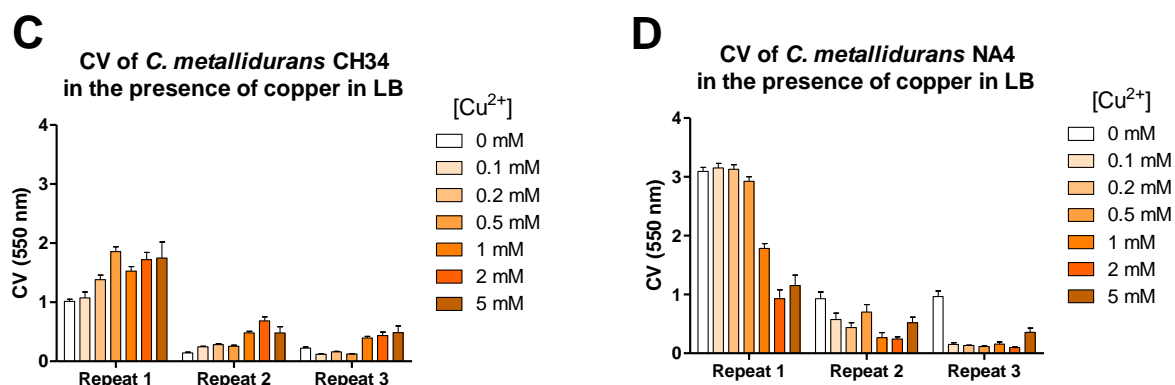


Figure 19: Crystal violet (CV) absorbance measurements for *C. metallidurans* CH34 (C) and NA4 (D) biofilms grown in LB medium appended with  $\text{CuSO}_4$

### CV TO OD RATIO

Figure 20 shows the CV/OD values of *C. metallidurans* CH34 in LB medium in the presence of copper. Repeats 1 and 3 show an overall increase in biofilm formation with increasing  $\text{Cu}^{2+}$  concentrations, repeat 2 shows rather deviant values in comparison to repeats 1 and 3. Also, the absolute CV/OD values for repeat 1 are about fourfold the values of repeats 2 and 3. Overall, this might indicate that  $\text{Cu}^{2+}$  has a stimulating effect on *C. metallidurans* CH34 biofilm growth in LB medium, at least up to a concentration of 5 mM. Also, these data stress the inconsistency of biofilm growth in LB medium.

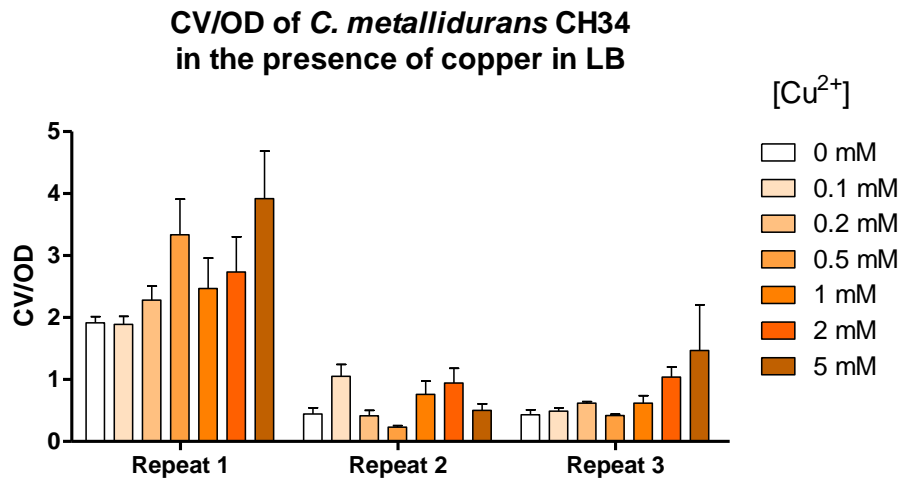


Figure 20: Ratio of CV absorbance value to optical density (OD) for *C. metallidurans* CH34 bacterial suspensions and biofilms grown in LB medium appended with  $\text{CuSO}_4$

Figure 21 shows the CV/OD values of *C. metallidurans* NA4 in LB medium in the presence of copper. Repeat 1 shows an increase in followed by a decrease in biofilm formation with increasing  $\text{Cu}^{2+}$  concentrations. This might indicate that copper has an inhibiting effect on *C. metallidurans* NA4 biofilm formation. Repeats 2 and 3 show deviant CV/OD values. Again, the CV/OD values for repeat 1 are much larger than for repeats 2 and 3. Indicating the inconsistency of biofilm growth in LB medium. Also, the overall *C. metallidurans* NA4 biofilm CV/OD values are higher than for CH34, indicating that NA4 forms more biofilm compared to CH34.

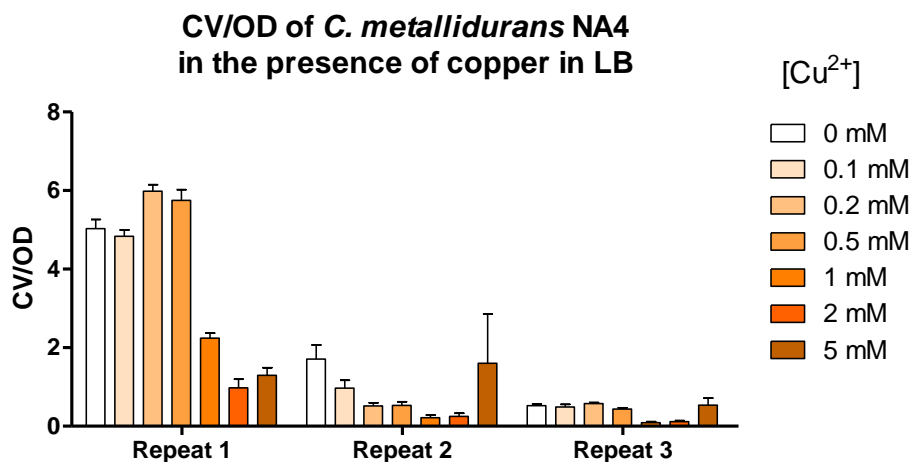


Figure 21: Ratio of CV absorbance value to optical density (OD) for *C. metallidurans* NA4 bacterial suspensions and biofilms grown in LB medium appended with  $\text{CuSO}_4$

## 4.1.2 BIOFILM FORMATION IN TRIS-BUFFERED MINERAL (284) MEDIUM

The influence of silver and copper ions on *Cupriavidus metallidurans* CH34 and NA4 biofilms in 284 medium is assessed using 96 well plates. Also, the minimum inhibitory concentration (MIC) was assessed to determine the lowest concentration of Cu or Ag which prevents visible growth of *Cupriavidus metallidurans* CH34 or NA4. 284 medium is a defined medium that is used in various studies where reproducibility was required. Whereas LB medium, which was previously mentioned, is an undefined medium.

### 4.1.2.1 SILVER

#### OPTICAL DENSITY (OD) MEASUREMENTS

The optical density of the bacterial suspensions of *C. metallidurans* CH34 and NA4 in the presence of silver was measured after 7 days of incubation in 284 medium at 600 nm as shown in Figure 22 and Figure 23.

For both *C. metallidurans* CH34 (Figure 22) and NA4 (Figure 23) an overall decrease in OD is observed with increasing silver concentrations. However, a slight increase of OD values is also observed in some repeats in the presence of 0.5  $\mu\text{M}$  and 1  $\mu\text{M}$  of silver, followed by a decrease at higher silver concentrations. This might indicate that  $\text{Ag}^+$  has an inhibiting effect on *C. metallidurans* CH34 and NA4 growth. When comparing 0  $\mu\text{M}$  silver and 5  $\mu\text{M}$  silver, CV values are significantly halved for all repeats. This decrease in OD for 5  $\mu\text{M}$   $\text{Ag}^+$  might indicate the MIC. Both repeats show a similar progression.

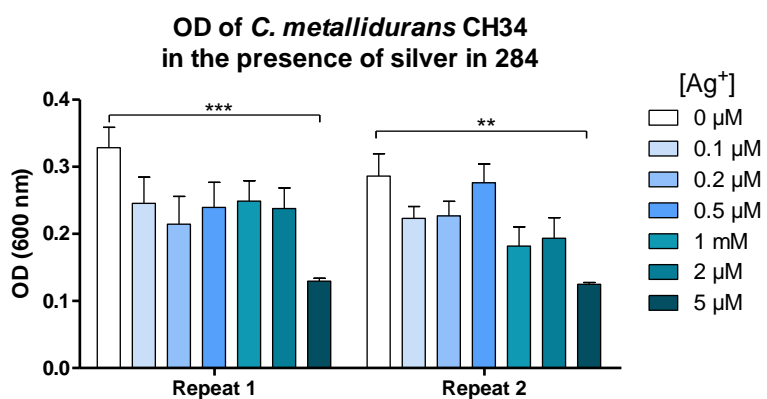


Figure 22: Optical density (OD) measurements for *C. metallidurans* CH34 bacterial suspensions grown in 284 medium appended with  $\text{AgNO}_3$  (\* =  $P \leq 0.05$ , \*\* =  $P \leq 0.01$ , \*\*\* =  $P \leq 0.001$ )

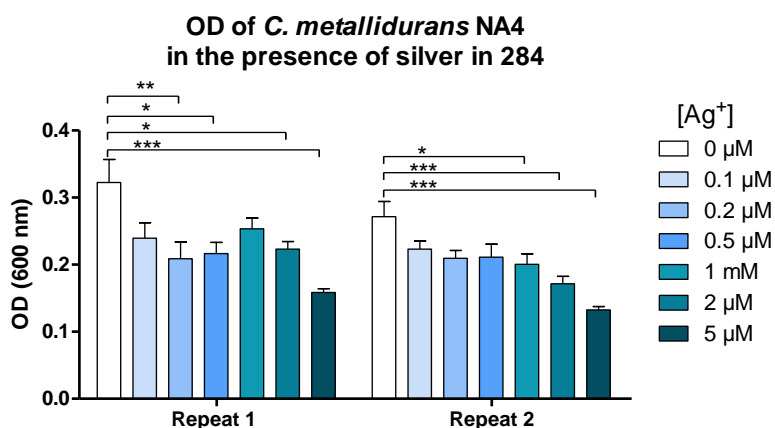


Figure 23: Optical density (OD) measurements for *C. metallidurans* NA4 bacterial suspensions grown in 284 medium appended with  $\text{AgNO}_3$  (\* =  $P \leq 0.05$ , \*\* =  $P \leq 0.01$ , \*\*\* =  $P \leq 0.001$ )

### CV MEASUREMENTS

A CV staining was performed to quantify biofilm formation of *C. metallidurans* CH34 and NA4 at 550 nm after 7 days of incubation in 284 medium in the presence of silver as shown in Figure 24 and Figure 25.

For *C. metallidurans* CH34 (Figure 24) biofilm formation remains relatively stable throughout the silver concentration range. Both repeats show similar absolute CV values, but their development is slightly different. In the presence of the highest concentration of silver (5  $\mu\text{M}$ ), a significant decrease is observed for both repeats. This might indicate an inhibiting effect of 5  $\mu\text{M}$   $\text{Ag}^+$  on biofilm formation.

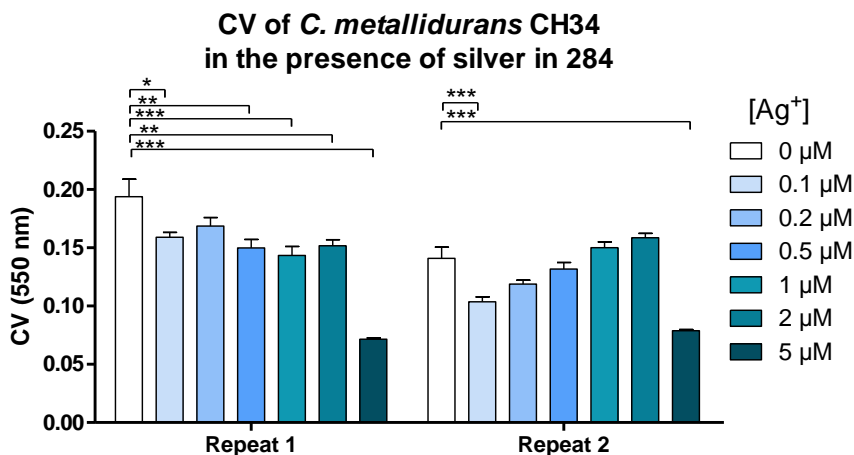


Figure 24: CV absorbance values for *C. metallidurans* CH34 biofilms grown in 284 medium appended with  $\text{AgNO}_3$  (\* =  $P \leq 0.05$ , \*\* =  $P \leq 0.01$ , \*\*\* =  $P \leq 0.001$ )

For *C. metallidurans* NA4 (Figure 25) CV values of repeat 1 show an overall decrease, whereas they remain relatively stable for repeat 2. At a silver concentration of 5  $\mu\text{M}$ , the CV values show a significant decrease for both repeats compared to a silver concentration of 0  $\mu\text{M}$ . Again, this might indicate an inhibiting effect of 5  $\mu\text{M}$   $\text{Ag}^+$  on biofilm formation. When comparing biofilm CV values of *C. metallidurans* CH34 (Figure 24) to NA4 (Figure 25), an overall increase is observed for *C. metallidurans* NA4. Indicating that NA4 forms more biofilm than CH34.

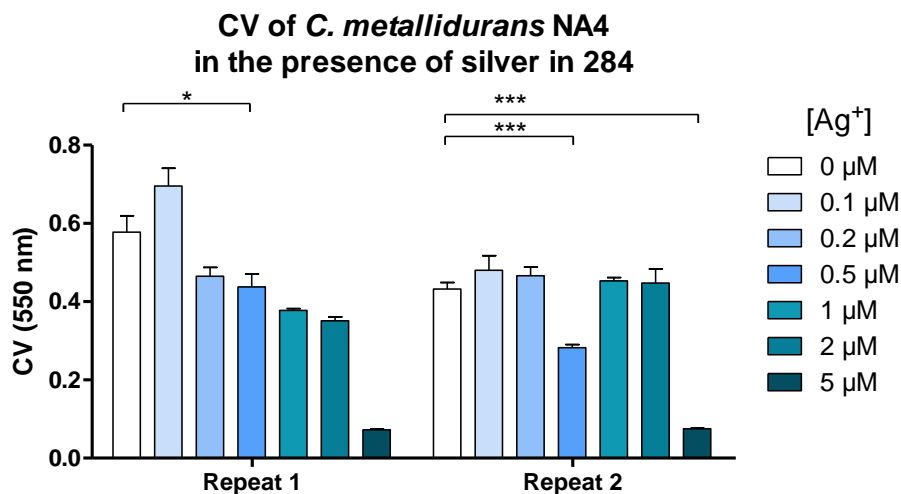


Figure 25: CV absorbance values for *C. metallidurans* NA4 biofilms grown in 284 medium appended with  $\text{AgNO}_3$  (\* =  $P \leq 0.05$ , \*\* =  $P \leq 0.01$ , \*\*\* =  $P \leq 0.001$ )

### CV TO OD RATIO

Figure 26 shows the CV/OD values of *C. metallidurans* CH34 (A) and NA4 (B) in 284 medium in the presence of silver.

For *C. metallidurans* CH34 (Figure 26A), biofilm formation remains rather stable throughout both repeats. Only for repeat 2, an increase is observed for 1  $\mu\text{M}$  and 2  $\mu\text{M}$   $\text{Ag}^+$ .

*C. metallidurans* NA4 (Figure 26B) repeats 1 and 2 show a similar progression. First, an increase in CV/OD values is observed, followed by a decrease. This might indicate that  $\text{Ag}^+$  exhibits an inhibiting effect on *C. metallidurans* NA4 biofilm formation, especially at a concentration of 5  $\mu\text{M}$ . A stimulating effect on biofilm formation is observed for 0.1  $\mu\text{M}$  and 0.2  $\mu\text{M}$ . Also, the overall *C. metallidurans* NA4 CV/OD values are about threefold the CH34 values. Indicating that *C. metallidurans* NA4 forms biofilms to a greater extent than CH34.

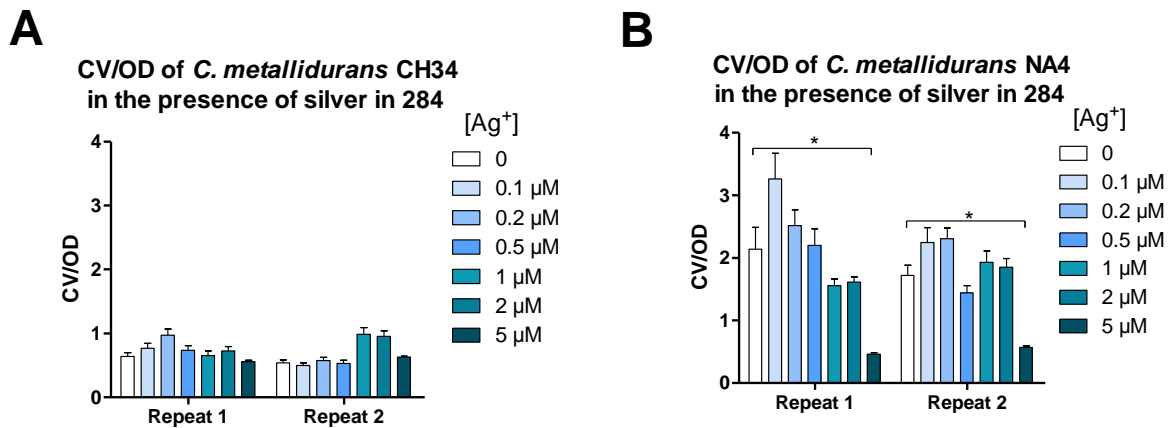


Figure 26: Ratio of CV absorbance value to optical density (OD) for *C. metallidurans* CH34 (A) and NA4 (B) bacterial suspensions and biofilms grown in 284 medium appended with  $\text{AgNO}_3$  (\* =  $P \leq 0.05$ , \*\* =  $P \leq 0.01$ , \*\*\* =  $P \leq 0.001$ )

#### 4.1.2.2 COPPER

##### OD MEASUREMENTS

The optical density of the bacterial suspensions of *C. metallidurans* CH34 and NA4 in the presence of silver was measured after 7 days of incubation in Tris-buffered mineral (284) medium at 600 nm as shown in Figure 27 and Figure 28.

For *C. metallidurans* CH34 (Figure 27), a similar progression is observed for both repeats. First, an overall decrease of bacterial growth is observed for 0.1 mM  $\text{Cu}^{2+}$ , followed by an increase for 0.2 mM and 0.5 mM  $\text{Cu}^{2+}$ , again followed by a decrease from 0.5 mM  $\text{Cu}^{2+}$  onwards. A significant decrease is observed between 0 mM and 5 mM  $\text{Cu}^{2+}$  for both repeats, indicating an overall inhibiting effect of copper on *C. metallidurans* CH34 growth.

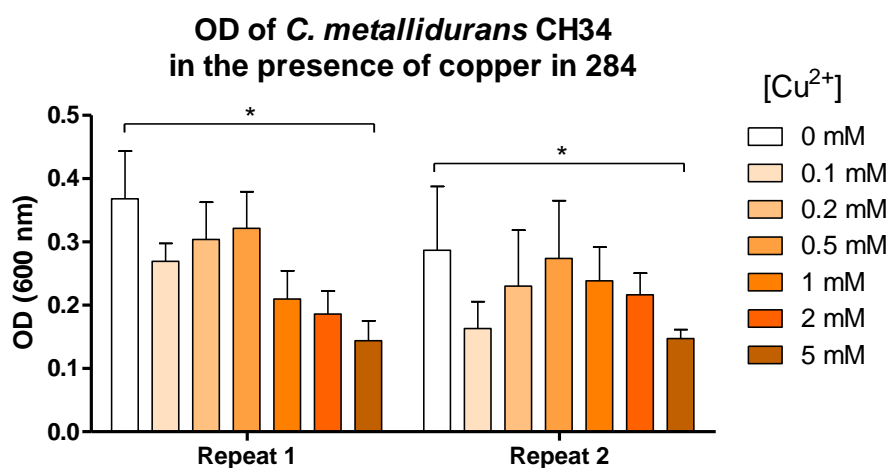


Figure 27: Optical density (OD) measurements for *C. metallidurans* CH34 bacterial suspensions grown in 284 medium appended with  $\text{CuSO}_4$  (\* =  $P \leq 0.05$ , \*\* =  $P \leq 0.01$ , \*\*\* =  $P \leq 0.001$ )

For *C. metallidurans* NA4 (Figure 28), a similar progression is observed for both repeats. First, an overall decrease of bacterial growth is observed for 0.1 mM  $\text{Cu}^{2+}$ , followed by an increase for 0.2 mM, 0.5 mM, and 1 mM  $\text{Cu}^{2+}$ , again followed by a decrease from 2 mM  $\text{Cu}^{2+}$  onwards. A significant decrease is observed between 0 mM and 5 mM  $\text{Cu}^{2+}$  for both repeats, indicating an overall inhibiting effect of copper on *C. metallidurans* NA4 growth.

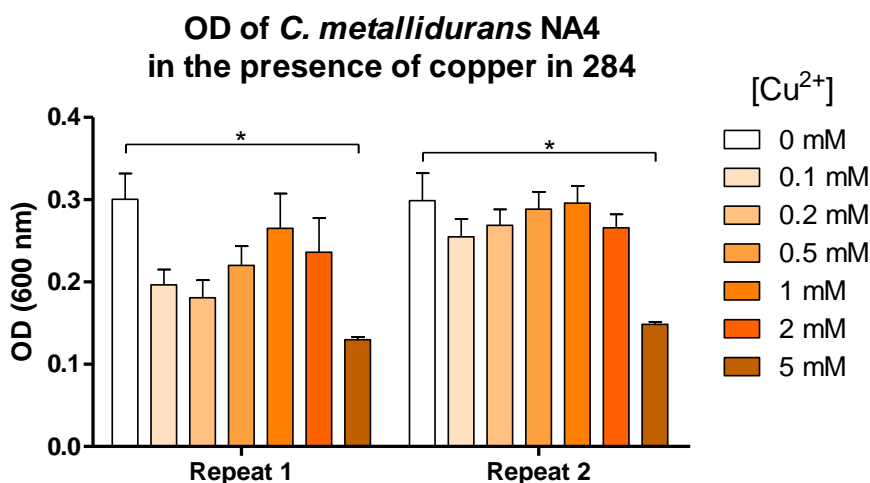


Figure 28: Optical density (OD) measurements for *C. metallidurans* NA4 bacterial suspensions grown in 284 medium appended with  $\text{CuSO}_4$  (\* =  $P \leq 0.05$ , \*\* =  $P \leq 0.01$ , \*\*\* =  $P \leq 0.001$ )

### CV MEASUREMENTS

For *C. metallidurans* CH34 (Figure 29), both repeats show similar absolute CV values and progression. At lower concentrations (0 mM – 0.5 mM Cu<sup>2+</sup>), biofilm formation remains relatively stable. A significant increase in CV values is observed for both 1 mM and 2 mM Cu<sup>2+</sup>. This indicates a stimulating effect of 1 mM and 2 mM Cu<sup>2+</sup> on biofilm formation. At a concentration of 5 mM Cu<sup>2+</sup>, CV values decrease again, indicating an inhibiting effect.

#### CV of *C. metallidurans* CH34 in the presence of copper in 284

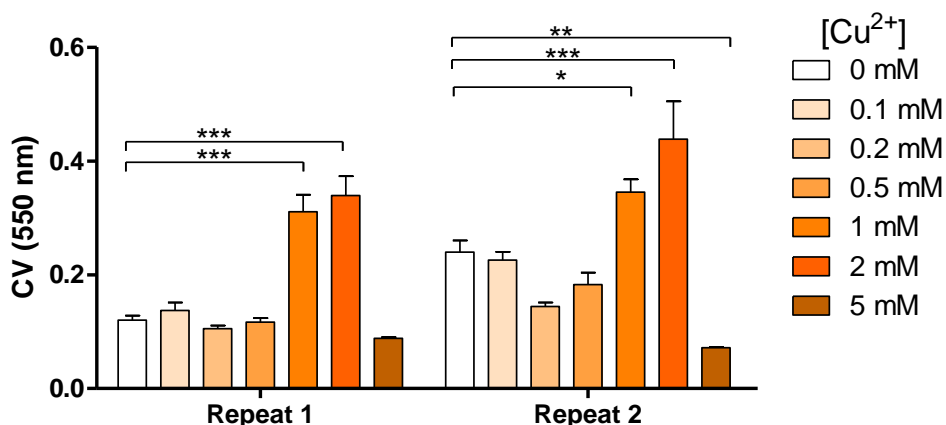


Figure 29: CV absorbance values for *C. metallidurans* CH34 biofilms grown in 284 medium appended with CuSO<sub>4</sub> (\* = P ≤ 0.05, \*\* = P ≤ 0.01, \*\*\* = P ≤ 0.001)

For *C. metallidurans* CH34 (Figure 30), both repeats show similar absolute CV values and progression. However, the CV value for 0 mM Cu<sup>2+</sup> of repeat 2 is twofold the value of repeat 1. An increase of biofilm formation is observed for 0.1 mM and 0.2 mM Cu<sup>2+</sup>, indicating a stimulating effect. From 0.5 mM Cu<sup>2+</sup> onwards, CV values decrease with increasing Cu<sup>2+</sup> concentrations, indicating an inhibiting effect on biofilm formation.

#### CV of *C. metallidurans* NA4 in the presence of copper in 284

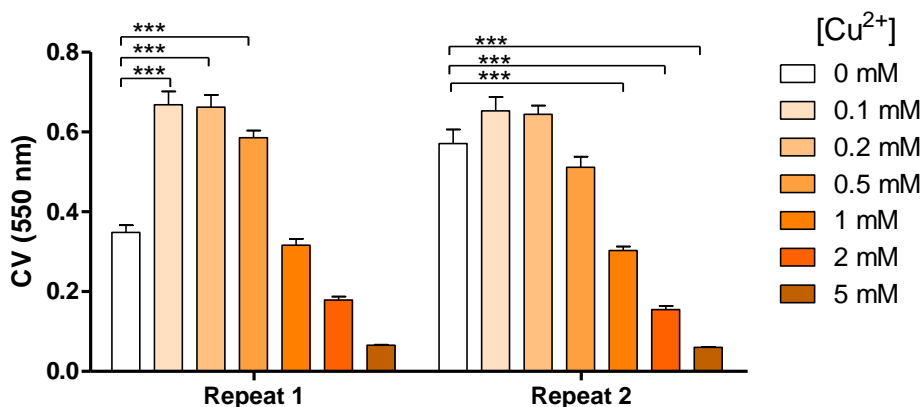


Figure 30: CV absorbance values for *C. metallidurans* NA4 biofilms grown in 284 medium appended with CuSO<sub>4</sub> (\* = P ≤ 0.05, \*\* = P ≤ 0.01, \*\*\* = P ≤ 0.001)

### CV TO OD RATIO

Figure 31 shows the CV/OD values of *C. metallidurans* CH34 (A) and NA4 (B) in 284 medium in the presence of copper.

For *C. metallidurans* CH34 (Figure 31A), both repeats show similar absolute CV/OD values and progression. At lower concentrations (0 mM – 0.5 mM  $\text{Cu}^{2+}$ ), biofilm formation remains relatively stable. An increase is observed for 1 mM and 2 mM  $\text{Cu}^{2+}$  for both repeats, followed by a decrease at 5 mM  $\text{Cu}^{2+}$ . This might indicate a stimulating effect of 1 mM and 2 mM  $\text{Cu}^{2+}$ , and an inhibiting effect of 5 mM  $\text{Cu}^{2+}$  on biofilm formation.

*C. metallidurans* NA4 (Figure 31B), shows a similar progression although CV/OD values of repeat 1 are slightly higher than those of repeat 2. For both repeats, a significant increase in biofilm formation is observed for 0.1 mM  $\text{Cu}^{2+}$ , indicating a stimulating effect. From 0.5 mM  $\text{Cu}^{2+}$  onwards, a decrease in CV/OD values is observed with increasing  $\text{Cu}^{2+}$  concentrations, indicating an inhibiting effect on biofilm formation.

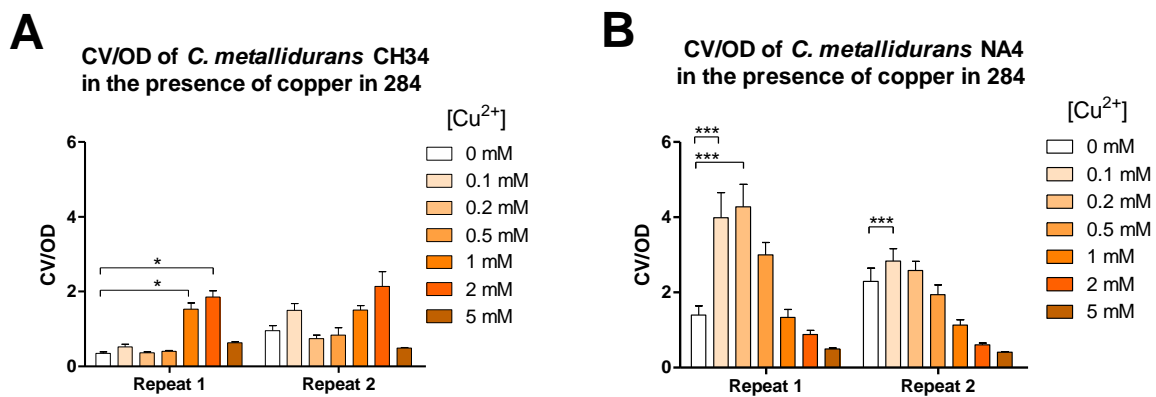


Figure 31: Ratio of CV absorbance value to optical density (OD) for *C. metallidurans* CH34 (A) and NA4 (B) bacterial suspensions and biofilms grown in 284 medium appended with  $\text{CuSO}_4$  (\* =  $P \leq 0.05$ , \*\* =  $P \leq 0.01$ , \*\*\* =  $P \leq 0.001$ )



## 4.2 SCANNING ELECTRON MICROSCOPY (SEM)

### 4.2.1 CUPRIAVIDUS METALLIDURANS CH34 BIOFILM STRUCTURE

#### 4.2.1.1 LB MEDIUM

Table 3 gives an overview of the most prominent features of *C. metallidurans* CH34 biofilms grown in LB medium appended with either CuSO<sub>4</sub> or AgNO<sub>3</sub>. Biofilms are scored according to their extent of EPS formation, cell clustering, and the presence of separate cells. To gain qualitative insights in biofilm formation in the presence of copper and silver, a broad concentration range of Cu<sup>2+</sup> and Ag<sup>+</sup> was used. This range partly matches the previously used concentrations (OD and CV experiments), but also very high concentrations (50 mM Cu<sup>2+</sup>) were used to observe their qualitative effect on biofilm structure.

If *C. metallidurans* CH34 biofilms were grown in LB medium without adding metal ions, EPS structures and clustering of cells are observed, indicating the presence of an actual biofilm. In the presence of Cu<sup>2+</sup>, EPS structures were also clearly observed. These EPS structures are not visible for *C. metallidurans* CH34 biofilms grown in the presence of Ag<sup>+</sup>. For both Cu<sup>2+</sup> and Ag<sup>+</sup>, clustering of cells was observed rather than the presence of separate cells. In the presence of 50 mM Cu<sup>2+</sup>, structures that might be copper crystals are observed. All SEM images are added in Appendix 3.

Table 3: Analysis of SEM imaging of *C. metallidurans* CH34 grown in LB medium appended with CuSO<sub>4</sub> or AgNO<sub>3</sub>

LB	EPS formation	Clustering of cells	Separate cells
<i>C. metallidurans</i> CH34			
0 mM Cu <sup>2+</sup>	++	+	-
0.5 mM Cu <sup>2+</sup>	+	+	-
5 mM Cu <sup>2+</sup>	++	+	-
50 mM Cu <sup>2+</sup>	-	+	-
2 μM Ag <sup>+</sup>	-	+	-
20 μM Ag <sup>+</sup>	-	-	+
200 μM Ag <sup>+</sup>	-	+	-

(++ = very present, + = present, +/- = slightly present, - = absent)

Figure 32 shows a comparison of scanning electron microscopy images of *C. metallidurans* CH34 biofilms grown in LB medium in the presence of 0 mM Cu<sup>2+</sup> and 5 mM Cu<sup>2+</sup>. In both conditions, EPS structure is present.

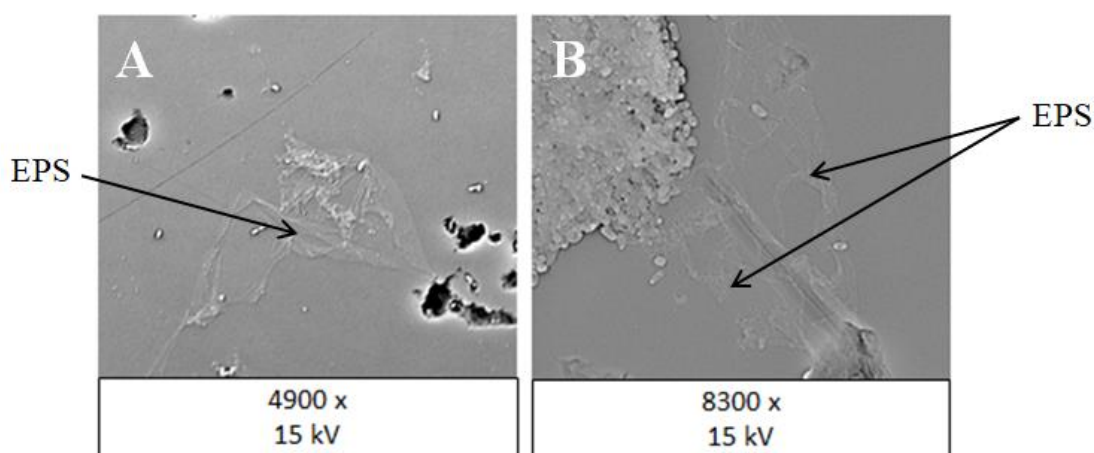


Figure 32: Scanning electron microscopy biofilm structures of *C. metallidurans* CH34 biofilms grown in LB medium in the presence of A) 0 mM Cu<sup>2+</sup> and B) 5 mM Cu<sup>2+</sup>

#### 4.2.1.2 284 MEDIUM

Table 4 gives an overview of the most prominent features of *C. metallidurans* CH34 biofilms grown in 284 medium appended with either CuSO<sub>4</sub> or AgNO<sub>3</sub>. Biofilms are scored according to their extent of EPS formation, cell clustering, and the presence of separate cells.

If *C. metallidurans* CH34 biofilms are grown in 284 medium without adding metal ions, EPS structures and clustering of cells are observed. In the presence of Cu<sup>2+</sup>, EPS structures seem to be more prominently present. These EPS structures are not visible for *C. metallidurans* CH34 biofilms grown in the presence of Ag<sup>+</sup>. For both Cu<sup>2+</sup> and Ag<sup>+</sup>, an overall clustering of cells is observed rather than the presence of separate cells, which might indicate biofilm formation

In the presence of 5 mM Cu<sup>2+</sup>, structures that might be copper crystals were observed. All SEM images are added in Appendix 3.

Table 4: Analysis of SEM imaging of *C. metallidurans* CH34 grown in 284 medium appended with CuSO<sub>4</sub> or AgNO<sub>3</sub>

284	EPS formation	Clustering of cells	Separate cells
<i>C. metallidurans</i> CH34			
0 mM Cu <sup>2+</sup>	+	+	+
0.05 mM Cu <sup>2+</sup>	-	-	+
0.5 mM Cu <sup>2+</sup>	++	+	-
5 mM Cu <sup>2+</sup>	+	+	-
0 μM Ag <sup>+</sup>	++	+	+
0.02 μM Ag <sup>+</sup>	-	+	-
0.2 μM Ag <sup>+</sup>	-	-	+
2 μM Ag <sup>+</sup>	-	+/-	+

(++ = very present, + = present, +/- = slightly present, - = absent)

Figure 33 shows a comparison of scanning electron microscopy images of *C. metallidurans* CH34 biofilms grown in 284 medium in the presence of 0.5 mM  $\text{Cu}^{2+}$  and 5 mM  $\text{Cu}^{2+}$ . For 0.5 mM  $\text{Cu}^{2+}$  (Figure 33A), EPS structure and clustering of cells are observed. For 5 mM  $\text{Cu}^{2+}$  (Figure 33B), structures that might be copper crystals are observed.

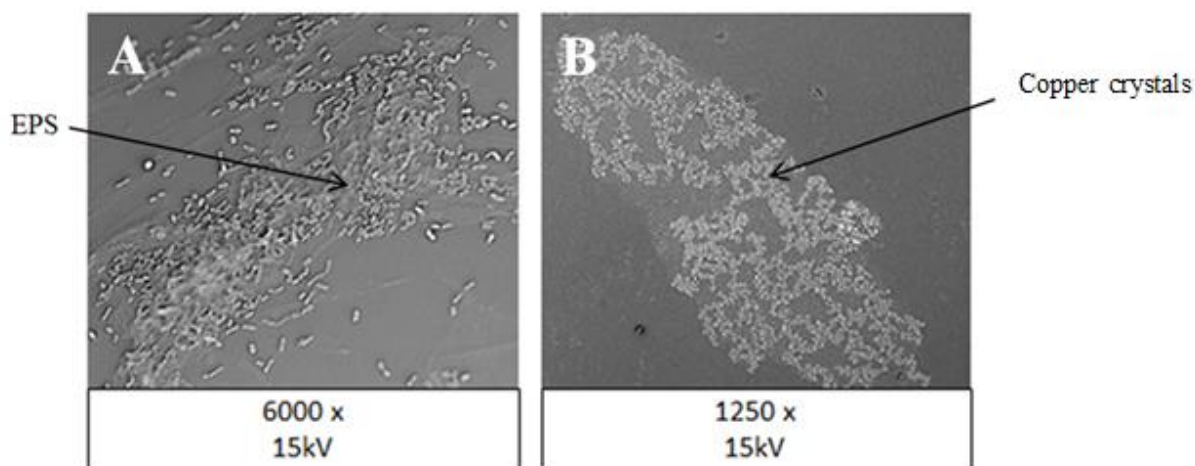


Figure 33: Scanning electron microscopy biofilm structures of *C. metallidurans* CH34 biofilms grown in 284 medium in the presence of A) 0.5 mM  $\text{Cu}^{2+}$  and B) 5 mM  $\text{Cu}^{2+}$

#### 4.2.1.3 LB VS. 284 MEDIUM

The structure of *C. metallidurans* CH34 biofilms visualised by SEM are shown Figure 34. For both *C. metallidurans* CH34 grown in LB medium (Figure 34A) and 284 medium (Figure 34B), the EPS structure is clearly visible as a matrix surrounding the cells with a widespread filament-like structure. This EPS structure is described in previous studies for *Pseudomonas* and *Streptococcus* strains as a very porous matrix of fibrils that cross-connect bacteria [78, 79].

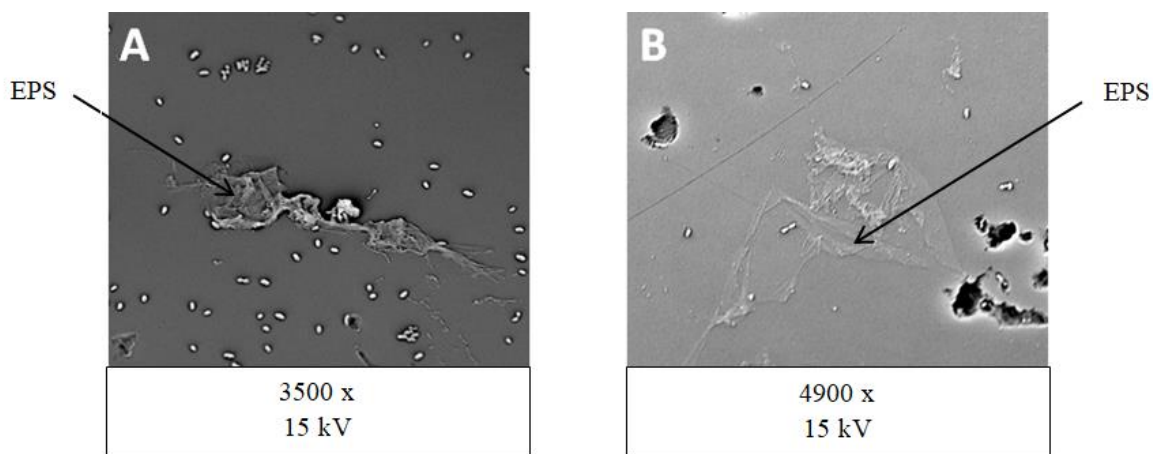


Figure 34: Scanning electron microscopy biofilm structures of *C. metallidurans* CH34 biofilms in the absence of metal ions: A) Biofilms grown in LB medium, B) Biofilms grown in 284 medium

## CUPRIAVIDUS METALLIDURANS NA4 BIOFILM STRUCTURE

### 4.2.1.4 LB MEDIUM

Table 5 gives an overview of the most prominent features of *C. metallidurans* NA4 biofilms grown in LB medium appended with either CuSO<sub>4</sub> or AgNO<sub>3</sub>. Biofilms are scored according to their extent of EPS formation, cell clustering, and the presence of separate cells.

If *C. metallidurans* NA4 biofilms are grown in LB medium without adding metal ions, EPS structures are present but rather difficult to observe in comparison to *C. metallidurans* CH34. Clustering of cells is prominently present. In the presence of Cu<sup>2+</sup>, EPS structures are also rather difficult to observe or absent at higher concentrations. Whereas for *C. metallidurans* NA4 biofilms grown in the presence of Ag<sup>+</sup>, EPS structures are observed, this is in contrast with the results of section 4.2.1.1 for *C. metallidurans* CH34 where no EPS structure was observed in the presence of Ag<sup>+</sup>. For both Cu<sup>2+</sup> and Ag<sup>+</sup>, clustering of cells as well as separate cells are observed. In the presence of 50 mM Cu<sup>2+</sup> clustering of cells is absent. All SEM images are added in Appendix 3.

Table 5: Analysis of SEM imaging of *C. metallidurans* NA4 grown in LB medium appended with CuSO<sub>4</sub> or AgNO<sub>3</sub>

LB	EPS formation	Clustering of cells	Separate cells
<i>C. metallidurans</i> NA4			
0 mM Cu <sup>2+</sup>	+/-	++	-
0.5 mM Cu <sup>2+</sup>	+/-	++	-
5 mM Cu <sup>2+</sup>	+/-	+	-
50 mM Cu <sup>2+</sup>	-	-	+
0 μM Ag <sup>+</sup>	+/-	++	-
2 μM Ag <sup>+</sup>	+	+	-
20 μM Ag <sup>+</sup>	+	+	-
200 μM Ag <sup>+</sup>	+/-	+	-

(++ = very present, + = present, +/- = slightly present, - = absent)

Figure 35 shows a comparison of scanning electron microscopy images of *C. metallidurans* NA4 biofilms grown in LB medium in the presence of 0 mM Cu<sup>2+</sup> and 5 mM Cu<sup>2+</sup>. For 0 mM Cu<sup>2+</sup> (Figure 35A), EPS structure and clustering of cells are observed. For 5 mM Cu<sup>2+</sup> (Figure 35B), clustering of cells is observed at a higher magnification (18500x).

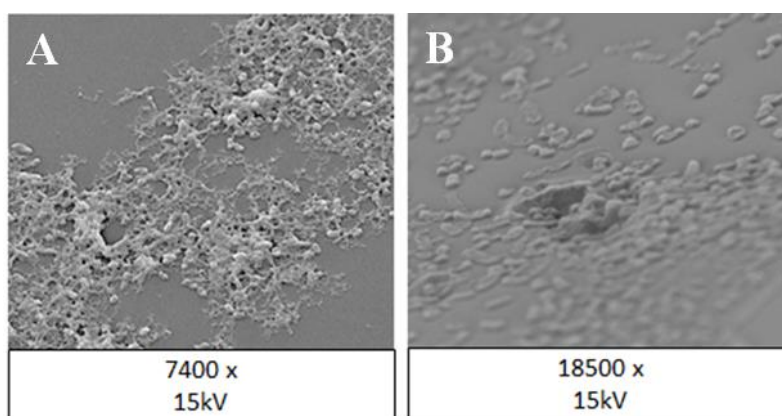


Figure 35: Scanning electron microscopy biofilm structures of *C. metallidurans* NA4 biofilms grown in LB medium in the presence of A) 0 mM Cu<sup>2+</sup> and B) 5 mM Cu<sup>2+</sup>

#### 4.2.1.5 284 MEDIUM

Table 6 gives an overview of the most prominent features of *C. metallidurans* NA4 biofilms grown in 284 medium appended with either CuSO<sub>4</sub> or AgNO<sub>3</sub>. Biofilms are scored according to their extent of EPS formation, cell clustering, and the presence of separate cells.

If *C. metallidurans* NA4 biofilms are grown in 284 medium without adding metal ions, EPS structures are present but rather difficult to observe. Clustering of cells is also observed. In the presence of Cu<sup>2+</sup>, EPS structures are also rather difficult to observe. Whereas for *C. metallidurans* NA4 biofilms grown in the presence of Ag<sup>+</sup>, EPS structures are absent. For both Cu<sup>2+</sup> and Ag<sup>+</sup>, clustering of cells as well as separate cells are observed. All SEM images are added in Appendix 3. These results are in line with the observations of section 4.2.1.2 for *C. metallidurans* CH34.

Table 6: Analysis of SEM imaging of *C. metallidurans* NA4 grown in 284 medium appended with CuSO<sub>4</sub> or AgNO<sub>3</sub>

284	EPS formation	Clustering of cells	Separate cells
<i>C. metallidurans</i> NA4			
0 mM Cu <sup>2+</sup>	+	+	-
0.05 mM Cu <sup>2+</sup>	-	+	+
0.5 mM Cu <sup>2+</sup>	+/-	+/-	+
5 mM Cu <sup>2+</sup>	+	+	+
0 μM Ag <sup>+</sup>	-	+/-	+
0.02 μM Ag <sup>+</sup>	-	+	+
0.2 μM Ag <sup>+</sup>	-	+/-	+
2 μM Ag <sup>+</sup>	-	-	+

(++ = very present, + = present, +/- = slightly present, - = absent)

Figure 36 shows a comparison of scanning electron microscopy images of *C. metallidurans* NA4 biofilms grown in 284 medium in the presence of 0 mM Cu<sup>2+</sup> and 5 mM Cu<sup>2+</sup>. For 0 mM Cu<sup>2+</sup> (Figure 36A), biofilm formation is observed. For 5 mM Cu<sup>2+</sup> (Figure 36B), increased EPS structure development is observed.

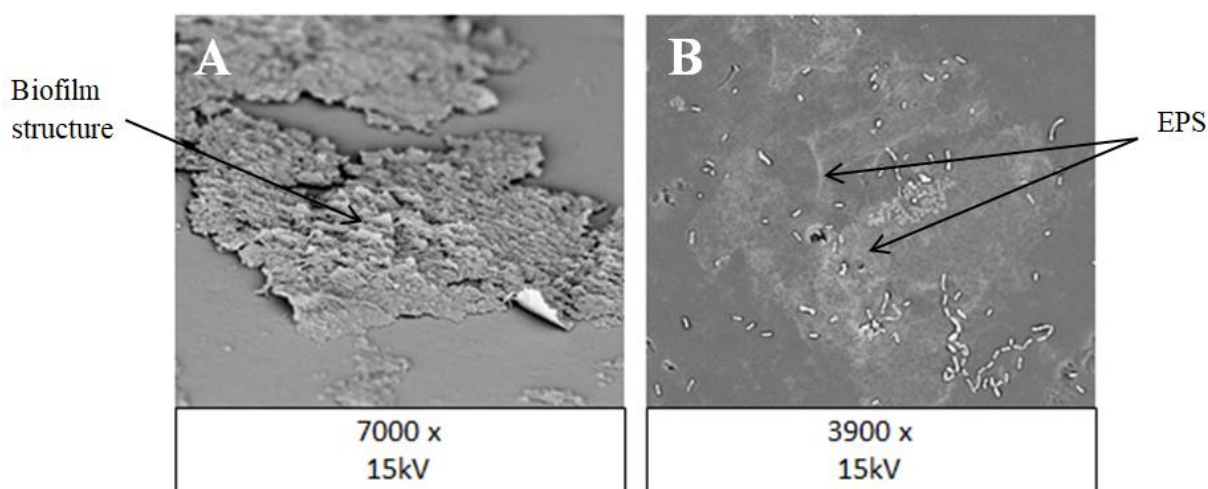


Figure 36: Scanning electron microscopy biofilm structures of *C. metallidurans* NA4 biofilms grown in 284 medium in the presence of A) 0.5 mM Cu<sup>2+</sup> and B) 5 mM Cu<sup>2+</sup>

#### 4.2.1.6 LB VS. 284 MEDIUM

The structure of *C. metallidurans* NA4 biofilms visualised by SEM are shown in Figure 37. For both *C. metallidurans* CH34 grown in LB medium (Figure 37A) and 284 medium (Figure 37B), the EPS structure is not visible, but clustering of cells is prominently present, leading to a more dense biofilm structure. There is a slight increase in the tendency of cells to form clusters in LB medium in comparison to 284 medium. Also, in LB medium EPS formation is observed in the presence of silver, this was not observed in 284 medium.

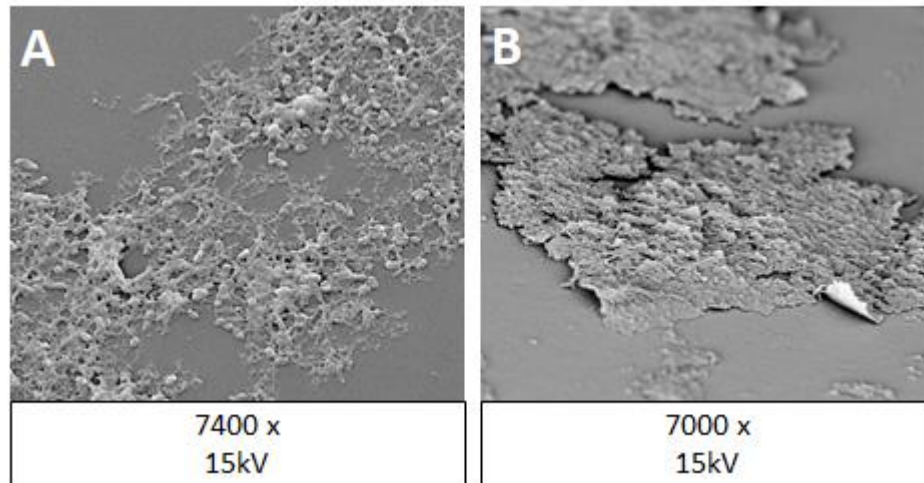


Figure 37: Scanning electron microscopy biofilm structures of *C. metallidurans* NA4 biofilms. A) NA4 biofilms grown in LB medium, B) NA4 biofilms grown in Tris-buffered mineral medium

## 4.2.2 MBEC™ ASSAY

### 4.2.2.1 BIOFILM FORMATION IN LB MEDIUM

#### CV MEASUREMENTS

The influence of silver and copper ions on the growth and development of *C. metallidurans* biofilms was assessed using a MBEC™ Assay (section 3.5.1). CV staining provides a quantitative analysis of biofilm formation.

Figure 38 shows the results of the CV measurements of *C. metallidurans* CH34 biofilm formation on MBEC™ Assay pegs in LB medium appended with CuSO<sub>4</sub> and AgNO<sub>3</sub>. An increase of CV values is observed with increasing Cu<sup>2+</sup> concentrations, indicating a stimulating effect of copper on biofilms. This effect was also observed in the previously obtained data. However, even at a concentration of 50 mM Cu<sup>2+</sup> a stimulating effect is observed. The CV values of *C. metallidurans* CH34 biofilms in the presence of Ag<sup>+</sup> were low for both concentrations, but show a slight decrease in the presence of 200 μM Ag<sup>+</sup>. This is in line with the observed SEM data, where no EPS structure (and thus biofilms) were observed in the presence of silver.

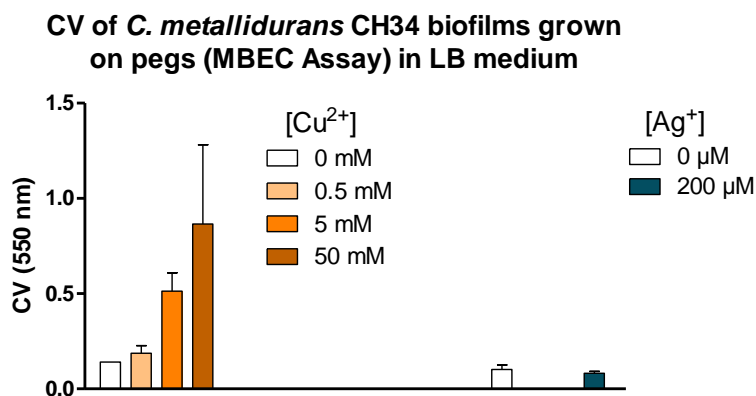


Figure 38: Crystal violet (CV) absorbance measurements for *C. metallidurans* CH34 biofilms grown on MBEC Assay pegs in LB medium appended with CuSO<sub>4</sub> and AgNO<sub>3</sub>

Figure 39 shows the results of the CV measurements of *C. metallidurans* NA4 biofilm formation on MBEC™ Assay pegs in LB medium appended with CuSO<sub>4</sub> and AgNO<sub>3</sub>. The CV values in the presence of Cu<sup>2+</sup> are rather stable, only at 5 mM Cu<sup>2+</sup> an increase is observed. An increase in CV values is observed with increasing concentrations of Ag<sup>+</sup>, only at 200 μM Ag<sup>+</sup> a decrease is observed. When comparing CV values of *C. metallidurans* CH34 (Figure 38) and NA4 (Figure 39), the overall CV values for NA4 are twofold the values for CH34.

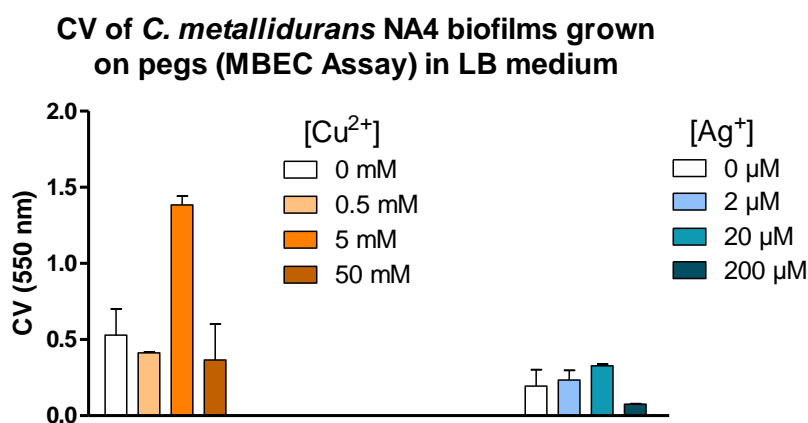


Figure 39: Crystal violet (CV) absorbance measurements for *C. metallidurans* CH34 biofilms grown on MBEC Assay pegs in LB medium appended with CuSO<sub>4</sub> and AgNO<sub>3</sub>

#### 4.2.2.2 BIOFILM FORMATION IN 284 MEDIUM

##### CV MEASUREMENTS

The influence of silver and copper ions on the growth and development of *C. metallidurans* biofilms was assessed using a MBEC™ Assay (section 3.5.1).

Figure 40 shows the results of the CV measurements of *C. metallidurans* CH34 biofilm formation on MBEC™ Assay pegs in 284 medium appended with CuSO<sub>4</sub> and AgNO<sub>3</sub>. The CV values in the presence of Cu<sup>2+</sup> are rather stable, only at 5 mM Cu<sup>2+</sup> an increase is observed. A slight overall decrease in CV values is observed with increasing concentrations of Ag<sup>+</sup>.

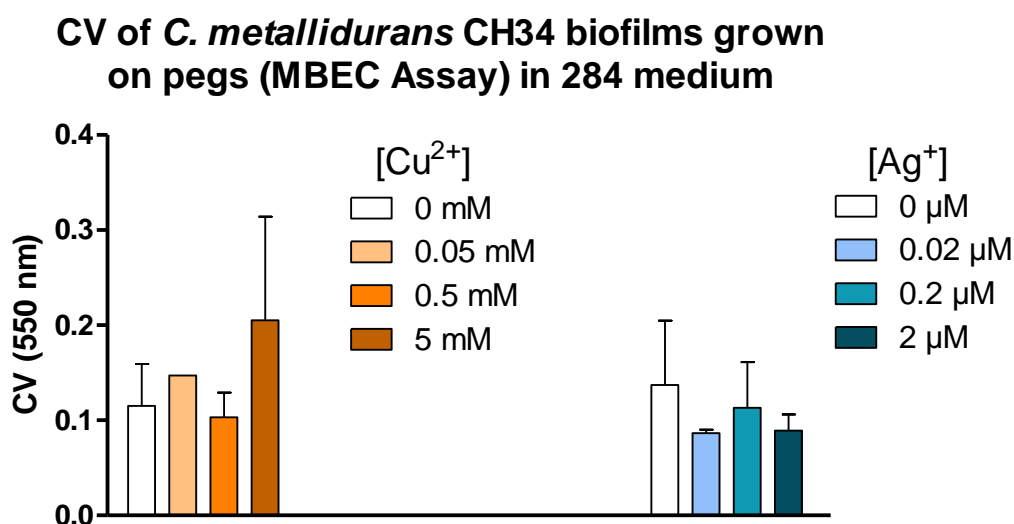


Figure 40: Crystal violet (CV) absorbance measurements for *C. metallidurans* CH34 biofilms grown on MBEC Assay pegs in 284 medium appended with CuSO<sub>4</sub> and AgNO<sub>3</sub>

Figure 41 shows the results of the CV measurements of *C. metallidurans* NA4 biofilm formation on MBEC™ Assay pegs in 284 medium appended with CuSO<sub>4</sub> and AgNO<sub>3</sub>. The CV values in the presence of Cu<sup>2+</sup> are rather stable, only at 5 mM Cu<sup>2+</sup> an increase is observed. CV values in the presence of Ag<sup>+</sup> are stable throughout all concentration.

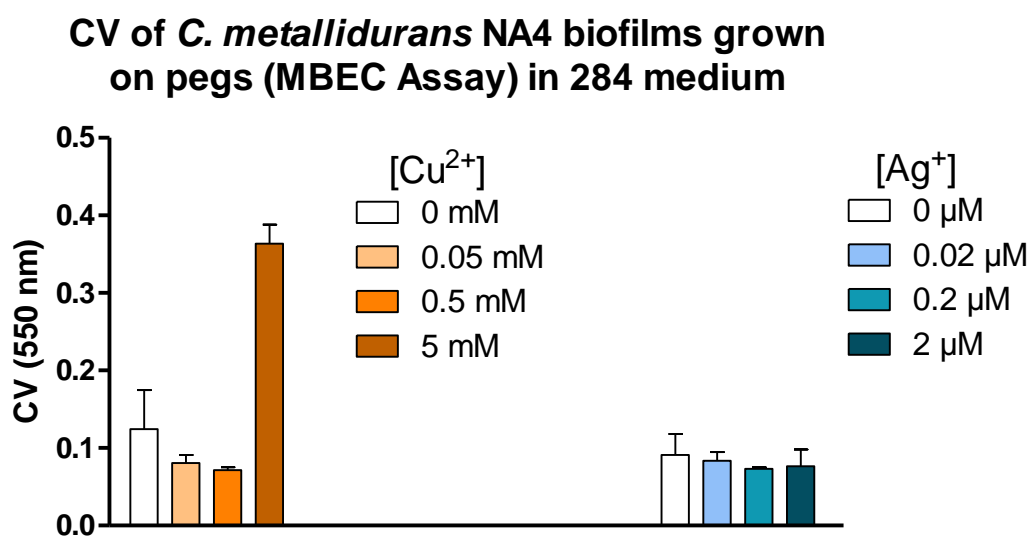


Figure 41: Crystal violet (CV) absorbance measurements for *C. metallidurans* NA4 biofilms grown on MBEC Assay pegs in 284 medium appended with CuSO<sub>4</sub> and AgNO<sub>3</sub>



### 4.3 BIOFILM RNA EXTRACTION

RNA extraction of *C. metallidurans* CH34 and NA4 biofilms grown in 96 well plates in LB medium appended with  $\text{AgNO}_3$  and  $\text{CuSO}_4$  was performed. Figure 42 shows the RNA concentrations ( $\text{ng}/\mu\text{L}$ ) of biofilms from 12 pooled wells that were obtained for each condition (*C. metallidurans* CH34 and NA4 biofilms grown in the absence of metal ions, in the presence of  $5 \text{ mM Cu}^{2+}$ , or in the presence of  $50 \mu\text{M Ag}^+$ ). For both *C. metallidurans* CH34 and NA4 no significant RNA yield differences were observed between the different conditions. A notable difference are the higher concentrations of RNA extraction of *C. metallidurans* CH34 biofilms in comparison to NA4 biofilms.

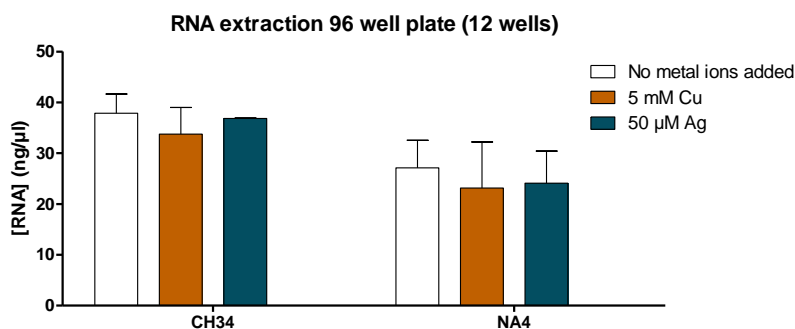


Figure 42: RNA extraction of *C. metallidurans* biofilms grown in LB medium. Per RNA extraction, biofilms of 12 individual wells were pooled and RNA concentration data were compared for no added metal ions,  $5 \text{ mM Cu}$ , and  $50 \mu\text{M Ag}$

Figure 43 shows the CV value (A) and RNA concentration to CV (biofilm quantity) ratio (B) of *C. metallidurans* CH34 and NA4 biofilms. When observing CV values of RNA extraction of *C. metallidurans* biofilms (Figure 43A), NA4 forms more biofilm compared to CH34. When comparing the *C. metallidurans* CH34 RNA/CV values to where no metal ions were added (Figure 43B), RNA extraction of biofilms in the presence of silver showed higher values, and RNA extraction of biofilms in the presence of copper showed lower values. For *C. metallidurans* NA4 biofilm RNA extraction, all values for different metal ion concentrations are relatively stable, but much lower than for *C. metallidurans* CH34. These data might be an indication that the RNA extraction of *C. metallidurans* CH34 biofilms show a higher RNA yield compared to biofilms of *C. metallidurans* NA4. This might be either caused by an increased RNA presence in the bacterial cells of *C. metallidurans* CH34, or by a facilitated RNA extraction of *C. metallidurans* CH34 biofilms.

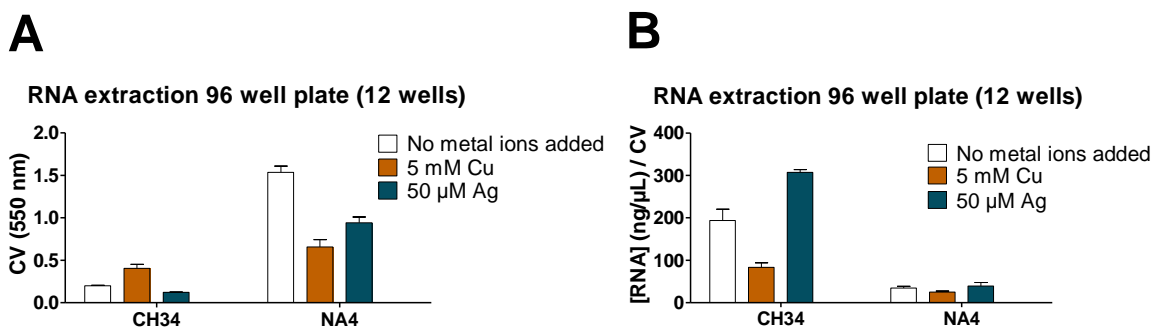


Figure 43: (A) CV absorbance value and (B) Ratio of RNA concentration ( $\text{ng}/\mu\text{L}$ ) to CV absorbance value for the RNA extraction of *C. metallidurans* CH34 and NA4 biofilms grown in LB medium appended with  $\text{CuSO}_4$  and  $\text{AgNO}_3$

## 5. DISCUSSION

### 5.1 PLANKTONIC GROWTH AND BIOFILM FORMATION IN THE PRESENCE OF SILVER AND COPPER

#### 5.1.1 BIOFILM FORMATION IN LB MEDIUM

To verify repeatability, three repeats of this experiment have been carried out.

##### 5.1.1.1 *C. METALLIDURANS CH34*

For both the OD and CV measurements in the presence of  $\text{Cu}^{2+}$  or  $\text{Ag}^+$ , the obtained data are not repeatable (4.1.1). Between repeats, the data show deviant progressions and deviant absolute values. The CV to OD (CV/OD) ratios showed overall similar progress of the curve values, but the absolute values vary strongly. For *C. metallidurans* CH34 in LB in the presence of  $\text{Cu}^{2+}$ , CV/OD values show an overall increase in biofilm formation with increasing concentrations of  $\text{Cu}^{2+}$  (4.1.1.2). This might indicate that  $\text{Cu}^{2+}$  stimulates biofilm formation and growth.

For the MIC determination, *C. metallidurans* CH34 absolute OD and CV values dropped in the presence of  $100 \mu\text{M Ag}^+$  (4.1.1.1). This indicates that both bacterial and biofilm growth are reduced to nearly zero at this concentration, therefore indicating the MIC. However, more repeats would need to be carried out in order to support these conclusions. All attempts to determine the MIC in LB medium in the presence of  $\text{Cu}^{2+}$  still showed visible growth of both bacteria and biofilm (4.1.1.2). In order to determine the  $\text{Cu}^{2+}$  MIC, higher concentrations of  $\text{Cu}^{2+}$  would need to be used ( $> 5 \text{ mM}$ ). The used  $\text{Cu}^{2+}$  concentrations were based on the MIC determinations of previous studies carried out in 284 medium as described in section 2.4.4.

##### 5.1.1.2 *C. METALLIDURANS NA4*

OD and CV measurement values of *C. metallidurans* NA4 in LB medium appended with  $\text{Ag}^+$  show an overall decrease with increasing concentrations of  $\text{Ag}^+$  (4.1.1.1). Even the CV to OD (CV/OD) ratio shows an overall decrease. This might indicate that *C. metallidurans* NA4 growth and biofilm formation are inhibited with increasing concentrations of  $\text{Ag}^+$ . For the OD and CV measurements in the presence of  $\text{Cu}^{2+}$ , the obtained data are not repeatable (4.1.1.2). Between repeats, the data shows deviant progressions and deviant absolute values. The CV to OD (CV/OD) ratios show inconsistent progressions and the absolute values vary strongly.

The MIC concentration of both  $\text{Cu}^{2+}$  and  $\text{Ag}^+$  could not be determined since higher concentrations need to be used.

##### 5.1.1.3 INTERACTIONS OF LB MEDIUM WITH COPPER OR SILVER

The observed data variations might be caused by interactions of  $\text{Cu}^{2+}$  or  $\text{Ag}^+$  with the medium. LB medium is an undefined medium containing digested casein proteins, such as tryptone and peptone.  $\text{Ag}^+$  is able to bind several amino acids, such as cysteine, lysine, arginine and methionine [80]. Proteins are also known to be able to bind  $\text{Ag}^+$ , therefore silver is even used as a protein stain [81]. In general, LB medium is an inappropriate choice for studies wherein reproducibility is required. In order to acquire reproducibility, is important that none of the components of liquid media become exhausted during growth of the culture. Only bacterial cultures in balanced growth have a reproducible chemical composition and average cell size. However, LB medium provides only a limited amount of carbohydrates or other utilizable carbon sources [82, 83].

These effects are not observed for 284 medium. Tris buffer is known to barely interact with metal ions, in contrast to other buffers. It does however contain  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , which might form precipitates with  $\text{Cu}^{2+}$  or  $\text{Ag}^+$  ( $\text{CuPO}_4$  and  $\text{AgPO}_4$ ). However,  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  is only present at a minimal concentration required for bacterial growth. Due to the uptake by bacteria,  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  is nearly absent as such. Also, it contains a rather small amount of  $\text{NH}_4\text{Cl}$ , which is able to bind  $\text{Cu}^{2+}$  or  $\text{Ag}^+$ , nonetheless to a much lesser extent than proteins or amino acids.

## 5.1.2 BIOFILM FORMATION IN 284 MEDIUM

### 5.1.2.1 *C. METALLIDURANS* CH34

For both the OD and CV measurements in the presence of  $\text{Cu}^{2+}$  or  $\text{Ag}^+$ , the obtained data are repeatable (4.1.2). When comparing repeats, the data show similar progressions and absolute values.

In the presence of silver (4.1.2.1), OD measurement values are similar throughout both repeats.

However, CV values are slightly deviant for  $\text{Ag}^+$  concentrations of 0.1  $\mu\text{M}$  and 0.2  $\mu\text{M}$ . CV to OD (CV/OD) ratios remain stable throughout both repeats, therefore the influence of  $\text{Ag}^+$  on *C.*

*metallidurans* CH34 biofilm formation in 284 medium could not be determined. The MIC of silver for *C. metallidurans* CH34 is estimated around 5  $\mu\text{M}$ , since a decrease in OD values is observed.

In previous studies however, the MIC of silver on *C. metallidurans* CH34 has been determined. One of these studies found a MIC of 0.5  $\mu\text{M}$   $\text{Ag}^+$  (Table 2). Though, this was not observed in the results of section 4.1.2.1. This could be due to the differences in experimental setups. In the previously mentioned experiment, *C. metallidurans* CH34 was exposed to metal ions during a timespan of 4 days. Whereas in the experiments conducted for this Master's thesis, the bacteria were exposed to metal ions for 7 consecutive days. Moreover, the inhibiting silver concentration was tenfold this value (5  $\mu\text{M}$   $\text{Ag}^+$ ), this was in line with the study carried out by Mijndonck et al. [3]. In this study, the MIC was also determined after 7 days. This study is more in line with the conducted experiments and supports the obtained data.

In the presence of copper (4.1.2.2), OD and CV measurement progress and absolute values are similar throughout both repeats. OD values remain stable up to a concentration of 2 mM  $\text{Cu}^{2+}$ . At a concentration of 5 mM  $\text{Cu}^{2+}$ , OD values drop significantly. This might indicate that bacterial growth is inhibited at a concentration of 5 mM  $\text{Cu}^{2+}$  (MIC). CV values increase with increasing  $\text{Cu}^{2+}$  concentrations, but decrease severely at a concentration of 5 mM  $\text{Cu}^{2+}$ . Significant increases are observed for 1 and 2 mM  $\text{Cu}^{2+}$ . This might indicate a stimulating effect of copper concentrations, especially 1 and 2 mM  $\text{Cu}^{2+}$ , on biofilm formation. At higher concentrations (5 mM  $\text{Cu}^{2+}$ ), bacterial growth is inhibited and therefore biofilm growth decreases. The previous statements are supported by the CV to OD (CV/OD) ratio data. CV/OD values increase with increasing  $\text{Cu}^{2+}$  concentrations, up to a concentration of 2 mM  $\text{Cu}^{2+}$ . At a concentration of 5 mM  $\text{Cu}^{2+}$ , a decrease is observed. As mentioned previously, this could indicate a stimulating effect of copper concentrations, especially 1 and 2 mM  $\text{Cu}^{2+}$ , on biofilm formation. At higher concentrations (5 mM  $\text{Cu}^{2+}$ ) biofilm growth is inhibited.

### 5.1.2.2 C. METALLIDURANS NA4

In the presence of silver (4.1.2.1), OD measurement values are similar throughout both repeats. However, CV values are slightly deviant for  $\text{Ag}^+$  concentrations of 0.1  $\mu\text{M}$  and 0.5  $\mu\text{M}$ . CV to OD (CV/OD) ratios first show an increase followed by a decrease with increasing  $\text{Ag}^+$  concentrations. This could indicate that silver exhibits a stimulating effect on *C. metallidurans* NA4 biofilm growth at low concentrations, and an inhibiting effect at higher concentrations ( $> 2 \mu\text{M}$ ). The MIC of silver for *C. metallidurans* NA4 is estimated around 5  $\mu\text{M}$ , since a decrease in OD values is observed. Furthermore, CV and CV/OD values drop to nearly zero in the presence of 5  $\mu\text{M}$   $\text{Ag}^+$ , supporting the previous findings.

In the presence of copper (4.1.2.2), OD and CV measurement progress and values are similar throughout both repeats. OD values show a decrease at a concentration of 0.1 mM  $\text{Cu}^{2+}$ , followed by an increase. At a concentration of 5 mM  $\text{Cu}^{2+}$ , OD values drop significantly. This might indicate that bacterial growth is inhibited at a concentration of 5 mM  $\text{Cu}^{2+}$ .

CV values increase significantly with increasing  $\text{Cu}^{2+}$  concentrations, especially for 0.1 mM and 0.2 mM  $\text{Cu}^{2+}$ . From 0.2 mM  $\text{Cu}^{2+}$  onwards, CV values decrease again. This might indicate a stimulating effect of copper concentrations, especially 0.1 and 0.2 mM  $\text{Cu}^{2+}$ , on biofilm formation. At higher concentrations ( $> 0.5 \text{ mM Cu}^{2+}$ ) biofilm growth is inhibited.

The previous statements are supported by the CV to OD (CV/OD) ratio data. CV/OD values of *C. metallidurans* NA4 increase significantly at a concentration of 0.1 mM and 0.2 mM  $\text{Cu}^{2+}$ . From 0.5 mM  $\text{Cu}^{2+}$  onwards, a decrease is observed. As previously mentioned, this might indicate a stimulating effect of  $\text{Cu}^{2+}$ , especially 0.1 and 0.2 mM  $\text{Cu}^{2+}$ , on biofilm formation. At higher concentrations ( $> 0.5 \text{ mM Cu}^{2+}$ ) biofilm growth is inhibited.

### 5.1.3 OVERVIEW OF COPPER AND SILVER EFFECT ON C. METALLIDURANS BIOFILMS IN 284 MEDIUM

Table 7 summarizes the above described conclusions of the influence of  $\text{Cu}^{2+}$  and  $\text{Ag}^+$  on *C. metallidurans* CH34 and NA4 biofilm formation in 284 medium. Stimulating and inhibiting concentrations on the CV/OD ratio are determined with regard to the CV/OD value where no metal ions were added.

**Table 7: A summary of the optimal and inhibiting concentrations of copper and silver ions on *C. metallidurans* CH34 and NA4 biofilm growth in 284 medium**

284 medium <i>C. metallidurans</i> strain	[ $\text{Cu}^{2+}$ ]		[ $\text{Ag}^+$ ]	
	Stimulation	Inhibition	Stimulation	Inhibition
CH34	1 – 2 mM	5 mM	/	5 $\mu\text{M}$
NA4	0.1 – 0.2 mM	2 – 5 mM	0.1 – 0.2 $\mu\text{M}$	5 $\mu\text{M}$

(/=no optimal or inhibiting concentration could be determined)

*C. metallidurans* CH34 biofilm growth stimulation seems to occur at higher concentrations (1 – 2 mM  $\text{Cu}^{2+}$ ) in comparison to NA4 (0.1 – 0.2 mM  $\text{Cu}^{2+}$ ). Since *C. metallidurans* NA4 is known to easily form biofilms, biofilm formation might already be induced at lower concentrations of copper. It might be possible that, since CH34 is not really known for its biofilm inducing genes, higher concentrations of copper are required to activate these genes. However, this assumption would need to be supported by studying genomics or expression patterns of CH34.

#### 5.1.4 COMPARISON OF *C. METALLIDURANS* CH34 AND NA4 BIOFILM FORMATION

Although *C. metallidurans* bacterial growth and biofilm development assessment in LB medium showed inconsistent data, all measurements indicate higher OD, CV and CV/OD values for *C. metallidurans* NA4 in comparison to *C. metallidurans* CH34. This indicates that both bacterial growth and biofilm formation occur to a greater extent in *C. metallidurans* NA4.

When comparing *C. metallidurans* CH34 to NA4, OD measurement values (bacterial growth) are similar. This indicates that bacterial growth occurs to a similar extent. All measurements indicate higher CV (biofilm formation) and CV/OD (biofilm formation normalized to planktonic cell density) values for *C. metallidurans* NA4 in comparison to *C. metallidurans* CH34. This indicates that biofilm formation occurs to a greater extent in *C. metallidurans* NA4. This effect was also observed in LB medium.

To conclude, it can be stated that *C. metallidurans* NA4 is able to form biofilms to a greater extent than *C. metallidurans* CH34. This effect was observed in both LB medium and 284 medium.

When comparing the OD values of *C. metallidurans* CH34 (Figure 27) and NA4 (Figure 28) in the presence of copper, a slightly higher OD value is observed for CH34 (0 mM Cu<sup>2+</sup>). This might indicate that bacterial growth of CH34 occurs to a greater extent. However, CV values of *C. metallidurans* NA4 are much higher than those of CH34, indicating that biofilm formation of NA4 occurs to a greater extent. Moreover, OD values of *C. metallidurans* CH34 decrease more rapidly with increasing concentrations of copper in comparison to NA4. This might be due to the fact that NA4 gained more resistance since they form more biofilm.

Also, we cannot verify an actual inhibiting effect of silver on *C. metallidurans* NA4 since they are known to be able to enter a 'dormant' (more persistent) state. This state allows them to survive in the presence of silver without proliferating. This dormant state might be responsible for the observed decrease in OD values, which can incorrectly be considered as an inhibition of NA4 growth.

## 5.2 SCANNING ELECTRON MICROSCOPY

Biofilms are hydrated, gel-like substances. When undergoing dehydration for SEM imaging, artefacts are introduced. This causes alterations in the biofilm structure. Also, not all samples are equally gold-coated, therefore resolutions vary between the different images.

### 5.2.1 SEM IMAGING

#### 5.2.1.1 LB MEDIUM

##### *C. METALLIDURANS* CH34 BIOFILMS

If *C. metallidurans* CH34 biofilms are grown in LB medium (4.2.1.1), an EPS structure is observed both in the presence and absence of  $\text{Cu}^{2+}$ . In the presence of  $\text{Ag}^+$  however, this EPS structure could not be observed. This might indicate that copper exhibits a stimulating effect on biofilm formation, whereas silver inhibits biofilm formation. This is in line with the previously obtained results. All *C. metallidurans* CH34 bacterial cells tend to form clusters, the presence of separate cells is rather rare for both  $\text{Cu}^{2+}$  and  $\text{Ag}^+$ , indicating the actual formation of biofilms. However, the previous results showed that there was an inhibiting effect on biofilms in the presence of 5 mM  $\text{Cu}^{2+}$ , whereas for the SEM imaging a developed biofilm structure was still observed in the presence of this concentration.

##### *C. METALLIDURANS* NA4 BIOFILMS

If *C. metallidurans* NA4 biofilms are grown in LB medium (4.2.1.4), an EPS structure is observed both in the absence and presence of  $\text{Cu}^{2+}$  and  $\text{Ag}^+$ , but to a lesser extent in comparison to *C. metallidurans* CH34. All *C. metallidurans* CH34 bacterial cells tend to form clusters, the presence of separate cells is rather rare for both  $\text{Cu}^{2+}$  and  $\text{Ag}^+$ , indicating the actual formation of biofilms. At a concentration of 50 mM  $\text{Cu}^{2+}$ , structures resembling copper crystals were observed.

#### 284 MEDIUM

#### 5.2.1.2 284 MEDIUM

##### *C. METALLIDURANS* CH34 BIOFILMS

If *C. metallidurans* CH34 biofilms are grown in 284 medium (4.2.1.2), the same observations were made as for LB medium. EPS structure is observed both in the presence or absence of  $\text{Cu}^{2+}$ , but is absent in the presence of  $\text{Ag}^+$ . This might be due to the fact that copper exhibits a stimulating effect and silver exhibits an inhibiting effect on biofilm formation. Again, this is in line with the previously obtained results. Overall, clustering of cells was observed more than separate cells, indicating the actual formation of biofilms. For 5 mM  $\text{Cu}^{2+}$ , structures resembling copper crystals were observed. This concentration is probably lower compared to LB medium because no known interactions occur between 284 medium and  $\text{Cu}^{2+}$ . In LB medium however, interactions with  $\text{Cu}^{2+}$  lower the available  $\text{Cu}^{2+}$  concentration.

##### *C. METALLIDURANS* NA4 BIOFILMS

If *C. metallidurans* NA4 biofilms are grown in 284 medium (4.2.1.5), an EPS structure is observed both in the presence and absence of  $\text{Cu}^{2+}$ . In the presence of  $\text{Ag}^+$  however, this EPS structure could not be observed. This might indicate a that copper exhibits a stimulating effect on biofilm formation, whereas silver inhibits biofilm formation. Again, this is in line with the previously obtained results. Both clustering of cells and separate cells were observed. But in general, more dense and layered biofilms were observed.

### 5.2.1.3 *C. METALLIDURANS* CH34 AND NA4 COMPARISON

Table 8 provides an overview of *C. metallidurans* CH34 and NA4 biofilm dissimilarities visualised by SEM imaging.

**Table 8: An overall comparison of *C. metallidurans* CH34 and NA4 biofilm parameters observed by SEM imaging**

Parameter	<i>C. metallidurans</i> CH34	<i>C. metallidurans</i> NA4
<b>EPS</b>	EPS structure is prominently present and developed to a greater extent	EPS structure is present, but to a lesser extent
<b>Cell clustering</b>	Cell clustering is prominently observed	Cell clustering is prominently observed, and layers of cells are visible
<b>Biofilm formation</b>	Biofilm formation is observed. Biofilms are characterised by the presence of a large EPS structure embedded with clusters of cells	Biofilm formation is observed. Biofilms are characterised by less developed EPS structures and layers of large clusters of cells, leading to a more dense biofilm with a greater mass

### 5.2.2 MBEC™ ASSAY

First of all, the biofilms grown on TiO<sub>2</sub> coated pegs could not be used for CV staining, since the CV stains the coating as well. Therefore, only data obtained from uncoated pegs was shown. Also, some pegs were not available for CV measurements due to inappropriate handling. This caused gaps in certain graphs (Figure 38: Crystal violet (CV) absorbance measurements for *C. metallidurans* CH34 biofilms grown on MBEC Assay pegs in LB medium appended with CuSO<sub>4</sub> and AgNO<sub>3</sub> Figure 44).

MBEC™ Assays were performed to show a similarity with the biofilms visualised using the SEM. This is important since the biofilms for the MBEC Assay were grown in the bottom of the same microtiter plate of which the pegs on the lid were used for SEM. Since SEM visualisation of biofilms in 96 well plates can not be carried out, it is important to demonstrate that biofilm formation in the 96 well plates (of the MBEC Assay) occurs to a similar extent. Unfortunately, no OD measurements were performed on the MBEC Assay 96 well plate, therefore only CV data can be used to compare both the SEM (biofilms grown on pegs) and MBEC Assay (biofilms grown in 96 well plate) biofilms. The ratio of CV peg to CV bottom values is added in Appendix 4.

#### 5.2.2.1 BIOFILM GROWTH IN LB MEDIUM

##### CV MEASUREMENTS

CV measurements of *C. metallidurans* CH34 biofilms grown on pegs in LB medium appended with Cu<sup>2+</sup>, show an increase in CV values with increasing concentrations of copper. The highest CV values were obtained for 50 mM Cu<sup>2+</sup>, indicating that even higher concentrations would need to be used for MIC determination in LB. These results support the previous findings that copper exhibits a stimulating effect on biofilm growth. Also, this was observed for SEM, except for the fact that EPS structures were no longer observed in the presence of 50 mM Cu<sup>2+</sup>. For Ag<sup>+</sup> however, only values close to zero were observed. There was a slight decrease in biofilm formation. This is also in line with the SEM data, which pointed out that Ag<sup>+</sup> inhibits biofilm formation and EPS development (4.2.1.1).

CV measurements of *C. metallidurans* NA4 biofilms grown on pegs in LB medium appended with Cu<sup>2+</sup>, only show an increase in CV value for 5 mM Cu<sup>2+</sup>. This result is in contrast to previously discussed results, since 5 mM Cu<sup>2+</sup> caused a severe decrease in biofilm formation (Figure 21). However, this was in line with the observed SEM data, since EPS structure was still observed at 5 mM Cu<sup>2+</sup> (4.2.1.4)

For  $\text{Ag}^+$ , CV values slightly increased up to a concentration of 20  $\mu\text{M}$ . CV values decreased at a concentration of 200  $\mu\text{M}$   $\text{Ag}^+$ . Again, these results are in line with the observed SEM data, since EPS structure was observed up until a concentration of 20  $\mu\text{M}$   $\text{Ag}^+$ . However, this is in contrast to the results obtained from the 96 well plate assays, since an inhibiting effect on biofilm formation was observed in the presence of silver (4.2.1.4).

Furthermore, CV values were significantly higher for *C. metallidurans* NA4 in comparison to *C. metallidurans* CH34, indicating that NA4 forms biofilms to a greater extent. These findings are in line with the previously described results.

### 5.2.2.2 BIOFILM GROWTH IN 284 MEDIUM

#### CV MEASUREMENTS

CV measurements of *C. metallidurans* CH34 biofilms grown on pegs in 284 medium appended with  $\text{Cu}^{2+}$ , show an increase in CV values with increasing concentrations of copper. This is in line with the previous results stating that copper has a stimulating effect on biofilm formation. The highest CV values were obtained for 5 mM  $\text{Cu}^{2+}$ . This is in contrast to the previously obtained results, where 5 mM  $\text{Cu}^{2+}$  was considered as inhibiting (Table 7). However, these results are in line with the SEM data, where an increase in EPS development is observed for 5 mM  $\text{Cu}^{2+}$ , and EPS development is still observed at 50 mM  $\text{Cu}^{2+}$  (4.2.1.2). In the presence of  $\text{Ag}^+$ , a decrease of CV values of *C. metallidurans* CH34 biofilms was observed with increasing concentrations of  $\text{Ag}^+$ . This is in line with the previously obtained results. In general, these results support the previous findings that copper exhibits a stimulating effect on biofilm growth and silver exhibits an inhibiting effect. Furthermore, SEM data showed the absence of EPS structure in the presence of silver (4.2.1.2).

CV measurements of *C. metallidurans* NA4 biofilms grown on pegs in 284 medium appended with  $\text{Cu}^{2+}$ , only show an increase in CV value for 5 mM  $\text{Cu}^{2+}$ . Again, this is in contrast to the previously obtained results, where 5 mM  $\text{Cu}^{2+}$  was considered as inhibiting (Table 7). Still, this is in line with the SEM data where an increase in EPS structure is observed with increasing copper concentrations. For  $\text{Ag}^+$ , all CV values showed a slight decrease with increasing silver concentrations. Again, this is in line with the previously obtained data. Furthermore, this is supported by the SEM data, that show the absence of EPS development in the presence of silver.



## 5.3 BIOFILM RNA EXTRACTION

### 5.3.1 96 WELL PLATES

RNA extraction was performed to verify the differences between planktonic and biofilm culture transcripts and the potential effect on these transcripts due to the presence of copper and silver ions. Due to a lack of time, these experiments could not be conducted.

The obtained data showed an increase in RNA yield of *C. metallidurans* CH34 biofilms in comparison to *C. metallidurans* NA4 biofilms. This could be caused by the more dense structure of NA4 biofilms, leading to a more demanding lysis. Also, the higher yield could be due to the fact that CH34 biofilms contain RNA at a higher concentration.

When RNA concentration to CV ratios were determined, a severe increase in value was observed for 5 mM Cu<sup>2+</sup>. This could indicate an interference of Cu<sup>2+</sup> with one or more steps of the RNA extraction protocol. However, an increase in RNA yield is observed in the presence of 50 μM Ag<sup>+</sup>. This might be an indication that small concentrations of silver stimulate one or more steps of the RNA extraction protocol, or that silver leads to a higher RNA concentration in the bacterial cell. For *C. metallidurans* NA4, all RNA yields were very low.

Also, RNA integrity values were measured (data not shown). These values were insufficiently high to use the RNA for example for cDNA creation. Therefore, the RNA extraction protocol for biofilms needs to be optimized.

### 5.3.2 GLASS SLIDES

RNA extraction of biofilms formed on glass slides was carried out. However, the amount of biofilm formed on one slide was too big to perform the RNA extraction protocol. Due to this large amount, the membranes of the Spin Columns clogged and the RNA could not be properly eluted from the column. Therefore, no quantitatively representative results were obtained. In order to solve this problem while still using the Promega SV Total RNA Isolation System, a longer lysis step could be performed.

Another option could be the use of the Qiagen RNeasy PowerBiofilm Kit, designed for the RNA extraction of biofilms. This kit uses a novel lysis method that is a combination of pre-treatment, mechanical and chemical lysis to ensure high RNA yields from biofilm samples. The RNeasy PowerBiofilm Kit has been designed to solve the ineffective cell lysis of biofilm samples.

### 5.3.3 RNA EXTRACTION OF BACTERIAL SUSPENSIONS

Additionally, RNA extraction of bacterial suspensions was performed to detect transcriptional differences between planktonic cells and biofilm cells (3.6.1). RNA extraction was performed successfully (data not shown), but due to a lack of time these differences could not be studied.

## 6. CONCLUSION

For all *C. metallidurans* CH34 and NA4 biofilms grown in LB medium, no repeatable data were obtained. If reproducibility is required, the use of Tris-buffered mineral (284) medium is preferred over LB medium. However, if *C. metallidurans* CH34 and NA4 biofilms were grown in 284 medium, more repeatable data were obtained for both OD and CV measurements.

### ***C. metallidurans* CH34**

*C. metallidurans* CH34 growth is inhibited or reduced at a concentration of 5  $\mu\text{M}$   $\text{Ag}^+$  and 5 mM  $\text{Cu}^{2+}$ . In the presence of silver, CV/OD ratios of *C. metallidurans* CH34 were quite stable in a concentration range of 0 – 5  $\mu\text{M}$   $\text{Ag}^+$ . This might indicate that there is no significant effect of  $\text{Ag}^+$  on biofilm formation of *C. metallidurans* CH34 up to a concentration of at least 5  $\mu\text{M}$   $\text{Ag}^+$ . In the presence of copper, CV/OD ratios of *C. metallidurans* CH34 show an overall increase with increasing  $\text{Cu}^{2+}$  concentrations. For 5 mM  $\text{Cu}^{2+}$  however, a significant decrease is observed. This might indicate a stimulating effect of increasing copper concentrations on biofilm formation up to a concentration of 5 mM, which has an inhibiting effect. *C. metallidurans* CH34 biofilms are characterized by the presence of an extensively developed EPS structure embedded with clusters of cells.

### ***C. metallidurans* NA4**

*C. metallidurans* CH34 growth is inhibited or reduced at a concentration of 5  $\mu\text{M}$   $\text{Ag}^+$  and 5 mM  $\text{Cu}^{2+}$ . In the presence of silver, CV/OD ratios of *C. metallidurans* NA4 showed an overall decrease. This might indicate that there is an inhibiting effect of  $\text{Ag}^+$  on NA4 biofilm formation. CV/OD ratios of *C. metallidurans* NA4 showed an increase at a concentration of 0.1 mM and 0.2 mM  $\text{Cu}^{2+}$ , followed by a decrease. When a concentration of 5 mM  $\text{Cu}^{2+}$  was reached, a significant decrease was observed. This might indicate a stimulating effect of copper on *C. metallidurans* NA4 biofilm formation at concentrations of 0.1 mM and 0.2 mM, and an inhibiting effect at a concentration of 5 mM. CV/OD ratios of *C. metallidurans* NA4 show an increase followed by a decrease, also indicating a stimulating effect of increasing copper concentrations on biofilm formation up to a concentration of 2 mM, which has an inhibiting effect.

Overall it can be stated that:

- Silver inhibits *C. metallidurans* bacterial and biofilm growth;
- Copper inhibits *C. metallidurans* bacterial and biofilm growth. Except for 1 mM and 2 mM  $\text{Cu}^{2+}$ , a stimulating effect on *C. metallidurans* CH34 biofilm growth is observed. This stimulating effect on *C. metallidurans* NA4 biofilm growth is also observed for 0.1 mM and 0.2 mM  $\text{Cu}^{2+}$ ;
- For *C. metallidurans* CH34 and NA4 a MIC of 5  $\mu\text{M}$   $\text{Ag}^+$  was observed. Also, an inhibiting effect of 5 mM  $\text{Cu}^{2+}$  was observed, but higher copper concentrations would need to be assessed in order to be able to determine the MIC. *C. metallidurans* biofilm growth was also inhibited or reduced at 5  $\mu\text{M}$   $\text{Ag}^+$  and 5 mM  $\text{Cu}^{2+}$ .

Moreover, *C. metallidurans* NA4 is able to form biofilms to a greater extent than *C. metallidurans* CH34 in the absence and presence of low concentrations of metal ions. This effect was observed in both LB medium and 284 medium. This might cause NA4 to be more persistent to low concentrations of metal ions since the bacteria are shielded due to the gradient of stressors that is established along the biofilm as explained in section 2.2.

Scanning electron microscopy visualised structural properties of biofilms. EPS formation was observed for *C. metallidurans* CH34 both in the absence and presence of copper, but was not observed in the presence of silver. This highlights the previously mentioned inhibiting effect of silver on biofilm formation. *C. metallidurans* CH34 biofilm cells form clusters. For *C. metallidurans* NA4, the same characteristics were observed. Although *C. metallidurans* CH34 biofilm EPS structures were more developed, cell clustering was observed to a greater extent in *C. metallidurans* NA4, as well as layering of cells. This causes *C. metallidurans* NA4 biofilms to have a more dense structure and might explain why *C. metallidurans* NA4 biofilms have been observed to a greater extent than CH34 biofilms when quantified using CV staining.

The MBEC Assay CV measurements supported the SEM visualisation of biofilms. The stimulating effect of copper on *C. metallidurans* biofilm formation was observed as an increase in CV values, and the inhibiting effect of silver on *C. metallidurans* biofilm formation was observed as a decrease in CV values. This was supported by the presence of EPS structure in the presence of copper and the absence of EPS structure in the presence of silver, visualised by SEM.

In order to ensure enhanced and prolonged water decontamination, concentrations of silver or copper as disinfectant need to be strictly monitored and sufficiently high since lower concentrations might stimulate biofilm formation.

RNA extraction of *C. metallidurans* biofilms was performed successfully. However, a RNA extraction protocol needs to be optimized to obtain an increased RNA yield and quality. It was observed that for the RNA extraction of *C. metallidurans* CH34 biofilms a higher RNA yield was obtained in comparison to *C. metallidurans* NA4 biofilms. Also, an increase in RNA yield was observed for *C. metallidurans* CH34 biofilms grown in the presence of silver.

To confirm the previously obtained results, more repeats would need to be carried out in 284 medium. Also, *C. metallidurans* biofilms would need to be assessed in a broader concentration range of silver and copper ions. Additionally, it could be further investigated if *C. metallidurans* CH34 and NA4 are capable of forming biofilms on other surfaces, such as stainless steel, that are more relevant for spaceflight settings.

Future research would need to be carried out to fill the information gaps. First of all, biofilm RNA extraction needs to be optimized. The RNA could be used to verify the differences between planktonic and biofilm culture transcripts and the potential relative effect on these transcripts due to the presence of copper and silver ions in the biofilm. The focus would lie on genes known to have a function in general stress response, metal resistance, and biofilm formation and motility. Genomic studies could be performed to identify the actual biofilm formation genes in *C. metallidurans* CH34 and NA4 and their expression patterns. Transcriptional expression could be assessed in the presence of increasing concentrations of copper and silver to investigate if they respond in a dose-responsive way. This could elucidate the actual mechanisms and dynamics of biofilm formation and the influence of silver and copper ions.

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# APPENDIX

## 1. RNA Isolation protocol

### Sample preparation

1. Grow an overnight bacterial culture in the appropriate media and at the appropriate temperature. The following day, dilute the culture 1:50 and grow until the OD<sub>600</sub> is 0.6–1.0.
2. Transfer 2 mL of culture to a 2 mL microcentrifuge tube. Centrifuge for 2 minutes at 10,000 × g.
3. Carefully remove the supernatant, leaving the pellet as dry as possible.
4. Resuspend the pellet in 100 µl of freshly prepared TE containing lysozyme (3mg/mL).
5. Incubate the resuspended pellet at room temperature for 10 minutes.
6. Add 75 µl of RNA Lysis Buffer (RLA).
7. Add 350 µl of RNA Dilution Buffer (RDA). Mix by inversion. Do not centrifuge.
8. Add 200 µl 95% ethanol to the cleared lysate, and mix by pipetting 3–4 times. Transfer this mixture to the Spin Column Assembly. Centrifuge at 12,000–14,000 × g for one minute.
9. Take the Spin Basket from the Spin Column Assembly, and discard the liquid in the Collection Tube. Put the Spin Basket back into the Collection Tube.
10. Add 600 µl of RNA Wash Solution to the Spin Column Assembly. Centrifuge at 12,000–14,000 × g for 1 minute.
11. Empty the Collection Tube as before and place it in a rack.
12. For each isolation to be performed, prepare the DNase incubation mix by combining:
  - 40 µl Yellow Core Buffer,
  - 5 µl 0.09 M MnCl<sub>2</sub>,
  - 5 µl of DNase I.

Add 50 µl of the DNase mixture to the spin column and incubate for 15 minutes exactly at room temperature.

13. Add 200 µl of DNase Stop Solution to the Spin Basket, and centrifuge at 12,000–14,000 × g for 1 minute.
14. Add 600 µl RNA Wash Solution and centrifuge at 12,000–14,000 × g for 1 minute.
15. Empty the Collection Tube, and add 250 µl RNA Wash Solution and centrifuge at 12,000–14,000 × g for 2 minutes.
16. Discard the liquid from the collection tube and transfer the spin column to a new microcentrifuge tube.
17. Add 100 µl Nuclease-Free Water to the membrane. Be sure to completely cover the surface of the membrane with the water. Place the Spin Basket Assemblies in the centrifuge with the lids of the Elution Tubes facing out. Centrifuge at 12,000–14,000 × g for 1 minute. Remove the Spin Basket and discard.
18. Cap the Elution Tube containing the purified RNA and store at –80°C.

This protocol is based on the SV Total RNA Isolation System Protocol that can be found at <https://be.promega.com/resources/protocols/technical-manuals/0/sv-total-rna-isolation-system-protocol/>



## 2. Agilent RNA 6000 Nano Kit Quick Start Guide

### Preparing the Gel-Dye Mix

1. Allow the RNA dye concentrate (blue) to equilibrate to room temperature for 30 min.
2. Vortex RNA dye concentrate (blue) for 10 s, spin down and add 1  $\mu\text{L}$  of dye into a 65  $\mu\text{L}$  aliquot of filtered gel.
3. Vortex solution well. Spin tube at 13000g for 10 min at room temperature. Use prepared gel-dye mix within one day.

### Loading the Gel-Dye Mix

1. Put a new RNA chip on the chip priming station.
2. Pipette 9  $\mu\text{L}$  of gel-dye mix in the well marked .
3. Make sure that the plunger is positioned at 1 mL and then close the chip priming station.
4. Press plunger until it is held by the clip.
5. Wait for exactly 30 s then release clip.
6. Wait for 5 s. Slowly pull back plunger to 1 mL position.
7. Open the chip priming station and pipette 9  $\mu\text{L}$  of gel-dye mix in the wells marked .
8. Discard the remaining gel-dye mix.

### Loading the Marker

1. Pipette 5  $\mu\text{L}$  of RNA marker (green) in all 12 sample wells and in the well marked with the ladder.

### Loading the Ladder and Samples

1. Pipette 1  $\mu\text{L}$  of prepared ladder in well marked .
2. Pipette 1  $\mu\text{L}$  of sample in each of the 12 sample wells. Pipette 1  $\mu\text{L}$  of RNA Marker (green ) in each unused sample well.
3. Put the chip horizontally in the IKA vortexer and vortex for 1 min at 2400rpm.
4. Run the chip in the Agilent 2100 Bioanalyzer instrument within 5 min.

### Further Information

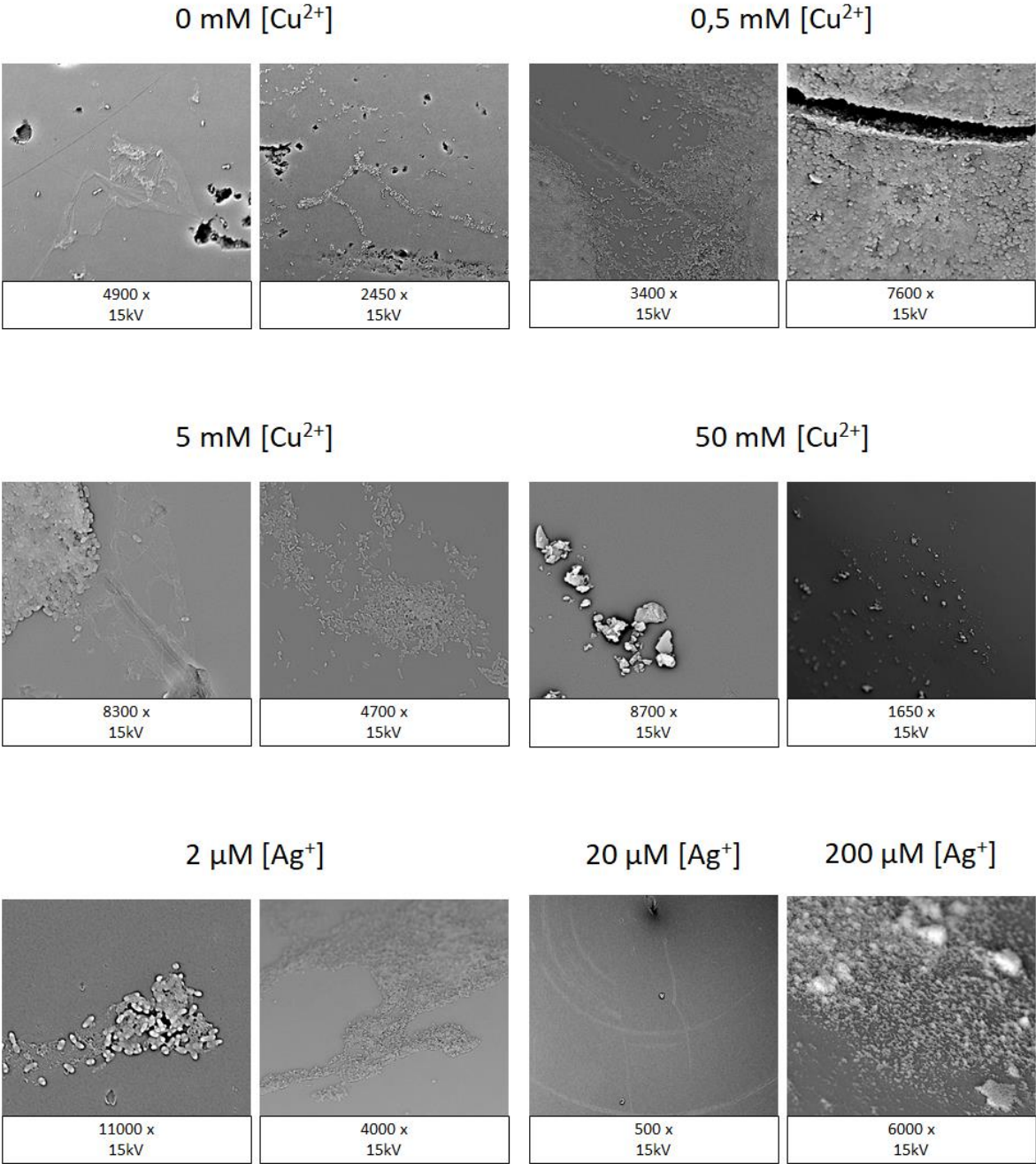
Visit the 2100 Bioanalyzer site at <http://www.agilent.com/genomics/bioanalyzer>. You can find useful information, support and current developments about the products and the technology



### 3. SEM imaging results

#### 3.1 Biofilms grown in LB medium

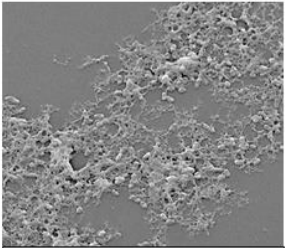
##### 3.1.1 *C. metallidurans* CH34



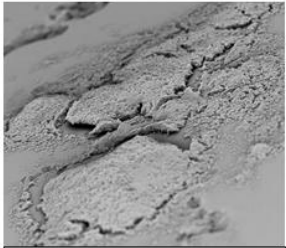


3.1.2 *C. metallidurans* NA4

0 mM [Cu<sup>2+</sup>]



7400 x  
15kV

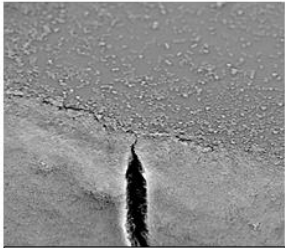


3500 x  
15kV

0,5 mM [Cu<sup>2+</sup>]

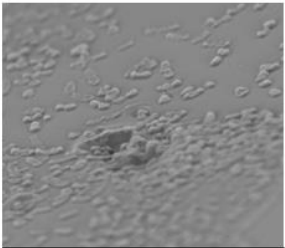


5600 x  
15kV

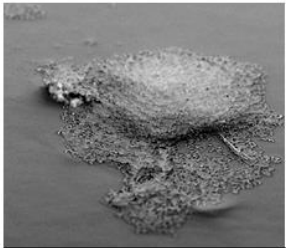


4600 x  
15kV

5 mM [Cu<sup>2+</sup>]

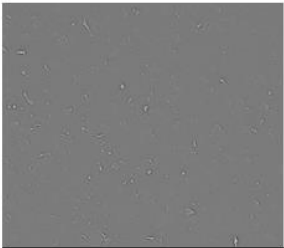


18500 x  
15kV

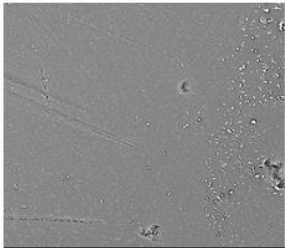


4200 x  
15kV

50 mM [Cu<sup>2+</sup>]

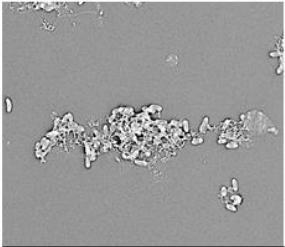


4900 x  
15kV

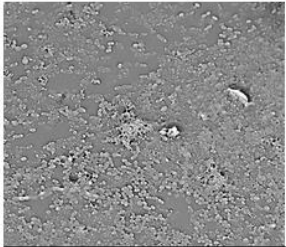


3500 x  
15kV

0 μM [Ag<sup>+</sup>]

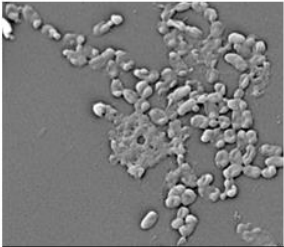


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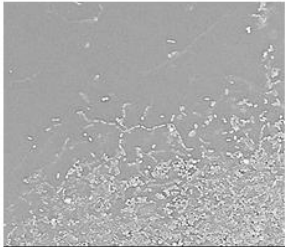


5200 x  
15kV

2 μM [Ag<sup>+</sup>]

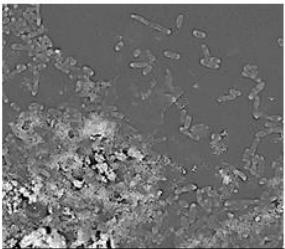


19000 x  
15kV

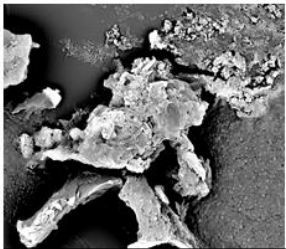


3200 x  
15kV

20 μM [Ag<sup>+</sup>]

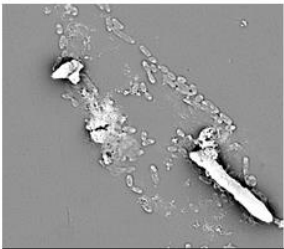


8900 x  
15kV

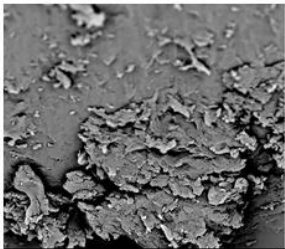


6400 x  
15kV

200 μM [Ag<sup>+</sup>]



1900 x  
15kV

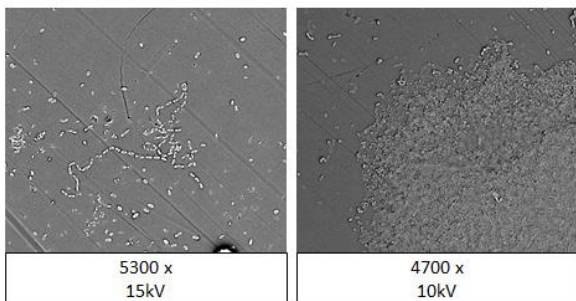


2150 x  
15kV

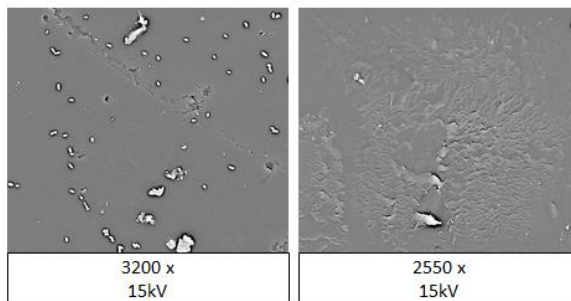
## 3.2 Biofilms grown in 284 medium

### 3.2.1 *C. metallidurans* CH34

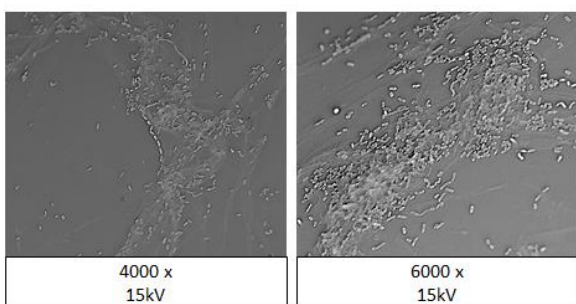
0 mM [Cu<sup>2+</sup>]



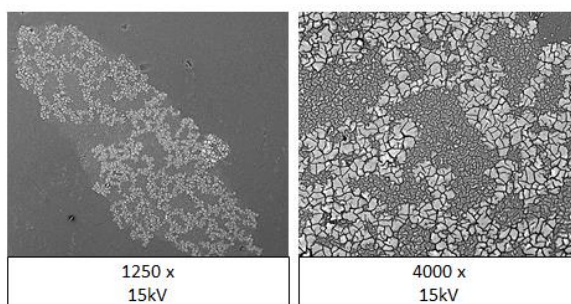
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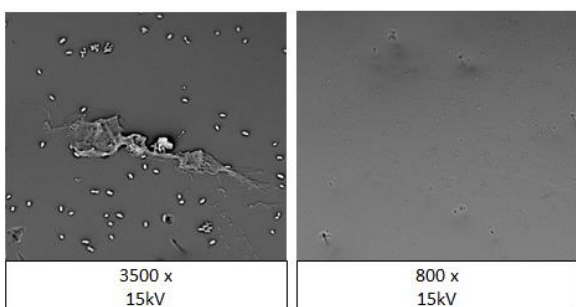
0,5 mM [Cu<sup>2+</sup>]



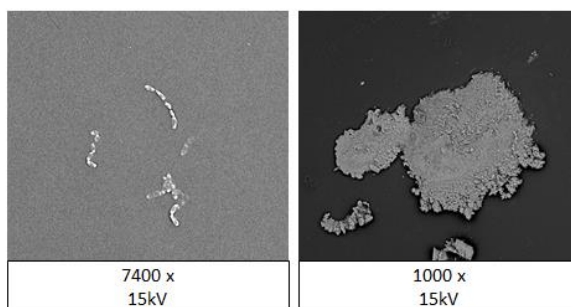
5 mM [Cu<sup>2+</sup>]



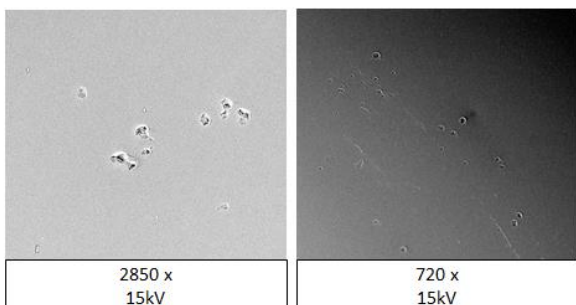
0 μM [Ag<sup>+</sup>]



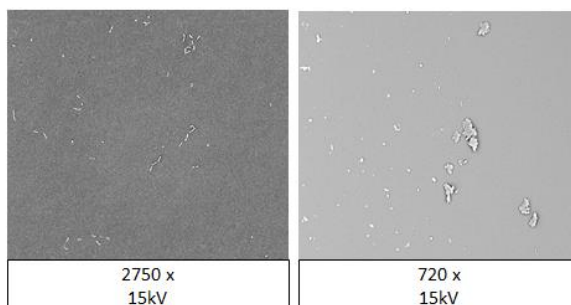
0,02 μM [Ag<sup>+</sup>]



0,2 μM [Ag<sup>+</sup>]

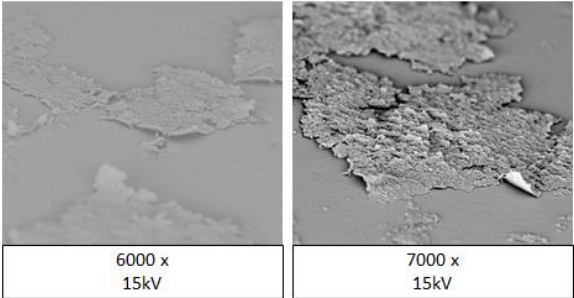


2 μM [Ag<sup>+</sup>]

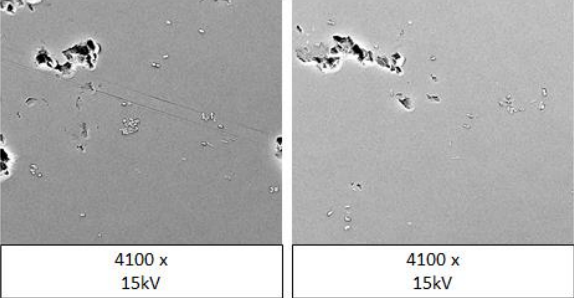


3.2.2 *C. metallidurans* NA4

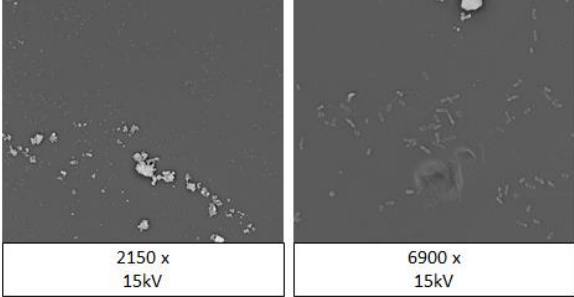
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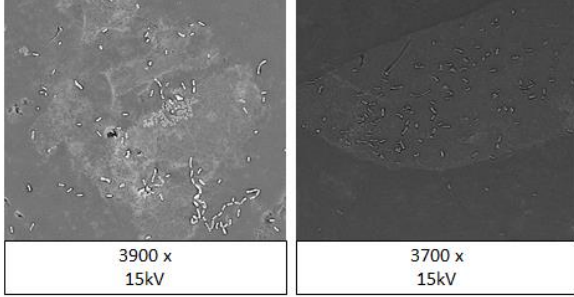
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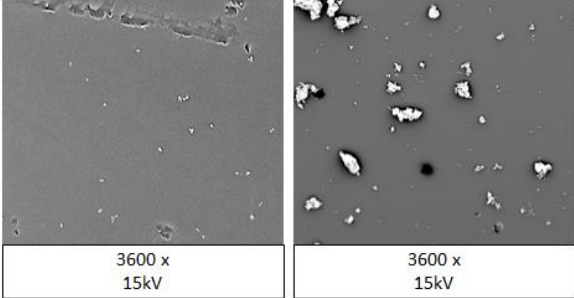
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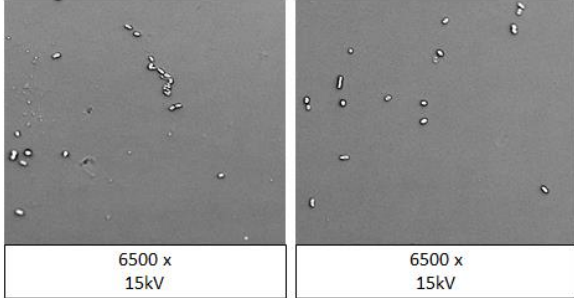
5 mM [Cu<sup>2+</sup>]



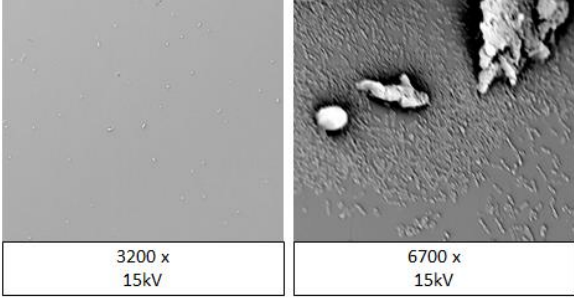
0 μM [Ag<sup>+</sup>]



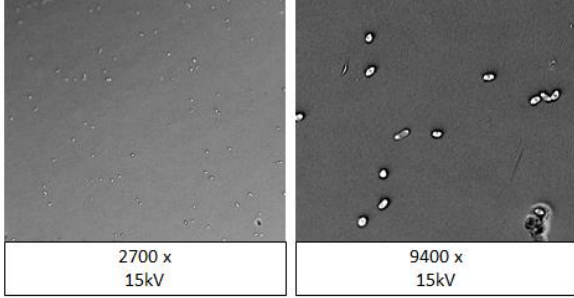
0,02 μM [Ag<sup>+</sup>]



0,2 μM [Ag<sup>+</sup>]



2 μM [Ag<sup>+</sup>]



## 4. MBEC Assay CV ratio data

### 4.1 BIOFILM FORMATION IN LB MEDIUM

Figure 44 shows the results of the CV measurements of the MBEC™ Assay peg biofilms to the CV measurements of the MBEC™ Assay bottom plate biofilms ratio for *C. metallidurans* CH34 biofilms grown in LB medium appended with  $\text{CuSO}_4$  and  $\text{AgNO}_3$ . An increase these values is observed with increasing  $\text{Cu}^{2+}$  concentrations. The values of *C. metallidurans* CH34 biofilms in the presence of  $\text{Ag}^+$  were low for both concentrations that were measured.

#### CV PEGS/CV BOTTOM MBEC Assay of *C. metallidurans* CH34 biofilms in LB

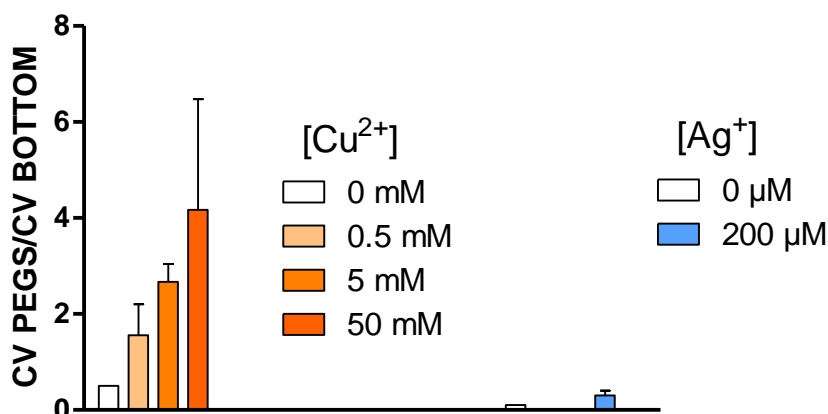


Figure 44: Ratio of CV of peg biofilms to CV of bacterial suspensions (MBEC assay) of *C. metallidurans* CH34 in LB medium appended with  $\text{CuSO}_4$  and  $\text{AgNO}_3$

Figure 45 shows the results of the CV measurements of the MBEC™ Assay peg biofilms to the CV measurements of the MBEC™ Assay bottom plate biofilms ratio for *C. metallidurans* NA4 biofilms grown in LB medium appended with  $\text{CuSO}_4$  and  $\text{AgNO}_3$ . These values in the presence of  $\text{Cu}^{2+}$  are show a decrease for 0.5 mM and 50 mM  $\text{Cu}^{2+}$ , only at 5 mM  $\text{Cu}^{2+}$  an increase is observed. The values of *C. metallidurans* NA4 biofilms in the presence of  $\text{Ag}^+$  were low for all measured concentrations.

#### CV PEGS/CV BOTTOM MBEC Assay of *C. metallidurans* NA4 biofilms in LB

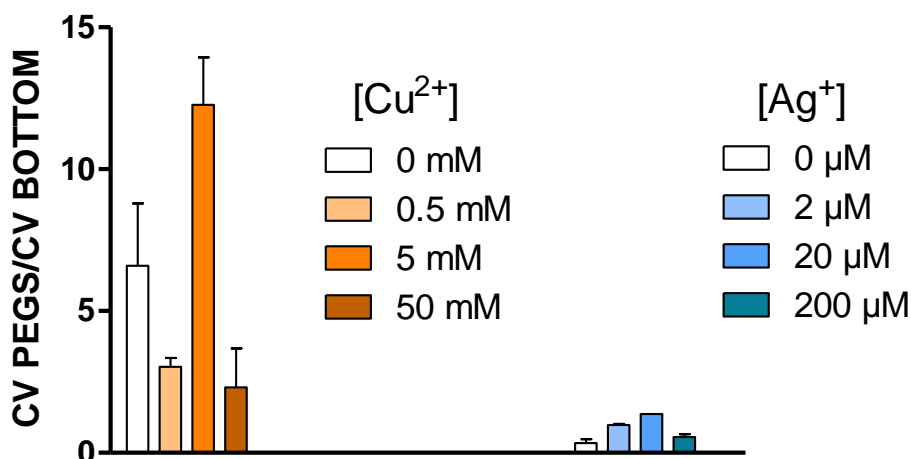


Figure 45: Ratio of CV of peg biofilms to CV of bacterial suspensions (MBEC assay) of *C. metallidurans* NA4 in LB medium appended with  $\text{CuSO}_4$  and  $\text{AgNO}_3$

#### 4.2 BIOFILM FORMATION IN 284 MEDIUM

Figure 46 shows the results of the CV measurements of the MBEC™ Assay peg biofilms to the CV measurements of the MBEC™ Assay bottom plate biofilms ratio for *C. metallidurans* CH34 biofilms grown in 284 medium appended with CuSO<sub>4</sub> and AgNO<sub>3</sub>. CV ratio values show an overall increase with increasing concentrations of Cu<sup>2+</sup>. An increase in CV ratio values is observed with increasing concentrations of Ag<sup>+</sup>, only at 2 μM Ag<sup>+</sup> a decrease is observed.

#### CV PEGS/CV BOTTOM MBEC Assay of *C. metallidurans* CH34 biofilms in 284

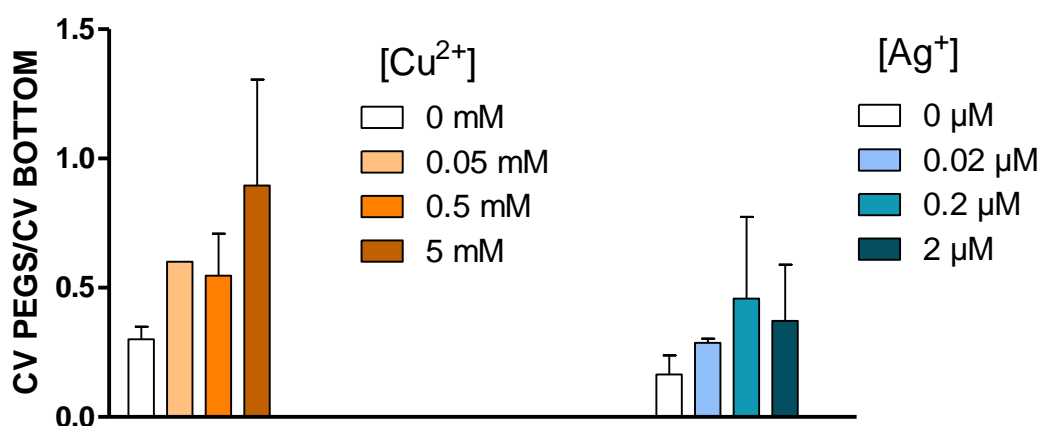


Figure 46: Ratio of CV of peg biofilms to CV of bacterial suspensions (MBEC assay) of *C. metallidurans* CH34 in 284 medium appended with CuSO<sub>4</sub> and AgNO<sub>3</sub>

Figure 47 shows the results of the CV measurements of the MBEC™ Assay peg biofilms to the CV measurements of the MBEC™ Assay bottom plate biofilms ratio for *C. metallidurans* NA4 biofilms grown in 284 medium appended with CuSO<sub>4</sub> and AgNO<sub>3</sub>. CV ratio values in the presence of Cu<sup>2+</sup> show a decrease for 0.05 mM and 0.5 mM Cu<sup>2+</sup> and an increase for 5 mM Cu<sup>2+</sup>. The CV ratio values increase slightly with increasing Ag<sup>+</sup> concentrations.

#### CV PEGS/CV BOTTOM MBEC Assay of *C. metallidurans* NA4 biofilms in 284

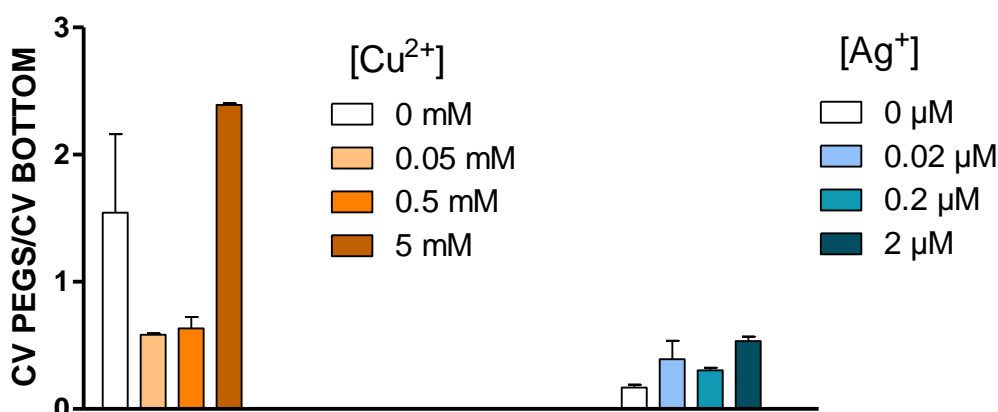


Figure 47: Ratio of CV of peg biofilms to CV of bacterial suspensions (MBEC assay) of *C. metallidurans* NA4 in 284 medium appended with CuSO<sub>4</sub> and AgNO<sub>3</sub>