PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE

SCHOOL OF ENGINEERING

THE ROLE OF A MICROBIAL BIOFILM ON CORROSION AND COPPER RELEASE IN PLUMBING SYSTEMS

CARLOS ALEJANDRO GALARCE GUTIÉRREZ

Thesis submitted to the Office of Graduate Studies in partial fulfillment of the requirements for the Degree of Doctor in Engineering Sciences

Advisor:

GONZALO E. PIZARRO

Santiago de Chile, June 2021

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To my mother, because she always pushed me to be better.

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LIST OF PUBLICATIONS

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Galarce, C., Vargas, I. T. & Pizarro, G. E. *FT-IR characterization of bacterial isolates related to corrosion of copper drinking water pipes.* Submitted to Journal of Environmental Health Science and Engineering.

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ABSTRACT

THE ROLE OF MICROBIAL BIOFILM ON CORROSION AND COPPER RELEASE IN PLUMBING SYSTEMS

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CARLOS ALEJANDRO GALARCE GUTIÉRREZ

Drinking water distribution systems (DWDS) are extreme environments, considering the oligotrophic conditions in them. Simultaneously, these systems are designed to maintain a low microbial burden since the drinking water has disinfectant residual and uses a biocidal material as copper to build them. Despite this, microorganisms can survive in them, mainly due to biofilm development.

Biofilms are one of the most widely distributed modes of life on Earth, in which a high cell density is composed of many species, and cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS). EPS is one of the essential components of biofilms because it helps create a barrier that controls the diffusional processes and chemical reactions.

The safety of drinking water can be affected by the microbial settlement over the copper pipe's inner wall. The processes that the microorganisms modify are diverse, as an example: i) biocorrosion process or microbiologically influenced corrosion, which increase the copper release up to levels hazardous for the people; ii) enhancing the growth of other microorganisms into copper pipes since the biofilm acts as nutrient storage, and iii) storing of metals on the biofilm surface, due to sorption capacities on it.

Despite the severe impact on public health than those situations can create, this topic has not been appropriately studied. This thesis proposes to study the characteristics and changes that the biofilm makes on the copper release and its influence on water quality. The experiments included field and laboratory approaches for a) determining the impact of microbial biofilm in the copper release and other water quality parameters, b) showing new evidence of how the biofilm development modifies the biocorrosion, and c) knowing the potential of the native bacterial isolate as storage of metals potentially hazardous.

The strategy to establish the biofilm role in the corrosion and copper released on drinking water systems included field and laboratory approaches. The analysis of copper pipes extracted from field sampling did not directly relate to the exposure time, corrosion rate, and amount of copper released in the water. Moreover, the outcomes revealed that biocorrosion is a dynamic biologic process that can be characterized neither a unique aging time nor extrapolation of a short time experiment, especially when the system study is a plumbing system.

On the other hand, the studies done under laboratory conditions emphasize the need to put the environment where the bacteria are involved. Our results indicate that the first isolate, identified as *Variovorax sp*, showed a striking loss of biofilm formation capacity when interacting with the second isolate, identified as *Ralstonia pickettii.*

Similarly, the characterization functional of EPS groups involved in the copper accumulation from bacterial isolate showed how the amine group modifications (by methylation) on the surface of bacteria isolated, increasing up to 30 % the copper sorption concerning the same bacterial isolate without modification. These results suggest the need to understand the environmental context to predict how each microorganism can act.

Finally, this thesis provides evidence that supports the idea of potential hazardous microbial contamination on the drinking water due to biofilm detachment and the accumulation of the metal by the microbial sorb capacity and later release. This knowledge will be the beginning to create a protocol on biofilm monitoring to reduce the water quality deterioration by the copper contamination and microorganisms, avoiding the negative effect on public health.

Keywords: Microorganisms, Electrochemistry, Corrosion, Copper release, Plumbing Systems

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RESUMEN

ROL DE LA BIOPELÍCULA EN LA CORROSIÓN Y LIBERACIÓN DE COBRE EN SISTEMAS DE TUBERÍAS

Tesis presentada a la Oficina de Estudios de Posgrado en cumplimiento parcial de los requisitos para el Grado de Doctor en Ciencias de la Ingeniería

CARLOS ALEJANDRO GALARCE GUTIÉRREZ

Los sistemas de distribución de agua potable son considerados como ambientes extremos dadas las condiciones oligotróficas que existen en las tuberías. Al mismo tiempo, estos sistemas están diseñados para mantener una baja cantidad de microorganismos, pues el agua en su interior mantiene residuos de desinfectante, lo que reduce la carga microbiana, a la vez que se emplea un material biocida como el cobre para la construcción de estos sistemas. A pesar de ello, los microorganismos pueden sobrevivir en este ambiente, debido principalmente al desarrollo de biopelículas.

Las biopelículas son uno de los modos de vida más ampliamente distribuidos en la Tierra, en el que una alta densidad celular está compuesta por muchas especies, y las células están frecuentemente incrustadas en una matriz autoproducida de sustancias poliméricas extracelulares (EPS). El EPS es uno de los componentes esenciales de las biopelículas porque ayuda a crear una barrera reactiva que controla los procesos difusivos y las reacciones químicas con el medio ambiente.

La seguridad del agua potable puede ser afectada debido al asentamiento de microorganismos en la superficie interna de la tubería de cobre. Los procesos que modifican los microorganismos son diversos, como por ejemplo: i) el proceso de biocorrosión o fenómeno conocido como corrosión influenciada por microorganismos, el cual incrementa

la liberación de partículas de cobre al agua en niveles perjudiciales para la salud de la población; ii) favorecer el crecimiento de otros microorganismos, ya que puede actuar como reservorio de nutrientes para ellos y ; iii) actuar como depósito de metales en su superficie debido a las capacidades de sorción en la superficie de la biopelícula.

A pesar del grave impacto en la salud pública que estas situaciones pueden generar, este tema no ha sido investigado adecuadamente. Por lo anterior, en esta tesis se propone estudiar las características y cambios que genera la biopelícula sobre la liberación de cobre y su influencia en la calidad del agua potable. Para ello se trabajará en experimentos de campo y de laboratorio para: a) establecer el impacto de la comunidad formadora de biopelícula en la liberación de cobre al medio y en otros parámetros de calidad de agua; b) entregar nueva evidencia de cómo el desarrollo de una biopelícula modifica la biocorrosión; y c) saber cuánto potencial posee aislados bacterianos nativos como reservorio de metales potencialmente nocivos.

La estrategia empleada para establecer el rol de la biopelícula en la corrosión y liberación de cobre en los sistemas de tuberías de cobre incluyó estudios de campo y en el laboratorio. El análisis de las tuberías de cobre recogidas en el estudio de campo no mostró una relación constante entre: el tiempo de exposición, la velocidad de corrosión y la cantidad de cobre liberado en el agua. Además, los resultados revelaron que la biocorrosión es un proceso biológico dinámico, el cual no puede caracterizarse por un único tiempo de envejecimiento o por la extrapolación de experimentos a corto plazo, especialmente en estudios sobre un sistema de tuberías real.

Por otro lado, los estudios realizados en condiciones de laboratorio enfatizan la necesidad de establecer el contexto ambiental donde las bacterias están involucradas. Nuestros resultados indican que, la bacteria asilada, identificada como *Variovorax sp*, mostró una pérdida de capacidad de formación de biofilm al interactuar con el segundo aislado bacteriano, identificado como *Ralstonia pickettii.*

Del mismo modo, la caracterización de los grupos funcionales del EPS implicados en la acumulación de cobre de los aislados bacterianos, expuso cómo las modificaciones de los grupos aminos (por metilación) en la superficie de los aislados bacterianos, aumentaban hasta un 30% la sorción de cobre, en comparación con el mismo aislado bacteriano sin modificación. Estos resultados apoyan de nuevo la necesidad de entender el contexto ambiental para predecir cómo pueda actuar cada microorganismo.

Por último, esta tesis aporta pruebas que apoyan el potencial peligro de contaminación microbiana del agua potable debido a: i) desprendimiento de biopelículas y ii) la acumulación de metales y su posterior liberación debido a la capacidad de sorción microbiana. Este conocimiento será la base para generar un protocolo de monitoreo y acción sobre las biopelículas, con el fin de reducir el deterioro de la calidad de agua por contaminación con cobre y microorganismos, con el fin de evitar los efectos negativos en la salud pública.

Palabras clave: Microorganismos, Electroquímica, Corrosión, Liberación de cobre, sistemas de tuberías

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1. INTRODUCTION

Microorganisms are considered the primary changing agents in the environment (Lytle and Nadagouda 2010). The role and action of each one fluctuate depending on the environmental conditions, the phase of growth, and other microorganisms around it. An exciting phenomenon where microorganisms are involved in the corrosion of copper pipelines in drinking water systems. In this process, products of chemical copper corrosion are mixed with various biological substances produced and released by microorganisms, which affect the water quality. Currently, there is a vast knowledge of chemical reactions and physicochemical parameters involved in copper corrosion. However, the impact of microbial influence still is incomplete. To the best of our knowledge, no systemic research over time has been conducted to determine *in situ* the microbial and water quality changes in a real premise plumbing. This work aims to use an integrated approach in a field study, using an experimental copper pipes system to acquire real data that allow us to link the different parameters with copper corrosion.

Moreover, it was studied the interactions of two native isolated microorganisms to analyze their influence on copper corrosion. These findings will contribute to understanding the local microbial component involved in copper pipes corrosion. In future steps, we will be able to improve the guidelines for the control strategies to preserve the water quality and reduce copper corrosion problems.

1.1. State of the art: Corrosion of copper pipes.

Providing fresh and safe water is the priority of authorities and supply companies worldwide due to the impact that it has on public health (Mohod and Dhote 2013; Stern et al. 2007; Zietz 2003). Nowadays, several procedures and infrastructure to analyze, handle, and distribute the water have been developed to obtain high-quality water. For decades, copper has been the material selected for piping used in distribution water systems (Merkel and Pehkonen 2006; Vargas et al. 2017) due to its excellent mechanical properties and

bactericide capacity (Keevil 2004; Luo et al. 2017). However, despite the functional characteristics and the broad experience of using copper pipes in water distribution systems, the use of copper presents several problems (Vargas et al. 2017).

A constant problem is the corrosion process, which releases metal to the water affecting water quality. The mechanism involves redox reactions where the electrons are shared from the anode (where the oxidation reaction is carried out, i.e., the electrons are given) to the cathode (where the reduction reaction is done, i.e., the electrons are received) (Huttunen-Saarivirta, Rajala, and Carpén 2015; Huttunen-Saarivirta et al. 2017; Hector A. Videla and Herrera 2009; Héctor A Videla and Herrera 2005). In this case, the copper pipe is the anode, and the oxygen or residual chlorine in the water is the cathode, which can be modified by abiotic or biotic factors.

The corrosion process is a complex phenomenon and is continually changing. During this process, different chemical reactions occur in different spatial and temporal scales simultaneously (Merkel and Pehkonen 2006). This situation leads to a constant change of the internal pipe conditions which interferes with a good understanding of the theoretical and/or predictive models. The internal conditions are even more variable when microorganisms are involved in the process than for abiotic conditions.

The microorganisms act as a reactive barrier when they are established on the copper surface. The microorganisms attached on the surface are defined as a biofilm, which is created by microbial aggregates embedded in a self-produced matrix of extracellular polymeric substances (EPS) (Flemming et al. 2016). Biofilm can modify the metal-solution interface reactions inducing changes in the ion concentrations, pH, oxygen levels, and cuprosolvency of the metal (Beech and Sunner 2004; Critchley et al. 2001).

As can be shown, copper corrosion is influenced consequently by the action of microorganisms, which generate a detriment of water quality and diverse problems as the

infrastructure rehabilitation associated with the aging of the system and the growth of biofilms (Vargas et al. 2017) Therefore, it is necessary to understand the changes produced by local microorganisms in the copper pipes that influence corrosion to resolve the unwanted effects on the water quality and damage to the infrastructure.

1.1.1. Chemical contribution of copper corrosion in the drinking water system

The chemical contribution of copper release in the water distribution system is the result of multiple and simultaneous processes (Merkel and Pehkonen 2006) that can be classified into three categories: i) electron transfer reactions, ii) copper speciation reactions, and iii) mass transfer processes. In this way, electron transfer reactions could be associated with the first step of corrosion, where the electrochemical phenomenon of anodic and cathodic half-reactions happens (Vargas et al. 2014). The process has been controversial because diverse mechanisms of copper release have been proposed to explain the phenomenon. Nevertheless, the understanding of reactions involved and the products generated suggest three possible mechanisms, as it is mentioned by Vargas *et al.* (Vargas et al. 2017). The related reactions proposed for electron transfer reactions are the following:

Simultaneous mechanism

$$2Cu^0 + H_2O \rightarrow Cu_2O + 2\bar{e} + 2H^+ \qquad \text{(Eq. 1)}$$

$$Cu^0 \rightarrow Cu^{2+} + 2\bar{e} \qquad \text{(Eq. 2)}$$

Sequential mechanism

$$2\,Cu^0 + H_2O \rightarrow Cu_2O + 2\bar{e} + 2H^+ \qquad \text{(Eq. 3)}$$

$$Cu_2O + 2H_2O \rightarrow Cu^{2+} + H_2 + 2\bar{e} + 2OH^- \qquad \text{(Eq. 4)}$$

Redeposition mechanism

$$Cu^0 \rightarrow Cu^{2+} + 2\bar{e} \quad \text{(Eq. 5)}$$

$$Cu^0 + Cu^{2+} + H_2O \rightarrow 2Cu_2O + 2H^+ \quad \text{(Eq. 6)}$$

Likewise, the cathodic half-reaction is explained by the dissolved oxygen (DO)reduction:

$$\frac{1}{2}O_2 + 4\bar{e} + 4H_2O \rightarrow 4OH^- \quad \text{(Eq. 7)}$$

The beginning of the process starts with the formation of a thin layer of cuprite (Cu_2O). Based on thermodynamic calculations and kinetic studies, it proposed that the initial step consists of the formation and precipitation of double layers of cuprite (Cu_2O) over the metal surface (Ives and Rawson 1962). The first layer would be compact with a 2µm thickness. The second layer is porous (Merkel and Pehkonen 2006). Due to the limited stability of cuprite in the presence of oxygen, solids with divalent copper are also formed. The formation of cuprous oxide is considered a significant retarding factor in copper corrosion (Shim and Kim 2004). In abiotic conditions, copper corrosion stabilizes over time; however, the necessary time differs depending on water composition and pH (Feng, Teo, Siow, Tan, et al. 1996; Shim and Kim 2004).

Simultaneously, the copper speciation reactions are governed by precipitation-dissolution, complexation, and acid-base reactions, which depend on the environmental conditions. For example, *Pehkonen et al*. (Pehkonen, Palit, and Zhang 2002) reported how the pH and DO influence the stability, thickness, and release of copper. For thermodynamic reasons, malachite is, in most cases, the dominant divalent copper compound in the presence of dissolved inorganic carbon (Harrison, Nicholas, and Evans 2004; Merkel and Pehkonen 2006).

Finally, the last process is mass transfer, which is controlled by the copper ions flux. Many investigations have indicated that the limiting step of copper corrosion in pipelines is

the diffusion of copper cations. This process can trigger the copper release in the form of soluble copper and particles (nano or microparticles) of by-products of copper (Vargas et al. 2010), which can increment the copper to the bulk water from the surface of the pipe (Vargas et al. 2017).

1.1.2. Microbial influence on copper corrosion

The action of microorganisms has been considered an essential factor in the release of copper into drinking water systems (Vargas et al. 2017). The microorganisms living in Drinking Water Distribution Systems (DWDS) can attach to pipes surfaces (Douterelo et al. 2017; Kelly et al. 2014), forming mixed-species biofilms (Videla and Herrera 2005) and promoting pipe corrosion (Lytle and Nadagouda 2010). This phenomenon is known as Microbiologically Induced Corrosion (MIC) or biocorrosion (Beech 2004; S. Liu et al. 2016), which has been reported worldwide as responsible for considerable damages to infrastructure and human health (Mohod and Dhote 2013; Stern et al. 2007; Vargas et al. 2017; Zietz 2003).

Biofilms are defined as aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that adheres to each other and/or a surface (Flemming et al. 2016). The biofilm lifestyle is distinct from that of free-living bacterial cells (Flemming et al. 2016). Bacterial biofilms create a physically different habitat which provides shelter and a source of nutrients (Brözel and Cloete 1991; Cloete 2003; Jungfer et al. 2013; Elhariry et al. 2012), which fundamentally modify both the physicochemical environment of the biofilm and interactions among the organisms therein (Flemming et al. 2016).

It is possible to find a wide variety of bacteria in drinking water systems. The majority of the them corresponds to Gram-negative bacteria, mainly Proteobacteria (Bohus et al. 2010; Pavissich et al. 2010). The wide microbial diversity is strongly influenced by the environment, where the most important factors are: the water treatment (Jungfer et al. 2013; Hwang et al. 2012), the material of the water distribution network (Lin et al. 2013; R. Liu and Junge Zhu 2013), and water quality parameters such as sulfate and the amount of organic matter among others (Marc Edwards and Sprague 2001; Pehkonen, Palit, and Zhang 2002).

Environmental influences creates a drive for renewing continuously the microbial community that conforms to the biofilm (Sun et al. 2014; Gomez-Alvarez, Revetta, and Domingo 2012; Gomez-Alvarez et al. 2015). These microbial changes can occur in the space of a few stagnation hours (Lautenschlager et al. 2010). However, only slight changes in richness and evenness of the populations during stagnation have been observed (Douterelo et al. 2017; Douterelo et al. 2016).

The development of biofilm is a successional process (Martiny et al. 2003) where the first stage is the attachment of different clumps of bacteria on the surface recruited from the bulk water population forming a monolayer on the surface (Martiny et al. 2003; Kragh et al. 2016). After the initial attachment, secondary colonization of bacteria that benefit from a protective environment in the biofilm and/or feed on the remnants of other bacteria occurs. In this secondary community, better resource or space competitors may exclude less competitive organisms (Martiny et al. 2003). However, it should be mentioned that there is a previous selection in DWDS over the bacteria that will participate in the biofilm development process because the kind of bacteria depends on the water supply conditions (Sun et al. 2014) and the material used to build the DWDS (H. Wang et al. 2014).

The microorganisms that conform to the biofilm influence the complexity and dynamism of the conceptual model of MIC for copper pipes. The addition of a new member into the biofilm could change the global activity of this structure, which would be modified the copper biomineralization, complexation of copper, and biocorrosion (Figure 1) (Vargas et al. 2017; Pizarro et al. 2014).

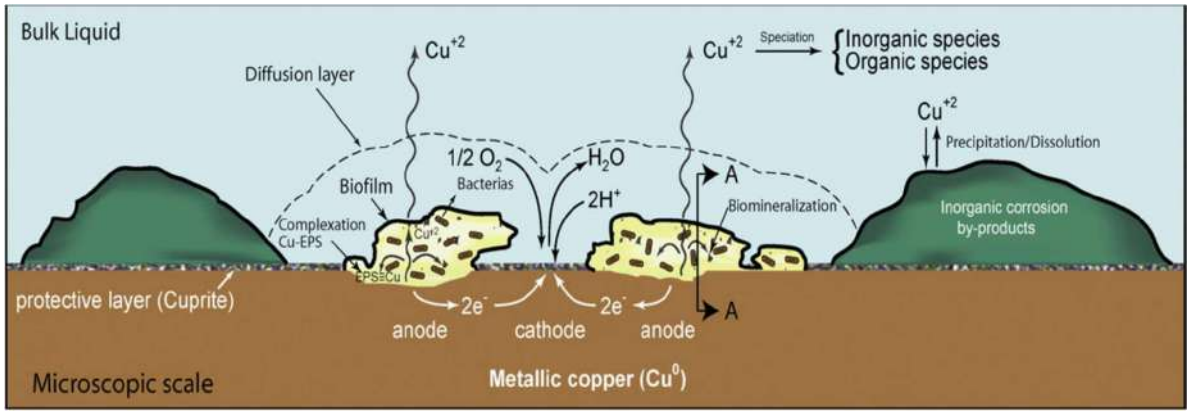

Figure 1. Conceptual model and main processes considered for corrosion pipes under the presence of biofilm during stagnation conditions (Pizarro et al. 2014).

There is a high selective pressure produced by the environment and competitive microorganisms within the copper pipe, which can reduce the type of bacteria. Nowadays, it is still complicated to isolate and determine the specific function of each bacterium that are part of the biofilm. Few studies about the succession and structure of biofilm to long term in DWDS have been done. One of them was conducted in a model DWDS where little pieces of stainless steel were exposed for three years of operation in a batch system. The outcomes showed the development of a biofilm where a diverse community of microorganisms dominated the biofilm structure depending on the stage of development (Martiny et al. 2003). However, these efforts to understand copper corrosion processes and the conditions used did not reflect on the real environment.

In the same way, microbial colonization of the pipe surface has been proposed to follow a sort of ecological succession (Keevil 2004). Pavissich et al. 2010 studied the effect of bacterial communities associated with MIC using culture dependent and independent analysis to know the colonization process. The outcomes showed a relatively higher bacterial richness and different structure in the biofilm from the distribution system concerning the short-term laboratory test (Pavissich et al. 2010). These results indicate two crucial points in the copper corrosion in DWDS study. The first point is associated to the biofilm formation, which is indicated as the key element for both the establishment and promotion of corrosion and the survival of microbial communities in copper corrode plumbing (Pavissich et al. 2010); and the second point is related to the capacity to mimic the environment since the reproducibility of the main influencing factors have not been achieved on biocorrosion yet.

There is much information about the wide variety of bacteria on DWDS in exact times and different conditions; however, the central message of all of them is the need for more field study with a global and experimental point of view to know and understand the local microbial community to develop an appropriate strategy of microbial control.

In summary, the biofilm settlement produces changes in the corrosion process within copper pipes under drinking water conditions and increases copper release. Over time this will decrease water quality. Despite these precedents, the shifting on copper release and microbial community under actual conditions still needs more research. In consequence, considering that the complexity of biocorrosion of copper pipes is influenced by the progressive, temporal, and spatial changes, the following research questions arise:

i. How biofilm aging relates to the corrosion rate under actual drinking water conditions?
ii. How the native bacteria modify their electrochemical behavior when they interact with another kind of bacteria?
iii. Which are the superficial changes in the EPS groups in native microorganisms that act as a metal reservoir?

1.2. Working hypothesis

The biofilm acts as a reactive barrier that is controlling the corrosion process. The structure of biofilm is linked highly with the members that built it and its response to environmental conditions. However, little information is available about the local microbiology that lives in the copper pipes. Considering that how the native microorganisms influence the corrosion process remain to be elucidated and the interaction among the biofilm´s members is still poorly understood, this thesis hypothesizes that:

i. The development of a mature biofilm increments on direct form the copper release and corrosion rate over time under actual drinking water conditions.
ii. The development of a group of bacteria (consortium) intensifies the individual electrochemical response; and
iii. The exposition of specific groups of EPS on bacterial membrane enhances the sorption properties of the surface.

1.3. Objectives

The general objective of this work was to understand the biofilm action on copper release to the water from a microbiological and electrochemical point of view.

The following specific objectives were developed:

i. To evaluate the relation of biofilm complexity with corrosion rate under actual drinking water conditions in the long-term. (Chapter I)

ii. To evaluate the corrosive capability of different bacteria isolated from a biofilm settled down on copper pipes. (Chapter II)

iii. To evaluate the sorption capacity of isolated bacteria from a biofilm attached to the copper pipe surface. (Chapter III)

1.4. Structure of the thesis

In the first part of this study, the relation between biofilm complexity and the corrosion rate was analyzed using an experimental system connected for two years to premise plumbing, in a location that showed severe corrosion events. The physicochemical parameters were monitored in each sample time to determine the stability of the system. Moreover, the copper released into the pipes was monitored with flushing experiments and determination of corrosion rate with electrochemical techniques. The changes of biofilm composition were evaluated through complete DNA analysis of microbial community attached to copper surface pipes using DGGE analysis. The outcomes are presented in Chapter I of this thesis, Section III. The results derived from the completion of Objective i, which is contained in the paper "Dynamics of Biocorrosion in Copper Pipes under Actual Drinking Water Conditions," published in *Water* in April 2020.

In the second part of this study, the corrosive capability of different bacteria isolated from a biofilm settled down on copper pipes was evaluated under simulated environmental conditions. The approach involved the use of electrochemical impedance spectroscopy as the primary technique to analyze the corrosive bacterial behavior. These findings are presented in Chapter II, Section IV. The results, derived from the completion of Objective

iii, are contained in the article “Effect of Hazardous Bacteria Isolated from Copper Plumbing System on Microbiologically Influenced Corrosion of Copper,” published in *International Journal of Electrochemical Science*, in January 2019.

Finally, in the third part of the thesis, the sorption capacity of external EPS of bacteria isolated from the local biofilm was evaluated. The relevance of EPS in the formation of a mature biofilm structure and its capacity to accumulate metal is a crucial point in the understanding of biofilm activity in the corrosion process. The approach included the microbial analysis of each bacterium isolated and their surface analysis by the FTIR technique. The results were submitted to the *Journal of Environmental Health Science and Engineering* in the paper “FT-IR characterization of bacterial isolates related to corrosion of copper drinking water pipes” and is presented in Chapter III, Section V. These results completed Objective iii and provided information to complete Objective ii.

General conclusions are presented at the end of the document, which were deduced from the results showed in each Chapter. It should be note that each Chapter corresponds to a published article.

2. CHAPTER I: DYNAMICS OF BIOCORROSION IN COPPER PIPES UNDER ACTUAL DRINKING WATER CONDITIONS

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Abstract: Deficient disinfection systems enable bacteria to form in drinking water; these can invade plumbing systems even if the pipes are composed of antibacterial materials such as copper. Severe copper corrosion by microorganisms and their subsequent release into the water system are evidenced by the blue water phenomenon. Proper monitoring and control can reduce such undesirable effects on water quality. However, a lack of data from analysis under actual conditions has limited the development of useful predictive tools and preventive strategies. In this work, an experimental aging system was connected to a drinking water network affected by the blue water phenomenon. The microbially influenced corrosion (MIC) was evaluated by studying the dynamics of the formed bacterial community and its relationship with copper corrosion and the release of copper. The results suggest that the conformation and composition of the biofilm attached to the surface influence the measured parameters. The corrosion rate was variable throughout the sampling time, with the highest value recorded after one year of aging. The composition of biofilms also changed with time; however, the genus *Pseudomonas* was ubiquitous over the sampling time. No relationship between the corrosion rate and the biofilm age was observed, thereby suggesting that MIC is a dynamic phenomenon that requires further study.

Keywords: copper; corrosion rate; biocorrosion; biofilm

2.1. Introduction

Water utilities invest time and money toward controlling the presence of undesirable microorganisms in drinking water systems (Isabel Douterelo et al. 2016). The microbial contamination of a water distribution network begins with microbial attachment on a pipe surface (I. Douterelo et al. 2017; Kelly et al. 2014), which then forms a mixed-species biofilm (Héctor A Videla and Herrera 2005). Biofilm formation is a crucial element for both the promotion of corrosion and the survival of microbial communities in corroded copper plumbing (Pavissich et al. 2010; Beech and Sunner 2004) because the biofilm's structure can modify interfacial metal-solution reactions, inducing changes in the ion concentrations, pH, dissolved oxygen (DO) levels, and organic and inorganic material detachment (Beech and Sunner 2004; Vargas et al. 2017).

Copper pipes in the drinking water service are highly valued in domestic plumbing systems owing to their corrosion resistance (I. Vargas et al. 2017; Lee and Kim 2012) and antiseptic properties (Keevil 2004; Luo et al. 2017). However, bacteria living in copper plumbing systems have reported (Isabel Douterelo et al. 2016; I. Vargas et al. 2017; Reyes et al. 2008; Lehtola et al. 2004). Microorganism settlement can promote and enhance copper corrosion in pipes (I. Douterelo et al. 2017; Kelly et al. 2014), causing a phenomenon known as microbiologically influenced corrosion (MIC) or biocorrosion (Beech and Sunner 2004; S. Liu et al. 2016). Biocorrosion leads to two problems: high levels of copper in drinking water, which is a public health concern (Beech 2004; Kip and van Veen 2015; P. J. Bremer, Webster, and Brett Wells 2001; Hector A. Videla and Herrera 2009), and infrastructure failure owing to localized corrosion (I. Vargas et al. 2017). Moreover, owing to the development of extracellular polymeric substances (EPS) by the biofilm, an unknown amount of copper can be stored or sorbed on the biofilm´s surface (Beech 2004; I. T. Vargas et al. 2010) , which increases the risk of contaminating the water with undesirable copper concentrations.

Consumption of water containing copper has adverse effects on human health, ranging from stomach distress to the liver or kidney failure (Stern et al. 2007; Zietz 2003; Mohod and Dhote 2013; Ehi-Eromosele and Okiei 2012). The World Health Organization (WHO) limits the copper concentration in drinking water to 2 mg/L (Gorchev and Ozolins 2011). Nevertheless, extreme copper corrosion cases can lead to copper concentrations of up to 20 mg/L (Marc Edwards, Jacobs, and Taylor 2000) manifested as blue coloring, which is referred to as the blue water phenomenon.

The difficulty in accessing the internal surface of copper pipes within operational networks creates challenges in the study of biofilms (Isabel Douterelo et al. 2016). Most of the available information on biofilms in copper pipes have been collected by using sampling points or by assessing a few selected microorganisms under controlled laboratory conditions. However, neither of these methods represents the dynamics of diverse communities within real networks (Pavissich et al. 2010; I. Vargas et al. 2017; I. T. Vargas et al. 2014; Calle et al. 2007). *Pseudomonas* is one of the most common types of bacteria found in the biofilm that develops in plumbing systems (I. Douterelo et al. 2017; Kelly et al. 2014; Dunne 2002). It has been reported that its adhesion capacity promotes biofilm development (Dunne 2002; Kostakioti, Hadjifrangiskou, and Hultgren 2013), and the secretion of enzymes with oxidative activity as a catalase is associated with the corrosive process (Landoulsi et al. 2008). Although these studies conducted under laboratory conditions have provided valuable information, the conditions often differ significantly from those occurring in premise plumbing, particularly in the biofilm growth cycle and temporal variability (Kelly et al. 2014).

In this study, an experimental copper pipe aging system is connected to a drinking water network affected by MIC. The main objective is to investigate the bacterial community changes and the association of MIC with the dissolved copper in water. Chemical, hydrodynamic, and microbiological evaluations were conducted during a two-year

experiment to address the dynamic nature of the process and to provide a comprehensive study of copper MIC under actual conditions.

2.2. Materials and Methods

2.2.1. Sample collection

An aging system consisting of six parallel copper pipes every 1 m in length with an internal diameter of 1.95 cm (3/4 inches) and a volume of 300 mL (Olivares et al. 2014) was installed in the yard of a residence in Olmué, a rural community in Chile (32°59'43" S, 71°11'08" W; Figure 2A), that was affected by the blue water phenomenon (Figure 2B). In Figure 1B, it is possible to appreciate the change in the watercolor (with a green-blue tone), which is the result of corrosion. The water was supplied from a well located near the residence without a disinfection system. The experimental aging system was connected to the plumbing system (Figure 2C) and was flushed three times a day (every 8 h) for 5 min. Two of the copper pipes were collected and replaced for each sample time of 1, 3, 8, 12, and 24 months. The exposure was initiated starting from August 2012 (winter season) to April 2015 (fall season). The water samples were collected, stored at 4 °C, and rapidly transported to the laboratory for analytical and experimental procedures. At each collection time, the water quality parameters were measured to analyze the stability of the system.

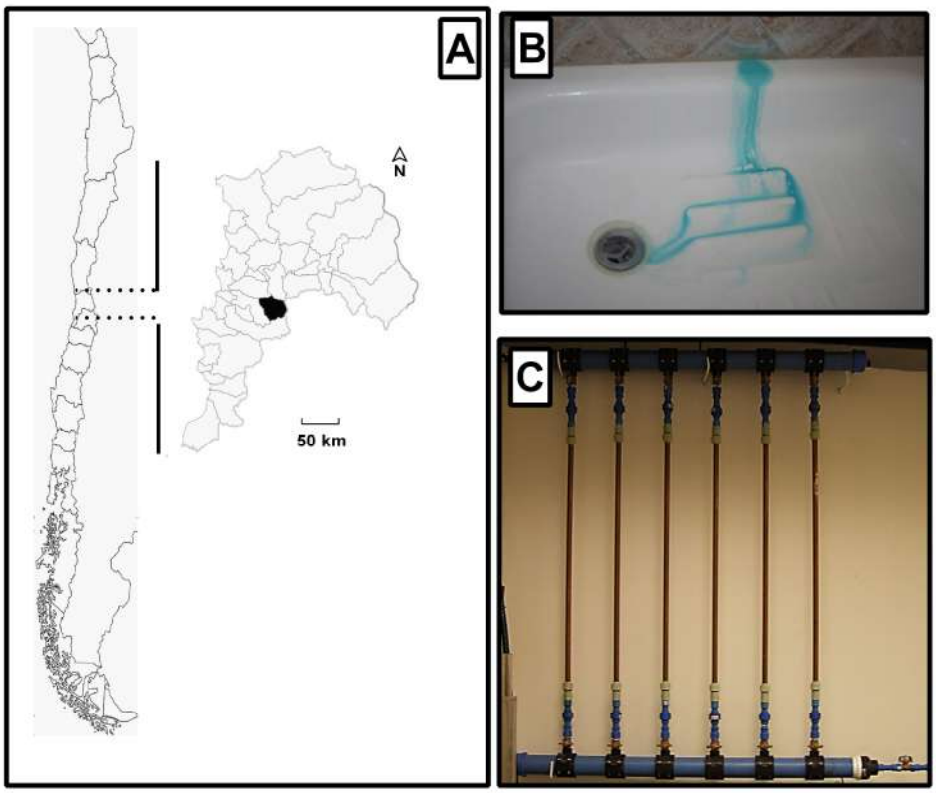

Figure 2. (A) Map of Chile highlighting the location of the sampling point in the central region. (B) Blue-water phenomenon observed in local premise plumbing. (C) Sampling system installed in the local premise plumbing system.

2.2.2. Flushing experiments

The amount of copper released was studied using the system described in Vargas et al. (I. T. Vargas et al. 2010), which consisted of a tank filled with water from the field site connected to a 1 m polyvinyl chloride (PVC) pipe followed by the sampled copper pipe. The pipe was flushed at a constant laminar flow rate of 0.48 L/min until a volume of 5 L liters passed through the system. During flushing, 12 sequential samples of 100 mL were taken at 100, 300, 500, 700, 1000, 1300, 1600, 1900, 2200, 2500, 3500, and 4500 mL. The collected samples were filtered using a 0.45 μm pore size membrane and all samples were acidified with nitric acid for preservation. The samples were analyzed by an inductively coupled

plasma-optical emission spectrometer (ICP-OES; model 7300, PerkinElmer, Waltham, MA, USA) to measure the copper concentration. Two pipes were used for each sampling time.

2.2.3. Water analysis

The water quality parameters were measured at each sample collection. Other parameters, such as pH, temperature, conductivity, and DO were measured in situ. Chemical analysis was conducted in the laboratory. Anions such as chloride, fluoride, sulfate, nitrate, nitrite, and phosphate were analyzed in the laboratory by using an ion chromatograph (IC; Metrohm, model 882 Compact IC plus, Herisau, Switzerland). The mobile phase was 3.2 mM of sodium carbonate and 1 mM of sodium bicarbonate at a flow rate of 0.7 mL/min. A Metrosep 4/5 Guard precolumn was used to support the employed Metrosep A Supp 5-250/4.0 column. The total organic carbon (TOC) was measured using Shimadzu (TOC-L CPH analyzer equipped with an autosampler (ASI-L) and a nitrogen module (TNM-L). The concentrations of copper and iron were measured using ICP-OES (model 7300, PerkinElmer, Waltham, MA, USA).

2.2.4. DNA extraction

The copper pipes were aseptically cut into segments 25 cm in length. The inner surface of each pipe was carefully washed and filled with Milli-Q autoclaved water to avoid biofilm loss. The biofilms were released by sonication in a bath of ice water at a frequency of 40 kHz (Branson 2510 Ultrasonic Cleaner, Danbury, CT, USA). After 5 min, the sonication was paused, and the pipes were manually agitated for 10 s and then sonicated for an additional 5 min. The biofilm extracts were pelleted in 50 mL sterile polypropylene tubes by centrifugation at 12,452g for 10 min. Supernatants were then withdrawn, leaving 2 mL for pellet resuspension. The pellet suspension method was employed for DNA extraction using the Power Soil® DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions (Rojas et al. 2017). Gel-Red stain was used in agarose electrophoresis to verify the obtained DNA integrity.

2.2.5. Denaturing Gradient Gel Electrophoresis

About 10 ng of extracted DNA was used as the template for polymerase chain reaction (PCR), in which 16S ribosomal DNA (rDNA) specific primers were used. For denaturing gradient gel electrophoresis (DGGE) analysis, we used primers 358f-GC and 907r (Muyzer and Smalla 1998) with a 40 bp CG clamp attached to the 5' end of the forward primer required for DGGE methodology, which amplified the fragments to a size of approximately 550 bp. The 25 µL PCR mixtures contained each deoxynucleoside triphosphate at a concentration of 100 mM, 1.5 mM MgCl2; each primer at a concentration of 0.3 mM, 2.5 U of Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA); and the PCR buffer supplied with the enzyme (Diéz et al. 2001). The PCR program specified an initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. During the last cycle of the program, the length of the extension step was increased to 10 min (Diéz et al. 2001). An aliquot of the PCR product was electrophoresed on a 1.0% agarose gel and stained with Gel-Red, and the concentration was estimated by using the Low DNA Mass Ladder as a standard (Gibco BRL, Thermo Fisher Scientific, Waltham, MA, USA). DGGE gel containing 0.6% polyacrylamide was produced at 0.75 mm in thickness with urea-formamide differential concentrations of 40%–70% as DNA denaturant agents [33]. The DGGE was run at 100 V per 16 h using SYBR Gold Stain (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA) at 0.01% for 30 min, and the results were revealed using an ultraviolet (UV) transilluminator (Bio-Rad Technologies, Berkeley, CA, USA).

2.2.6. Sequence analysis

To identify the bacterial community, the profile bands with the highest intensity were excised, and their DNA was amplified using the previously described primers without the 40 bp GC clamp (Diéz et al. 2001). Then, the PCR products were confirmed by electrophoresis and were sent for sequencing (Macrogen Inc., Seoul, Korea). The recovered

sequences were then aligned and compared with the database of the National Center for Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST; NCBI) algorithm. The 16S rRNA phylotypes retrieved from the DGGE band sequences together with the reference taxa, and the closest relatives from GenBank, included only in published studies or cultures, were aligned using MAFFT version 7.123b software. The retrieved sequences from the present study were analyzed against the phylogenetic tree containing all sequences in the Silva database (http://www.arb-silva.de/).

Phylogenetic reconstruction using maximum-likelihood search strategy with 10,000 bootstrap replicates was performed subsequently for each gene dataset using the FastTree version 2.1.9 SSE3 software.

2.2.7. Scanning Electron Microscopy

Eight coupons of 1 cm × 1 cm from were cut aseptically from the field copper pipes for microscopic analysis. The samples were kept hydrated with the corresponding test water before preparation for scanning electron microscopy (SEM). The coupons were fixed with 2.5% (w/v) glutaraldehyde buffered with 0.1 M phosphate (pH 7). The samples were rinsed with sterile distilled water, postfixed with 1% (w/v) osmium tetroxide for 1 h and dehydrated in 50%–100% serial ethanol and 100% acetone baths, as described by Pavissich et al. (Pavissich et al. 2010). After dehydration, the coupons were dried to a critical point and were coated with gold. The morphology and structures formed on the inner surfaces of the copper pipes were analyzed by using an SEM instrument (LEO 1420VP, LEO Electron Microscopy, Ltd., Cambridge, UK) with an energy dispersive spectroscopy (EDS) system including an Oxford 7424 solid-state detector for elemental analysis.

2.2.8. Electrochemical test

A three-electrode cell was used for the electrochemical tests. Several coupons of 1 cm × 1 cm were cut randomly from the field pipes for use as working electrodes. The counter electrode was composed of graphite bar, and the reference electrode was composed of silver/silver chloride (sat. KCl). Electrochemical measurements were completed with a potentiostat (CHI Instruments 750D, Austin, TX, USA). Artificial tap water, similar to that reported by Feng et al.(Feng, Teo, Siow, Tan, et al. 1996) (Table 1), was used as an electrolyte to compare the electrochemical responses.

Table 1. Chemical composition of simulated tap water used as an electrolyte in the electrochemical tests.

Parameter	Simulated Tap Water Concentration
Chloride (Cl^-)	11.4 mg/L
Sulfate (SO_4^{-2})	90 mg/L
HCO_3^-	98 mg/L
pH	7.5
Conductivity	680 µS/cm
Temperature	23 °C

Linear polarization (LP) was used to evaluate the corrosion rate at each sample time with a scan rate of 0.01 V s^{-1}, whereas electrical impedance spectroscopy (EIS) was used to check the LP values and to support the proposed model of the superficial corrosion process (Feng, Teo, Siow, Tan, et al. 1996). EIS measurements were conducted at the open circuit potential over a frequency range of 10.000 – 5 × 10^{-6} kHz using an alternating current (AC) amplitude of ±10 mV rms.

2.3. Results and Discussion

2.3.1. Water quality

The operational parameters have a strong influence on the MIC process (Jungfer et al. 2013; D. Yu et al. 2015; Lin et al. 2013; Hwang et al. 2012). Accordingly, the water source was monitored throughout the sampling. The values of the monitored parameters (corresponding to the beginning of the experiment) are summarized in Table 2. The pH, conductivity, and DO were stable with values of about 6.9 ± 0.06, 690 ± 40.8 μS/cm, and 8.3 ± 0.4 mg /L, respectively. Other parameters associated with microbial growth showed changes during the experiment. The TOC levels showed a constant increase from 0.35 to 3.04 mg/L) at the source. The same increase trend was observed for the total alkalinity, from 51 to 219 mg/L as $CaCO_3$, which increased to reach a plateau at the end of the study. The increase in total alkalinity and TOC over time can indicate possible contamination of the source water of the system by microbial growth (Serrano and Leiva 2017). This possibility is supported by the values of the sulfate, chloride, and nitrate levels, which were lower at the second sampling than those recorded at other times. A discrete and quick bacterial bloom likely occurred to trigger such changes. However, the microbial contamination in the source water of the system did not pose a problem in observing the microbial changes occurring on the inner wall of copper pipes. The changes in the bulk water communities do not exert an evident influence on the composition of the attached community, as discussed by Douterelo et al. (I. Douterelo et al. 2017). Another approach considered a dilution effect owing to a previous rain event; however, this effect was discarded because it did not feature the same change trend in all measured parameters.

Table 2. Physicochemical properties of the source water

Exposure Time (Month)/ Parameter (Unit)	1	3	8	12	24
Fluoride (mg/L)	0.019	0.035	0.152	0.035	-- [1]
Chloride (mg/L)	21.53	21.45	23.94	3.23	19.43
Alkalinity (mg/L as $CaCO_3$)	200	193	219	144.6	51.47
Total hardness (mg/L as $CaCO_3$)	145.7	142.29	139.2	150.2	147.74
Sulfate (mg/L)	155.2	155	163.6	34	149.46
Nitrite (mg/L)	0.016	0.009	0.015	0	-- [1]
Nitrate (mg/L)	10.22	11.1	9.44	1.32	10.07
Phosphate (mg/L)	0.71	0.3	0.36	0.17	0.41
TOC (mg/L)	2.1	1.67	3.04	0.57	0.35
DO (mg/L)	8.1	8	8.2	8.8	8.86
pH	6.84	7	6.96	7	6.93
Conductivity	704	673	708	736	629

[1] Data no measured.

The temperature of the sampling place varied widely from 2 to 30 °C owing to seasonal changes. Previous reports indicate the temperature as a relevant factor influencing biofilm development. Ling et al. (Ling et al. 2016) observed differences between the biofilm communities from household water and the tap water samples owing to seasonal changes in temperature. Similarly, Qian et al. (Qian et al. 2017) showed that temperature cycles affect biofilm formation such that higher temperature keeps the growth of nonadapted mesophilic bacteria under control (Qian et al. 2017). In our research, the temperature likely factored in the growth of the microbial community at the source of water, as reported by Ling et al. (Ling et al. 2016). However, the inner surfaces of the copper pipes resulted in selective pressure in the microbial composition because the microorganisms were able to attach to the metal surface, which hastened the biofilm production.

2.3.2. Scanning Electron Microscopy

Figure 3 shows the inner surface of a copper pipe affected by MIC. The superficial layer is composed of oxides and byproducts of copper corrosion, which can facilitate the generation of a microenvironment (I. T. Vargas et al. 2014). Each SEM image exposed diverse stages of biofilm development on the internal surfaces of the copper pipes. Figure 3 A, C shows low development in the mineral layer with microorganisms attached to the surface. The development of a heterogeneous biofilm likely enhanced the weak oxide layer with low protective capacity. Previous research revealed that the presence of a consortium of microorganisms with the ability for biofilm production promotes the precipitation of copper hydroxide with low protective function in comparison with the abiotic condition (Galarce et al. 2019). This situation and regular replacement of water in the copper pipes every 8 h might explain the low coverage of corrosion byproducts on the pipes exposed for 3 and 12 months.

On the contrary, a more complex surface was observed in the pipes exposed for 8 and 24 months (Figure 3 B, D), in which a more homogenous corrosion byproduct layer was developed that included microorganisms, suggesting early biofilm formation. Figure 3B shows the sample exposed for eight months, in which sea urchin-shaped precipitates formed. Even though a complete characterization of these precipitates was not conducted (i.e., X-ray powder diffraction analysis), the EDS analysis of the corrosion byproducts revealed a composition of carbon, oxygen, and copper. These results, together with the observed shapes and the high alkalinity of the water averaging 162 mg/L as CaCO3 (Table 2), suggest the presence of copper carbonates on the surface. It has been reported that malachite (Cu(OH)2CuCO3) has a passivating effect on the copper surface (I. Vargas et al. 2017; I. T. Vargas et al. 2014). The passivation characteristics of malachite would explain the increments of open circuit potential (OCP) values and the lower corrosion rate shown in the samples exposed for eight months (shown later). Similarly, diverse precipitates were observed in the sample exposed for 24 months (Figure 3D). In both cases, a low number of

bacteria were attached to the surface, particularly in the surface cracks of oxide layers. The diverse exposition areas likely promoted the copper corrosion at these sampling times. This evidence suggests that the microorganisms attached to the inner pipe surfaces and that their EPS modified the corrosion process and the release of copper owing to changes in the oxide layer that formed on the inner surface of the copper pipes during the exposure time.

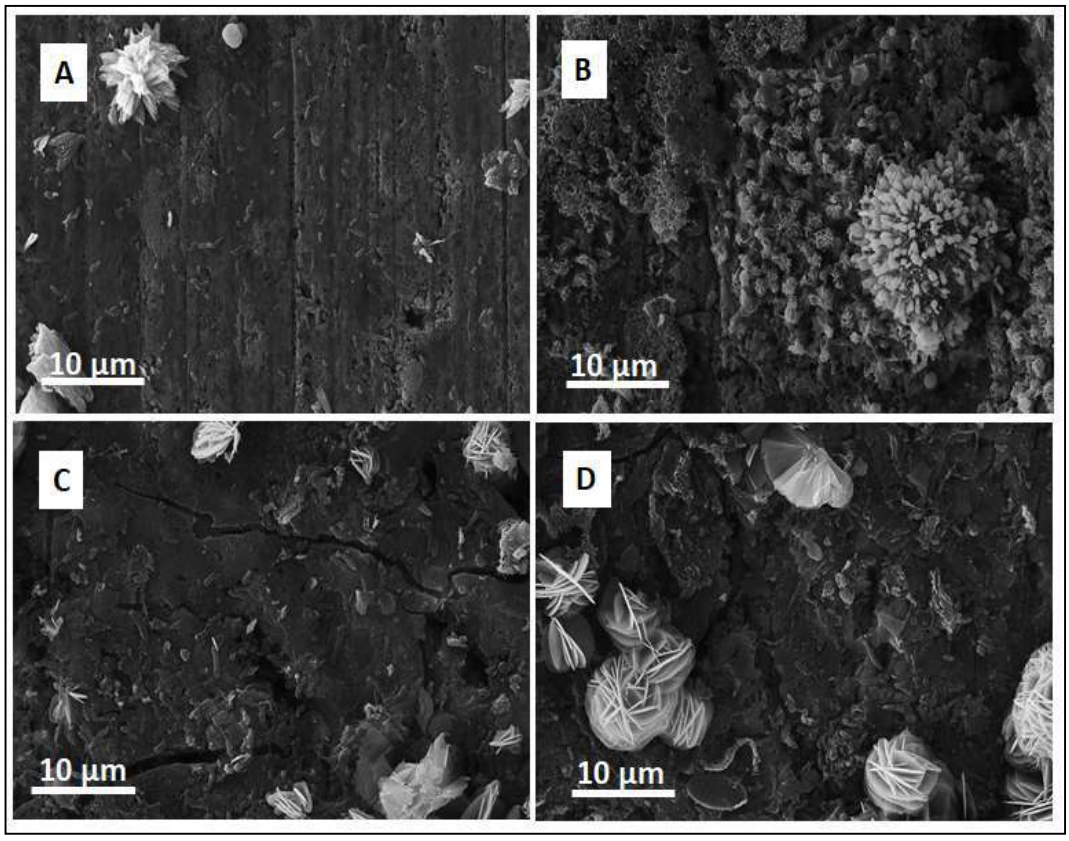

Figure 3. SEM images of copper after different periods of exposure: (A) 3 months; (B) 8 months; (C) 12 months; and (D) 24 months

2.3.3. Flushing Experiments

The environmental conditions present at the sampling site were enabling to study the development of an extreme (bio) corrosion event. Moreover, the experimental set-up allowed us to observe the behavior of release copper in actual premise plumbing. The results of the flushing experiments suggest that the microorganisms attached to the inner surfaces of the copper pipes modified the copper release process under actual biotic conditions. The released copper measured in the flushing experiments could be explained in two ways: (i) by modification of the oxide layer composition and (ii) by biosorption of copper by the EPS of the biofilm. Both can be inferred after comparing our results with the data previously reported in Vargas et al. (Vargas et al. 2010, 2014). Modification of the oxide layer can explain the higher levels of dissolved copper in our research compared with the amount released in our previous experiments in the same range of exposure time (Vargas et al. 2010, 2014). The values observed in this research were more than 10 times higher than reported previously. These differences can be attributed to exposure to uncontrolled environmental conditions, which allowed us to appreciate the development of a weak oxide layer into the copper pipe that did not observe in studies before.

The weak layer was likely influenced by both the chemical composition of the water source and the microbial community attached to the copper pipe interiors. The presence of sulfate, bicarbonate, and orthophosphate enhances the formation of brochantite and cupric phosphate during long periods, which would prevent the formation of insoluble tenorite or malachite phases (M. Edwards et al. 2001) from creating a weak oxide layer. Moreover, previous research indicates that the presence of a biofilm creates an acidic microenvironment that hastens the formation of soluble copper compounds as cupric hydroxide ($Cu(OH)_{2(s)}$)(Vargas et al. 2017; Vargas et al. 2014; Galarce et al. 2019; Webster et al. 2000).

The EPS influence on the biosorption of dissolved copper can be deduced from the data shown in Figure 4. After 1 L of water was extracted from the pipe, the total mass of the dissolved copper released reached a plateau for all-time series. Vargas et al. (I. T. Vargas et al. 2014) reported a constant release of copper for both abiotic and biotic conditions; however, our results did not show the same behavior. A large number of EPS might have been attached to the copper surface owing to biofilm growth at the same time, which would have enhanced the release of copper corrosion products, as reported previously (I. T. Vargas et al. 2014). The results indicate that the sorption capacity of the microorganisms attached to the inner surface of the copper pipes controls the release of copper at the end of the flushing experiment. Similar results were not observed in previous studies likely because the testing conditions did not provide consecutive sampling points for reviewing the microbial succession and its effects on copper corrosion. Moreover, the field conditions might have supplied the requirements or signals needed to change the EPS production, which would have modified the corrosion response.

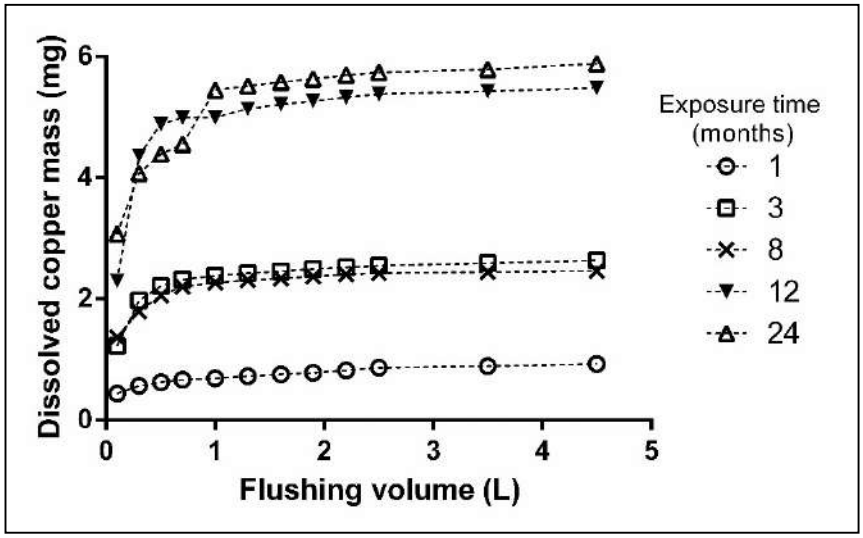

Figure 4. The average value of dissolved copper mass released from the copper pipe during flushing experiments.

On the contrary, Feng et al. (Feng, Teo, Siow, and Hsieh 1996) showed a direct relationship between the immersion time and the copper concentration during 21 days in neutral tap water. However, the analysis of dissolved copper did not show the same relationship in our research. The flushing experiments revealed three levels of dissolved copper in three discontinuous ranges of time after 1 L of water was passed through the pipe. The first level was reached in the pipe exposed for one month, with an average value of 0.5 mg of dissolved copper after 8 h of stagnation. The second level was achieved in the samples exposed for three and eight months, which had similar copper levels of about 2.2 mg and exceeded the WHO recommendation for the first liter of water flushed in the pipe (United States Environmental Protection Agency (EPA) 2009). Finally, an increment of dissolved copper was observed in the samples exposed for 12 and 24 months, both of which had an accumulated mass release of about 5 mg. Probably the shifts on copper release between the samples exposed to 8 and 12 months are the result of a different stage of biofilm development for each time. Additionally, an active replacement of biofilm (attachment/detachment) exposed the metal to the bulk water again, which might increase the release of copper after 8 months. The exposure under actual environmental conditions, allowed us to observe the different levels of copper released that was not appreciated in previous reports.

Consistently, after three months, the amount of dissolved copper mass and the peak concentration after passing 1 L of water through the pipe in the flushing experiments exceeded the WHO recommendation, which is considered to be harmful to humans (United States Environmental Protection Agency (EPA) 2009). Incorporation of the environmental and microbial aspects revealed in our research explains the differences from the results reported by Feng et al. (Feng, Teo, Siow, and Hsieh 1996). Moreover, it possible that the outcome of Feng et al. showed the initial process of corrosion, in which the chemical kinetics control the copper corrosion. Therefore, our results present biocorrosion as a dynamic process, whereby the biofilm´s characteristics can modulate the copper release.

2.3.4. Microbial Community Analyses

Analysis of the development of the biofilm attached to the inner copper pipe was conducted by DGGE fingerprinting (Figure 5). This approach revealed the manner in which microbial populations change with each tested condition. Thus, DGGE band profiles were assigned to the different phylotypes present on the DGGE gels, which enabled screening of the enriched microbial populations under the tested conditions. Knowledge of the type of bacteria attached to the surface for each sampling time will help to understand the stage of biofilm development and the possible mechanisms involved in the copper corrosion. The technique used in this analysis enabled the identification of several of the most abundant/dominant members of bacteria. The limited DNA concentrations obtained for most of the samples over time prevented a more detailed analysis.

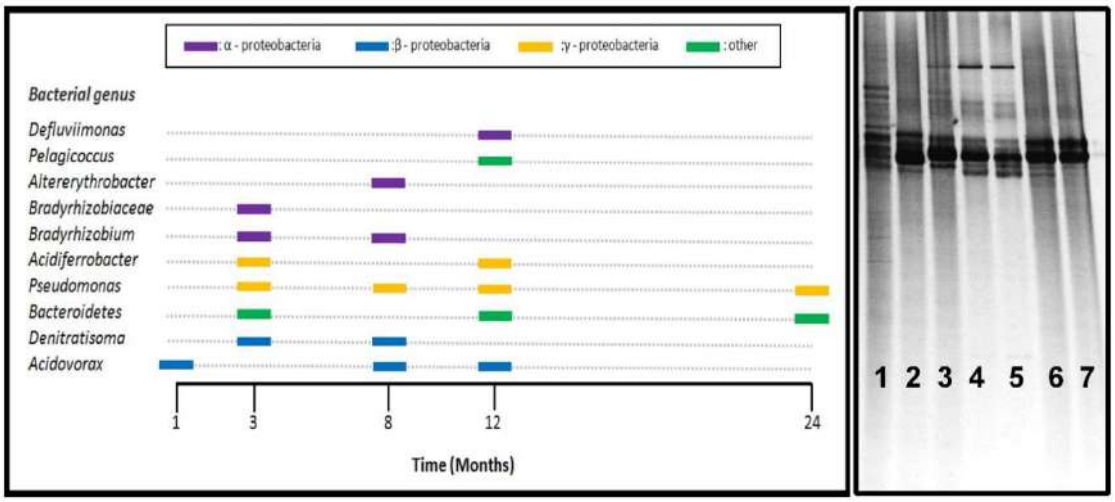

Figure 5. (Left) Diagram showing the distribution of bacterial phylotypes over time. (Right) Denaturing gradient gel electrophoresis (DGGE) profile of 16S rRNA of total bacterial community from bulk (line 1) and biofilm from inner copper pipes (line 2: 1 month; lines 3 and 4: 3 months; line 5: 8 months; line 6: 12 months; and line 7: 24 months). Summary of electrochemical results. OCP evolution (square) and variation of corrosion rate (circle) over time.

The results indicate the occurrence of active biofilm succession/dynamics during the testing time (Figure 3 and Figure 5). In drinking water systems, changes in the microbial community occur after even a few hours of stagnation (Lautenschlager et al. 2010); however, the populations that are part of the biofilm's core essentially do not change during the stagnation (Isabel Douterelo et al. 2016; Gomez-Alvarez, Revetta, and Domingo 2012; Revetta et al. 2010; Buse et al. 2014; J. Lu et al. 2014). For this reason, the stagnation time was ruled out as the primary change factor. Contamination of the water source could also explain the microbiological changes in the biofilm; however, this possibility was also discarded on the basis of Douterelo et al.(I. Douterelo et al. 2017) , who showed that the local biofilm community prevailed rather than the planktonic community or an external selective pressure (I. Douterelo et al. 2017). This means that the inclusion of a new biofilm's members depends on the selection process of the microbial community (or core) that develops the biofilm. Abiotic factors such as temperature, water quality, and aging time (Lautenschlager et al. 2010; Gomez-Alvarez et al. 2015; G. Liu et al. 2017) had stronger influences on the active renewal of biofilm because they have a greater effect on the physicochemical and biological process in the environment.

The DGGE fingerprints of the biofilm communities attached to the inner surfaces of the copper pipes showed that members of the genus Pseudomonas (Gammaproteobacteria) were the most persistent phylotypes during the sampling time (Figure 5). Formation of a mature biofilm can depend on the presence of Pseudomonas because they have a strong affinity and easy adherence to surfaces, which facilitates the attachment of other microorganisms (I. Douterelo et al. 2017; Dunne 2002; Kostakioti, Hadjifrangiskou, and Hultgren 2013). The mechanisms of Pseudomonas associated with copper corrosion are related to the capacity of the biofilm formation and its enzymatic activity. Regarding the latter, Pseudomonas has both oxidase and catalase activity, which generate a constant cycle of oxygen renewal on the surface of a copper pipe, thereby promoting an increase in the cathodic current and thus an increase in the overall corrosion process (Baeza et al. 2013; Hallberg, Hedrich, and Johnson 2011; Busalmen, Vázquez, and de Sánchez 2002). Our

results suggest that Pseudomonas form the main core of biofilm owing to their prevalence and other characteristics. The control of Pseudomonas attachment on a metallic surface would be an appropriate strategy for reducing or controlling the copper corrosion. Other studies have reported a similar prevalence of Pseudomonas in drinking water systems concerning corrosion and biofilm production (I. Douterelo et al. 2017; Hwang et al. 2012; Nercessian et al. 2010; Ren et al. 2015; Revetta et al. 2013; Cha and Cooksey 1991).

On the contrary, the metabolism of most of the other phylotypes present in these communities and recovered by DGGE are known to be involved in the nitrogen cycle; these include *Defluviimonas, Bradyrhizobium, Denitratisoma*, and some members of Bacteroidetes (Fahrbach et al. 2006; Foesel, Drake, and Schramm 2011; Giongo et al. 2008; Marcondes de Souza et al. 2014). Kelly et al. (Kelly et al. 2014) reported differences in bacterial abundance related to the availability of inorganic nutrients in drinking water, specifically nitrogen, and found a significant correlation between the bacterial cell numbers and the nitrate concentration (Kelly et al. 2014). The nitrification could promote metal corrosion owing to pH reduction, which increases the solubility of minerals on the surfaces of materials (White, Tancos, and Lytle 2011). Moreover, some nitrate-reducing bacteria (NRB) strains have the ability to induce NRB-assisted corrosion in other materials under anoxic conditions (Kato 2016); thus, similar behavior can be expected for copper. For these reasons and from a practical perspective, specific control of the nitrogen (nitrite and nitrate) concentration in the water would be an appropriate strategy for avoiding biofilm development and copper pipe corrosion (Melchers 2015, 2014).

Other phylotypes present at various sampling times include members of the Bacteroidetes phylum. Bacteroidetes members are commonly found in drinking water systems (Ling et al. 2016; Revetta et al. 2013; Kahlisch et al. 2010). They are a very diverse heterotrophic bacterial group with aerobic and anaerobic members that are able to degrade biopolymers (Navarro-Noya et al. 2013) related to degradation of the complex compounds derived from dead biomass (Cao et al. 2016). These phylotypes could influence copper

corrosion through the stimulation of a cathodic reaction by consuming electrons as the metabolic energy sources (Kato 2016). In oxic environments, a cathodic reaction represents a reduction in oxygen, although nitrate reduction is a major microbial metabolism process in anoxic environments. Bacteroidetes members likely have less of an influence on copper pipe corrosion when they occur deep in the biofilm, where the oxygen concentration is limited.

The biofilm development stages did not show a direct relationship with copper corrosion. Vargas et al. (I. T. Vargas et al. 2014) presented evidence of the differences between the biofilm community structures of field pipes and pipes tested in the laboratory. The results suggest a probable ecological succession over time, which was resolved in the present work. This conclusion is important because the order in which surfaces are colonized by either aggressive or protective bacteria influence the outcome with regard to MIC (Fallowfield et al. 2011). Moreover, Vargas et al. (I. T. Vargas et al. 2014) proposed that the age of the biofilm determines its effects on copper corrosion. However, our results indicate that the type of bacteria present on the surface is more important because the corrosion rate is strongly influenced by the kind of oxide on the surface, which depends on the specific activity of the microbial community.

To conclude the microbial analysis, a few groups of microorganisms were detected in specific sampling times (Figure 5). These bacteria, such as those of genus *Acidiferrobacter*, are able to decrease the water pH (Hallberg, Hedrich, and Johnson 2011; Bellenberg et al. 2014), which helps to increase both the corrosion rate and amount of copper released. However, the *Acidovorax* phylotype has shown a positive influence on copper corrosion and the formation of EPS (Bellenberg et al. 2014). The secretion of EPS and enzymes creates a hospitable zone for the establishment of areas with different potentials, thereby promoting an increase in the cathodic current and, thus, an increase in the overall corrosion process (Landoulsi et al. 2008; Baeza et al. 2013). The type of enzyme secreted is more relevant in stress events because under such conditions, the hydrogen peroxide (H_2O_2)

concentration increases, which triggers the secretion of catalase or peroxidase. The activity of these enzymes produces an increment of local oxygen concentration, which increases the kinetics of the cathodic reaction and, thus, the global corrosion process(Hector A. Videla and Herrera 2009).

Finally, our microbiological analysis is the first temporal local study to address the changes in microbial biofilm communities, which allows for evaluation of the temporal prevalence of the diverse phylotypes described previously in water distribution systems (Isabel Douterelo et al. 2016; I. Douterelo et al. 2017; Gomez-Alvarez et al. 2015; Revetta et al. 2013; Isabel Douterelo et al. 2014; I. Douterelo, Sharpe, and Boxall 2013; Fish et al. 2015; Martiny et al. 2003).

2.3.5. Electrochemical Tests

The evolution of the copper corrosion was evaluated through electrochemical techniques. Because of its simplicity, OCP has been used in numerous studies of MIC (Dexter et al. 1991). The changes in potential observed with this technique according to a plot of potential versus time enables the detection of an accelerated attack caused by bacteria (Dexter et al. 1991). In this work, the OCP measurements showed a positive trend during the pipe aging. This result means the metal surface was ennobled, which indicates the formation of a semi-protective film composed of oxides and microorganisms that can influence the reaction of oxygen reduction on the metal surface (Dexter et al. 1991) (Figure 6A). The most positive OCP value was slightly higher than zero ($0.03\ V \pm 7 \times 10^{-4}$) and was reached in the sample aged for eight months, whereas the most negative value was achieved in the sample aged one month ($-0.22\ V \pm 0.025$). The pitting potential was not detected during the study because the potential did not exceed the critical value of 0.10–0.17 V, as reported by Cornwell et al. (Cornwell, Wildsmith, and Gilbert 1973).

On the contrary, the results of linear polarization resistance (LPR) used to calculate the corrosion rate indicated a volatile value (Figure 6B). In the present study, the corrosion rate was almost seven times higher than for the unused pipes, from 3.4 mpy to 20.2 mpy. The highest value was reached in two sampling times, but likely the mechanism was different for each one. Probably, after three months of exposure, several micro galvanic cells were formed on the pipe's surface, producing a high corrosion rate. On the other hand, in the sample aged for one year, the high value of the corrosion rate is due to the attachment/detachment process of biofilm settle down. The variability of the data could be attributed to multiple patterns in the pipe, where the microorganisms are distributed heterogeneously on the copper pipe surface.

It should be noted that the OCP values showed a trend to become stable during the exposure time, but the corrosion rate did not show the same behavior. Moreover, samples with more positive OCP values did not show a lower corrosion rate, which suggests that a reactive barrier produced by microorganisms, EPS, and corrosion products formed during the pipe aging controlled the copper pipe corrosion.

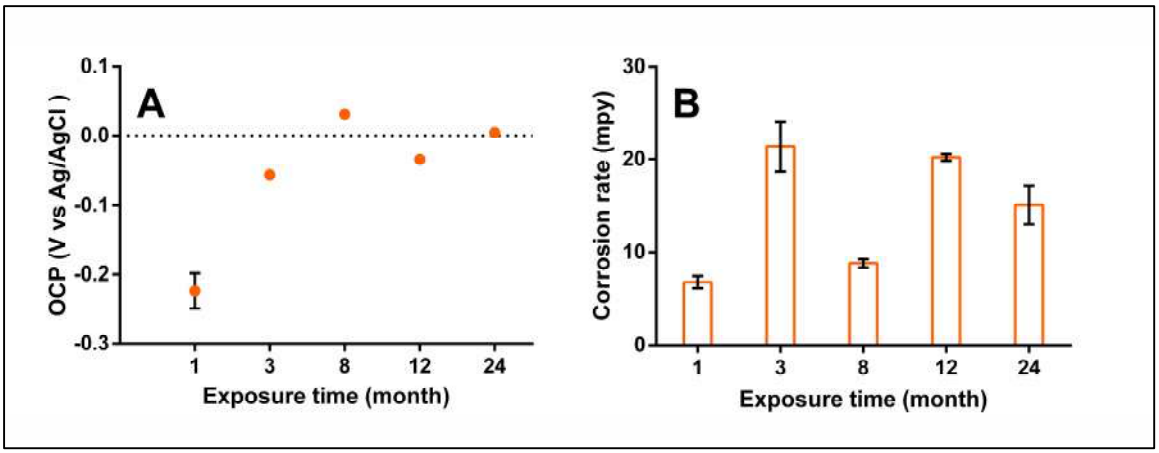

Figure 6. Summary of electrochemical results. OCP evolution (A), and variation in the corrosion rate (B) over time.

Our results suggest that the corrosion rate was modified by the presence of microorganisms. Similar results were reported by Vargas et al. (I. T. Vargas et al. 2014), in which the corrosion rate and the amount of copper released were compared under biotic and abiotic conditions. The assessment of both conditions suggests that the age of the biofilm determines its effect on copper corrosion (I. T. Vargas et al. 2014). However, the possible role of diverse biofilm members should also be considered because a small change in the biofilm members can produce a relevant change in the corrosion rate, as is described in this research.

Figure 7 shows the most representative Nyquist diagrams of copper pipes at different exposure times. The results indicate that a copper ion diffusion process controlled the corrosion, as implied by the Warburg impedance observed in the Nyquist plots. However, the diffusion process showed different behaviors at each sample time. At the initial times, the diffusional behavior observed was close to that reported by Feng et al. (Feng, Teo, Siow, Tan, et al. 1996) at a pH of approximately 5 under the abiotic condition with 24 h of immersion (Feng, Teo, Siow, Tan, et al. 1996), although a slight variation in the Warburg impedance angle was indicated. Frateur et al. (Frateur et al. 1999) discussed the changes in Warburg impedance during the immersion time. Their results showed changes in the Warburg angle from 45° to 22.5° with increases in the immersion time under abiotic conditions (Frateur et al. 1999), which was attributed to the electrode behavior as a semi-infinite porous medium. The impedance results in the present study reflect the development of oxide layers with a different type of porosity owing to the oxide layer composition of, which included a combination of biofilm and diverse corrosion products. This is supported by the angle values (

Figure 7) and SEM images (Figure 3). A specific electrochemical behavior for each sampling time cannot be assigned directly because a heterogeneous surface was observed,

which produces variations on the magnitude of Z_{Re} and Z_{im} values. However, the shape of the curve presented in Figure 6 is representative of each time.

Moreover, our results question the possibility of establishing a direct relationship between the corrosion rate and the age of biofilm using EIS measurements because the diversity of the pores on the reactive barrier and the chemical composition of the oxides could be modified by the action of a specific microorganism. The porosity modification on the reactive barrier can change the effective area of measurement and its apparent steady state; thus, measurements by EIS could not be compared for different sampling times. The resolution of these concerns, further studies must be conducted to determine the types of oxides produced and to examine the changes in porosity of the oxide layer caused by microbial activity.

Finally, owing to the dynamic process of MIC, the inclusion of additional parameters for the physical model did not help to solve the problem by allocating an appropriated equivalent circuit because each new parameter lacked physical representation and was determined with less confidence; similar results were reported by Frateur et al. (Frateur et al. 1999). In our case, the changes in the surface could have caused short-term fluctuation of the electrochemistry at the metal–biofilm interface, which in turn might have caused a localized attack under the biofilm. Thus, biofilm can hinder the detection of potentially important smaller-scale dynamics (Dexter et al. 1991; Martiny et al. 2003).

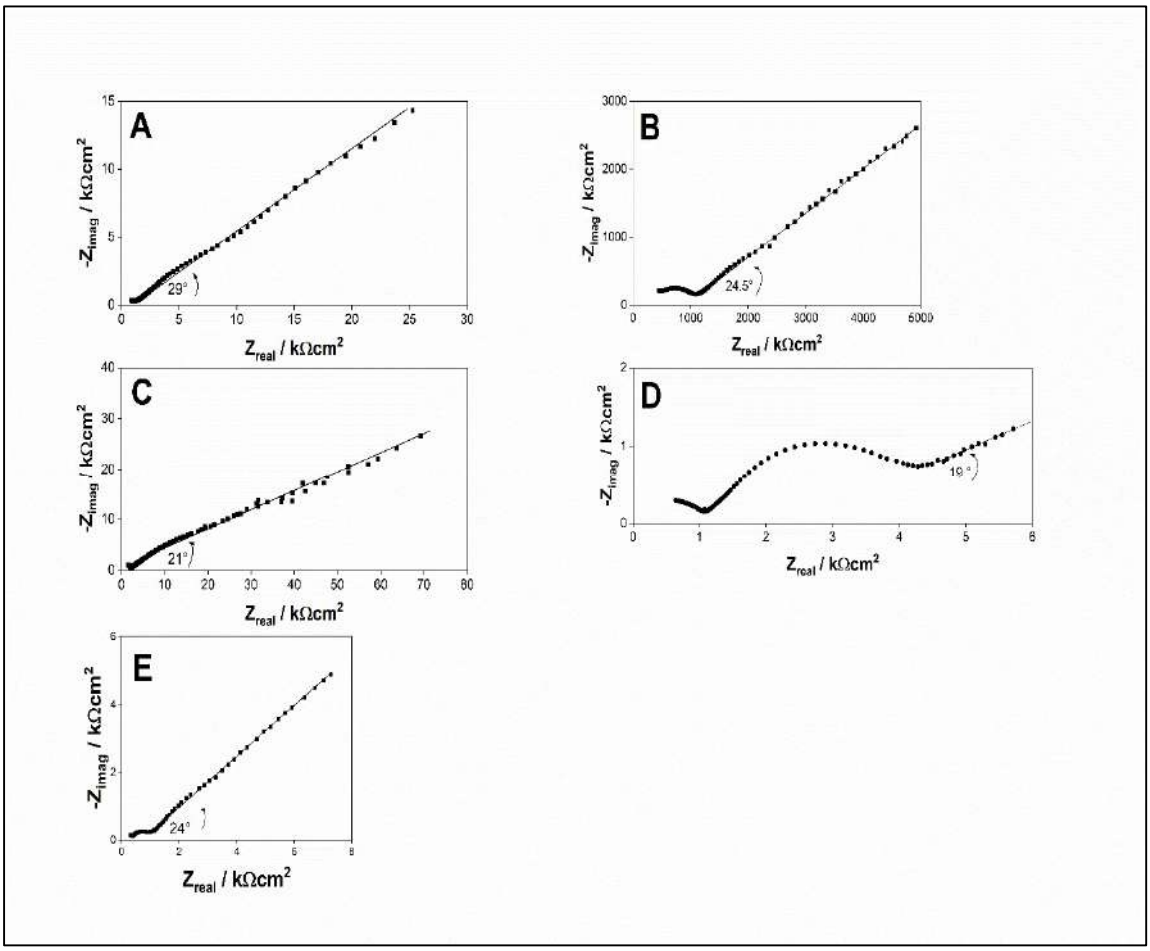

Figure 7. Summary of more representative electrochemical impedance results of samples exposed to A) 1 month, B) 3 months, C) 8 months, D) 12 months, and E) 24 months of aging.

2.4. Conclusion

This work highlights the relevance of studying MIC as a dynamic process. The results do not show a constant relationship among the exposure time, corrosion rate, and amount of copper released into the water. This finding revealed that the variability of a dynamic biological process cannot be characterized by a single aging time or by extrapolation of short-term experiments, particularly in an actual plumbing system. Indeed, our results pose new questions and challenges in understanding the selection process that defines biofilm composition, structure, and corrosivity. Further research is required to understand the specific succession/interaction mechanisms among members of the biofilm. This could be accomplished by using advanced sequencing techniques or by considering appropriate time scales and environmental parameter monitoring over time. Finally, a better understanding of MIC processes will enable the development of appropriate control strategies, which will prevent plumbing deterioration and reduce the risk of drinking water contamination by released copper.

2.5. Acknowledgments

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3. CHAPTER II: EFFECT OF HAZARDOUS BACTERIA ISOLATED FROM COPPER PLUMBING SYSTEM ON MICROBIOLOGICALLY INFLUENCED CORROSION OF COPPER

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Abstract

Plumbing systems can be affected by Microbiologically Induced Corrosion (MIC). Through this process, microorganisms can modify water quality and jeopardize consumers' health by releasing metal from pipes' surface into the water. While it is known that microorganisms' interactions increase their electrochemical effect on the metal surface, the effect of mixed communities and their interactions remain poorly understood. In this work, we investigated two hazardous bacteria isolated from a copper plumbing system, *Variovorax sp,* and *Ralstonia pickettii*. Electrochemical impedance spectroscopy (EIS) showed a changing of oxide layer properties depending on immersion time. At short times, a capacitive behavior was observed at the low-frequency range, transiently including an additional inductive loop. At long times of exposure, the capacitive behavior disappears, and a Warburg behavior is present at the low-frequency. Interestingly, the corrosion was inhibited in pure culture tests, but this effect was reduced when the bacteria formed a consortium. In fact, EIS data show that the highest inhibitor activity was presented by *Variovorax sp* pure culture, with 3.5-fold reduction in the corrosion rate compared with abiotic condition, and around of 2-fold when copper was exposed to *Ralstonia pickettii* and the consortium. XPS showed the formation of a different by-product of corrosion in the samples exposed to bacterial action. Moreover, SEM images revealed different bacterial growth behavior at the end of the test

period. This research highlights the relevance of understanding the interactions of drinking water microbial communities.

Keywords: Microbial corrosion; biofilm; copper; impedance; pathogen bacteria

3.1. Introduction

Microbiologically Induced Corrosion (MIC) or biocorrosion is the change in the corrosion process by the presence or activity of microorganisms (ASTM International G15-08 2010). This has a critical impact on on drinking water systems thus affecting infrastructure and human health (I. Vargas et al. 2017). The complexity of the interplay between microorganisms, metal surface, and products of abiotic corrosion, and their different reaction kinetics, has prevented a clear understanding of the process.

Historically, copper has been used to build household plumbing systems due to its antiseptic properties (Keevil 2004; Luo et al. 2017). However, bacteria have been found living in copper plumbing systems (Isabel Douterelo et al. 2016; J. Lu et al. 2014; Coenye et al. 2003; Lehtola et al. 2004; S. Liu et al. 2016; Reyes et al. 2008). Many of those are Gram-negative bacteria growing as a biofilm (Beale et al. 2013; Lin et al. 2013; S. Liu et al. 2016; Lytle and Nadagouda 2010; Vargas et al. 2014). This biological structure provides protection to various types of stresses such as disinfectants, pH, copper ions and temperature changes (Cloete 2003; Jungfer et al. 2013). One of the most important components of biofilm is the Extracellular Polymeric Substance (EPS), which helps to create a barrier that controls the diffusional process and chemical reactions with the environment. This barrier can favor the settlement of pathogenic bacteria, and thus, potential health hazards (Jost Wingender and Hans-Curt Flemming 2011; S. Liu et al. 2016). This risk has led to an increase of the chlorine levels used to disinfect and reduce the bacterial load, reaching levels above the range suggested by WHO guidelines and adding yet another health concern (Schwering et al. 2013).

The blue water phenomenon is the most extreme case of copper corrosion (Marc Edwards, Jacobs, and Taylor 2000). This occurs when high levels of copper are released into the water, giving a characteristic blue-green color (Critchley, Pasetto, and O'Halloran 2004; Marc Edwards, Jacobs, and Taylor 2000). Unfortunately, little information and understanding are available about of how bacteria influence this phenomenon.

This research presents with an opportunity to understand how biocorrosion occurs in a copper plumbing affected by blue water phenomenon. Here, two bacterial strains were isolated from such a system. One of them, *Variovorax sp*, has shown a high capacity to grow as a biofilm over copper pipes (Pavissich et al. 2010; Reyes et al. 2008), and it is able to tolerate high copper (127 $mg*l^{-1}$) (Pavissich et al. 2010) and chlorine concentrations (16 $mg*l^{-1}$ when growth like a biofilm) (Schwering et al. 2013). Moreover, a recent study reports the presence of transferable genetic elements, which could be involved in spreading antibiotic and disinfectant resistance genes to other bacteria nearby (Khan, Knapp, and Beattie 2016).

The second strain is *Ralstonia pickettii*, which is considered as an opportunistic pathogen (Armbruster et al. 2012; Sun et al. 2014). This species has been isolated from highly controlled clean environments such as hospitals (Coenye et al. 2003; Michael P Ryan and Adley 2013; M. P. Ryan, Pembroke, and Adley 2007) and ultrapure industrial water systems (Mijnendonckx et al. 2013; M. P. Ryan, Pembroke, and Adley 2007; Michael P Ryan and Adley 2013). In water systems, *R. pickettii* strains often form biofilms, making them more resistant to biocides and complicating their eradication (Mijnendonckx et al. 2013). Moreover, it has been reported that *R. pickettii* has antibiotic resistance genes (Khan, Knapp, and Beattie 2016) which confer a wide resistance to many antimicrobial agents (Michael P Ryan and Adley 2013). Furthermore*, R. pickettii* has shown a high resistance to copper concentrations (around 200 to 600 $mg*l^{-1}$) (Konstantinidis et al. 2003; Xie et al. 2010; Yang et al. 2010), allowing the bacterial growth in the copper plumbing system.

This study aims to understand how these two copper-tolerant bacteria modify the corrosion process in a copper plumbing system. Specifically, we will examine how bacterial interactions could affect the water quality by producing a higher release of corrosion by-products and/or pathogenic biofilm detachment.

Therefore, the electrochemical behavior of copper was evaluated through electrochemical impedance spectroscopy and surface analysis, when exposed to both, *Variovorax sp*, and *Ralstonia pickettii*. The results allowed to estimate the effect on copper corrosion produced by each bacterium and when they interacted forming a microbial consortium.

3.2. Materials and Methods

3.2.1. Isolation and Identification of Microorganisms

Copper pipe samples were taken from the suburbs of Olmué, Chile (33°00′S 71°12′O), a rural area that ha shown severe copper corrosion in their plumbing system. *Bacteria* were isolated from the inner wall of one-year-old copper pipes, using lysogeny broth medium (Difco™). The individual strains of *Variovorax sp* and *Rasltonia pickettii* and their consortium were cultured in a modified MSVP (Minerals Salts Vitamins Pyruvate) medium (Teitzel and Parsek 2003), with a concentration of pyruvate of $4{,}4 \cdot 10^{-4}$ M to ensure a minimum source of organic matter. MSVP was selected because it has been shown to minimize the complexation of heavy metals (Teitzel and Parsek 2003).

Bacterial strain identification was done through partial sequencing of 16S rRNA and alignment with the BLAST GenBank(Alvarado G. et al. 2017). 16S rRNA gene w amplified by PCR with the following settings: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The last extension step was longer (10 min). An aliquot of the PCR product was electrophoresed in a 1.0 % agarose gel (Cleaver, Scientific Ltd) and stained with

GelRed™(Biotium). Afterward, the concentration of the PCR product was estimated by comparing with a standard (Low DNA Mass Ladder; Gibco BRL) (Alvarado G. et al. 2017). The PCR product was sent to Macrogen Inc. for sequencing analysis.

3.2.2. Electrochemical Measurements

A typical three-electrode electrochemical cell was used. It consisted of a cylinder of PMMA (poly (methyl methacrylate) fixed on the copper sample surface, providing a working electrode area of 31,17 cm^2. A calomel electrode and platinum grid used as reference and counter electrode, respectively. The surface of copper was polished with fine sandpaper (2400-grit waterproof) and then it was cleaned with distilled water and acetone (ASTM G1-03 2017). Copper coupons were sterilized with alcohol, washed with distilled water and exposed to UV light for 15 min before performing the electrochemical test using a Biologic VSP device, operating at the corrosion potential.

Electrochemical impedance spectroscopy (EIS) was performed using a peak-to-peak sinusoidal potential perturbation of 10mV and a frequency range extended from 100 kHz to 3 mHz, with 8 points per decade. Three independent samples were analyzed for each time and conditions. The immersion time was 1, 5, 7, 14, 21, 28, 35 and 60 days. All measurements were obtained under static conditions. |Z| value was corrected as described by Tran *et al*. (Tran, Tribollet, and Sutter 2016). Results were analyzed through the fitting of a proposed equivalent circuit (EC) (Figure 8) using Zview® software. EC core was previously reported by Webster *et al*.,2000(Webster et al. 2000). The quality of fitting to the equivalent circuit was judged by chi-square ($\chi 2$) value. The capacitance elements in the electrical equivalent circuit were replaced by constant phase elements (CPE). The elements of the circuit are as follows: R_{bulk} is the solution resistance, R_{ct} is the charge-transfer resistance for copper dissolution, Cdl is the double-layer capacitance, W is a diffusion component representing diffusion of copper ions through the copper oxide film and/or biofilm, R_{pore} is the pore resistance, C_{film} is the oxide film/or biofilm capacitance, R_{ox} is the oxide resistance and C_{ox} is the oxide capacitance.

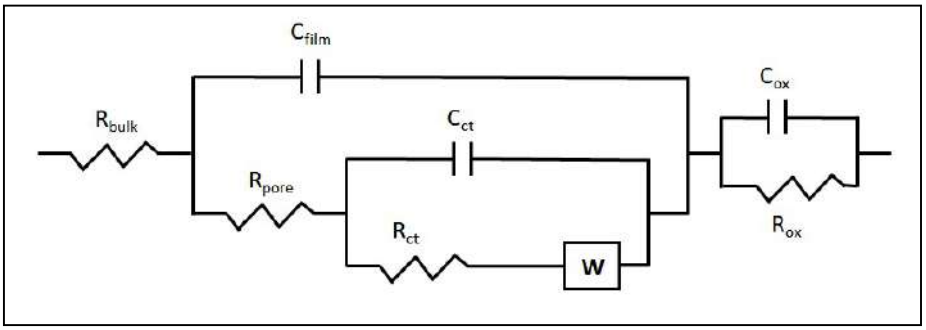

Figure 8. Equivalent circuit used to model to EIS data

3.2.3. Oxide Thickness Calculations

The thickness of the oxide layer (δ) was determined using two approaches the Cole-Cole plot and the power law model formula (Hsu and Mansfeld 2001; Hirschorn et al. 2009; Barrès et al. 2017; Benoit et al. 2016) after exposure to both sterile and inoculated MSVP medium. The thickness of the oxide layer was obtained according to Equation 8 (Eq. 8), where the permittivity of vacuum (ε_0) was considered as 8.8542 x10^{-14} F/cm, the dielectric constant of the oxide film was (ε), and C_∞ corresponds to the capacitance value obtained in the high frequency range of the Cole-Cole plot (Barrès et al. 2017).

$$\boldsymbol{\delta = \frac{\varepsilon \varepsilon_0}{C_\infty}} \qquad \text{(Eq. 8)}$$

Cole-Cole plots were obtained using the modulus of impedance corrected by ohmic-resistance (R_e), as previously described (Barrès et al. 2017). In the present work, R_e was obtained as numerical graphical analysis. However, the amount of corrosion by-products and/or metabolic residues of bacterial consortium could have influenced the precision of the R_e value. This effect is better explained by Nguyen *et al.*(Nguyen et al. 2017), where small changes in the determination of R_e produce significant changes in the value of $|Z|_{(f=5\ mHz)}$. However, the action of each isolated bacteria indicated that the $|Z|_{(f=5\ mHz)}$ values registered were higher than in other conditions (i.e., low corrosion was produced), but the thickness

values estimated were lower. The values calculated were compared using power-law model (Figure S 1).

3.2.4. Scanning Electron Microscopy

The topography and structures formed on the samples' surfaces were analyzed by scanning electron microscopy (SEM) at the end of the experiment. The images were acquired by using an LEO 1420VP microscope (Cambridge, UK). According to the methodology described by Pavissich *et al.* (Pavissich et al. 2010), coupons of 1.0 x 1.0 cm2 were used for microscopic analyses. Coupons were fixed with 2.5 % (w/v) glutaraldehyde to avoid the detachment of bacteria from the surface. Samples were rinsed with sterile distilled water, postfixed with 1% (w/v) osmium tetroxide for 1 h and dehydrated in serial ethanol (50 – 100%) and acetone (100%) baths. Finally, after dehydration, coupons were dried to a critical point and coated with an ultrathin gold layer.

3.2.5. X-ray Photoelectron Spectroscopy

X-ray Photoelectron Spectroscopy (XPS) was used to determine the nature of the corrosion by-products formed on the copper surface. XPS spectra were recorded on an XPS-Auger PerkinElmer electron spectrometer Model PHI 1257, which included an ultra-high vacuum chamber, a hemispherical electron energy analyzer, and an X-ray source that provided unfiltered K radiation from its Al anode (hv= 1486.6 eV). Pressure of the main spectrometer was in the range of 10^{-6} Pa during data acquisition. The binding energy (BE) scale was calibrated using the peak of adventitious carbon, which was set to 284.8 eV [Handbook of XPS]. The samples were studied without preparation.

3.3. Results and Discussion

3.3.1. Electrochemical measurements

3.3.1.1. Open Circuit Potential (OCP)

Figure 9 shows the OCP responses for all systems as a function of exposure time. The results show how the OCP values moved closer to zero at all conditions, which can be explained by a decrease in the corrosion currents densities associated with the formation of an oxide layer. However, at the end of the experiment, the OCP values were only positive in inoculated media.

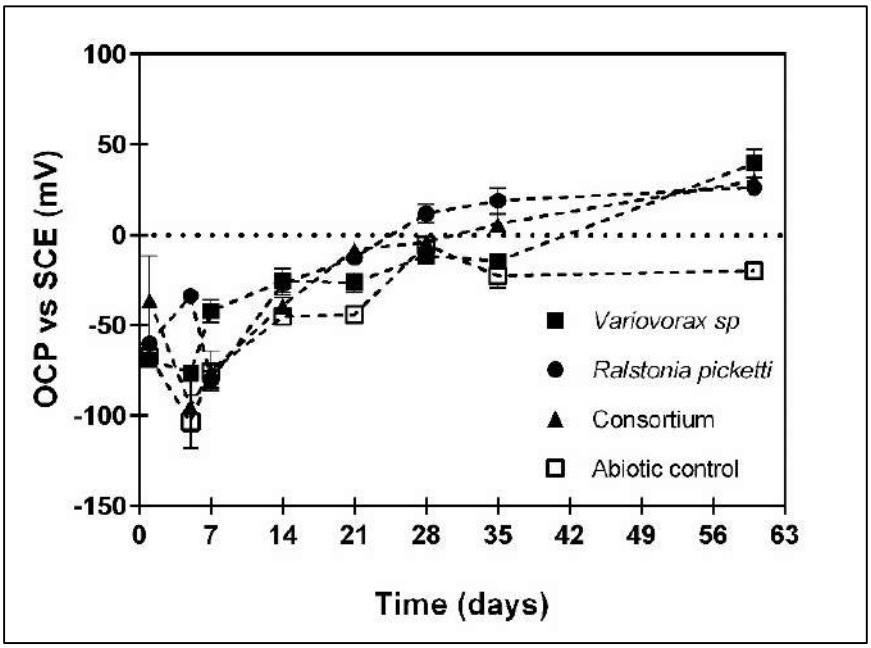

Figure 9. Open circuit potential of copper after exposure to MSVP inoculated with bacteria and abiotic control. Standard deviation bars are shown.

3.3.1.2. Impedance Results

The EIS results evidenced two different behaviors in function of exposure time. Figure 10 shows the Nyquist diagrams of copper after being exposed to MSVP in abiotic condition for 7 days. Nyquist diagrams revealed one capacitive loop at the range of high and medium frequencies, which can be associated with the formation of a porous film of Cu_2O. On the other hand, the inductive loop observed at low frequencies could be related to a relaxation process of adsorbed species due to the dissolution of copper (Sánchez et al. 2012).

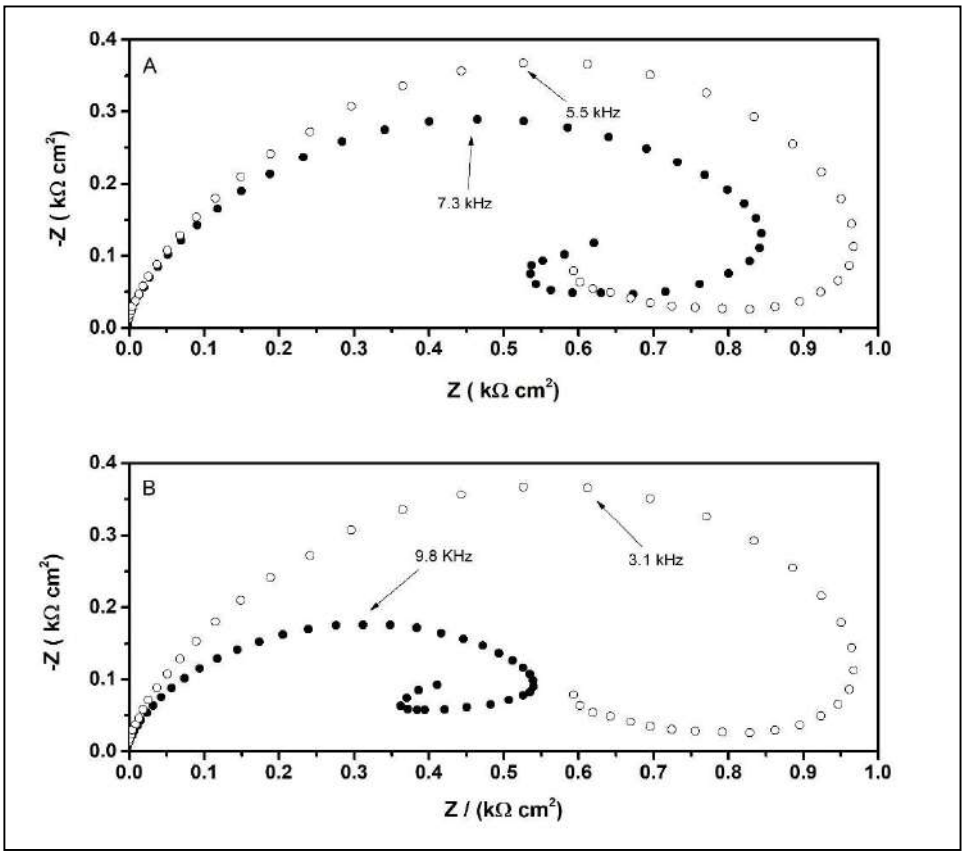

Figure 10. Nyquist diagrams of copper after exposure to MSVP inoculated with the bacterial consortium (A) and abiotic condition (B). Exposure time: 1 day (●) and 5 days (○).in abiotic condition.

After one week of exposure, EIS results revealed a different behavior in all conditions. At low frequencies, a Warburg behavior appeared (Figure 11). This is attributed to the oxygen-mass transport through the oxide film, similar to other metals (Mirzaeian and Hall 2010; Stratmann and Mûller 1994; Y. Wang et al. 2015).

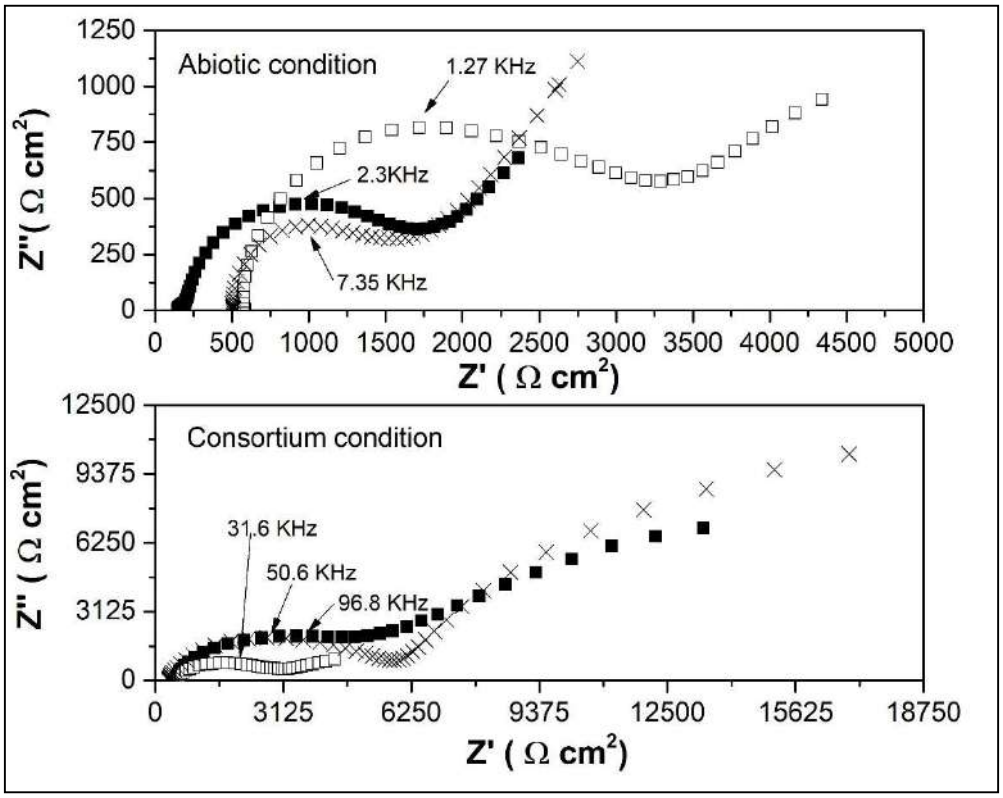

Figure 11. Nyquist diagrams of copper after exposure to abiotic conditions in MSVP as a function of exposure time. After 7 days (■), 21 days (□) and 35 days (×).

The impedance modulus at a low-frequency domain ($|Z|_{(f=5\ mHz)}$) was used as an approximation of the polarization resistance of the system for the analysis of impedance data. The values were greater than the abiotic control while the exposure time was longer, which might indicate a decrease in the corrosion rate, which is in accordance with the OCP results. Copper coupons exposed to *Variovorax sp*. and the bacterial consortium, showed a

decrease in the $|Z|_{(f=5\ mHz)}$ values between days 7 and 28, from 270 to 130 Ωcm^2 for *Variovorax sp*. and from 460 to 190 Ωcm^2 for the consortium. (Figure 12). This behavior could be attributed to the oxide dissolution reaction. In contrast, *R. pickettii* kept stable values of around 200 Ωcm^2, between 7 to 28 days of exposure. The difference between OCP data and impedance results can be explained by the interference of microbial on electrochemical turnover reactions that occur over the metal surface. The abiotic condition also had stable values, around 75 Ωcm^2, until the 35 days of exposure (Figure 12).

After 60 days of exposure, the $|Z|_{(f=5\ mHz)}$ peak average was reached for all conditions (Figure 5). In the abiotic condition, the $|Z|_{(f=5\ mHz)\ values}$ were lower than all the inoculated samples, which suggests that the bacteria have an inhibitory effect on the copper corrosion. The comparison of $|Z|_{(f=5\ mHz)}$ suggests that *Variovorax sp* pure culture was 1.8 times less corrosive than the consortium, and 3.5 time less corrosive than abiotic condition. On the other hand, *R. pickettii* was 11% less corrosive than the consortium, and 2.4-fold less corrosive that the abiotic condition. This indicate that the consortium is more corrosive than each bacterial pure culture. In summary, two aspects can be distinguished of electrochemical measurements. First, *Variovorax sp and R. pickettii* reduce the copper corrosion under the conditions used, which it is depending on exposure time after to compare with the abiotic condition, and second, bacterial behavior was different when microorganisms were put together to create a consortium.

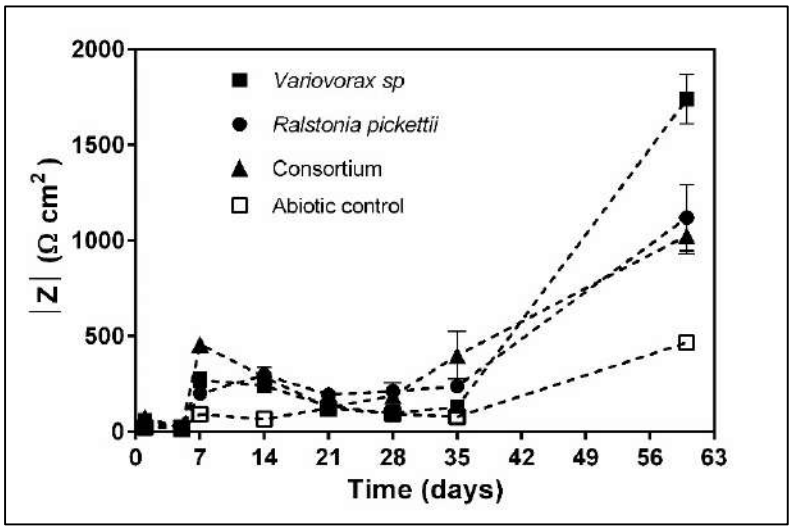

Figure 12. Average impedance modulus values of copper in MSVP medium as a function of exposure time. Standard deviation bars are showed. The modulus was estimated at five mHz.

EIS results can be mainly explained by the action of bacteria. Therefore, their mechanisms to offset the copper concentration are key to evaluate. For instance, *R. pickettii* possess a combination of adsorption and bioaccumulation capabilities, in conjunction with resistance mechanisms including influx and efflux pumps (Yang et al. 2010). Nevertheless, the discharge of some bacterial exudate (e.g. catalase or organic acid) in the interface oxide/metal (Baeza et al. 2013) cannot be ruled out, since it could also help in creating an environment that inhibits copper corrosion. However, its lower biofilm formation capacity on a copper surface resulted in it being less protected than *Variovorax sp.* On the contrary, *Variovorax sp.* can tolerate more copper due to its biofilm formation capacity. EPS could have affinity by cupric ions (Geesey et al. 1988), which could reduce the concentration of free copper ions in the surrounding environment, allowing bacterial settlement. Therefore, the changes observed in the $|Z|_{f=5\,mHz}$ values between 7 to 28 days may be related to bacterial attachment/detachment, suggesting that the difference in biofilm formation capacity could be an advantage to colonize the inner pipe. Notwithstanding, our data does

not show discernible kinetics for bacterial detachment, so this hazardous phenomenon merit further study. All in all, the impedance results indicated that copper corrosion was strongly affected by the microorganisms, as results showed an increase of $|Z|_{f=5\,mHz}$ values in comparison with the abiotic control (Figure 12).

The contribution of each component in the system was evaluated through a mathematical model. The data from Nyquist plots from day 7 to end time was fitted by using the equivalent circuit (EC) described in Figure 8. The results indicate that at the higher frequency relaxation, the n values were between 0.6 to 0.8, which are considered typical of the pure capacitive behavior of nonhomogeneous surfaces (Webster et al. 2000). No differences were observed in the resistance of oxide (R_{ox}) and bulk (R_{bulk}) among all conditions.

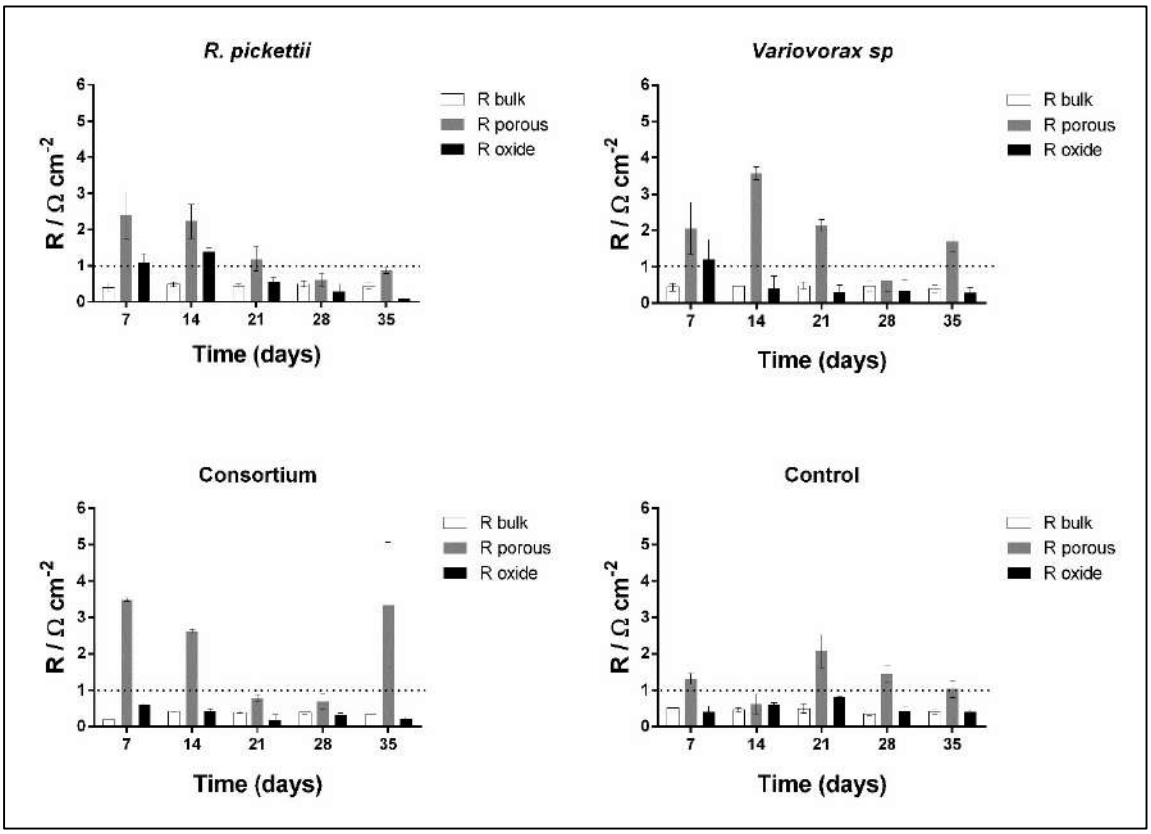

Figure 13. Average data values obtained for charge transfer resistances (R) until 35 days of exposure.

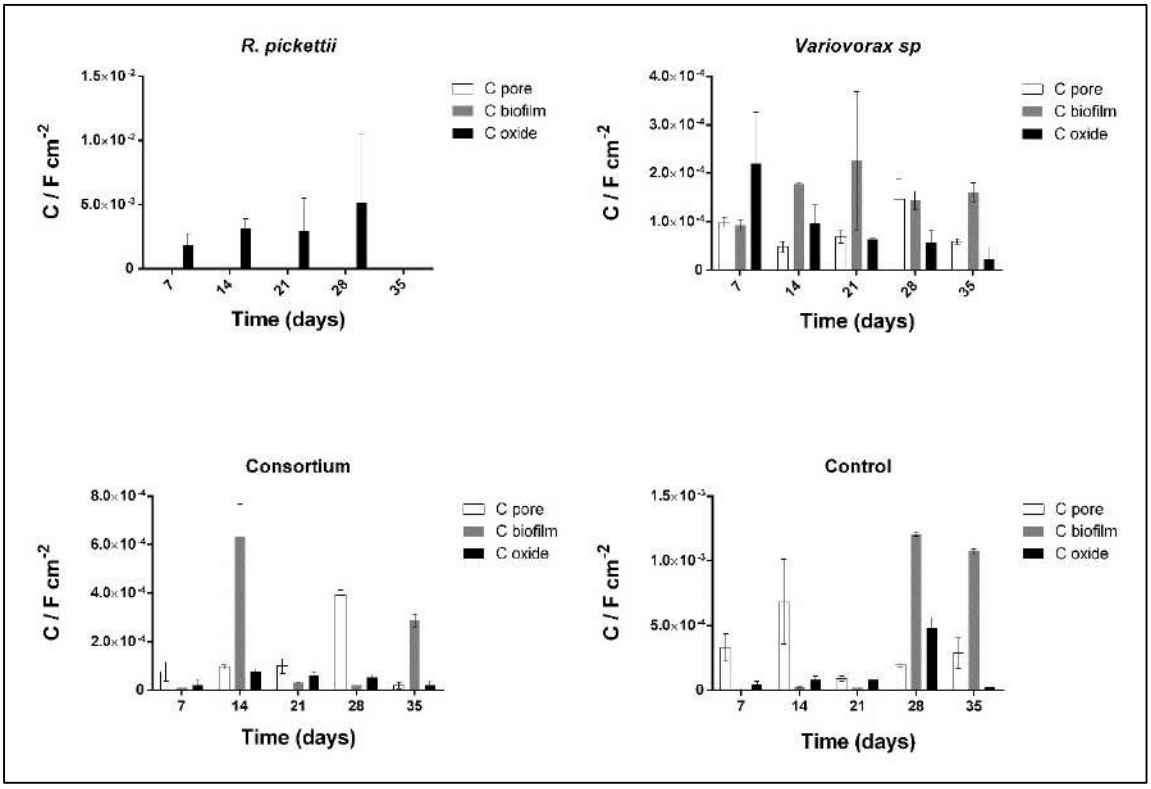

Figure 14. Average data values obtained for capacitances (CPE) until 35 days of exposure.

However, the distribution of resistance of pore (R_{pore}) changed depending on the time and condition studied (Figure 13). The highest values of R_{pore} were observed in the inoculated samples. The analyses of capacitances in *R. pickettii* samples showed a high influence of oxide, where the C_{ox} until 2 order of magnitude values than C_{film} (average $8x10^{-6}$) and C_{pore} (average $4x10^{-5}$) (Figure 14). These large differences in CPE oxide values indicate that the oxide films behave very differently in terms of charge transfer resistance

(R_{oxide}), while the resistances across the conditions are similar. This suggests that the bacterial action on the surface changed the oxide layer properties.

The thickness of the oxide layer (δ) was determined through the study of a Cole-Cole plot to evaluate the influence of this variable. The δ average values are shown in Figure 15. The results show each value obtained from Cole-Cole plot matched within the range calculated by the power-law model at each time and condition. Copper inoculated with the bacterial consortium showed the highest thickness values after 60 days of exposure. This method revealed the high variability of the oxide layer thickness on the copper coupons, especially in the case of the consortium after 60 days.

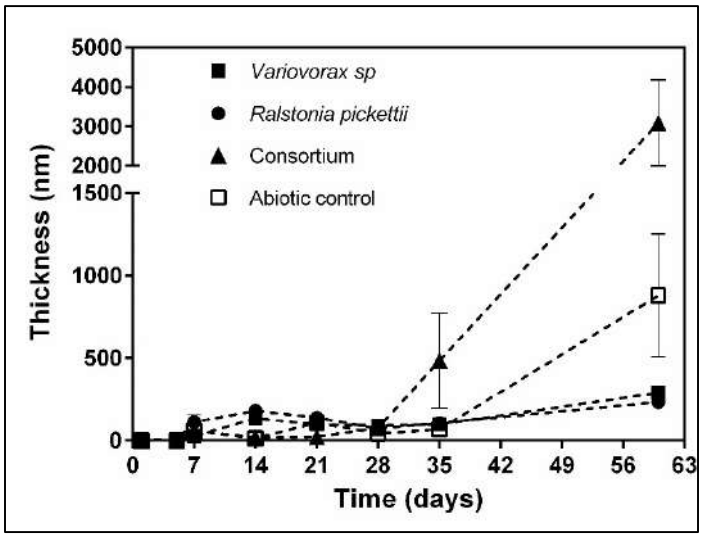

Figure 15. Variation of oxide layer thickness during the exposure time**.**

3.3.2. SEM Analysis

The surface of copper coupons after 60 days of exposure time in MSVP, in abiotic and biotic conditions, are shown in Figure 16. The abiotic condition shows a porous oxide layer, with surface cracks and ruts, together with some pitting on the surface (Figure 16.D). These conditions could promote the high reactivity of the surface, increasing the metal dissolution reaction. On the other hand, copper exposed to *Variovorax sp*. was covered by a biofilm (Figure 16.A). The picture also shows pitting and cracks over the surface. The irregular oxide layer of the surface suggests that copper released was the product of big detachments of copper oxide and bacteria.

A completely different picture was observed when copper was exposed to *R. pickettii*. The surface had deep grooves and cracks, but bacteria were nearly absent from the surface compared to the copper exposed to *Variovorax sp.* (Figure 16.B). Regarding copper exposed to the bacterial consortium, the surface showed damage to the conditions observed in the samples exposed to *R. pickettii,* but no bacteria were observed on the surface (Figure 16.C).

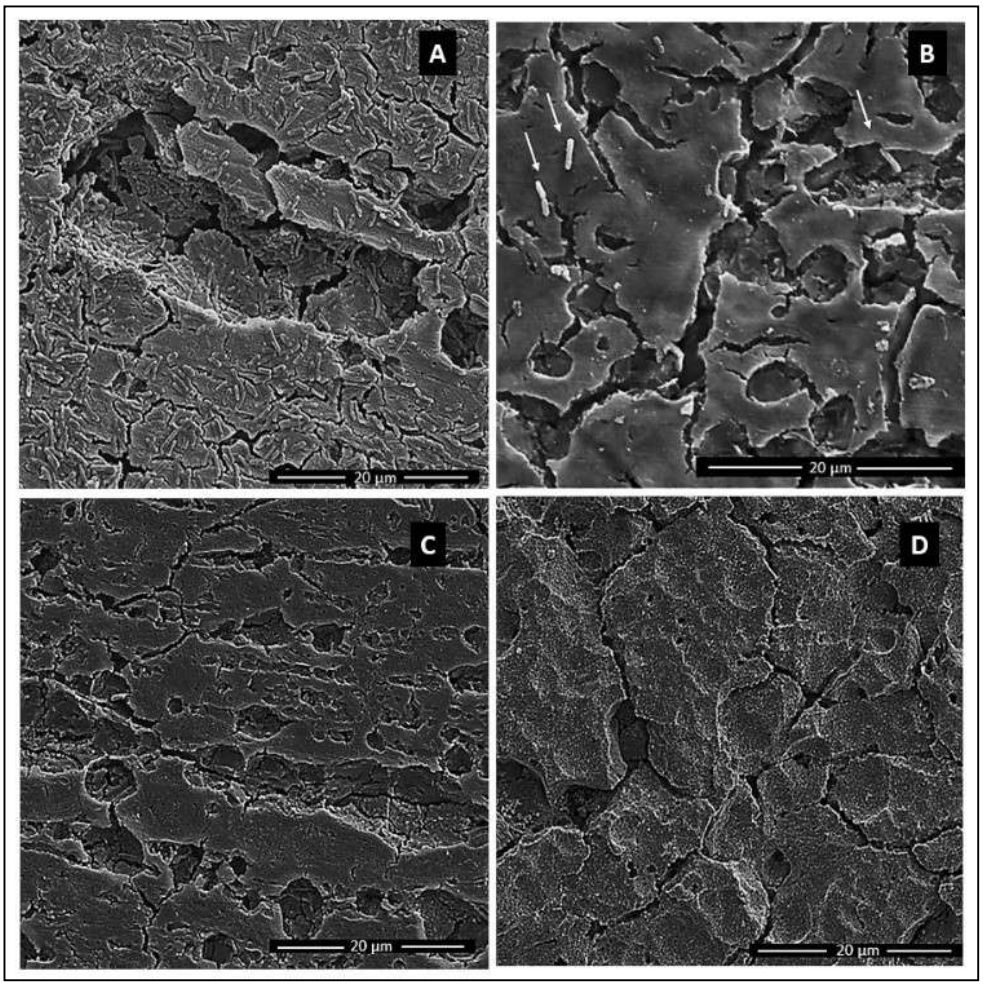

Figure 16. SEM images of copper coupons after 60 days of exposure time in MSVP. Copper inoculated with *Variovorax sp* (A), *Rasltonia pickettii* (B), Consortium (C), and Abiotic condition (D).

It is likely that the different EIS responses observed are caused by growing strategies of each bacterium, either as planktonic cells or as a biofilm. *Variovorax sp* showed a biofilm formation capacity on copper surface (Figure 16.A), which was also observed in previous studies (Reyes et al. 2008; Pavissich et al. 2010). On the contrary, *R. pickettii* did not show a good biofilm formation capacity over the surface at the end of the exposure time (Figure 16.B). In fact, it only a few bacteria were found when an extensive inspection was done.

This suggests that *R. pickettii* prefers to grow in the planktonic form in the study conditions. In previous reports, *R. pickettii* has been identified on biofilms in plastic water piping (M. P. Ryan, Pembroke, and Adley 2007), however, this was not observed on copper. Copper toxicity did not appear to affect this, as bacteria in the samples were alive when tested with a live/dead assay (Waines et al. 2011) (data not shown) This is consistent with reports of *R. pickettii* living in high dissolved copper conditions (Konstantinidis et al. 2003; Xie et al. 2010).

SEM images revealed no presence of bacteria on the copper surface exposed to the bacterial consortium. This suggests that the structure and the life cycle of biofilm is different when composed of two or more bacteria (Flemming et al. 2016). Inhibition of bacterial development due to the interaction between them has been reported (Héctor A Videla and Herrera 2005; Hector A. Videla and Herrera 2009; Philip J Bremer and Geesey 1991). This may be caused by a quorum sensing signal among the bacteria that conform the microbial community, which can grow as planktonic cells or as biofilm (S. Liu et al. 2016; Ramalingam 2012; Shrout and Nerenberg 2012).

The observed biofilm's detachment could be a hazardous condition when these two bacteria interact on a plumbing system, as they could increase the bacterial load of drinking water, *R. pickettii* may have acted as an activator of biofilm replacement, so we only observed the results of the biofilm detachment with SEM images (Figure 16.C). Nevertheless, the predation between the bacteria cannot be excluded (Flemming et al. 2016), since it was not possible to determine the consortium's species composition.

3.3.3. Surface Chemical Composition

Copper oxide layer composition developed over the metallic surface was obtained through the XPS technique after 60 days. XPS of the high resolution of copper revealed that the Cu2p signal had four components. The important ones were: Cu (0) +Cu(II) or Cu metallic + CuO at binding energy of 932.8 - 933.1 eV, Cu(I) or Cu2O at binding energy of

933.8 – 934.2 eV, and $Cu(OH)_2$ "hydroxylated copper" at binding energy of 935.1 – 935.8 eV. This last signal was only detected for samples with bacteria. A similar result was observed by a previous study using XAS (Calle et al. 2007). The cuprite (Cu_2O) was the predominant species in all conditions (Figure 17). No significant statistical differences among the samples exposed to bacteria were showed.

Cuprite (Cu_2O) has been reported as a passivating agent in copper corrosion and it grows over time (Shim and Kim 2004). Furthermore, the literature suggests the cuprite film limits the diffusion of copper ions controlling copper corrosion (I. Vargas et al. 2017). However, this research indicate that biological activity was the main factor to affect copper corrosion, because of the similar percentages of cuprite found in all conditions by XPS analyses. Perhaps this amount of cuprite was not enough to protect copper from corrosion in the experimental conditions utilized. On the other hand, the percentage of copper hydroxide ($Cu(OH)_2$) was higher in the copper exposed to bacteria. This could be another indication of the relevance of biological activity on copper corrosion since thermodynamics do not predict its presence in these conditions (pH=7). However, thermodynamic calculations based on bulk chemistry may not be accurate, since microbial activity can change the local chemical environment, inhibit oxide growth kinetics and decrease oxide film thickness (I. T. Vargas et al. 2014). Probably, bacteria change the electric potential or pH of their surrounding metal surface, which allows copper hydroxide production to be thermodynamically feasible as reported by Calle et al. (Calle et al. 2007). Therefore, pH could have shifted to values near 8, a point where copper hydroxide can be generated as reported by *Obrecht et al* (Obrecht and Pourbaix 1967). On the other hand, other studies that used EIS suggested that biofilms on copper create an acid microenvironment (Webster et al. 2000; Vargas et al. 2017). However, each research is specific for each microorganism used. This situation indicate the needed to develop standardized tests for evaluate MIC (Wade et al. 2017).

The behavior of copper in abiotic conditions was in agreement with the results presented by *Ives et al* (Ives and Rawson 1962) where a non-defined porous oxide layer was formed (Figure 16.D). However, its thickness determined using both the Cole-Cole and power-law model were less than in other studies (Feng, Teo, Siow, Tan, et al. 1996; Shim and Kim 2004). Medium components such as organic matter or orthophosphate could modify the protective character of the oxidation film on the copper surface (I. T. Vargas et al. 2009; Pehkonen, Palit, and Zhang 2002) . Despite the impact of the medium in accelerating corrosion, it is clear the bacteria have an inhibitory effect on the electrochemical behavior of copper. A corrosion-inhibitory effect has been reported for another bacteria (Kip and A van Veen 2015), however, few reports have shown electrochemical results of bacterial interaction as it was done in this study. EIS results helped to understand how the biofilm acts as diffusion barrier and produced experimental evidence of possible interactions between bacterial species in a biofilm, underscoring the need to further develop this research.

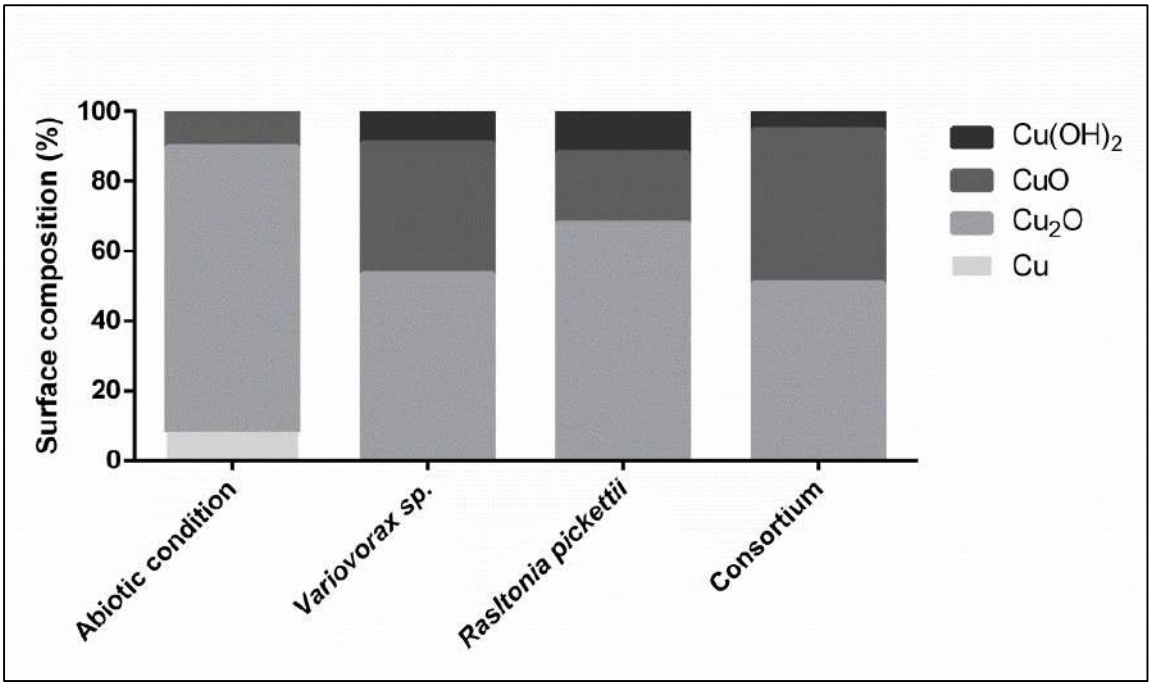

Figure 17. Surface composition of copper samples after 60 days of exposure.

3.4. Conclusion

This study shows the effect of *Variovorax sp* and *Ralstonia pickettii* on copper corrosion. Both bacteria inhibit copper corrosion in the tested conditions, increasing the protective properties of cuprite layer due to the development of biofilm and other copper oxides on the surface during the exposure time. However, further studies are required to understand the interaction between these bacteria, as highlighted by the striking loss of *Variovorax sp*'s biofilm formation capacity when interacting with *Ralstonia pickettii*. This research provides evidence to support the potential danger of tap water microbial contamination due to biofilm detachment of bacterial consortiums.

SUPPLEMENTARY MATERIAL

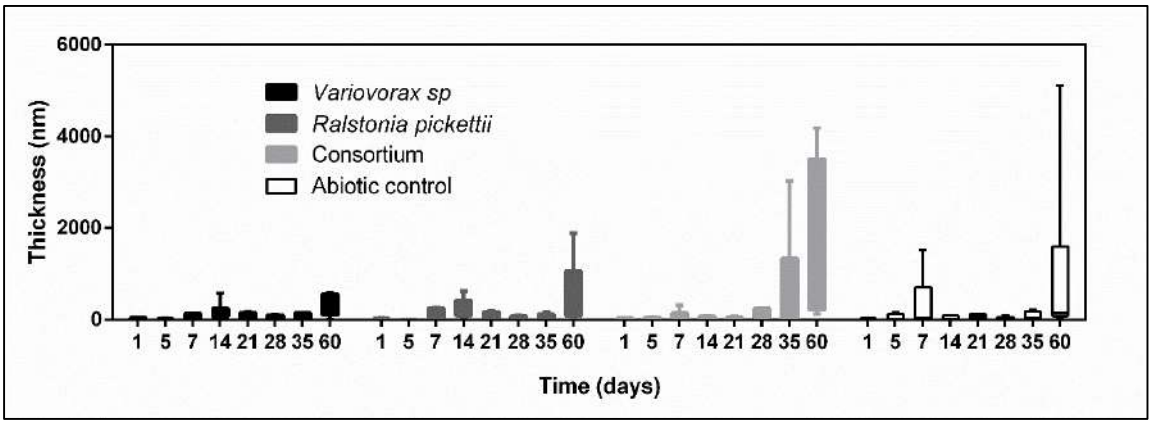

Figure S 1. Range of thickness of oxide layer during exposure time using power-law model.

3.5. Acknowledgements

Special thanks to Nadine Pébère for all advice and methodological support in the determination of oxide layer thickness.

4. CHAPTER III: FT-IR CHARACTERIZATION OF BACTERIAL ISOLATES RELATED TO CORROSION OF COPPER DRINKING WATER PIPES

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Abstract

The formation of extracellular polymeric substances (EPS) is critical for the development of biofilms in diverse areas. Despite decades of research, we still have not developed a full understanding of the composition and function of EPS components. This situation is especially relevant when the microbial expression of EPS modifies the metal concentration of tap water. In this study, we isolated two microorganisms from a copper plumbing system. The functional groups involved in copper biosorption were characterized by potentiometric titration, modification of functional groups by chemical treatment, and FTIR analysis. The results indicate that the isolate corresponding to Variovorax sp. has a greater tolerance of copper (2 mM) in the culture medium than that of Ralstonia pickettii. The observation of growth suggests that the previous stage of biofilm formation (as flocs) provided an advantage to Variovorax sp. Moreover, methylation of amines by chemical modification increased the copper biosorption by 30% and 34% for Variovorax sp. and Ralstonia pickettii, respectively. However, the chemical surface analysis did not show significant differences between the bacterial isolates. This research highlights the relevance of understanding both the environmental context of the microorganisms, and the cellular phase of development in the EPS expression.

4.1. Introduction

Drinking water distribution systems (DWDS) can be seen as extreme environments, considering the oligotrophic conditions that exist where a disinfectant residual is maintained, as is common (I. Douterelo, Sharpe, and Boxall 2013). Despite this, microorganisms can survive in them, mainly due to biofilm development. Biofilms are one of the most widely distributed modes of life on Earth, in which a high cell density is composed of many species, and cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) (Flemming et al. 2016). EPS is one of the essential components of biofilms because it helps to create a barrier that controls the diffusional processes and chemical reactions with the environment (Galarce et al. 2019; I. T. Vargas et al. 2014).

The production of EPS is essential for biofilm development because of its vital role in the process of cell attachment to metal surfaces (Beech and Sunner 2004; Beech et al. 2002; Beech 2004). The EPS content varies depending on the bacterial species and growth conditions (Ras et al. 2011; Miqueleto et al. 2010; De Philippis et al. 1991; Beech 2004). One of the fundamental properties of EPS is their ability to form complexes with metal ions, which involves interactions between the metal ions and anionic functional groups that are common in the protein and carbohydrate components of exopolymers (Beech 2004; Beech and Sunner 2004). The affinity of EPS for different metals can be strong, depending on the environmental conditions (Comte, Guibaud, and Baudu 2008; Yue et al. 2015; J. Wang et al. 2014). The capacity and affinity of binding metal ions of EPS in the biofilm create two potential problems in the copper pipes of DWDS. First, the affinity for metal ions in different oxidation states in the biofilm matrix can result in substantial shifts in the standard reduction potentials (Beech and Sunner 2004), which can promote the corrosion process by the formation of microenvironments and subsequent localized corrosion. Second, the biofilm developed on the pipes of the DWSD can release harmful metal ion concentrations into the water, creating a potential health hazard (Calle et al. 2007; Galarce et al. 2020).

In copper pipes, microorganisms and the EPS matrix accumulate soluble copper by mineralization and sorption of labile copper (I. T. Vargas et al. 2014), which increases the copper concentration when a detachment of corrosion by-products occurs, increasing the copper released into the drinking water (Olivares et al. 2014; I. T. Vargas et al. 2010; Calle et al. 2007). Despite efforts to understand the sorption process in copper pipes, there are still some mechanisms that are not completely understood. The critical point is to determine the functional groups present in the EPS of the microorganisms that are part of the biofilms in copper pipes.

The development of a reactive barrier on the surface of a copper pipe can compromise water safety. A previous study on copper release in plumbing systems indicated that the surface serves as a storage compartment of labile copper, which may be released under flow conditions when a reactive barrier is attached to it (Calle et al. 2007). Stagnation experiments and an analysis with a hydrodynamic model indicated that diffusion was not the principal mechanism that governs the release of copper from copper pipes (Calle et al. 2007). Pizarro *et al.* (2014) (Pizarro et al. 2014) developed a mathematical model which suggests that the complexation of Cu-biomass and hydrodynamic mechanisms are the most important factors for copper release into water. Therefore, knowledge of how a chemical modification of a bacterial EPS can enhance the accumulation and subsequent release of copper to a DWDS is a big step in developing strategies for reducing this microbial hazard.

The development of a stronger understanding of the biosorption capacity of microorganisms in a DWDS is necessary if we consider the study developed by Kragh *et al*. (2016)(Kragh et al. 2016), which provided new evidence of how free-floating biofilm aggregates can have a profound local effect on biofilm development when attaching to a surface. These results also highlight a further development in the understanding of the microenvironment of local bacteria under actual conditions, since if favorable conditions exist in the pipe, chemical modification of functional groups can occur. This situation would increase both the metal sorption and the potential danger of copper contamination of tap

water due to metal accumulation in the pipe. This study used two copper-tolerant species of bacteria isolated from the inner wall of a one-year-old copper pipe, extracted from a one-year-old DWDS that was affected by a severe case of microbially influenced corrosion (MIC). These bacteria are *Variovorax* sp. and *Ralstonia pickettii*, which have been reported both as copper-tolerant and as biofilm-forming colonizers of the inner walls of pipes (Pizarro et al. 2014; Pavissich et al. 2010; Galarce et al. 2019). Additionally, both bacteria represent high-potential health hazards, which makes it essential to understand how their EPS groups can be involved in the complexation of copper, and the risk of its accumulation in the water distribution system. Therefore, the functional groups syndicated with an active role in copper biosorption were identified by potentiometric titration, chemical treatment, and FTIR analysis. The results of this study should contribute to a better understanding of the role of biomass in the accumulation of copper in MIC-affected pipes used in DWSD.

4.2. Materials and Methods

4.2.1. Sample Collection and Bacterial Strains

Biological samples were obtained from a microbial biofilm formed on the inner wall of a copper pipe that had been tested for one year in an actual household DWDS affected by MIC (Galarce et al. 2019). Biofilm suspension samples were grown on R2A agar plates at 30°C for four days, and the isolates were subsequently purified and stored at 4°C. Pure DNA was extracted using the Wizard DNA extraction kit (Promega, Madison, WI, USA), and identification was performed by partial sequencing of 16S rRNA gene amplicons using Sanger sequencing. The strains were taxonomically classified using the BLAST database. Polymerase chain reaction (PCR) conditions for the 16 rRNA gene were as described by Galarce *et al.* (2019)(Galarce et al. 2019).

4.2.2. Culture conditions and biomass preparation

Pure bacterial isolates were grown in sterile Erlenmeyer flasks using 150 mL of minimal salt vitamin (MSV) medium at pH 6.5, which contained (per liter): 1 g of $(NH_4)_2SO_4$, 0.06 g of $MgSO_4 \cdot 7H_2O$, 0.06 g of $CaCl_2$, 0.02 g of KH_2PO4, 0.03 g of $Na_2HPO_4 \cdot 7H_2O$, 2.383 g of HEPES, 1 mL of 10 mM $FeSO_4$, and 1 mL of a trace vitamin solution. The trace vitamin solution contained (per liter): 20 mg of biotin, 20 mg of folic acid, 50 mg of thiamine HCl, 50 mg of D-(+)-calcium pantothenate, 1 mg of vitamin B12, 50 mg of riboflavin, 50 mg of nicotinic acid, 100 mg of pyridoxine HCl, and 50 mg of p-aminobenzoic acid. Pyruvate ($5.45 \cdot 10^{-2}$ M) was added as the carbon source, creating an MSVP medium (Teitzel and Parsek 2003). Once inoculated, the bottles were kept for four days on a shaker at 50 rpm, at room temperature (23 ± 2°C).

For the superficial and functional groups study, 150 mL of saturated cultures were transferred to 50-mL centrifuge tubes and centrifuged at 5000 rpm for 10 minutes, with the supernatant discarded after each new centrifugation step. The concentrated biomass was washed three times in Milli-Q water and dried through sublimation using a FreeZone 1 model instrument (LabConco)(Fang et al. 2014). Finally, the dried samples of bacteria were stored in a vacuum desiccator for further use.

Bacterial growth performance at different copper concentrations was evaluated using aliquots of each isolate extracted from saturated cultures, which were exposed to a new MSVP medium with two copper concentrations (0.2 and 2 mM). The high copper concentration tolerated by *Variovorax* sp. was used to compare the performance between the bacteria. The copper value was selected from prior research carried out by Pavissich *et al*. (2010)(Pavissich et al. 2010). The growth curves were measured in microplates using an EPOCH 2 instrument (BioTech) at an absorbance of 600 nm (A600).

4.2.3. Morphological characterization

The samples were assayed by the Gram stain protocol, using immobilized live cells from exponentially growing cultures (Horikoshi and Bull 2011). Floc morphology was determined using a light microscope (CX31, Olympus) at 100× magnification. The bacterial size was estimated using live cells from exponentially growing cultures and dyed with acridine orange. The cellular size was determined by scanning electron microscopy (SEM) (LEO 1420VP microscope Cambridge, UK).

4.2.4. Superficial chemical analysis of each isolate

A biomass chemical analysis of each isolate was carried out through the characterization of its pH zero charge and functional groups, which were analyzed by chemical treatment and FTIR analysis.

4.2.4.1. Potentiometric titration and point of zero charge determination.

The point of zero charge was determined using the method described by Fiol *et al*. (2009)(Fiol and Villaescusa 2009). The procedure used a 0.03 M KNO_3 solution to obtain a constant ionic strength in pH_{pzc} determinations. A dried bacterial mass of ≤ 10 g/L was placed in contact with a 0.03 M KNO_3 solution. The mixture was agitated for 24 h in an orbital shaker at 200 rpm, until the pH of the sample was constant. Vigorous agitation was needed to ensure the homogenization of the bacterial suspension. Subsequently, the bacterial suspensions were titrated by adding 0.05 mL of HNO_3 (0.1 M) under continuous agitation, for the acid part. The pH value was recorded as a function of the added volume of the titration solution. The same treatment and procedure were used for a blank solution (0.03 M KNO_3). For the basic part, the titration was performed with KOH (0.1 M). Potentiometric titrations, as well as pH measurements, were carried out with a Titrino 794 (Metrohm) instrument at 25°C.

A similar procedure was carried out to determine the acidic and basic sites on the biomass of the isolated bacteria. The total acidic sites were neutralized using NaOH (0.1 mol/L), and the basic sites with HCl (0.1 mol/L), using the Titrino 794 (Metrohm) instrument at 25°C. The potentiometric titration curve was obtained by plotting the volume of the titrant against the recorded pH.

4.2.4.2. Modification of functional groups by chemical treatment of the biomass

After analyzing the potentiometric titration curves of the dried bacterial biomass of both bacterial isolates, three modifications were carried out: methylation of amines, and modifications of sulfhydryl and carboxyl groups. After each treatment, the biomass was washed with ultra-pure water and filtered using Whatman no. 42 filter paper. The modified biomass was then dried until a constant weight was obtained using a Freezone 1 (LabConco) instrument.

Methylation of amines was carried out by treating 1 g of biomass with 20 mL of pure formaldehyde and 40 mL of pure formic acid, as reported by Ramrakhiani *et al*. (2011)(Ramrakhiani, Majumder, and Khowala 2011). The reaction is described in Equation 9 (Eq.9).

$$\boldsymbol{RCH_2NH_2 \rightarrow RCH_2N(CH_3)_2 + CO_2 + H_2O} \quad \text{(Eq. 9)}$$

Modification of the sulfhydryl group was carried out by dithiopyridine treatment, and the carboxyl group was modified using concentrated HCl and ethanol. Both procedures were carried out using the same specifications as reported by Ramrakhiani *et al*. (Ramrakhiani, Majumder, and Khowala 2011).

4.2.5. FTIR spectroscopy

FTIR spectra of modified and non-modified functional groups of the biomass were obtained using the IRPrestige-21 SHIMADZU FTIR instrument. The samples were pressed into spectroscopic quality KBr pellets with a sample/KBr ratio of approximately 1/100. The FTIR spectra were recorded in the 4000–300 cm^{-1} region (Schmitt and Flemming 1998). The IR spectra were collected at room temperature, and were obtained by co-adding 128 scans with 4 cm^{-1} spectral resolution (Gerbino et al. 2011).

4.2.6. Copper sorption experiments

Batch experiments were carried out at room temperature (23 ± 2°C) in stoppered glass tubes. The experiments consisted of shaking a fixed mass of ≤ 0.3 g of each bacterial isolate with 20 mL of 3 mg/L Cu (II) solution adjusted to the pH range 4-6, in triplicate. This pH range was chosen because under the experimental conditions used, copper is in solution as free Cu^{2+}, and total copper precipitation is not achieved at the highest pH value investigated (Fiol and Villaescusa 2009). The tubes were placed in a rotary mixer (LSI-3016r, Labtec) and agitated at 50 rpm for 24 h to ensure that equilibrium was reached. After agitation, the solid was removed by filtration through a 0.45 μm cellulose filter paper (Millipore Corporation). Total copper was measured using the bicinchoninate method (HACH #8506) with a HACH DR/2010 portable spectrophotometer (I. T. Vargas et al. 2009).

4.3. Results and Discussion

4.3.1. Microbial characterization

As shown in Figure 18, both bacterial species were rod-shaped and gram-negative (data not shown). The size of the first bacterium was ~ 4 μm (Figure 18.A), and the second was ~ 2 μm (Figure 18.B). Molecular characterization based on 16S rRNA identified the more prominent bacterium as *Variovorax* sp. and the other as *Ralstonia pickettii*.

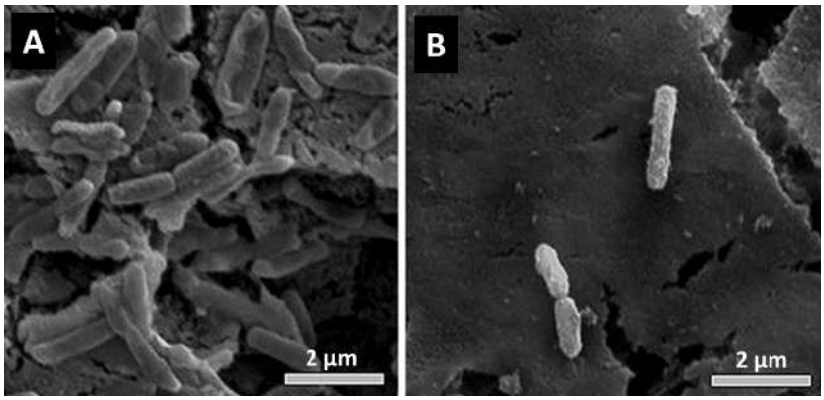

Figure 18: SEM images of isolated bacteria: *Variovorax* sp. (A) and *Ralstonia pickettii* (B).

These two bacteria are good models for studying the microbial effect on copper pipe corrosion in DWSD, since both are (i) copper-tolerant, which allows them to respond to an adverse environment; (ii) classified as potential health hazards, owing to pathogenic genomic information; and (iii) prevalent in drinking water pipelines (Pavissich et al. 2010; Galarce et al. 2019).

Previously, the electrochemical behaviors of both bacteria were reported by Galarce *et al*. (2019)(Galarce et al. 2019). This research showed an inhibitory effect on copper corrosion by both bacteria, owing to biofilm formation and precipitation of copper oxides, which was not found in abiotic conditions. It has been reported that both bacteria can grow in the presence of copper (Khan, Knapp, and Beattie 2016; Konstantinidis et al. 2003; Xie et al. 2010; Yang et al. 2010; Pavissich et al. 2010; Galarce et al. 2019), but there is no information about how their growth is affected by the copper concentration.

The results showed different growth behaviors between the two isolates as the copper concentration increased in the culture medium. The growth curve of *Variovorax* sp. presented a more prolonged and slower growth pattern than *Ralstonia pickettii* in culture conditions without copper or with a low copper concentration (0.2 mM). The growth curve of *Variovorax* sp. did not show a noticeable peak after 100 h under any conditions studied (Figure 19.A). On the other hand, the growth peak of *Ralstonia pickettii* appeared close to

20 h for conditions without copper or with a low copper concentration (0.2 mM) (Figure 19.B). The comparison among the growth curves with high copper concentration (2 mM) in the culture media showed a better response by *Variovorax* sp. than *Ralstonia pickettii*, because the shape of the curve of *Variovorax* sp. showed a delayed and reduced bacterial growth, but for *Ralstonia pickettii* no growth was observed (Figure 19.B).

The analysis of growth curves suggests that *Ralstonia pickettii* has a better tolerance at low dissolved copper concentrations, in comparison with *Variovorax* sp., and prefers to grow in the planktonic form. This is inferred from the higher value of OD_{600} using 0.2 mM of copper in the culture medium. *Ralstonia pickettii* can grow under dissolved copper concentrations lower than 0.2 mM, owing to the capacity of its resistance system. This bacterium has a combination of adsorption and bioaccumulation capabilities, in conjunction with resistance mechanisms, including influx and efflux pumps (Yang et al. 2010). It is likely that the resistance system is over-saturated at higher copper concentrations and the medium begins to become toxic to the bacteria. This can probably explain why *Ralstonia pickettii*, despite its metabolic machinery and reported high tolerance of copper (Michael P Ryan and Adley 2013; Khan, Knapp, and Beattie 2016; Yang et al. 2010), showed a worse capacity to grow under high copper concentrations than *Variovorax* sp. in this study.

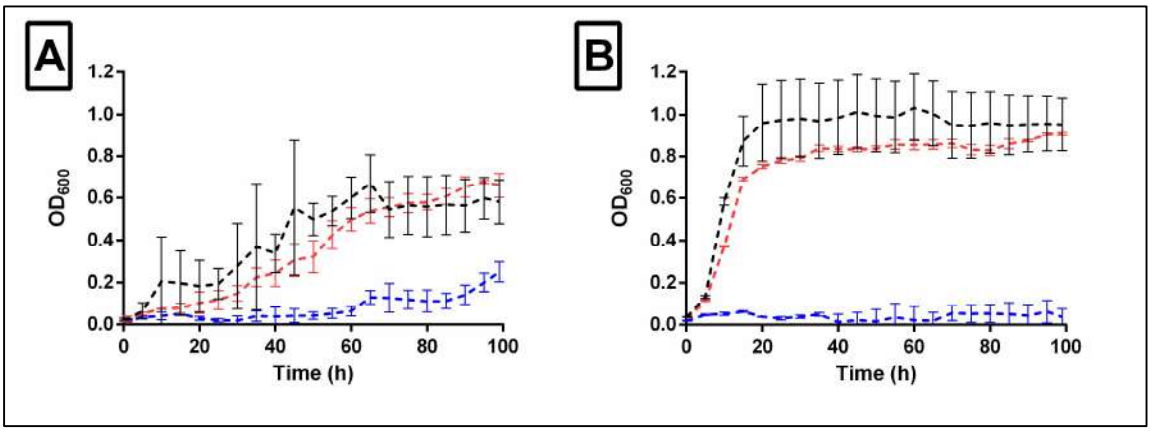

Figure 19: Growth curves of *Variovorax* sp. (A) and *Ralstonia pickettii* (B). Black line: without copper in the medium; red line: with 0.2 mM of copper; blue line: with 2 mM of copper. Standard deviation bars are shown.

On the other hand, *Variovorax* sp. showed better tolerance to dissolved copper (2 mM). This better capacity is probably due to both EPS production and the type of bacterial association in planktonic form. In this study, *Variovorax* sp. tended to create flocs, which would explain the shape of the growth curve under the conditions used. The floc formation probably provided a structure that gave protection against copper. Previously, Charles *et al.* (2017) reported the reduction of pH stress for some bacteria in alkaline environments owing to floc formation. That study showed that floc formation and EPS production are survival strategies under hyperalkaline conditions (Charles et al. 2017).

The floc structure of *Variovorax* sp. varied depending on culture time; moreover, the irregular size and shape of the flocs made them difficult to measure and quantify. Therefore, it was not possible to make an appropriate photographic record (Figure 20). It is possible that floc formation was a previous stage of biofilm development, a capacity previously reported in *Variovorax* sp. (Pavissich et al. 2010; Khan, Knapp, and Beattie 2016; Galarce et al. 2019).

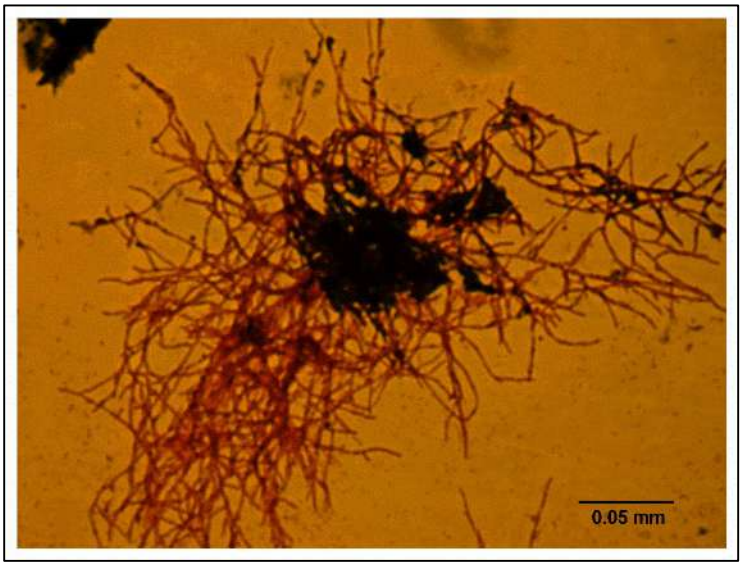

Figure 20: Optical microscope image of *Varivorax* sp. flocs at 100×

4.3.2. Potentiometric titration and point of zero charge determination

The test for the determination of acidic and basic sites and functional groups present on the cell walls of the biomass was performed by potentiometric titration. The results provide a rough characterization of the biomass. Only the basic curve displayed two inflection points. The corresponding pKa values suggest that the binding functional groups are present on the cell walls of the biomass of both isolated bacteria (Figure 21). According to the potentiometric titration curve, it may be inferred that the acidic group was comparable to the carboxylic group, and the alkaline group was comparable to the values reported for amines and sulfhydryl (thiol) (Table 3).

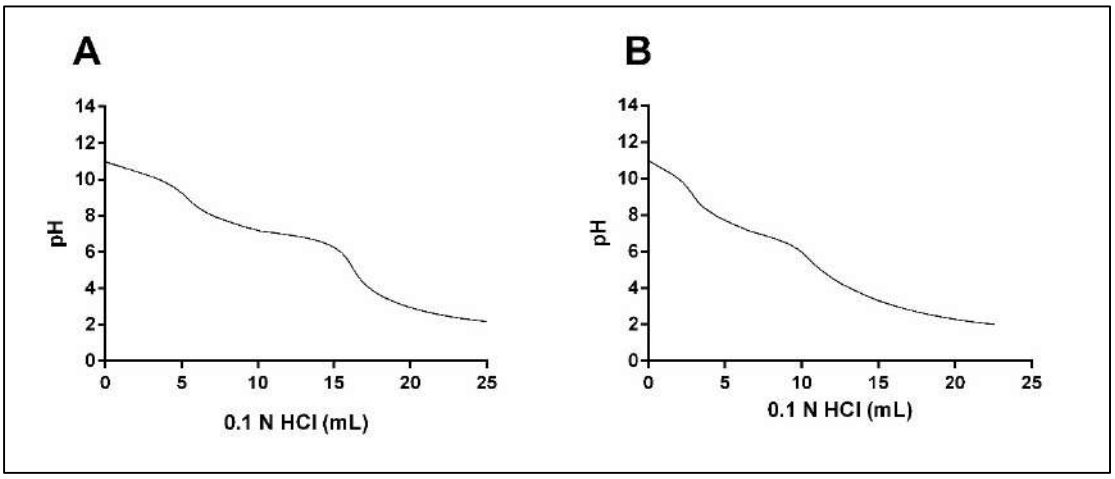

Figure 21: Potentiometric titration curves of biomass obtained without copper in the medium. Determination of basic sites and functional groups of *Variovorax* sp. (A) and *Ralstonia pickettii* (B).

The point of zero charges did not show differences between the two analyzed isolates; in both cases it was nearly a neutral value. Both curves displayed two inflection points, which correspond to pKa values suggesting the presence of binding functional groups on the cell walls of the biomass (Ramrakhiani, Majumder, and Khowala 2011). The pKa values are listed in Table 3. These results suggest that sulfhydryl, amine and carboxyl groups are responsible for the absorption process.

Stehr *et al.* (1995) reported that EPS production gives protection at different levels, including an extensive capacity for binding particulate and dissolved materials, as well as the cells of other bacterial species. The mechanism involved includes changing the pH values or salt concentrations of the medium (Stehr et al. 1995). Our results suggest that the development of an organized structure would probably improve the response to copper, because the isoelectric point of the bacterial surface would then be similar to that of copper.

Table 3: Summary of functional groups determination and PCZ values using potentiometric titration.

Species	**PCZ**	**pH initial**	**pH final**
Variovorax sp.	7.11	5.6	7.92
Ralstonia pickettii	7.06	5.6	7.4
pk_a values	**Functional groups**		
4.29	Carboxyl		
8.48	Sulfhydryl(thiol) and amine		

Note: Functional groups determination by potentiometric titration reported previously (Ramrakhiani, Majumder, and Khowala 2011; Fein et al. 2005).

4.3.3. Chemical modification of the functional groups of the biomass

The functional groups on the binding sites were investigated through chemical modification, where the potential site was altered by introducing a new functional group that might have a higher affinity for metal binding. The main effects of the functional group modification of the biomass are shown in Table 4.

Table 4: Effect of chemical modification on the biosorption of dissolved copper.

Isolate/Modification	**Methylation of amino**	**Sulfhydryl groups**	**Non modificated**
Variovorax sp	30% ± 1.6	6% ± 1.3	3.3 ± 0.33
Rasltonia pickettii	34% ± 1	3.3% ± 0.19	4.5 ± 0.84

Note: The data show the average remotion percentage of three independent measurements.

The methylation of amino groups produced an increase in the biosorption of copper in both bacterial isolates. *Variovorax* sp. enhanced the biosorption by 30% at the equilibrium phase, while *Ralstonia pickettii* increased the biosorption by approximately 34%. In comparison, the non-modified biomass showed a biosorption value of 3.3% for *Variovorax* sp. and 4.5% for *Ralstonia pickettii*. No relevant differences were observed after the carboxyl and sulfhydryl modifications. The increase in biosorption may be due to the acidic conditions, which might have caused the exposure and availability of binding sites other than the amine groups on the cell surface, as has been reported previously (Ramrakhiani, Majumder, and Khowala 2011).

An increase in the biosorption of dissolved copper can create a severe problem in drinking water systems. Galarce *et al*. (2020)(Galarce et al. 2020) measured the copper dissolved in flushing experiments with copper pipes of different ages. The results showed values more than 10 times higher than those reported previously. This increase was produced by the sorption capacity of the microorganisms attached to the inner surfaces of the copper pipes, which controlled the release of copper at the end of the flushing experiment. This suggests that specific localized conditions in the surrounding surface produced by the microorganisms themselves can enhance metal biosorption and increase its later release. In this study, specific chemical modifications of the biomass substantially increased its biosorption, which supports the argument presented above. For this reason, further research should be conducted to determine how the biosorption of local microorganisms can be changed and what the magnitude of these changes is.

4.3.4. Characterization using FTIR spectroscopy.

FTIR spectra of isolated bacterial biomasses were obtained to verify the interactions between the copper and the functional groups on the bacterial surfaces. The FTIR spectra of *Variovorax* sp. and *Ralstonia pickettii* are shown in Figure 22 and Figure 23, respectively.

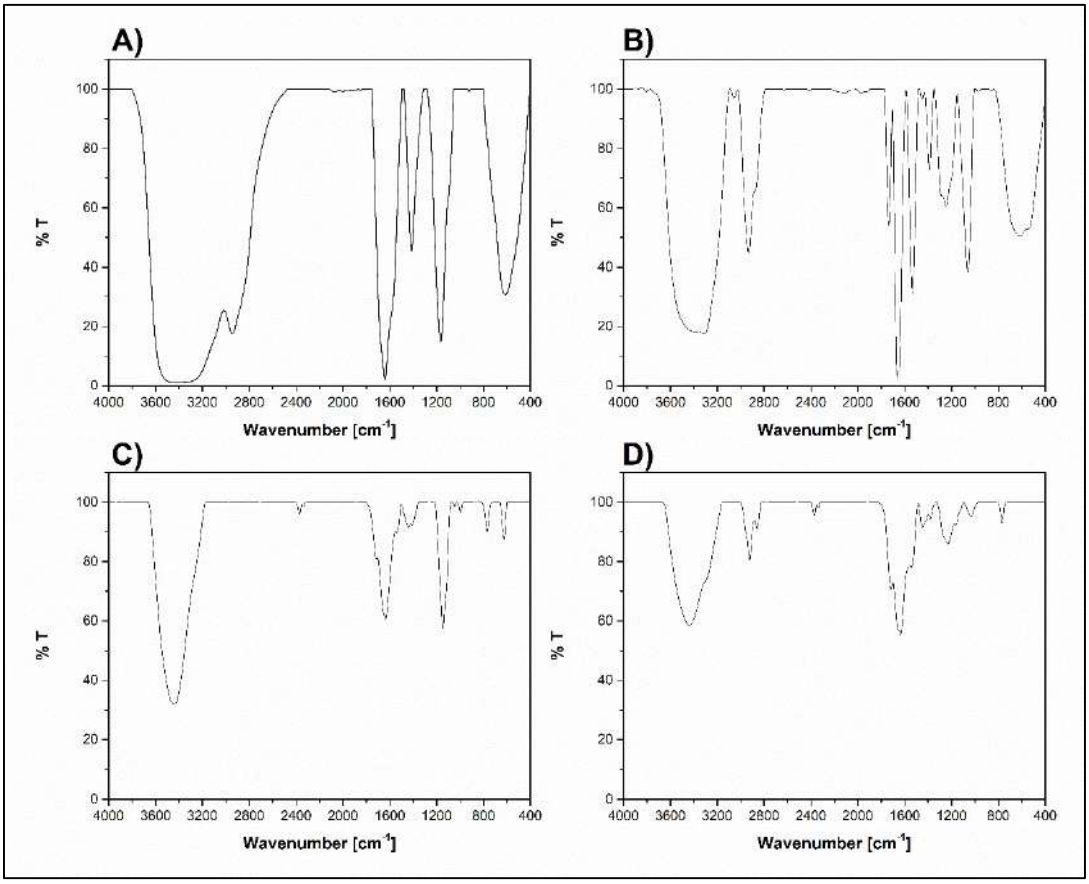

Figure 22: FTIR spectra of *Variovorax* sp. before and after methylation of amine groups. (A) FTIR spectrum of *Variovorax* sp. biomass growth without copper in the medium; (B) FTIR spectrum of *Variovorax* sp. biomass growth with copper in the medium; (C) after chemical treatment and before contact with copper; and (D) after chemical treatment and in contact with copper.

The surface characteristics of *Variovorax* sp. grown without copper or with low copper concentration (0.2 mM) in the culture medium are shown in Figure 22. A and 22.B, respectively. The definition of the peak close to 2850 cm^{-1} and the emergence of peaks between 1600 and 1200 cm^{-1} reveal that the phosphate groups and fatty acids were exposed on the surface in response to the chemical modification of the amine groups.

The *Variovorax* sp. biomass with the modified amine group exhibited FTIR spectra with a band ranging from 1500 to 1650 cm^{-1}, which specifies primary, secondary and tertiary amines, and ammonium salts of carboxylic acid compounds, in solid-state samples (D. Lu et al. 2009; Gerbino et al. 2011; Ramrakhiani, Majumder, and Khowala 2011). Moreover, the presence of a peak around 1200 cm^{-1}, which indicates the asymmetric P=O stretch, shows the participation of phospholipids from the cell membrane (C. Yu and Irudayaraj 2005) (Figure 22.C). On the other hand, after methylation of the amino group, and contact with copper (Cu (II)), there was an apparent disappearance of the peak at 1200 cm^{-1} corresponding to phosphate groups in Cu(II) - loaded bacterial biomass (Figure 22.D). This shift indicates the complexation of the phosphate group's coordination with copper (Ramrakhiani, Majumder, and Khowala 2011). The appearance of a peak at 2850 cm^{-1} could reveal the exposure of fatty acids after methylation, allowing copper to sorb onto the surface. Similar results were observed in the biomass without treatment, but only the modified biomass increased the absorption of dissolved copper. It is likely that the chemical modification exposed the functional groups, facilitating the binding with copper.

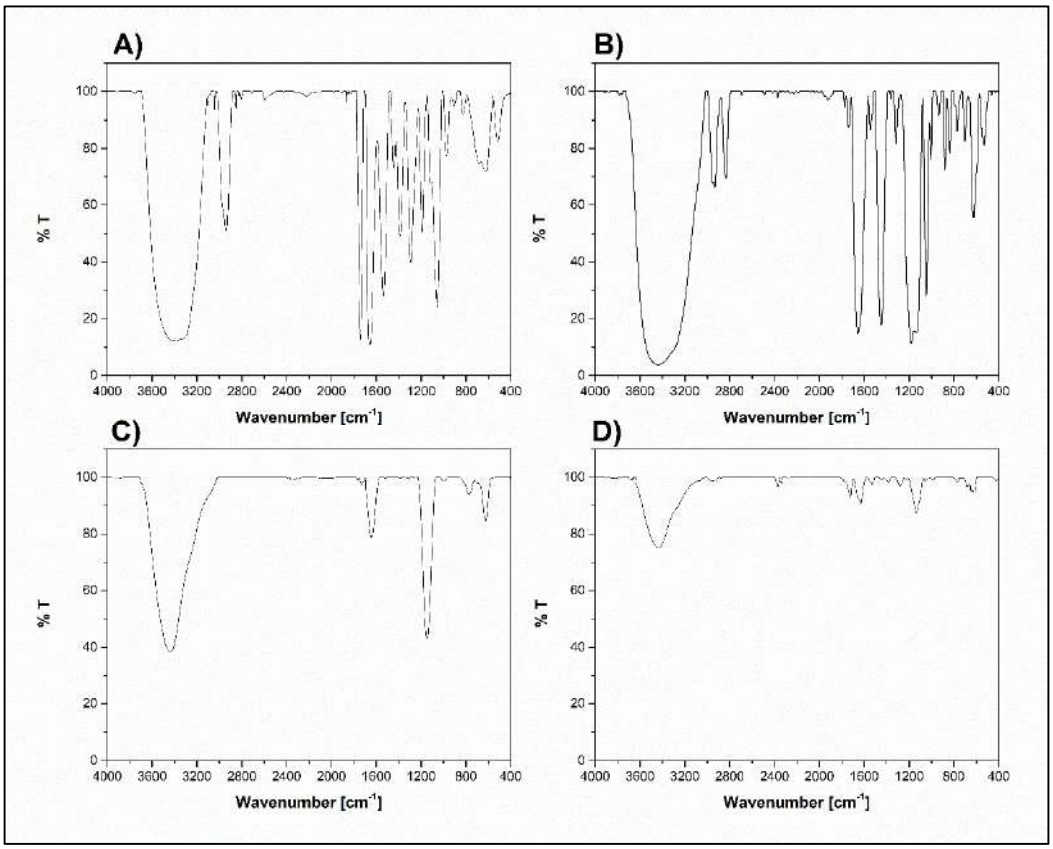

Figure 23: FTIR spectra of *Ralstonia pickettii* before and after methylation of amine groups. (A) FTIR spectrum of *Ralstonia pickettii* biomass growth without copper in the medium; (B) FTIR spectrum of *Ralstonia pickettii* biomass growth with copper in the medium; (C) after chemical treatment and before contact with copper; and (D) after chemical treatment and in contact with copper.

In the case of *Ralstonia pickettii*, the FTIR spectra of the biomass showed a significant number of signals between 1600 and 1200 cm^{-1} without copper in the culture medium (Figure 23.A), and the presence of copper (Figure 23.B) indicated that the –NH, carbonyl (–CO) and disulfide groups were involved in the copper biosorption (Schmitt and Flemming 1998). After chemical modification, the FTIR spectra exhibited three clear peaks: at approximately 1600 cm^{-1}, 1200 $cm^{-1,}$ and 780 cm^{-1}. However, after exposing *Ralstonia pickettii* biomass to copper, these three peaks decreased in intensity, which suggests that the

improvement in biosorption involved the N–H of amines, C=O of amides, carboxyl, and phosphate groups (Gerbino et al. 2011; Schmitt and Flemming 1998; D. Lu et al. 2009; Ramrakhiani, Majumder, and Khowala 2011).

The FTIR analysis revealed that the main functional groups present on the surfaces of the bacterial isolates were amides, fatty acids, and phosphate groups. In previous reports, other compounds, such as polysaccharides, have shown a tendency to bind metal ions and to influence the stability of metal-ligand interactions (Geesey et al. 1988; Beech and Gaylarde 1991). In addition, other compounds have been reported as biofilm components (Ras et al. 2011; Fish et al. 2015; Comte, Guibaud, and Baudu 2008); however, we cannot assume the same specific role for each EPS found. A similar idea was expressed by Seviour *et al.* (2019), who recognized the importance of knowing the molecular composition and function assigned to individual EPS components, but without forgetting that extracellular polymers do not necessarily provide the same functions across all systems. In our study, the two bacteria showed different responses to the environment, but had similar surface features, which indicates the importance of bacterial aggregation, such as the formation of flocs, in addition to the features of each microorganism.

The identities of the extracellular substances (EPS) responsible for the establishment and function of biofilms are still poorly understood in relation to water treatment and supply technologies (Seviour et al. 2019). The implications of functional group modifications and the increase in copper sorption capacities can lead to an increase in both the metal concentration and the charge on the metal surface. In particular, the build-up of copper on the bacterial surface promotes corrosion, due to the inhibition of oxide formation (Luo et al. 2019). This situation affects the increase in copper corrosion in two ways: (i) by the creation of several microgalvanic cells, which is enhanced by the expanded cathodic zone; and (ii) through weakness of the passive layer, which results from both the precipitation of the less protective component, and changes in porosity (Galarce et al. 2019, 2020). Unfortunately, this still does not elucidate how the changes in biosorption capacities impact the

physicochemical equilibrium and contamination by nanoparticles (Pereira et al. 2015; I. T. Vargas et al. 2010)

The conceptual model related to the MIC of copper pipes and the copper release in water integrates biological and chemical aspects, together with electron transfer between microorganisms and redox-active surface metal (I. T. Vargas et al. 2014; S. Liu et al. 2016). However, few models consider the emergent properties derived from the biofilm (as the modification of biosorption properties), which are not predictable from the study of free-living bacterial cells from the EPS matrix, as mentioned by Flemming et al. (2016)(Flemming et al. 2016). Currently, the behavior of the biofilm after modification of the membrane components has not been studied, due to the complexity associated with the removal of the biofilm from the surface. However, the evidence presented in this paper highlights how the sorption capacity of microorganisms can change when the environmental conditions shift.

4.4. Conclusions

A better understanding of the EPS will improve strategies for controlling biofilms in water and wastewater systems. In this study, two bacterial isolates usually found in DWDSs built with copper pipes were analyzed. A higher copper tolerance was observed in *Variovorax* sp. than in *Rasltonia pickettii*. The microbial organization of *Variovorax* sp. in planktonic form, as flocs, probably allowed greater tolerance of copper. The chemical modification of the amine group on the bacterial biomass exposed the functional groups of each isolate, increasing the biosorption by 30% and 34% for *Variovorax* sp. and *Ralstonia pickettii*, respectively. Nevertheless, no differences were observed in the surface microbial composition.

The evidence presented in this study emphasizes the need to establish the environmental changes and bacterial interactions that can occur when these bacteria are present. Finally, this research provides evidence to support the potential risk of copper contamination of tap water, produced by the increasing capacity of the bacteria studied to sorb and accumulate copper.

5. CONCLUSION AND PERSPECTIVES

5.1. General conclusions

This work studied the role of a microbial biofilm on corrosion and copper release in plumbing systems. A combination of both field and laboratory conditions were carried out to represent properly what happens into copper pipes of a drinking water system.

The main findings of this thesis were the following:

1) The results of copper pipes collected on the field study did not show a constant relationship among the exposure time, corrosion rate, and amount of copper released into the water. Moreover, the findings revealed that the variability of a dynamic biological process could not be characterized by a single aging time or by the extrapolation of short-term experiments, particularly in drinking plumbing systems.

2) The studies carried out under laboratory conditions emphasize the need to establish the environmental context of these bacteria. The first results that support the asseveration were shown in Chapter II, where the isolate identified as *Variovorax sp* showed a striking loss of biofilm formation capacity when interacting with the second isolate, identified as *Ralstonia pickettii*.

   In the same way, the characterization of functional EPS groups involved in copper accumulation from bacterial isolates, presented in Chapter III, exposed how the amine group modifications (by methylation) in the surface of bacteria isolates increased to 30% the copper sorption, in comparison with the same bacterial isolate without modification. These results support the need to understand the environmental context to predict how each microorganism can act.

3) Finally, this thesis provides evidence to support the potential hazardous of tap water microbial contamination due to i) biofilm detachment of bacterial consortiums and ii) metal accumulation and subsequent metal release owing to microbial sorption capacity.

In summary, a global analysis of the results showed in this Thesis leading us to reconsider the relevance of biofilm and its impact on biocorrosion. Chapter I highlighted the lack of direct relation between copper release, corrosion rate, and biofilm development. These results put a warning message about carrying out the corrosion tests and its interpretation, mainly when attributed characteristics to biofilm due to its age or stage of development. Additionally, the results showed in Chapter II support the idea of the inability to generalize the microbial action when it is analyzed its response to a specific range of time or environmental conditions.

5.2. Future work and connections with public policy

This research contributes to the knowledge of the biocorrosion process into copper pipes. However, further studies are required to elucidate the selection process that defines biofilm composition, its structure, and its corrosivity. Understanding specific succession/interaction mechanisms among the biofilm members will give us better predictive tools to prevent plumbing deterioration and reduce the risk of drinking water contamination by released copper.

Finally, in terms of public policy, the following considerations are raised. A critical foundation for the design of sustainable and resilient biofilm management on the drinking water system requires careful monitoring. To achieve that, more local data under actual conditions over time must be measured. These data must include the seasonal changes and the different primary colonizers that might begin the biocorrosion.

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