PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE

SCHOOL OF ENGINEERING

ENHANCING SELECTION FOR PERCHLORATE-REDUCING BACTERIA IN PRESENCE OF COMPETING ELECTRON ACCEPTORS

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Thesis submitted to Pontificia Universidad Católica de Chile and University of Notre Dame in partial fulfillment of the requirements for the Degree of Doctor in Engineering Sciences

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Santiago de Chile, June, 2019

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Abstract

by

Marcela N. Vega Muñoz

Perchlorate is a ubiquitous water contaminant that inhibits thyroid function. Standards for perchlorate in drinking water range from 2 to 18 μg L^{-1} in United States and Europe. A major natural source of perchlorate contamination is Chile saltpeter, found in the Atacama Desert in Chile.

This dissertation starts by providing a literature review on the unique situation of perchlorate contamination in Chile. The review discusses perchlorate sources, presence in environmental media and in the human population, possible steps to mitigate its health impacts, and opportunities for bioprospecting.

Microbial degradation is a promising strategy to remediate perchlorate, as it is reduced to innocuous chloride and oxygen. However, perchlorate is typically found in the μg/L range, and exerts a weak selective pressure for perchlorate-reducing bacteria (PRB). Also, nitrate can inhibit perchlorate reduction, so low nitrate levels are needed. Low nitrate levels can favor sulfate-reducing bacteria (SRB). Sulfate reduction has also been related to inhibition of perchlorate reduction. Thus, the overarching goal of this research was to devise strategies to enrich PRB when perchlorate is at low concentrations, together with inhibition of sulfate reduction. The proposed strategy was the addition of the perchlorate analogs chlorate or chlorite. Both are intermediates in the perchlorate reduction pathway, and could have a stronger selective pressure for PRB and also inhibit SRB.

The addition of chlorate and chlorite was tested in a hydrogen-based membrane biofilm reactor (H_2-MBfR). In this type of reactor, H_2 is supplied as electron donor through a hollow fiber membrane and bacteria grow on the surface using nitrate, perchlorate and others as electron acceptors. Chlorate was added for 30 days to a H_2-MBfR reducing oxygen, nitrate, perchlorate and sulfate. Before chlorate addition, nitrate and perchlorate were reduced to low levels, but after 17 days sulfate reduction took place, leading to a decrease in perchlorate reduction. When chlorate was added, it increased perchlorate reduction and decreased sulfate reduction. Interestingly,

analysis of the microbial community with 16S rRNA high-throughput sequencing suggested that the SRB (Desulfovibrionaceae) relative abundance increased. This was probably due to their role in sulfur cycling, although it cannot be ruled out that they played a role in chlorate reduction. To further understand the effect of chlorate on the microbial community, we tested chlorate addition in a H_2-MBfR reducing nitrate, perchlorate and sulfate with similar bulk concentrations as before, but the bulk chlorate concentration was 10 times higher. Although the effect on perchlorate could not be evaluated, chlorate exerted a strong selective pressure for PRB, doubling the abundance of *Dechloromonas*, a common genus of PRB. As before, our results suggest that chlorate addition inhibited sulfate reduction.

To understand the effect of chlorite, we initially determined the potential of chlorite to serve as an indirect electron acceptor and support bacterial growth, as it is dismutated to O_2 during perchlorate metabolism. This was successfully proven by first determining O_2 production and consumption rates after chlorite additions to PRB and CRB enrichments. Subsequently, the bacterial growth on chlorite was also demonstrated by measurements of the optical density of PRB and CRB cultures. Finally, we evaluated the selective pressure of chlorite in a H_2-MBfR reducing nitrate, perchlorate and sulfate. This showed that chlorite had a minor selective pressure.

This research provided evidence that adding chlorate and chlorite to a perchlorate H_2-MBfR improved perchlorate reduction. Although it should be further studied, the results suggest this strategy could be helpful, particularly in the case of chlorate.

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ACKNOWLEDGMENTS

First, I want to deeply thank my advisors and mentors, Dr. Ignacio Vargas and Dr. Robert Nerenberg, for their support and guidance through my PhD. Thanks for helping me to overcome difficulties during research, for your willingness to helping me with everything I needed, and for all you taught me. Both are great humans and researchers; I feel very lucky to have been part of their research groups.

I would also like to thank the committee members, Dr. Kyle Bibby, Dr. Rodrigo Labatut, Dr. Aura Ontiveros Valencia and Dr. Mario Vera for their valuable comments and suggestions to improve this work, and also for their willingness to help me. Special thanks to Dr. Aura Ontiveros Valencia, for her great research advice and for teaching me tools that were the core of this research.

Thanks to my research group at PUC, for their suggestions and disposition to hear about my research. I am also very grateful to current and former members, and visitors of my research group at ND. This wonderful and international group was always ready to help and support each other. Besides our long research discussions, I really enjoyed our lunches and talks about a wide variety of topics; I know the world a little better thanks to you. Particularly, thanks to Patricia Perez Calleja for her suggestions, for teaching me how to use microsensors and many other things. I am very glad to say I not only had great colleagues, but also great friends.

I want to give my profound gratitude to Tina (Kristine Mitchell) for all her help, honesty and willingness to answer every single question I made. She is an invaluable person to the CEEES department at Notre Dame. I also want to thank all the administrative staff at CEEES, particularly Mollie and Bonnie, and at DIHA, who helped me in Chile: Fernanda, Mauri, Carmen Gloria and María Inés.

This research would not have been possible without the financial support of CONICYT, the Vicerrectoría de Investigación of Pontificia Universidad Católica de Chile, and CEEES.

To my PUC friends Pauli, Javi and Vero, I am very happy I met such great friends. Thanks for all your help and for always cheering me up. Also, I am very thankful to my Chilean, and not so Chilean, friends-family at ND, for the numerous immemorable and fun moments we shared. Graduate school, and life in South Bend, would not have been the same without you. You are friends for life.

Finally, I want to thank the most important people in my life, my parents, Florencia and Ricardo, and my whole family. This would not have been possible without their unconditional love, education, endless support and encouragement. Saving the best for the very last, it is hard to find words to express my enormous gratitude to Rodrigo, my wonderful husband. Thank you for your infinite love and unwavering support with this and absolutely everything in life.

CHAPTER 1
Introduction

1.1 Background

Perchlorate (ClO_4^-) is a ubiquitous water contaminant of environmental concern due to its inhibitory effect on mammalian thyroid function (Srinivasan and Sorial 2009). Its high solubility, stability, and low sorption affinity make ClO_4^- highly mobile in aqueous systems, and also makes it a challenge to treat.

ClO_4^- contamination mainly results from the historic disposal of synthetic ClO_4^- to the subsurface and from the use of explosives, fireworks, and other perchlorate-containing products (Motzer 2001, Backus et al. 2005). However, ClO_4^- also is formed by natural photochemical processes (Bao and Gu 2004, Dasgupta et al. 2005). While these natural processes result in the widespread presence of trace levels ClO_4^- in the environment, there are a few cases where natural ClO_4^- exists at very high concentrations. One of the most notable is the Atacama Desert in Chile, where ClO_4^- exists in saltpeter deposits at concentrations up to the g kg^{-1} range (Jackson et al. 2015).

Standards for ClO_4^- in United States and Europe range 1 - 18 μg/L for drinking water (US EPA 2006, US EPA 2009, ANSES 2011, ANSES 2012, EFSA CONTAM Panel 2014). Besides drinking water, people can be exposed to ClO_4^- via fruits, vegetables, beverages, and dust. Accordingly, the European Union defined reference values for ClO_4^- in food (EFSA CONTAM Panel 2014).

Methods to treat ClO_4^- can be divided into physicochemical and biological processes. Physicochemical processes include ion-exchange (IX), carbon adsorption, membrane filtration (e.g. reverse osmosis, ultrafiltration or electrodialysis), and chemical and electrochemical reduction (Coates and Achenbach 2004, Srinivasan and Sorial 2009, Ye et al. 2012, Sijimol et al. 2015, Ma et al. 2016). IX is the most widely used technology, but its major disadvantage is that IX only separates ClO_4^- from water and generates a brine that requires further treatment or disposal.

Biological treatment technologies include microbial reduction and phytoremediation. Microbial perchlorate reduction is based on perchlorate reducing bacteria (PRB) which gain energy by reducing ClO_4^- to chlorate (ClO_3^-) and then to chlorite (ClO_2^-), a toxic intermediate. ClO_2^- is then disproportionated to molecular oxygen and chloride (Cl^-) (Figure 1.1) (Coates and Achenbach 2004, Srinivasan and Sorial 2009, Ye et al. 2012, Sijimol et al. 2015, Ma et al. 2016). However, this technology requires an exogenous electron donor (e.g., acetate, ethanol, H_2), which can increase the operational costs. Biological treatment of drinking water is not widely used in most countries, due to possible bacterial contamination of the treated water (Sharbatmaleki and Batista 2012, Choe et al. 2013). Nevertheless, biological reduction using inorganic electron donors are the most environmentally friendly alternative for ClO_4^- treatment, due to its low global warming footprint, and the use of biofilm reactors can reduce or eliminate downstream treatment to avoid microorganisms in the effluent (Choe et al. 2013).

1.2 Perchlorate reducing bacteria

PRB are generally facultative anaerobes, Gram negative, and belong to α, β, γ, ε subclasses of the Proteobacteria phylum (Figure 1.2) (Youngblut et al. 2016). They can use both organic and inorganic electron donors. As electron acceptors, most of them can also use oxygen (O_2), nitrate (NO_3^-), and ClO_3^- and, in general, grow faster on these than on ClO_4^-. Their optimal growth rates are typically obtained at neutral pH and mesophilic temperatures.

ClO_4^- is reduced by the perchlorate reductase enzyme (Pcr) to ClO_2^- and the chlorite dismutase enzyme (Cld) dismutates ClO_2^- to oxygen and chloride (Figure 1.1).

ClO_2^- is a toxic intermediate of ClO_4^- reduction pathway, so it is quickly transformed. The Cld is a unique heme enzyme and one of the few known to form O_2 as product. Genes for Cld are well conserved among PRB and functional analogs have been isolated from non-PRB (Maixner et al. 2008, Kostan et al. 2010, Mlynek et al. 2011). While a large family of Cld-like protein genes have been detected using genome screening, only a small percent of them show significant Cld activity (Ebihara et al. 2005, Maixner et al. 2008). Most of them have unknown enzymatic activities.

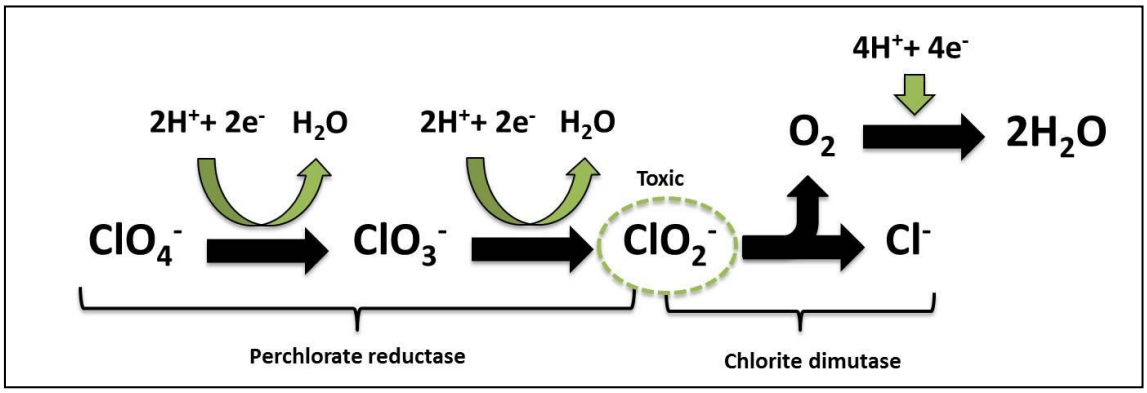

Figure 1.1. Biological pathway of perchlorate reduction. The perchlorate reductase enzyme catalyzes the reduction of perchlorate and chlorate to chlorite. The chlorite dismutase enzyme is responsible for chlorite removal. Adapted from Bardiya and Bae (2011).

It has been proposed that Cld in non PRB might protect cells from highly oxidizing compounds different from ClO_2^-, as Cld has catalase activity (Ebihara et al. 2005). Other studies suggest its presence in nitrifying bacteria is because ClO_3^- can be used as an electron acceptor in the nitrite oxidation reaction and, in the absence of Cld, ClO_2^- accumulation would destroy cytochromes and stop nitrite oxidation (Lees and Simpson 1957, Meincke et al. 1992, Spieck et al. 1998). In hypoxic environments, Cld could also provide the oxygen necessary for aerobic oxidation of nitrite (Maixner et al. 2008).

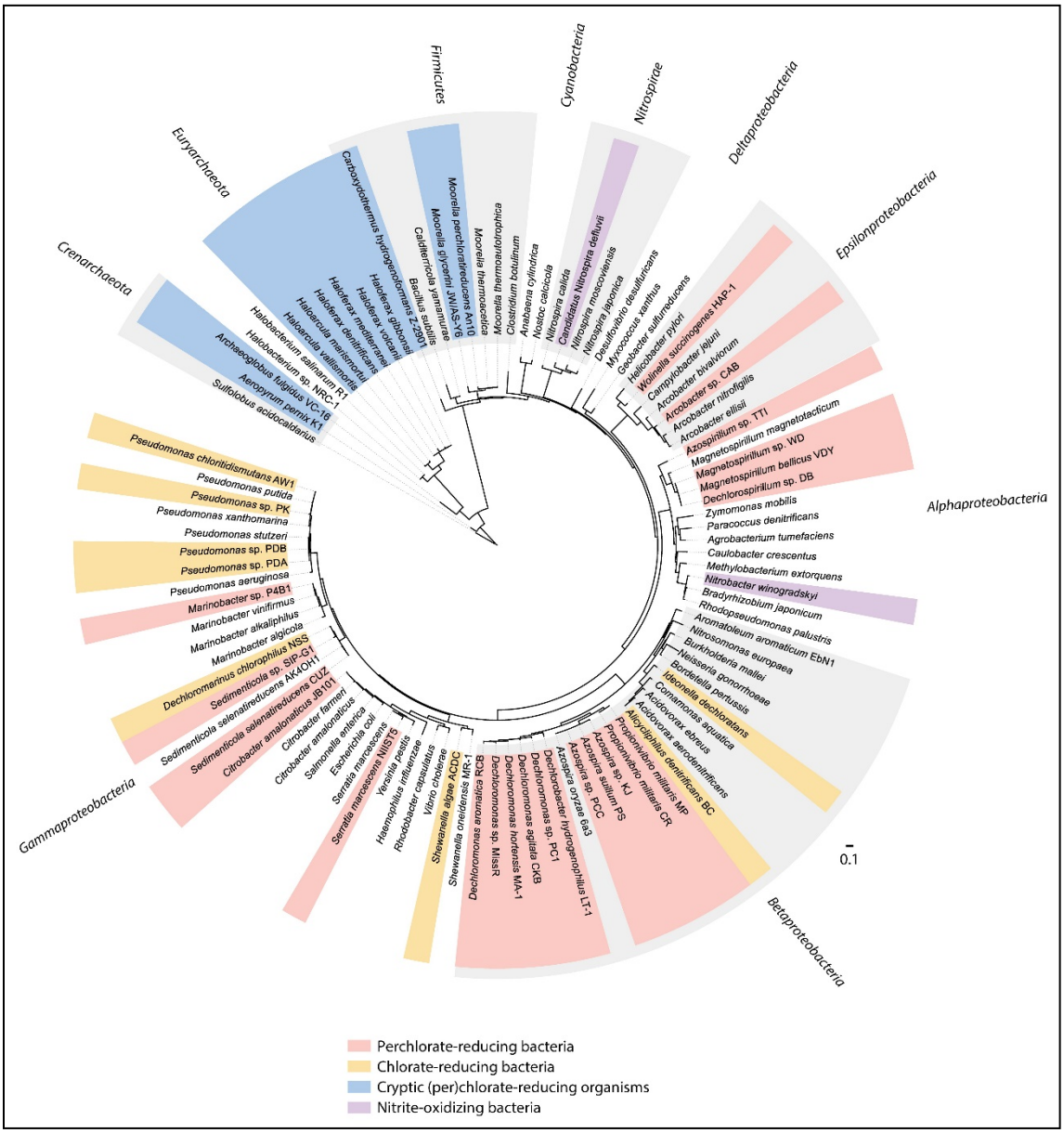

Figure 1.2. Phylogenetic tree of perchlorate and chlorate reducing bacteria (Youngblut et al. 2016). Bacteria at the order and phylum level are highlighted in colors: perchlorate-reducing bacteria (pink), chlorate reducing bacteria (yellow), cryptic perchlorate reducing organisms (blue) and nitrite-oxidizing bacteria (purple) containing catalytic chlorite dismutase.

1.3 Common electron acceptors in drinking water sources

ClO_4^- is one among several contaminant oxyanions present in water. Typical concentrations of ClO_4^- in U.S drinking water are 100 µg/L. In Chile, ClO_4^- concentrations range between 20 – 140 µg/L, although they can reach up to 2 mg/L (Crump et al. 2000, Calderón et al. 2014). Below are described other relevant oxyanions present in sources for drinking water.

1.3.1 Nitrate

NO_3^- is a common co-contaminant with ClO_4^- in surface water and groundwater. It is typically found at much higher concentrations than ClO_4^-, often up to 20 mg N/L. The NO_3^- standard from the U.S. EPA, and also from the World Health Organization (WHO) is 10 mg N/L (US EPA 2009, WHO 2011).

NO_3^- is reduced by microorganisms from a wide range of taxonomic domains, but in biological water treatment processes it is mainly reduced by denitrifying bacteria (DNB) (Lu et al. 2014). Most DNB belong to the Proteobacteria and, to lesser extent, to the Bacteroidetes. Most are in the α, β, γ, and δ subclasses of the Proteobacteria. DNB reduce NO_3^- in four consecutive steps, ending in nitrogen gas (N_2), as shown in Figure 1.3 (Zumft 1997).

DNB are facultative anaerobic, preferentially using O_2 as electron acceptor. Some DNB are believed to use nitrate reductase enzyme to reduce ClO_3^- or ClO_4^-, although the rates are very low and they do not appear to gain energy from these reactions (Logan et al. 2001).

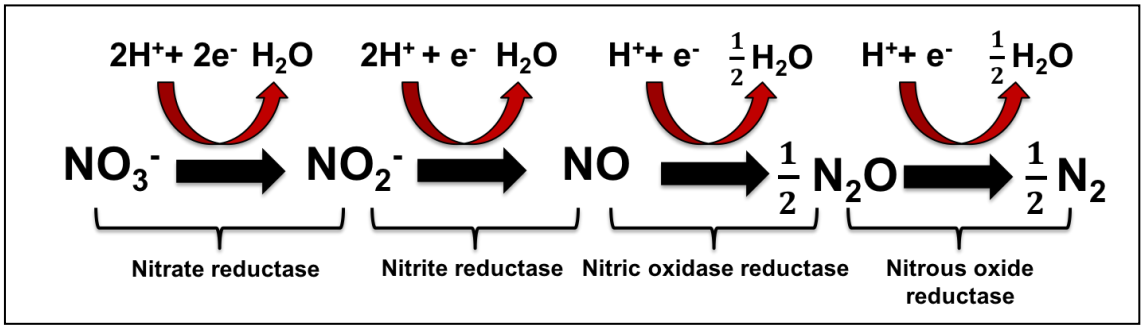

Figure 1.3. Denitrification metabolism pathway. NO_3^- is reduced in four steps to nitrogen gas (N_2).

1.3.2 Sulfate

Sulfate (SO_4^{2-}) is mainly of aesthetic concern in drinking water, as it provides an objectionable taste above 500 mg/L, and laxative effects above 1g/L. SO_4^{2-}. The US EPA and WHO regulate SO_4^{2-} with secondary standards, i.e., standards that are recommended to enhance water aesthetic quality. A level of 250 mg/L has been defined by the WHO and US EPA (WHO 2004, US EPA 2009). Typical SO_4^{2-} concentration in fresh water is around 20 mg/L and can range from 0 to 630 mg/L in rivers, and 0 to 230 mg/L in groundwater (WHO 2004).

SO_4^{2-} is used as a terminal electron acceptor by sulfate reducing bacteria (SRB), which reduce it in three steps (Figure 1.4). First, adenosine triphosphate (ATP) is used by the enzyme APT sulfurylase to activate SO_4^{2-} to adenosine phosphosulfate (APS), producing pyrophosphate (PPi) (Madigan et al. 2018). Subsequently, APS is reduced to sulfite (SO_3^{2-}), and this is reduced to hydrogen sulfide (H_2S). H_2S is a toxic and malodorous gas, which can be subsequently be oxidize back to S^0 or SO_4^{2-} by sulfur oxidizing bacteria (SOB).

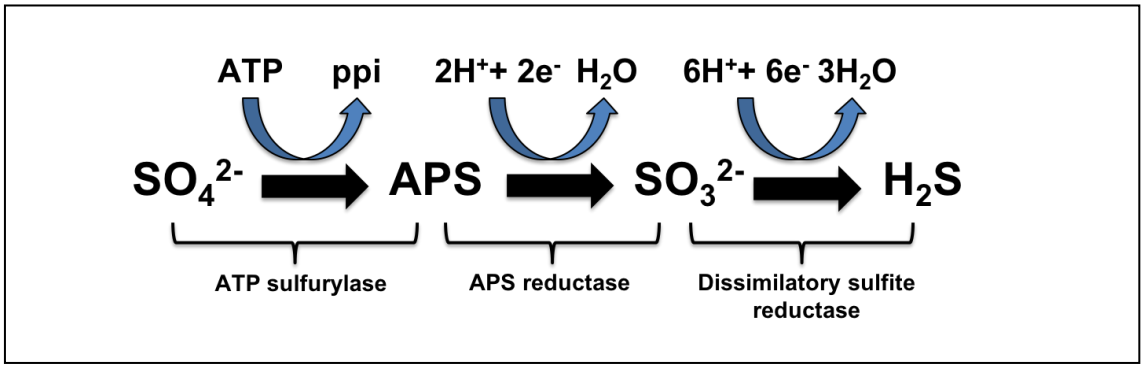

Figure 1.4. Sulfate reduction pathway for SRB. SO_4^{2-} in three steps. First, adenosine triphosphate (ATP) is used by the enzyme APT sulfurylase to activate SO_4^{2-} to adenosine phosphosulfate (APS), producing pyrophosphate (PPi). Following, APS is reduced to sulfite (SO_3^{2-}), and this is reduced to hydrogen sulfide (H_2S).

SRB are very versatile bacteria and are the microorganisms that reduce the greatest number of different terminal electron acceptors (Muyzer and Stams 2008, Barton and Fauque 2009). Some SRB are able to reduce O_2 (when it is present at low concentrations),

and also NO_3^- and (or) NO_2^- to ammonia (Ito et al. 2002, Lobo et al. 2007, Marietou 2016). For some SRB, NO_3^- can be the preferred electron acceptor. These bacteria are distributed in five phyla in the Bacteria domain and two phyla within Archaea, although most of them belong to Deltaproteobacteria (Muyzer and Stams 2008, Barton and Fauque 2009).

1.3.3 Chlorate

ClO_3^- is a water contaminant with a recommended standard of 0.7 mg/L, determined by the US EPA and WHO (US EPA 2016, WHO 2016). It is not usually present together with ClO_4^-, but some drinking water sources in Northern Chile have bothe ClO_4^- and ClO_3^- at levels of 6 – 7 mg/L (unpublished data).

As shown in Figure 1.1, PRB can use ClO_3^- as electron acceptor with the enzyme Pcr. However, there also are specialized chlorate reducing bacteria (CRB), which reduce ClO_3^-, but not ClO_4^-, using the specialized chlorate reductase enzyme (Clr). This enzyme differs from the perchlorate reductases (Steinberg et al. 2005). CRB also belong to the α-, β-, γ- and ε-proteobacteria, although they are mainly contained in the γ subclass.

ClO_3^- is an analog to ClO_4^- for PRB, meaning it is reduced by the same enzyme and can help select for PRB. PRB actually grow faster on ClO_3^- than on ClO_4^-. For this reason, ClO_3^- may have strong selective pressure to enrich PRB when ClO_4^- concentrations are low. However, ClO_3^- addition can also select for CRB, which do not contribute to ClO_4^- reduction.

1.3.4 Chlorite

ClO_2^- is a reactive compound that is toxic to bacteria, fungi, and algae. In infants and young children, it can produce anemia or neurological effects (US EPA 2009). Generally, ClO_2^- occurs in drinking water as a disinfectant by-product at less than 0.2 mg/L (WHO 2016). A provisional guideline for drinking water determined by the WHO is 0.7 mg/L of ClO_2^- and the standard level for drinking water in the U.S. is 0.8 mg/L (US EPA 2009, WHO 2016).

PRB and CRB produce ClO_2^- as an intermediate in their metabolic pathway, and concurrently transform it into O_2, which then is reduced to H_2O via the usual aerobic

pathway. While ClO_2^- itself is not an electron acceptor, the produced O_2 serves as a powerful electron acceptor for PRB and CRB (Melnyk and Coates 2015).

1.4 Hydrogen-based membrane biofilm reactor (H_2-MBfR)

Among biofilm reactors, such as packed-bed reactors, fluidized reactors, or different membrane bioreactors, the hydrogen-based membrane biofilm reactor (H_2-MBfR) (Figure 1.5) presents some strong advantages for biological drinking water treatment. The electron donor (H_2 gas) is transferred through a gas permeable hollow fiber, while the electron acceptor (e.g., O_2, NO_3^-) diffused into the biofilm from the bulk liquid. This makes the gas transfer highly efficient and avoids bubbling. In turn, this reduces the process cost and provides a safe environment by minimizing H_2 emission to the atmosphere. Bacteria grow as a biofilm in the outer wall of the membrane; thus, the microbial retention time is very high.

The H_2-MBfR is a good model for research biofilm. Autotrophic system populations are less diverse than heterotrophic ones, so it is easier to determine changes in microbial communities when different conditions are applied (Wan et al. 2016). Besides, H_2-MBfR has already been tested and successfully applied for denitrification (Xia et al. 2010, Martin and Nerenberg 2012) and it also has been studied for ClO_4^- reduction (Nerenberg et al. 2008, Ontiveros-Valencia et al. 2013b, Ontiveros-Valencia et al. 2014a). Nevertheless, a challenge with this type of reactors is the need to control biomass growth and microbial interactions within the reactor over long periods with successful ClO_4^- reduction (Ma et al. 2016, Martin et al. 2013). It also is desirable to maximize the abundance of PRB in order to help meet the very low standards for ClO_4^- in drinking water.

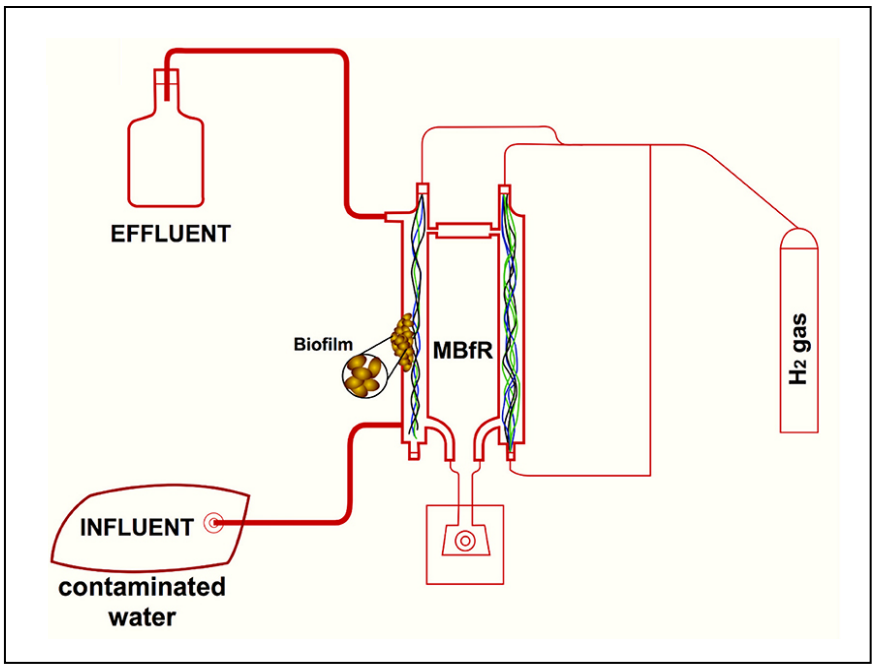

Figure 1.5. Schematic of a H_2 based membrane biofilm reactor (H_2-MBfR) (Zhou et al. 2019). The H_2-MBfR is composed of two glass units. One unit contains a bundle of membranes, while the other has only few membranes, which are used to collect biological samples.

Studies have shown that low levels of ClO_4^- (0.1 – 0.5 mg/l) are not enough to support substantial growth of PRB as sole electron acceptors (Nerenberg at al. 2008), and when NO_3^- is present at high concentrations, the dominant bacteria are DNB. ClO_3^- is a ClO_4^- analog, so it may help to enrich PRB and improve ClO_4^- reduction.

1.5 Microbial competition in a biofilm

The presence of different electron acceptors, such as O_2 and NO_3^-, can inhibit ClO_4^- reduction in several ways. First, most PRB can use O_2 and NO_3^- as electron acceptors. Although biological reduction of perchlorate is energetically favorable and similar to full NO_3^- reduction (Table 1.1) (Youngblut et al. 2016, Madigan et al. 2018), most PRB prefer O_2 and NO_3^- over ClO_4^-, so high concentrations of these acceptors will inhibit ClO_4^- reduction (Nerenberg et al. 2008, Ontiveros-Valencia et al. 2013b, Ontiveros-Valencia et al. 2014a). Second, high concentrations of O_2 and NO_3^- select for

bacteria that grow well on O_2 and NO_3^-, not necessarily for PRB. Under these conditions, the presence of PRB is only because they are effective facultative aerobes or denitrifiers.

SO_4^{2-} also presents challenges for ClO_4^- reduction. Reducing and NO_3^- in the presence of SO_4^{2-} may also lead to SO_4^{2-} reduction (Ontiveros-Valencia et al. 2013b, Ontiveros-Valencia et al. 2014a), even though NO_3^- and ClO_4^- have been shown to inhibit SO_4^{2-} reduction in batch systems and continuous souring columns (Engelbrektson et al. 2014, Carlson et al. 2015, Engelbrektson et al. 2018). Reduction of SO_4^{2-} is undesirable as it produces H_2S, a toxic, corrosive, and malodorous gas. Studies of a H_2-MBfR for ClO_4^- reduction in the presence of NO_3^- and SO_4^{2-} showed SO_4^{2-} reduction in almost all cases (from 10% to 80% reduction). This led to difficulties in reaching low ClO_4^- concentration in a one-stage H_2-MBfR when ClO_4^- was present at high concentrations, i.e., above 1 mg/L (Ontiveros-Valencia et al. 2013b, Ontiveros-Valencia et al. 2014a). Previous studies on H_2-MBfRs suggest that SRB have advantages over PRB in competing for space in the biofilm, because their results showed incomplete ClO_4^- reduction (Ontiveros-Valencia et al. 2013b).

Table 1.1. Standard reduction potentials

Redox couple	mV
O_2/H_2O	+816
ClO_4^-/Cl^-*	+797
ClO_3^-/Cl^-*	+792
$NO_3^-//N_2$	+750
SO_4^{2-}/HS^-	–217

*Biological reduction potential considering the formation and use up of O_2 (Youngblut et al. 2016).

Microbial competition for space and substrates within the biofilm depends on the growth and decay rates of each type of bacteria (Tang et al. 2012). Bacteria with higher growth rates will outcompete slow growing bacteria, as slow-growers are “pushed out” of the biofilm. However, the position in the biofilm, the biofilm thickness, and the concentrations of the electron acceptor in the bulk and the electron donor within the

biofilm, can influence the persistence and of some microbes. A diagram explaining the biofilm formation and evolution of an H_2-MBfR community is shown in Figure 1.6.

At the initial stage of H_2-MBfR biofilm development, bulk O_2, NO_3^-, ClO_4^- and SO_4^{2-} concentrations are high. DNB and PRB will be the first bacteria growing since they grow faster than SBR (Figure 1.6a), although, ClO_4^- reduction will not start after O_2 and NO_3^- are reduced to low levels. After O_2, NO_3^- and ClO_4^- are substantially removed, their concentration in the bulk liquid is much lower than initially, while the SO_4^{2-} concentration remains high (Figure 1.6b). Due to these low concentrations, O_2, NO_3^- and ClO_4^- will only reach the outer layer of the biofilm, while SO_4^{2-} fully penetrates the biofilm. This allows the growth of SRB in the deeper biofilm, where the H_2 concentrations are high and there is little competition from DNB and PRB. SRB are also more protected from detachment than SNB and PRB growing on the outer layers of the biofilm. This could explain the proliferation of SRB in the mentioned studies (Ontiveros-Valencia et al. 2013b).

The ecological interactions among members of the biofilm community mentioned above are relevant for achieving a feasible treatment for ClO_4^- reduction in water systems that may contain these different electron acceptors.

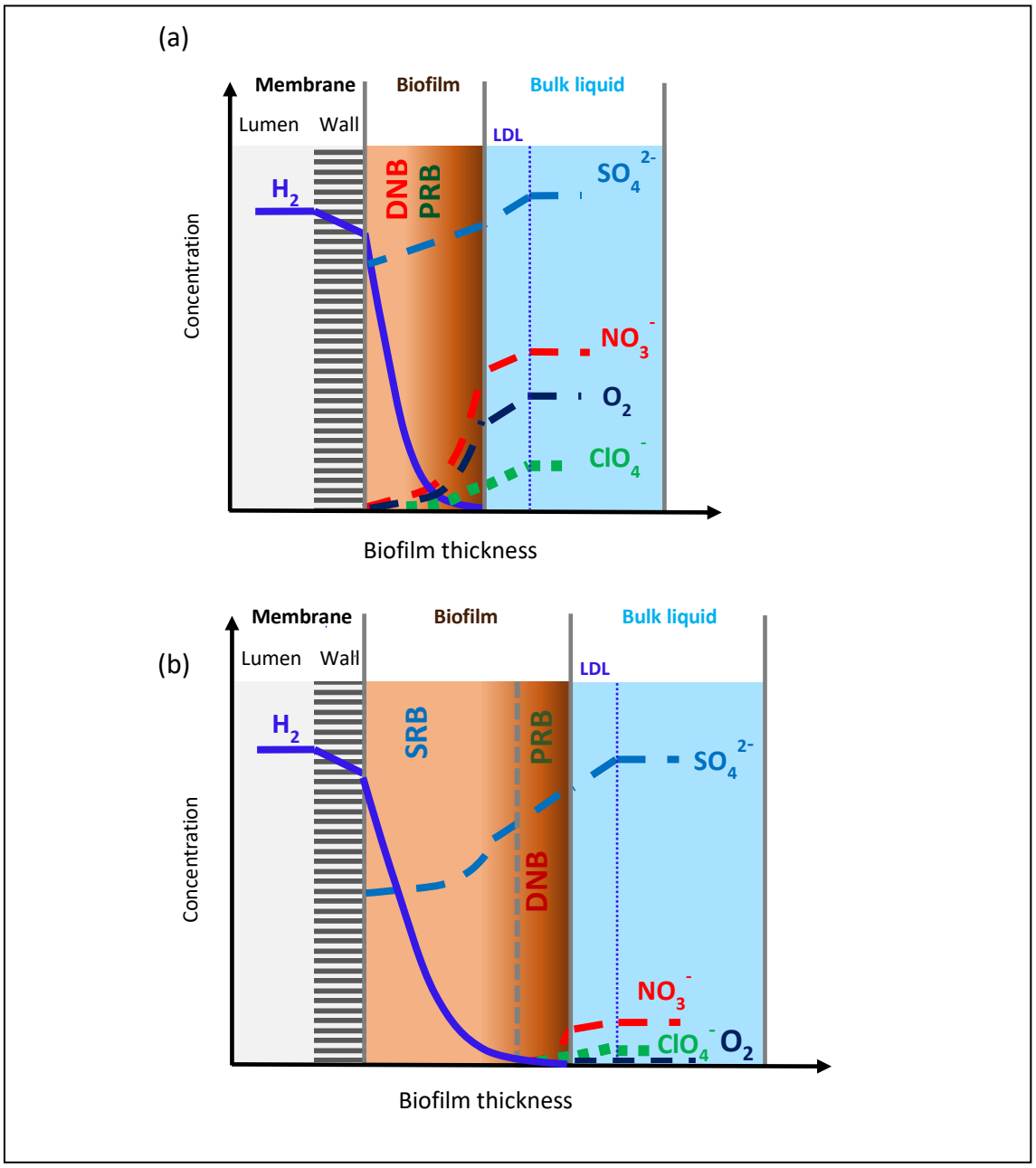

Figure 1.6. Microbial competition in a biofilm of a hydrogen-based membrane biofilm reactor (H_2-MBfR). (a) At initial stages of development, denitrifying bacteria (DNB) and perchlorate reducing bacteria (PRB) are favored. (b) Once the biofilm grows, nitrate ($NO3^{-}$) and perchlorate ($ClO4^{-}$) are reduced to low levels, and SRB start proliferating in the deeper biofilm.

1.6 Objectives and thesis organization

1.6.1 Hypotheses

ClO_3^- is an intermediate on the ClO_4^- reduction pathway of PRB, and PRB grow even faster on ClO_3^- than on ClO_4^-. It is hypothesized that, if ClO_3^- is added to a bioreactor with low levels of ClO_4^-, it will greatly enhance the abundance of PRB. This, in turn, will enhance the levels of ClO_4^- reduction. Also, ClO_3^- could help to inhibit SRB.

ClO_2^- also is an intermediate in the ClO_4^- reduction pathway of PRB. Although it does not serve directly as an electron acceptor, it is transformed by PRB into O_2 and Cl^-, and the O_2 can be further used by PRB. We hypothesize that ClO_2^- can help select for PRB. At the same time, the O_2 may inhibit the proliferation of SRB.

1.6.2 Objectives

The overarching goal of this research was to improve the performance of ClO_4^--reducing biofilm reactors when ClO_4^- is at much lower concentrations than competing acceptors, such as O_2, NO_3^-, and SO_4^{2-}.

The specific objectives of this dissertation are:

1. Perform a review of ClO_4^- contamination and treatment needs in Chile.
2. Explore ClO_3^- as a means to select for PRB and avoid SO_4^{2-} reduction in a H_2-MBfR reducing O_2, NO_3^-, and SO_4^{2-} and low concentrations of ClO_4^-, and study the microbial community developing under these conditions.
3. Evaluate the effect of ClO_3^- on SO_4^{2-} reduction on a H_2-MBfR reducing O_2, NO_3^-, SO_4^{2-} and ClO_4^-, which is at low concentrations.
4. Determine the ability of PRB and CRB to disproportionate ClO_2^- and use the O_2 produced to grow, and study the potential of ClO_2^- to enrich PRB and prevent sulfate reduction in a H_2-MBfR reducing O_2, NO_3^-, and SO_4^{2-} and low concentrations of ClO_4^-.

1.6.3 Dissertation structure

The following describes the organization of this dissertation. Chapter 2 contains a review of ClO_4^- in water and soil samples in Chile and the world. Chile is known for having high concentrations of natural ClO_4^-. However, only two studies of Chilean population exposure to this contaminant have been reported and the literature lacks national surveys about ClO_4^- contamination in different matrices, such as soil, drinking water and food. The purpose of this work was to describe ClO_4^- contamination in Chile by gathering information about ClO_4^- characteristics, its presence in Chile and the world, its origin, the regulations, and treatment systems. Also, we consolidated and reviewed information about ClO_4^- contamination in different matrices in Chile, i.e., soil, drinking water, and food, and provided guidelines for future work; and discussed future research needed for Chile to manage ClO_4^- contamination. This manuscript was published in *Environmental Engineering* (Vega et al. 2018).

Chapter 3 describes the study of ClO_3^- as a ClO_4^- analog, potentially enriching for PRB when the ClO_4^- concentrations are low (e.g. less than 0.6 mg/L), and also to help inhibit SRB. We tested the effect of ClO_3^- with two H_2-MBfRs in three stages. First, Reactors A (control) and B (experimental) were supplied with ClO_4^-, NO_3^-, SO_4^{2-}, O_2. In the second stage we continuously added ClO_3^- to Reactor B and in the third stage we operated both reactors without ClO_3^-. The performance results together with the microbial community analyses, provided a better understanding of how ClO_3^- can shape the performance of a H_2-MBfR and the presence of PRB and SRB.

In Chapter 4, we further studied the selective pressure of ClO_3^- on PRB. ClO_3^- was added to H_2-MBfRs reducing NO_3^-, ClO_4^-, and SO_4^{2-} at concentrations ten times lower than previously and also at a lower H_2 loading. As in Chapter 3, one reactor served as control (Reactor 1) and the other as experimental (Reactor 2) to which ClO_3^- was added. Although the effect on perchlorate could not be evaluated, ClO_3^- did exert a strong selective pressure to PRB, doubling the abundance of *Dechloromonas*, a well-known PRB. As before, our results suggest that addition of ClO_3^- inhibited sulfate reduction.

In Chapter 5, we explored ClO_2^- as an indirect electron acceptor and if it helps to select PRB and inhibit SRB. As PRB and CRB produce O_2 by dismutating ClO_2^-, they

can use the oxygen produced to grow, allowing PRB to compete for space by providing O_2 to the inner layers of the biofilm. This O_2 could also help to avoid SO_4^{2-} reduction. As ClO_2^- is a toxic compound, it could give a possible advantage to PRB over other bacteria. Here we first present ClO_2^- toxicity studies to PRB and CRB enrichments, and then we demonstrate they are able to grow on ClO_2^- as an indirect electron acceptor. Finally, the effect of ClO_2^- addition was tested in a H_2-MBfR with similar characteristics to the reactor described in Chapter 3, and the same three stages strategies. In this case, the effect of ClO_2^- on PRB and SO_4^{2-} reduction was minor.

Finally, Chapter 6 presents the summary of the main points from this research, together with the proposed studies and strategies to further the knowledge related to the effects of ClO_3^- and ClO_2^- on PRB and SRB.

CHAPTER 2

Perchlorate contamination in Chile: legacy, challenges, and potential solutions

This chapter was published in an altered format in *Environmental Research* (Vega et al., 2018)

2.1. Introduction

Perchlorate (ClO_4^-) is an ubiquitous water contaminant, of environmental concern due to its inhibitory effect on mammalian thyroid function (Srinivasan and Sorial 2009). Its high solubility, stability, and low sorption affinity make perchlorate highly mobile in aqueous systems, and also makes it a challenge to treat.

Perchlorate contamination mainly results from the disposal of synthetic perchlorate to the subsurface, a common practice prior to the existence of more stringent environmental regulations (Motzer 2001, Backus et al. 2005). It also may result from the use of explosives, fireworks, fertilizers and other perchlorate-containing products. These anthropogenic perchlorate sources, as well as exposure pathways, are fairly well characterized.

Perchlorate also is formed by natural photochemical processes (Dasgupta et al. 2005, Kang et al. 2006, Kang et al. 2008, Rao et al. 2010). While these natural formation processes result in the widespread presence of perchlorate in the environment, the levels are typically below those thought to present a health concern. However, there are a few cases where natural perchlorate exists at very high concentrations. One of the most notable is in the Atacama Desert in Chile, where perchlorate exists in saltpeter deposits at concentrations up to the g kg^{-1} range (Jackson et al. 2015). The potential threat of these

natural sources is not well understood, and treatment technologies relevant to Chile also are unknown.

This review provides an overview of perchlorate in the environment in Chile. We discuss how perchlorate in soils has resulted in contamination of different matrices, such as drinking water and food, and review evidence of human exposure. We discuss health effects and the need for perchlorate risk assessments in Chile. Finally, we suggest future research to address perchlorate in the environment in Chile. Chile may serve as a model for the few other locations with similar conditions. Chile also may provide a unique, natural "laboratory" for research on perchlorate-reducing microorganisms.

2.2. Background

2.2.1. Sources of perchlorate

Due to its high oxidization potential, perchlorate has been synthesized for use in numerous applications, such as rocket fuels, explosives, fireworks, air bags, munitions, signal flares and in a wide range of industrial products (Motzer 2001, Backus et al. 2005). As a result of its widespread use, perchlorate contamination in United States (U.S.), Europe, and other industrialized countries is widespread (Smith et al. 2001).

It has been shown that perchlorate can be naturally formed through serial reactions in the atmosphere involving UV exposure, ozone oxidation, and/or electrical discharges such as lightning (Dasgupta et al. 2005, Kang et al. 2006, Kang et al. 2008, Rao et al. 2010). After it forms in the stratosphere, it can reach soils through wet or dry deposition. In wet deposition, perchlorate dissolves into moisture droplets and is removed by precipitation. When perchlorate-containing precipitation reaches the ground, perchlorate maybe deposited in soils. However, given its high solubility and poor sorption properties, it is readily transported to surface water or groundwater sources (Rao et al. 2007, Rajagopalan et al. 2009). Dry deposition can occur in the form of eolian dust, atmospheric aerosols and gases (Andraski et al. 2014). These processes are thought to account for the trace perchlorate levels found in many environments. It may also explain high perchlorate

concentrations in arid environments, such as the Atacama Desert, as there is not enough precipitation to flush perchlorate from soils.

2.2.2. Perchlorate as an emerging contaminant

In the 1990s, improved analytical techniques allowed the detection of trace levels of perchlorate in surface waters and groundwaters in the U.S. (Trumpolt et al. 2005). This prompted a non-mandatory action by the United States Environmental Protection Agency (US EPA), setting a recommended reference dose of 0.7 μg kg^{-1} of body weight day^{-1}, equivalent to 24.5 μg L^{-1} for drinking water. In 2009 the US EPA set a new recommendation of 15 μg L^{-1} for drinking water (US EPA 2006, US EPA 2009). Similarly, in Europe the Directorate General for Health (DGS) in France and the French Agency for Food, Environmental and Health Occupational & Safety (ANSES) established a maximum drinking water concentration of 15 μg L^{-1} for adults and 4 μg L^{-1} for newborns (ANSES 2011, ANSES 2012). The European Food Safety Authority (EFSA) established a tolerable daily intake of 0.3 μg kg^{-1} of body weight (EFSA CONTAM Panel 2014). In addition to drinking water supplies, people can be exposed to perchlorate via fruits, vegetables, beverages, and dust. Accordingly, the European Union (EU) defined reference values for perchlorate in food (EFSA CONTAM Panel 2014).

2.2.3. Treatment systems for perchlorate

There are different systems to treat perchlorate-contaminated drinking water supplies. The most commonly used is ion exchange (IX). However, this system, as well as other physicochemical systems such as membrane filtration and activated carbon adsorption, produces perchlorate-containing waste streams that require further treatment and disposal. Biological reduction has been used as a complement/alternative for these systems. Contaminated soils have been treated using phytoremediation (Srinivasan and Sorial 2009, Ye et al. 2012, Sijimol et al. 2015, Ma et al. 2016). Treatment systems are discussed in more detail in Section 7, below.

2.3. Natural sources of perchlorate in Chile

Evidence of perchlorate in the natural environment was first reported in the 19th century by Beckurts, who analyzed nitrate deposits in the Atacama Desert (Beckurts 1886, Penrose 1910, Mueller 1968 , Ericksen 1983). The Atacama Desert is located in the northern part of Chile, from around 69.5º W to 70º W longitude and from 19.5º S to 26ºS latitude, delineating an extension of 128,000 km^2 (Ericksen 1981) (Figure 2.1). It is one of the driest deserts in the world, with an average rainfall of 1-2 mm per year. Another unique feature is the presence of major nitrate deposits, which led Chile to be a major exporter of nitrogen fertilizers, prior to the development of synthetic fertilizers. Generally, perchlorate is present together with nitrate deposits. Unlike areas with anthropogenic perchlorate contamination, high perchlorate concentrations in the Atacama Desert region are believed to have existed for millions of years (Ericksen 1983, Böhlke et al. 1997, Sturchio et al. 2009, Pérez-Fodich et al. 2014). Figure 2.1 indicates locations where perchlorate has been found in soils and water sources, as well as locations with perchlorate non-detects.

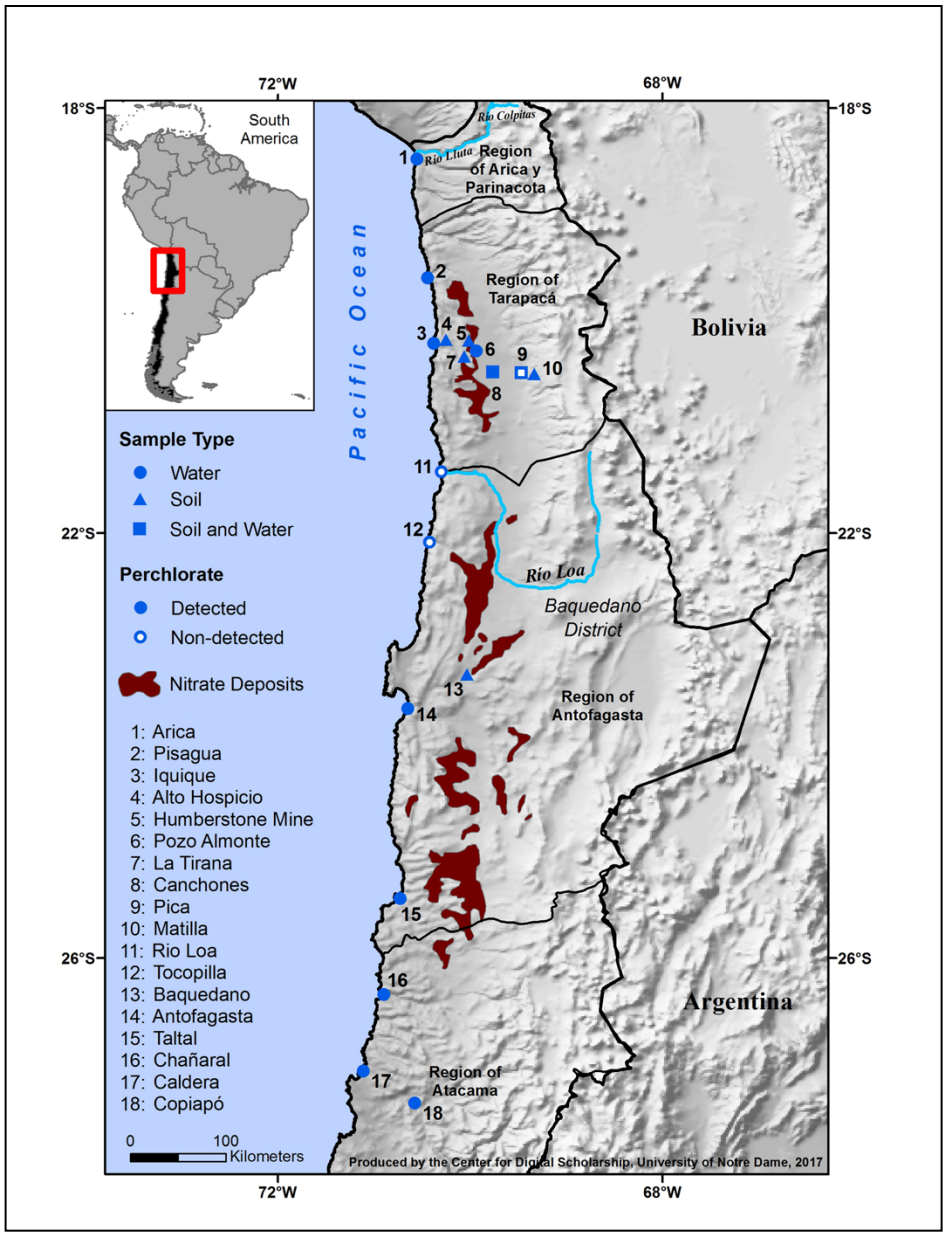

Figure 2.1. Location of natural perchlorate found in Atacama Desert, Chile. Presence of perchlorate in different matrices from cities of the Atacama Desert and places near the nitrate deposits. Adapted from Ericksen. (1983). In location 9, perchlorate was below detection in the soil sample (<40 µg/kg), but it was detected in the water sample. For locations 11 and 12, the detection limits were not defined.

2.3.1. Perchlorate in minerals, soils, and fertilizers

Nitrate deposits of Atacama Desert have been studied for more than a century, and a wide range of perchlorate concentrations have been reported (Table 2.1). Early reports

of perchlorate in crude Chilean caliche (nitrate deposits) were as high as 68 g kg^{-1} (Penrose 1910, Schilt 1979). In cooperation with the Instituto de Investigaciones Geológicas de Chile, Ericksen et al. (1981) measured perchlorate in samples from different nitrate ores of the Atacama Desert, with perchlorate concentrations ranging from non-detect to 5.7 g kg^{-1}. The Humberstone mine, one of the main producers of Chilean saltpeter, had the highest perchlorate concentrations.

Table 2.1. Natural sources of perchlorate in Chile reported in the last century

Location	Description	ClO_4^- (g kg^{-1})	Reference
Atacama Desert	Chilean Caliche	0 - 68	(Schilt 1979)
Tarapacá Region[a]	Nitrate Deposits	2 - 7.8	(Penrose 1910)
Atacama Desert	Nitrate ores	ND[b] - 5.7	(Ericksen 1981)
Baquedano District	Soil	0 - 0.2	(Prellwitz 2007)
Baquedano District	Soil	0.5×10^{-6} - 1	(Jackson et al. 2010, Jackson et al. 2015)
Pozo Almonte	Nonagricultural soil	0.29×10^{-3}	(Calderón et al. 2014)
Pica	Agricultural soil	ND[c]	(Calderón et al. 2014)
Canchones	Agricultural soil	0.47×10^{-3}	(Calderón et al. 2014)
Humberstone	Nonagricultural soil	0.0026	(Calderón et al. 2014)
Atacama Desert	Chilean fertilizers	0.5 - 4	(Urbansky et al. 2001, Urbansky et al. 2001)

[a] Now Region of Arica y Parinacota and Region of Tarapacá.
[b] ND: not detected. ND value was not defined.
[c] ND < 40 µg/kg

Lower concentrations of perchlorate have been reported in recent years. Concentrations of perchlorate up to 0.3 g kg^{-1} near the Baquedano District, where some

saltpeter mines were located, have been published (Prellwitz 2007, Jackson et al. 2010). Other recent studies have analyzed perchlorate concentrations in samples from around the Baquedano District, with concentrations ranging from $0.5x10^{-6}$ to 1 g kg^{-1} (Jackson et al. 2015). Calderón et al. (2014) analyzed contamination of agricultural and non-agricultural soils in northern Chile, revealing perchlorate concentrations as high as 2.6 mg kg^{-1}. The samples in Calderón's study were from different locations close to Pozo Almonte (locations 9-12 and 14 in Figure 2.1), 300 km north to the Baquedano District.

The different perchlorate concentration for similar locations may be due to differences in natural deposition, changes in the soil caused by mining activity in the area, or differences in analytical errors. Difficulties in obtaining reproducible results in soil samples have been mentioned by Urbansky (2001). This has been addressed by measuring samples in more than one laboratory and improving measurement methodologies (Urbansky et al. 2001, Collette et al. 2003). As a result, the more recent studies may be more reliable.

Fertilizers from Chile are also a possible source of perchlorate contamination. Chilean nitrate deposits have been used since 1830's to produce fertilizers, which have been used throughout Chile and exported to the world (Ericksen 1983, Dasgupta et al. 2006). Earlier perchlorate measurements of Chilean fertilizers showed concentrations ranging from 0.5 to 4 g kg^{-1} (Urbansky et al. 2001, Urbansky et al. 2001). The U.S. has been one of the major importers of Chilean fertilizers, which could account for perchlorate contamination in U.S. agricultural areas. (Urbansky et al. 2001, Dasgupta et al. 2006). In fact, isotope studies on groundwater and soil samples from Pomona, California, and Long Island, New York, have determined the presence of perchlorate from the Atacama Desert (Böhlke et al. 2009, Sturchio et al. 2014). However, this contamination is constrained to the historical use of the Chilean fertilizers, because in 2001 the Chilean producer (Sociedad Química y Minera de Chile SA, SQM) changed the production process to reduce the content of perchlorate to below 0.1 g kg^{-1} and currently they have one product with a perchlorate concentration below 0.05 g kg^{-1} (Urbansky et al. 2001, SQM). Besides, these fertilizers currently only represent a small portion of the total market of fertilizers in U.S. (Ericksen 1983, Dasgupta et al. 2006).

It also has been reported that fertilizers commercialized in EU, not derived from the Atacama Desert, also contained perchlorate up to 2.3 g kg^{-1} (EFSA CONTAM Panel 2014). Nevertheless, currently, only fertilizers with less than 0.1 g kg^{-1} of perchlorate are commercialized in the EU and there is a proposal for regulation of perchlorate in fertilizers with maximum 0.05 g kg^{-1} (EFSA CONTAM Panel 2014, European Commission 2016, European Commission 2016).

In Chile, the perchlorate content of fertilizers is not analyzed. It is unknown whether or not fertilizers from Chilean caliche or other sources used within the country have high perchlorate concentrations. Even though SQM has products with low perchlorate content, there is no information about many other products that they might sell in Chile.

2.3.2. Natural perchlorate distribution worldwide

In recent decades, high concentrations of perchlorate in soils and minerals have been found in several places besides Chile: the U.S., Antarctica, Canada, Bolivia, and recently in Southern Africa, United Arab Emirates (UAE) and northeastern China (Table 2.2). The highest perchlorate concentrations were found in potash ores samples from Carlsbad, New Mexico and in Bolivia, with a maximum concentration of 3.7 g kg^{-1} and 1.7 g kg^{-1}, respectively (Orris et al. 2003).

Table 2.2. Perchlorate concentration in natural sources worldwide

Location	Description	ClO_4^- (g kg^{-1})	Reference
Mojave Desert and Amargosa Desert	Soil	0.1×10^{-6} - 0.28	(Orris et al. 2003, Rao et al. 2007, Jackson et al. 2010, Lybrand et al. 2013, Andraski et al. 2014, Jackson et al. 2015)
High Plains in Texas	Unsaturated soil	1×10^{-6} - 116×10^{-6}	(Jackson et al. 2004)
Carlsbad, New Mexico	Potash ore deposits	0.025 - 3.7	(Orris et al. 2003)
Saskatchewan, Canada	Potash ore deposits	0.042	(Orris et al. 2003)
Bolivia	Playa crust	0.5 - 1.7	(Orris et al. 2003)
China	Soil	0.1×10^{-3} - 0.016	(Jackson et al. 2015)
United Arab Emirates	Soil	0.3×10^{-3}	(Jackson et al. 2015)
Antarctica Valleys	Soil	$\leq 25\times10^{-6}$ - 1.1×10^{-3}	(Kounaves et al. 2010, Jackson et al. 2015)
Southern Africa	Soil	0.2×10^{-6} - 45×10^{-6}	(Jackson et al. 2015)

All the places mentioned above have hyper-arid, arid or semi-arid climates. This reinforces the hypothesis of a natural source of perchlorate (Orris et al. 2003, Rajagopalan et al. 2006, Jackson et al. 2015). Still, Chile is among the countries with the highest concentrations of natural perchlorate.

2.3.3. Distinguishing natural from synthetic perchlorate

Anthropogenic perchlorate can be distinguished from natural perchlorate by their stable isotope ratios. Cl< and O isotopes have been used for perchlorate (Bao and Gu 2004, Sturchio et al. 2009, Poghosyan et al. 2016). Bao and Gu (2004) studied O isotope ratios of anthropogenic and natural perchlorate from Atacama Desert. They found that perchlorate from the Atacama Desert has as high positive ^{17}O anomaly values, as does

atmospheric O_3. This suggests that perchlorate in minerals likely results from the atmospheric formation (Bao and Gu 2004).

Sturchio et al. (2009) compared chlorine isotopes of samples from three different perchlorate sources: natural perchlorate from the southwestern U.S. (water and soil), natural perchlorate from the Atacama Desert (soil) and synthetic perchlorate. The three sources showed very different values for the $^{36}Cl/Cl$ ratio and $\delta^{37}Cl$ compared to each other, clearly discriminating between natural perchlorate from Atacama, the U.S. and synthetic perchlorate. Natural perchlorate from the Atacama Desert has low values of $\delta^{37}Cl$; in contrast, synthetic perchlorate has high values of $\delta^{37}Cl$, coinciding with the $\delta^{37}Cl$ values of the marine saline brine used to produce synthetic perchlorate. Samples from the southwestern U.S. have high values of ^{36}Cl, but the stratosphere is the only place on Earth with such high ^{36}Cl values. This indicates that natural perchlorate from the southwestern U.S. should come from atmospheric reactions. However, perchlorate from the Atacama Desert has much lower values of ^{36}Cl and $\delta^{37}Cl$. The reason for this difference may be that soil of the Atacama Desert is millions of years older than soil of the southwestern U.S. This time is several times the half-life of ^{36}Cl (Sturchio et al. 2009). Poghosyan et al. (2016) also studied chlorine isotopes in urine samples from Atlanta, U.S. and Taltal, Chile, and the results were consistent with the results of Sturchio et al. (2009). Isotope analyses have also been used to study nitrate and sulfate, showing that these minerals are produced by photochemical reactions in the atmosphere (Böhlke et al. 1997, Michalski et al. 2004).

Other parameters have been used to identify the source of perchlorate. These include the proximity to known perchlorate sources, the age of the deposits, lack of human intervention and, in some cases, a high correlation of ClO_4^- with NO_3^- (or other ions) and NO_3^- isotope signature (Rajagopalan et al. 2006, Rao et al. 2007, Lybrand et al. 2013, Jackson et al. 2015). This can show a high proportion of atmospheric origin and similar NO_3^-/ClO_4^- ratio values compared to NO_3^-/ClO_4^- ratios of atmospheric deposition. While a number of methods are available to assess the source of perchlorate, stable isotope methods based on ClO_4^- are likely to be the most reliable.

2.4. Potential exposure pathways in Chile

Since perchlorate is highly soluble and mobile in water, its presence in one environmental matrix (e.g., soils) can impact a variety of others. For example, perchlorate salts in soils can dissolve in precipitation and be conveyed to water bodies. The water bodies may then be used as drinking water sources, as irrigation water for crops, or as water sources for industrial applications. As a result, perchlorate from a single source can impact a wide range of human exposure pathways, resulting in higher amounts of perchlorate intake.

In this section, we discuss perchlorate in different matrices in Chile, mainly due to the presence of natural perchlorate in soils, and potential pathways of human exposure in Chilean and the world.

2.4.1. Perchlorate in surface water, groundwater and drinking water

Although there is relatively little information on perchlorate in water sources in and around the Atacama Desert, there have been a few important studies. This information is summarized in Table 2.3.

Table 2.3. Natural perchlorate occurrence in water courses in Chile

Location	Description	n	ClO_4^- (μg L^{-1})	Reference
Atacama Desert	Groundwater for industrial use	-	1,000 - 10,000	(Crump et al. 2000)
Atacama Desert	Natural spring for recreation	-	1,000	(Crump et al. 2000)
Atacama Desert	Groundwater for human consumption	-	50 - 150	(Crump et al. 2000)
Río Loa	River	-	ND[a]	(Crump et al. 2000)
Arica	Drinking water	3	4 - 12	(Crump et al. 2000)
Pisagua	Drinking water	3	4 - 12	(Crump et al. 2000)
Iquique	Drinking water	3	4 - 12	(Crump et al. 2000)
Tocopilla [b]	Drinking water	3	ND[a]	(Crump et al. 2000)
Antofagasta[b]	Drinking water	3	ND[a]	(Crump et al. 2000)
	Drinking water	25	ND[c]	(Crump et al. 2000)
	Drinking water	6	0.46	(Téllez et al. 2005)
Taltal[b]	Drinking water	3	110 - 120	(Crump et al. 2000)
	Drinking water	25	112	(Crump et al. 2000)
	Drinking water	62	113.9	(Téllez et al. 2005)
Chañaral[b]	Drinking water	3	4 - 12	(Crump et al. 2000)
	Drinking water	25	5.5	(Crump et al. 2000)
	Drinking water	53	5.82	(Téllez et al. 2005)
Caldera	Drinking water	3	4 - 12	(Crump et al. 2000)
Copiapó	Drinking water	3	4 - 12	(Crump et al. 2000)
Baquedano	Groundwater for industrial use	-	21,600	(Jackson et al. 2010)
Canchones	Irrigation/Possible human consumption	3	1,480	(Calderón et al. 2014)
Pica	Irrigation/Possible human consumption	3	744	(Calderón et al. 2014)

[a] ND: not detected. ND value was not reported
[b] Crump et al. (2000) reported that Tocopilla and Antofagasta's drinking water came from sources near Calama, 200 km northeast of Antofagasta; Chañaral obtained drinking water from 5 wells in Copiapó, 120 km south-east from Chañaral, and Taltal from wells in Agua Verde, 45 km east from Taltal.
[c] ND < 4 μg/L

Crump et al. (2000) reported perchlorate concentrations in groundwater near nitrate deposits in the Atacama Desert. Samples of groundwater used for industrial

processes had very high perchlorate concentrations, ranging from 1 to 10 mg L^{-1}. A natural spring used for recreational purposes had 1 mg L^{-1} of perchlorate. Groundwater used for human consumption had perchlorate concentrations ranging from 50 to 150 µg L^{-1}. The authors also mentioned a second survey, where tap water samples were collected from several cities of the North of the country. Taltal was the city with the highest perchlorate concentrations, presenting a range of 110 to 120 µg L^{-1}.

Crump et al. (2000) and Téllez et al. (2005) also measured perchlorate concentration in drinking water from those cities, obtaining similar results to the previous study (Table 2.3). A very high concentration of perchlorate (22 mg L^{-1}) was reported by Jackson et al. (2010), in a well presumably located near the small village of Baquedano in the Atacama Desert, but the use of this water source was not specified by the authors.

A recent study showed perchlorate contamination in several surface waters in the Atacama Desert, which are used to irrigate agricultural crops and probably also used for drinking water by approximately 16,000 people from the towns of Pica and Canchones, Region of Tarapacá. The perchlorate concentrations were 1.5 mg L^{-1} and 0.8 mg L^{-1}, respectively (Calderón et al. 2014).

The perchlorate levels found in several water sources in Chile are much higher than the US EPA or European recommendations for potable water. Also, information regarding perchlorate contamination is lacking for most water supplies in the Atacama region. Since the Atacama Desert highly water-scarce, alternative water supplies are not readily available.

2.4.2. Perchlorate in food

Perchlorate has been widely found in products for human consumption, such as beverages, fruits and vegetables, throughout the world. Industrial products may be impacted if they are based on perchlorate-containing water, as in the case of beverages. Studies show that plants can uptake perchlorate and accumulate it. This mainly occurs in leaves, where, depending on a variety of factors, they can accumulate up to 2 orders of magnitude of the perchlorate available in the irrigation source or soil, reaching mg kg^{-1}concentrations. Fruits from these plants can also incorporate perchlorate, in much lower concentrations (µg kg^{-1}) (Tan et al. 2004, Jackson et al. 2005, Estrada et al. 2017). Thus,

agricultural products may absorb perchlorate if they are irrigated by contaminated water, if they are grown in contaminated agricultural soil, or if perchlorate-containing fertilizers are used.

Table 2.4 shows the concentrations of perchlorate found in several food samples from Chile, mainly fruits or fruit products. El Aribi et al. (2006) published the most comprehensive study on perchlorate in food, where a variety of products from different countries in America and Europe were tested. They found Chilean green grapes, raspberries, and apricots had the highest concentrations of perchlorate among the 53 tested agricultural products. Apricots had the highest perchlorate concentration of Chilean products (146 $\mu g\ kg^{-1}$). Similar results for green grapes and red grapes have been reported (Wang et al. 2009). Red grapes samples presented a wide range of perchlorate concentrations. A survey of the United States Food and Drug Administration (US FDA 2005) also found a large degree of variation of perchlorate in green grapes (Table 2.4). This could be attributed to differences in local origins of the products within Chile. Also, a survey of perchlorate in food consumed in the United Kingdom found perchlorate in Chilean blueberries (Fera Science Ltd 2016).

Table 2.4. Perchlorate occurrence in Chilean food

Food	n	ClO_4^-	ClO_4^- average	Reference
		($\mu g\ kg^{-1}$)	($\mu g\ kg^{-1}$)	
Salmon	4	ND[a] - 1.42[b]	0.7[c]	(US FDA 2005)
Grapes	4	ND - 38.6	23.8[c]	(US FDA 2005)
Green grapes	3	-	22.0[d]	(El Aribi et al. 2006)
Apricots	3	-	146.7[e]	(El Aribi et al. 2006)
Raspberries	3	-	23.1[d]	(El Aribi et al. 2006)
Green grapes	6	26.6 - 62.1	45.5	(Wang et al. 2009)
Red grapes	6	1.96 - 40.5	9.9	(Wang et al. 2009)
Blueberries	1	13	-	(Fera Science Ltd 2016)
		($\mu g\ L^{-1}$)	($\mu g\ L^{-1}$)	
Beer	3	6.0 - 9.0[d]	7.23[c]	(El Aribi et al. 2006)
Red Wine	6	5.4 - 38.9[d,e]	19.85[c]	(El Aribi et al. 2006)
White Wine	2	10.8 - 16.1[d]	13.49[c]	(El Aribi et al. 2006)

[a] ND: not detected
[b] Below limit of quantification, above limit of detection
[c] Calculated with the information published by the author. ND was considered as zero for calculation
[d] Average of triplicates
[e] Average of duplicates

It is not known if the perchlorate-containing food products mentioned above were from the Atacama region, as the source of each of the Chilean products was not specified by the researchers. However, raspberries are not known to be produced in the Atacama Desert. Also, the production of apricots and blueberries in that region, in 2005 and 2011 respectively, were below 0.03% of the national production, so the samples are unlikely to be from there (ODEPA and CIREN 2005, ODEPA and CIREN 2011). Raspberries, apricots and blueberries are mainly grown in the south-central area of Chile, more than 600 km away from the Atacama Desert. Grapes are likely to come from the Atacama Desert, since this region accounts for 15% of the national fruit production, and 91% of the grapes from this region are exported (ODEPA and CIREN 2005, CIREN 2015).

The perchlorate in apricots, raspberries and blueberries may have resulted from the use of contaminated fertilizers or contaminated irrigation water. We have no information about fertilizers used by these farmers, but the fact that perchlorate has not been detected in tap water in southern Chile (data not shown) suggests fertilizers as the most likely sources of perchlorate. Nevertheless, the exact origin of fertilizers used in Chile is unknown. The Chilean fertilizers market is composed by national and imported fertilizers (ODEPA 2009) and fertilizers from other places besides the Atacama Desert are also known to contain low, but measurable perchlorate, as discussed previously (EFSA CONTAM Panel 2014). Perchlorate may also result from trace levels found in precipitation (Dasgupta et al. 2005), since the southern Chile has a rainy climate and plants and roots can accumulate perchlorate (Jackson et al. 2005). Yet this is unlikely, as most fruit and vegetables from around the world do not have perchlorate (US FDA 2005, El Aribi et al. 2006, Calderón et al. 2017).

El Aribi et al. (2006) also found perchlorate in Chilean wine and beer (Table 2.4). Samples of red and white wine were analyzed and showed a high variability of perchlorate concentrations (5.4 to 39 μg L^{-1}); beer had less variability, and presented a mean concentration of 7.2 μg L^{-1}. The wide range of perchlorate concentrations in wine could be related to the wide range of perchlorate in grapes used for its production or may also be explained by a diverse local origin. Wine in Chile is produced in different regions. Similar to fruit samples, wine and beer production are mainly in the center and south of the country, although the biggest beer-producing company in Chile owns a plant in Antofagasta (CCU 2006, SAG 2006).

Even though some products may have high concentrations of perchlorate, they do not necessarily translate into high levels of human intake since some products are consumed in small amounts (e.g., it is unlikely people will eat 1 kg of apricots or raspberries on a daily basis, but they should drink more than 1 L of water every day). The risk for perchlorate exposure should consider the different pathways and likelihood of exposure. This is important to tailor the risk assessment to Chile, as it presents high concentrations of natural perchlorate.

2.4.3. Other potential perchlorate exposure pathways

Products such as bleach, firecrackers or explosives, matches, and pharmaceuticals may contain perchlorate and be a source of exposure (Wan et al. 2015). For example, perchlorate was present in indoor dust from 0.02 to 104 mg kg^{-1} in a study spanning 11 countries. However, this contamination accounted for less than 5% of perchlorate intake for people in the region (Wan et al. 2015).

In Chile, there might be perchlorate in dust due to uses of contaminated household items, but also there could be presence of perchlorate in dust formed from perchlorate-containing Chilean soils. Although this may represent a lower amount of perchlorate intake compare to water or food sources, it should be accounted for when assessing human perchlorate exposure in Chile.

2.5. Evidence of exposure to perchlorate in Chilean population

Two studies have addressed perchlorate exposure in towns in the Atacama Desert. Exposure was assessed through the presence of perchlorate in human urine, serum and/or breast milk.

Gibbs et al. (2004) used urine and serum samples from children in Antofagasta, Chañaral and Taltal taken by Crump et al. (2000) to measure perchlorate. Perchlorate was only found in Taltal, with an average concentration of 5.6 µg L^{-1} in serum (n=27, limit of detection was 5 µg L^{-1} in urine and 1.5 µg L^{-1} in serum).

A second study determined perchlorate in urine and serum of pregnant women from the same three cities, in three stages of pregnancy: at the end of 16weeks, 30 weeks, and postpartum. For the postpartum sampling, they also collected breast milk samples (Téllez et al. 2005). The mean perchlorate concentration in urine samples in the first two stages was around 20, 70 and 130 µg L^{-1} in Antofagasta, Chañaral and Taltal respectively. Perchlorate in serum was only detected in in Taltal, at around 12 µg L^{-1}.

In postpartum samples, perchlorate was only detected in neonatal serum samples from Taltal with a concentration of 20 µg L^{-1} (Téllez et al. 2005).Breast milk samples contained 82, 18 and 96 µg L^{-1} of perchlorate in Antofagasta, Chañaral and Taltal, respectively. It is interesting that perchlorate concentrations in breast milk samples of

Antofagasta were so high, since perchlorate concentration in local tap water was very low (0.5 μg L^{-1}). After more detailed research, they found that one person had a very high perchlorate concentration (1,000 μg L^{-1}). Although the source of this amount of perchlorate was not found, there probably were other sources of perchlorate besides tap water. Additionally, a study of isotope composition of perchlorate found in human urine of samples from Taltal, reported a similar value (148 μg L^{-1}) to Tellez et al. (2005) (Poghosyan et al. 2016).

Perchlorate concentrations in these studies are around 30 times higher than perchlorate concentration found in urine samples from the U.S. (3.5 μg L^{-1}) (Blount et al. 2006). Particularly, considering a newborn may intake at least 0.5 L of breast milk per day, the intake of perchlorate in the cases of Antofagasta and Taltal are much higher than the recommended values by US EPA or ANSES.

These studies suggest that people in the Atacama Desert have a direct exposure to perchlorate, which is very likely to come from different sources. No additional human studies have been conducted in the more than 10 years, and some studies have shown that people could be exposed to much higher concentrations of perchlorate than those previously reported (Calderón et al. 2014). Thus, new epidemiological studies are warranted.

2.6. Health effects and regulations

Perchlorate is known to block the normal iodine/sodium exchange in the thyroid gland (Clewell et al. 2004). This is of particular concern for pregnant women, newborns, infants, and children, where thyroid function is key to normal growth and development. Thyroid inhibition can impair neurological development, causing mental diseases, hearing problems and language deficit (Glinoer 2007).

There are several studies that assessed the correlation between exposure to environmental perchlorate with changes in the production of thyroid hormones T3, T4 and TSH, but only few have investigated the relation between perchlorate levels in humans and the actual result of hypothyroidism (ANSES 2014, EFSA CONTAM Panel 2014). Some of this research shows a relationship between perchlorate levels and changes in the

production of thyroid hormones or the occurrence of different physiological characteristics. For example, Rubin et al. (2017) found an association between perchlorate concentration in the mother with an increase of birth weight in males and male preterm births. Taylor et al. (2014) indicated that high-end maternal perchlorate levels (~20 μg L^{-1} in urine) in pregnant women with iodine deficiency have an adverse effect on the cognitive development of offspring. Results from Steinmaus et al. (2013) suggests that environmental exposure to thiocyanate and to perchlorate (~12 μg L^{-1} in urine) together with low iodine intake can impact thyroid hormone production in humans. In a similar way, but with healthy pregnant women, Horton et al. (2015) shows the exposure to perchlorate together with nitrate and thiocyanate increased TSH, and suggests that perchlorate had the highest influence among the three disruptors.

The US EPA was first to set a recommendation level for perchlorate, after perchlorate was found near a Superfund site and then in multiple water sources (NRC 2005). They set a Reference Dose of 0.7 μg kg^{-1} day^{-1} of perchlorate and a provisional action level at 24.5 μg L^{-1} for drinking water in 2006. After further assessment of the risks of perchlorate, a new recommendation for drinking water was set at 15 μg L^{-1} (US EPA 2006, US EPA 2009). Under the Safe Water Drinking Act in 2011, the US EPA proposed regulating perchlorate, but regulations and analyses have not been established (US EPA 2011). Despite the lack of a federal regulation for perchlorate, several U.S. states have adopted their own standards for perchlorate in drinking water. Limits range from 2 μg L^{-1} in Massachusetts to 18 μg L^{-1} in New York (Brandhuber et al. 2009).

France has also adopted perchlorate standards for drinking water, based on the same US EPA Reference Dose of 0.7 μg kg^{-1} day^{-1} (ANSES 2011). In 2011, ANSES evaluated the health risk for perchlorate in drinking water, recommending a standard of 15 μg L^{-1} to protect adults (ANSES 2011). The DGS adopted 4 μg L^{-1} as maximum perchlorate concentration for infants younger than six months old (ANSES 2012). Besides, the EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) set an allowable daily intake of 0.3 μg kg^{-1} of body weight and the European Union agreed on different reference values for perchlorate in several food commodities (EFSA CONTAM Panel2014).

When assessing perchlorate health effects, the iodine intake of the studied population also should be considered, as in France (2014). The main source of iodine in food is iodized salt. Countries with lower iodine intake may be more susceptible to perchlorate effects. Iodine intake has declined in many developed countries, probably due to policies that discourage excessive salt consumption (Parker 2009). Chile is also working in that direction (Escobar 2010, Minsal 2016). In addition, many new, non-processed salt products are being used. These may contain perchlorate risk due to less iodine content, and salt products from Atacama Desert may be contaminated with perchlorate, even though they may contain iodate. Therefore, iodine intake should be included in research to address perchlorate risk to Chilean population.

Even though studies on human populations in the Atacama Desert did not find a correlation between perchlorate consumption and hypothyroidism (Crump et al. 2000, Téllez et al. 2005), there have been some concern about the experimental design issues of those studies (Steinmaus et al. 2013), and pregnant women with low iodine intake were not studied. In addition, the perchlorate exposure in many studies is not as high as levels of perchlorate found in water sources affecting other populations in the Atacama Desert.

2.7. Treatment systems for perchlorate in drinking water

There are several methods to treat perchlorate, which can be divided into physicochemical and biological technologies. Physicochemical technologies include ion-exchange (IX), carbon adsorption, membrane filtration (e.g. reverse osmosis, ultrafiltration or electrodialysis), and chemical and electrochemical reduction (Coates and Achenbach 2004, Srinivasan and Sorial 2009, Ye et al. 2012, Sijimol et al. 2015, Ma et al. 2016). A disadvantage of these technologies, except for chemical or electrolytic reduction, is they only separate perchlorate from water. The separated brine or concentrate requires further treatment or disposal. Chemical or electrochemical reduction reduce perchlorate to harmless compounds (chloride and water), but they require very harsh operating conditions, such as high temperatures and pressures.

Biological treatment technologies include phytoremediation and biological reduction. Phytoremediation can be used to treat perchlorate in groundwater and soils. It is based on plants and trees that accumulate perchlorate (Sijimol et al. 2015, Ma et al. 2016). Biological perchlorate reduction is based on perchlorate reducing microorganisms (PCRM), which typically gain energy by reducing perchlorate to chlorate (ClO_3^-) and then to chlorite (ClO_2^-), a toxic intermediate. ClO_2^- is transformed by disproportionation into molecular oxygen and chloride (Cl^-) via the chlorite dismutase enzyme (Figure 2.2) (Coates and Achenbach 2004, Srinivasan and Sorial 2009, Ye et al. 2012, Sijimol et al. 2015, Ma et al. 2016). The disadvantage of this technology is it usually requires an exogenous electron donor, increasing the operational costs (Sharbatmaleki and Batista 2012, Choe et al. 2013). Also, the use of bacteria for drinking water treatment is still debated in many countries.

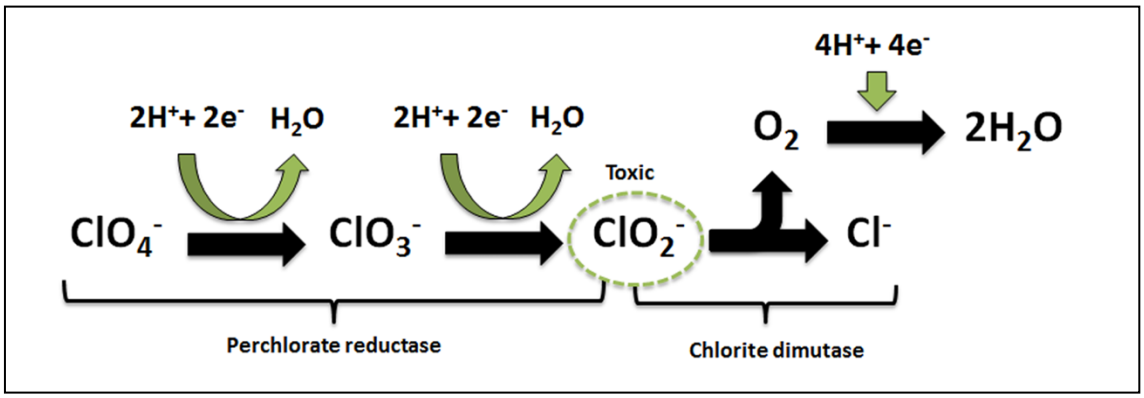

Figure 2.2. Biological pathway of perchlorate reduction. The perchlorate reductase enzyme catalyzes the reduction of perchlorate and chlorate to chlorite. The chlorite dismutase enzyme is responsible for chlorite removal. Adapted from (Bardiya and Bae 2011).

The environmental impact of ion-exchange technologies, biological reduction, and catalytic reduction technologies in the removal of perchlorate from drinking water has been recently assessed using a life cycle approach (Choe et al. 2013). The study concluded that biological treatment using hydrogen (H_2) as electron donor is the most environmentally friendly alternative to remove perchlorate from water due to its low impact and global warming footprint. The authors found that consumables are mainly

responsible for environmental impacts of each technology. The requirement of H_2 for biological reduction is 10 times smaller than of organic electron donors, and H_2 has lower environmental impacts than the inputs for IX and catalytic reduction.

Despite of its disposal disadvantage, IX is the most common treatment technology for perchlorate in groundwater and drinking water. There are two types of IX processes: those based on non-selective and selective resins. Non-selective resins remove perchlorate as well as other anions, which may be present at much higher concentrations. This requires frequent regeneration with concentrated brines, which in turn need further treatment before final disposal into the environment. Although the regeneration extends the lifetime of the resins, lowering the cost of the process, and the negative environmental impacts associated with brine disposal requires more attention and improvement. On the other hand, ion-exchange using perchlorate-selective resin exhibits better performance (Srinivasan and Sorial 2009, Ye et al. 2012). However, this selectivity makes the regeneration ineffective. Resins are typically incinerated after a single use, increasing costs and the environmental footprint of the treatment (Srinivasan and Sorial 2009, Ye et al. 2012, Choe et al. 2013, Ma et al. 2016). Because of the IX limitations, bio-regeneration of saline brines from non-selective resins and of spent selective resins has emerged as a good alternative to make IX process economically and environmentally more sustainable (Lehman et al. 2008, Srinivasan and Sorial 2009, Venkatesan et al. 2010, Sharbatmaleki and Batista 2012, Ye et al. 2012).

For the Atacama Desert area, selective resins may be a better option for perchlorate removal. With non-selective resins, there are two problems: a) loss water for the regeneration process would be infeasible because of high water scarcity in this area and b) natural water sources used in northern Chile already contain high concentrations of other anions (e.g., NO_3^-, SO_4^{2-}, IO_3^-), which will compete with perchlorate and will saturate the resin quickly, requiring more frequent regeneration. Reverse osmosis also could be a viable option, since it already is used in cities around the Atacama Desert. Anion competition and brine disposal would also be a problem (Srinivasan and Sorial 2009, Ye et al. 2012), although safe disposal could be addressed by biological reduction.

The target effluent standards for perchlorate, and other contaminants, need to be defined prior to choosing a treatment technology for water supplies in the Atacama Desert.

2.8. Recommended research and research opportunities in the Atacama Desert

2.8.1. Risk Assessment and Regulation

Chile has one of highest natural concentrations of perchlorate in the world, in both soil and water. Also, perchlorate has been found in Chilean food products, such as fruit and beverages. These are probably from the south of the country, where no high-level natural perchlorate sources have been reported. Because of these multiple pathways of high-concentration perchlorate exposure, much of the Chilean population may subject to health risks from perchlorate.

As with U.S. and Europe, Chile should assess exposure to perchlorate and develop recommendations for perchlorate levels in drinking water and other matrices. Some sources may not be a concern in themselves (e.g., raspberries, since the consumption is low). But combined with other sources, perchlorate intake may reach harmful levels. Therefore, comprehensive data on perchlorate in environment and food sources is needed. A systematic national survey of perchlorate in the environment (including water supplies, soils, and indoor dust), and in food, prioritizing areas with high levels of perchlorate, is an important first towards determining exposure levels and possible standards for drinking water and food products. These surveys should consider tracking the specific location of consumable products within Chile and the origin of the supplies used for their production, to understand the sources of perchlorate contamination and apply targeted remedial actions.

Contamination of water supplies and agricultural products is an important concern in the Atacama Desert where water resources are very scarce. Furthermore, environments contaminated with perchlorate in Chile also represent a global environmental concern, because many Chilean food products are exported, enlarging the exposed population.

Also, perchlorate has been found in products from other countries (El Aribi et al. 2006), so imported products could be another source of perchlorate contamination.

2.8.2. Biological perchlorate reduction: research opportunities

Atacama Desert has one of the most extensive reservoirs of perchlorate, with one of the highest natural perchlorate concentrations known today. Although these and other extreme natural environments in northern Chile may present a hazardous condition for the population, they are unexplored biological niches to find novel microorganisms adapted to extreme conditions (i.e. high salinity, nitrate concentrations, inorganic electron sources). If novel PCRM are found, they may allow improved biological treatment systems to remove this contaminant from soils and water sources. In the following paragraphs we highlight some challenges and opportunities for the Atacama Desert.

2.8.2.1. Biological perchlorate reduction in saline environments

Saline environments in northern Chile represent a potential source of novel PCRM that could be used to reduce perchlorate in solutions with high salinity, as the residual brine from IX systems used to remove perchlorate from drinking water.

PCRM have been studied in different reactor configurations to treat brines produced by the regenation of IX resins. This application is challenging, since perchlorate reduction by PCRM is inhibited at high salinities. Most studies show moderate perchlorate reduction rates at salinities of around 1% NaCl, and complete inhibition at concentrations above 4%. (Lehman et al. 2008, Chung et al. 2010). Nevertheless, salt tolerant PCRM have been isolated from marine enviroments, exhibing appreciable perchlorate reduction rates after aclimatation. (Logan et al. 2001, Okeke et al. 2002, Cang et al. 2004, Park and Marchand 2006). For example, Ryu et al. (2012) reported a salt tolerant consortia capable to reduce perchlorate at significant rates with a salt concentration as high as 7.5%.

Hydrothermal sources and rivers of northern Chile present high salinity content (up to 1.5% NaCl approximately) and biological activity related to perchlorate chemistry. In particular, the Colpitas River (Figure 2.3), a sub-basin of the Lluta river watershed in the XV Region of Arica and Parinacota, is of special interest because high concentrations of chlorate (482 mg L^{-1}) and perchlorate (up to 4 mg L^{-1}) have been observed, along with

positive PCRM isolation and the amplification of the chlorite dismutase gene (internal study, data not shown).

Figure 2.3. Hydrothermal source in the upper part of the Lluta River (Region of Arica y Parinacota). PCRM isolates, together with a positive indication of chlorite dismutase enzyme have been obtained from this hydrothermal source exhibiting chlorate and perchlorate concentration as high as 4.15 mg L^{-1}, and a conductivity of 25 mS cm^{-1}.

2.8.2.2. *Developing strategies to remove perchlorate from nitrate fertilizers*

Fertilizers containing perchlorate may be a source of soil, water and crop contamination where they are used, and consequently may contaminate crops for human consumption (Böhlke et al. 2009, Sturchio et al. 2014). Thus, reducing perchlorate concentration in these fertilizers could help to diminish perchlorate contamination. Biological reduction of perchlorate could help to accomplish this goal.

In general, biological perchlorate reduction is significantly inhibited by the presence of nitrate, an alternate electron acceptor and an important component of fertilizers (Chaudhuri et al. 2002, Bender et al. 2005). However, the mechanisms of

inhibition are not well understood. Also, it is not clear whether PCRM can denitrify with perchlorate reduction enzymes (Xu et al. 2004).

Early PCRM studies suggested that both perchlorate and nitrate reduction were catalyzed by the same nitrate reductase, since the presence of nitrate causes a longer lag in perchlorate reduction until nitrate is fully removed (Stouthamer 1967, Chaudhuri et al. 2002, Tan et al. 2004, Xu et al. 2004). Currently, sequential and simultaneous reduction of perchlorate and nitrate have been extensively reported for pure and enriched cultures (Chaudhuri et al. 2002, Xu et al. 2004, Bardiya and Bae 2005). Nevertheless, other studies confirmed the existence of completely separate pathways for perchlorate and nitrate reduction, which may allow for selective perchlorate reduction without denitrification (Bardiya and Bae 2011). Furthermore, Xu et al. (2015) found that nitrate reduction was inhibited by perchlorate when studying reduction of perchlorate by *Azospira* sp. KJ. However, it is not clear if the inhibition was competitive or non-competitive.

These studies of enzymatic pathways for the reduction of perchlorate and nitrate show there are diverse types of terminal reductases participating in these processes and possibly new, non-nitrate reducers PCRM may be found and used to eliminate perchlorate from Chilean fertilizers, avoiding contamination in places around the world.

2.8.2.3. Expanding the microbial diversity for perchlorate removal

Presence/absence of the chlorite dismutase (Cld) enzyme: The Cld is a heme enzyme that transforms chlorite to chloride and oxygen (Figure 2.2), making it a unique enzyme and one of the few known to form O_2 as product. Besides, chlorite is a toxic compound to bacteria, so it is essential for perchlorate reduction.

Chlorite reduction can also be carried out abiotically through reactions with strong reductants (Nerenberg 2013). This chemical reaction could allow perchlorate reducing microorganisms lacking the chlorite dismutase to grow on perchlorate without experiencing chlorite toxicity. This has been found for a recently discovered PCRM. Recently, researchers in the Netherlands reported a PCRM from the Archaeal domain (Liebensteiner et al. 2013). This novel PCRM, *Archaeoglobus fulgidus*, an obligate anaerobic hyperthermophile, does not metabolize nitrate. Interestingly, it also lacks a chlorite dismutase enzyme and relies on abiotic chlorite reduction with sulfide (Nerenberg

2013). Furthermore, another hyperthermophile member of the archaeal domain, *Haloferax mediterranei*, was found to be able to reduce perchlorate without having genes encoding for perchlorate reductase. Unlike *A. fulgidus*, *H. mediterranei* did have a putative gene for chlorite dismutase.

Aerobic perchlorate bioreduction: Perchlorate respiration typically occurs under anaerobic conditions since it has been determined that the presence of oxygen in growth media inhibits perchlorate reduction (Chaudhuri et al. 2002, Bardiya and Bae 2011). An exception to this rule was found in 2008, for strains of the *Arthrobacter* genus. This bacteria is able to reduce perchlorate under aerobic conditions (Shete et al. 2008). In addition, *Pseudomonas PDA* was capable to reduce chlorate, but not perchlorate, under aerobic conditions (Xu et al. 2004). Analyses made on the chlorate reductase enzyme from *Pseudomonas PDA* showed that it was different from typical anaerobic perchlorate reductases (Steinberg et al. 2005), suggesting different pathways for perchlorate/chlorate reduction under aerobic conditions. Aerobic respiration of compounds that are typically respired under anaerobic conditions (e.g. sulfate, nitrate) has been reported before (Canfield and Des Marais 1991, Carter et al. 1995).

These are examples of new types of metabolisms found in nature. The perchlorate-containing environments of northern Chile are complex biogeochemical systems, where biological and chemical reactions may allow activity of PCRM with special characteristics as the mentioned above. Bioprospecting PCRM in these places could emerge as an opportunity to learn more about the different metabolic pathways involve on perchlorate respiration. PCRM isolated under these conditions may have taxonomic or ecologic relevance in perchlorate-containing environments.

2.9. Conclusions

Perchlorate is a ubiquitous environmental contaminant. In most cases of concern, perchlorate contamination results from anthropogenic sources. The Atacama Desert in Chile is one of the few major natural sources of perchlorate, with some of the highest concentrations. Stable isotope data analyses support the theory that natural perchlorate in Chile results from atmospheric deposition of perchlorate in the desert environment over

millions of years. This natural existence in Chile has led to contamination of different sources such as soils, waters and food sources that not only impacts the Chilean population, but the population worldwide. Despite the health concerns from perchlorate exposure, there is very limited research in Chile with respect to perchlorate contamination. A comprehensive assessment the health risks of perchlorate, by studying its abundance in the environment and food sources, as well as the pathways of exposure, is highly desirable. A risk assessment could be used to develop a standard for perchlorate in drinking water.

Perchlorate contamination also presents opportunities to study biological perchlorate reduction. Novel microorganisms with special features may be found in the north of Chile, where the environment has extreme characteristics. These microorganisms could be a powerful research tool to expand knowledge regarding the metabolic pathways and community inter-relations of PCRM. This information would be helpful to design novel, sustainable and efficient alternatives to remove perchlorate from sources as water or fertilizers.

CHAPTER 3

Enhancing perchlorate reduction by adding chlorate to a hydrogen-based membrane biofilm reactor (H_2-MBfR)

3.1. Introduction

Perchlorate (ClO_4^-) is a water contaminant that is highly soluble and stable in aqueous solution. It can inhibit thyroid function, leading to hypothyroidism (Srinivasan and Sorial 2009). Recommended levels for ClO_4^- drinking water in United States and Europe range 1 - 18 μg/L (US EPA 2006, US EPA 2009, ANSES 2011, ANSES 2012, EFSA CONTAM Panel 2014).

Ion exchange (IX) is an effective means to remove ClO_4^-, but it creates a high-strength ClO_4^- brine that requires further treatment or special disposal. Microbial reduction by perchlorate reducing bacteria (PRB) is a more promising strategy, as it reduces ClO_4^- to innocuous chloride (Cl^-). PRB sequentially reduce ClO_4^- to chlorate (ClO_3^-) and chlorite (ClO_2^-), via the perchlorate reductase (Pcr) enzyme. Then ClO_2^- is transformed by dismutation into chloride (Cl^-) and oxygen (O_2). The O_2 is concurrently utilized by PRB as an electron acceptor (Coates and Achenbach 2004, Srinivasan and Sorial 2009, Ye et al. 2012, Sijimol et al. 2015, Ma et al. 2016).

PRB are able to use organic electron donors, such as acetate and lactate, or inorganic electron donors, such as hydrogen. Using an inorganic electron donor is the most environmentally friendly alternative due to its low global warming footprint (Choe et al. 2013). Also, the use of biofilm reactors can reduce or eliminate downstream treatment to avoid microorganisms in the effluent.

The hydrogen-based membrane-biofilm reactor (H_2-MBfR) has been successfully applied for denitrification (Martin and Nerenberg 2012) and ClO_4^- reduction (Nerenberg et al. 2008, Ontiveros-Valencia et al. 2013b, Ontiveros-Valencia et al. 2014a). In this type of biofilm reactor, the control of the biomass growth and microbial competition is necessary to achieve successful ClO_4^- reduction (Ma et al. 2016).

PRB are ubiquitous in natural environment, even in pristine areas with perchlorate contamination (Coates et al. 1999). This is probably because PRB are effective aerobes and denitrifiers. However, ClO_4^- is a highly energetic electron acceptor, and when O_2 and NO_3^- are limiting and ClO_4^- is present, PRB have a strong metabolic advantage over common, non-PRB. In fact, PRB have been shown to increase in abundance when ClO_4^- is present and O_2 and NO_3^- concentrations are low. Nerenberg et al. (2008) found that the relative abundance of PRB in a denitrifying H_2-MBfR was around 13% when the influent ClO_4^- was 100 µg/L and the bulk ClO_4^- was around 10 µg/L. The relative abundance increased to nearly 50% when the influent ClO_4^- was 10 mg/L and the bulk around 5 mg/L. However, when ClO_4^- needs to be removed to the low µg/L range, there is a weak selective pressure for PRB probably leading to low abundances. This, along with the significantly lower rates of ClO_4^- reduction at these concentrations due to their ClO_4^- affinities in the high µg/L to low mg/L range (Nerenberg et al. 2008), making it challenging to meet the recommended treatment levels for ClO_4^-.

An additional challenge is that the competing electron acceptors, such as O_2 and NO_3^-, can inhibit microbial ClO_4^- reduction in PRB. For effective perchlorate reduction, low NO_3^- and O_2 levels are needed in the reactors. However, low levels of O_2 and NO_3^-, and the presence of sulfate (SO_4^{2-}), favor sulfate-reducing bacteria (SRB) (Ontiveros-Valencia et al. 2013b, Ontiveros-Valencia et al. 2014a). Reduction of SO_4^{2-} is undesirable, since it produces H_2S, a toxic, corrosive and malodorous compound. Studies of a H_2-MBfR for ClO_4^- reduction in the presence of NO_3^- and SO_4^{2-} showed SO_4^{2-} reduction in almost all cases (from 10% to 80%) and difficulties reaching low ClO_4^- concentration in one stage H_2-MBfR when ClO_4^- was present at high concentrations (above 1 mg/L) (Ontiveros-Valencia et al. 2013b, Ontiveros-Valencia et al. 2014a). Previous work on H_2-MBfR with incomplete ClO_4^- reduction pointed out that SRB have advantages over PRB

by competing for space in the biofilm and possibly being located in the inner layers, away from detachment forces and closer to the electron donor (Ontiveros-Valencia et al. 2013b).

In order to enhance ClO_4^- reduction, and prevent he proliferation of SRB, we propose manipulating the microbial community to favor PRB and suppress SRB. For example, doubling the abundance of PRB could substantially reduce the effluent ClO_4^- concentration.

Adding high amounts of ClO_4^- would clearly increase the abundance of PRB, but would defeat the purpose of treatment. It could be added intermittently, but there still would be concerns about adding a toxic compound that would require special handling and control of any discharges. A better strategy would be adding a compound that selects for PRB, but is less toxic. A potential option is ClO_3^-, which can be considered an analog to ClO_4^-. An analog is used by the same enzyme, and potential also provides energy and therefore a selective pressure for the microorganism. ClO_3^- is an intermediate in the ClO_4^- reduction pathway and transformed by the Pcr enzyme. PRB actually grow faster on ClO_3^- than on ClO_4^- (Bardiya and Bae 2011). For example, Nerenberg et al. (2006) found that a pure culture of PRB grew around twice as fast on ClO_3^- than on ClO_4^-. Also, the half saturation constant for ClO_4^- was 0.14 mg/L, while for ClO_3^- it was below 0.014 mg/L, meaning that high growth rates could be obtained at much lower concentrations.

Based on the above, ClO_3^- may have a strong selective pressure for PRB when ClO_4^- concentrations are low. In addition, ClO_3^- is known as an inhibitor of SO_4^- reduction in batch systems (Carlson et al. 2015) and in a continuous soured columns (Engelbrektson et al. 2014, Engelbrektson et al. 2018). Thus, ClO_3^- could both help select for PRB and inhibit SRB. However, the effect of ClO_3^- on the performance of a continuous H_2-MBfR system has not been studied.

The goal of this study was to analyze the effects of ClO_3^- addition on the reduction of ClO_4^- in a H_2-MBfR and the development of the microbial community in presence of O_2, NO_3^- and SO_4^{2-} at common drinking water concentrations.

3.2. Materials and Methods

3.2.1. Reactors set up and culture medium

We operated two identical H_2-MBfRs composed of two glass tubes joined together with Norprene tubing and connectors. One of the glass tubes contained a bundle of 31 membranes (Mitsubishi MHF200TL) ("main bundle") and the other tube had 3 membranes (Mitsubishi MHF200TL) to take biological samples ("coupon"). Both groups of membranes had a length of 23.5 cm and were connected to the H_2 line at one end and sealed at the other. A schematic of the setup is shown in Figure 3.1 and the reactor characteristics are summarized in Table 3.1.

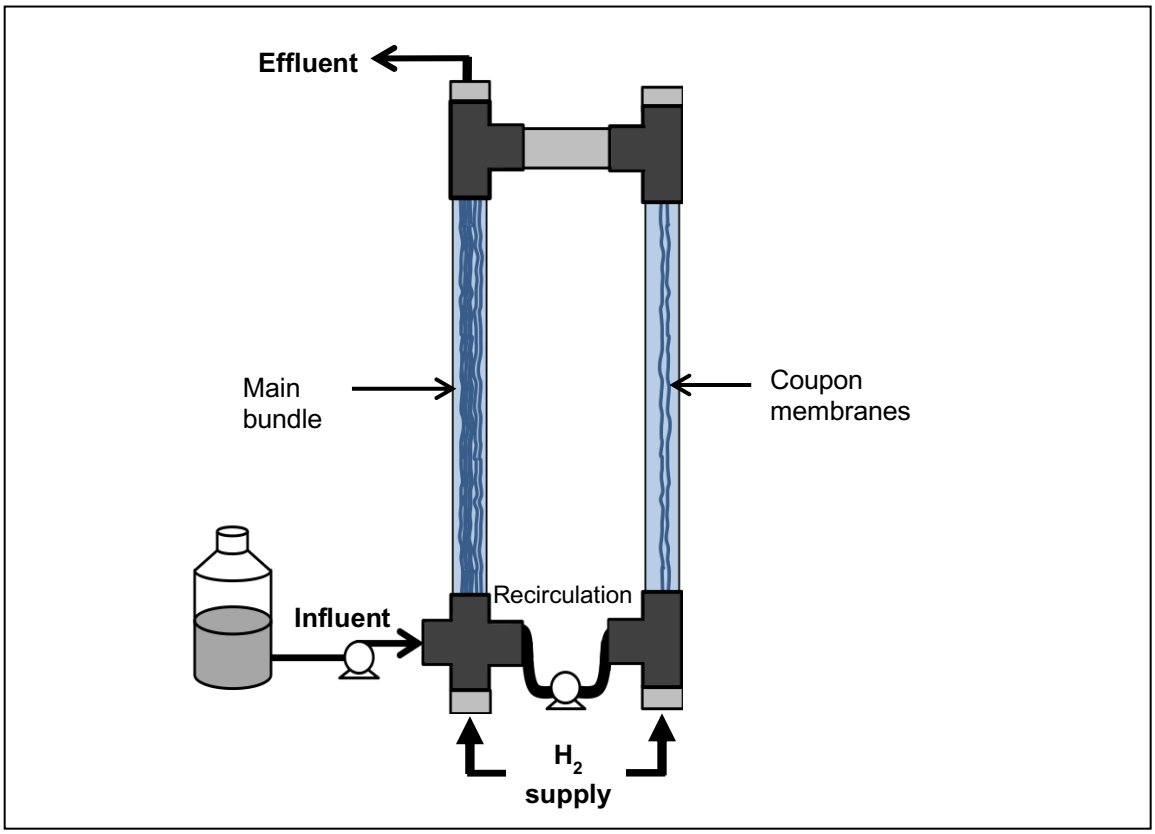

Figure 3.1. Scheme of a H_2 based membrane biofilm reactor. The reactor is composed of two glass tubes. One tube contains a bundle of 31 membranes ("main bundle") and the other tube had 3 membranes for biological sampling ("coupon"). Hydrogen is supplied from one end of the membranes, and the other end is sealed.

The influent media contained (g/L): 0.434 Na_2HPO_4, 0.128 KH_2PO_4, 0.2 $MgSO_4 \times 7H_2O$, 0.2 $NaHCO_3$, 0.001 $FeSO_4 \times 7H_2O$, 0.001 $CaCl_2$ and 1 mL of trace minerals solution. The trace minerals solution was composed of (mg/L): 100 $ZnSO_4 \times 7H_2O$, 30 $MnCl_2 \times 4H_2O$, 300 H_3BO_3, 200 $CoCl_2 \times 6H_2$, 10 $CuCl_2 \times 2H_2O$, $NiCl_2 \times 6H_2O$, 30 $Na_2MoO_4 \times 2H_2O$ and 30 Na_2SeO_3. The pH of the media was adjusted to 7.5.

Table 3.1. Reactors characteristics and operational conditions

Total membrane area	70.3 cm^2
Total volume	45 mL
H_2 pressure	5 psi
Influent flow	1 mL/min
Recirculation flow	100 mL/min
HRT	45 min
Temperature	Room temperature (~22°C)

3.2.2. Startup and stage experiments

Both reactors were inoculated with 1 mL of the same sample of activated sludge from the South Bend Wastewater Treatment Plant, South Bend, Indiana, USA. This inoculum was divided in aliquots with 20% glycerol and stored at -20 prior to used in inoculating the reactors. After inoculation, the reactors were run in recirculation mode for 16 h before starting continuous influent flow. The operational conditions are described in Table 3.1. The high recirculation flow rate created completely mixed conditions in the reactor bulk liquid. Based on past research with similar flows and influent concentrations, the hydrogen (H_2) pressure was expected to be sufficient to reduce the influent O_2, NO_3^-, and ClO_4^- (Nerenberg et al. 2008).

The reactors were operated in three stages as detailed in Table 3.2. In the first stage, Reactors A (control) and B (experimental) were supplied with an influent with 5 mg N/L NO_3^-, 80 mg/L SO_4^{2-}, and 8.4 mg/L O_2 for 25 days. ClO_4^- was supplied at 0.1 mg/L during the first 9 days, and then was increased to 0.6 mg/L. In the second stage, after NO_3^- and ClO_4^- reached steady state, ClO_3^- was continuously added at 5 mg/L to Reactor

B for 30 days. In the last stage, we stopped ClO_3^- addition to Reactor B was stopped for 15 days.

Table 3.2. Influent concentrations in each stage of Reactors A and B.

Conditions	Stage 1 Days 0 – 25		Stage 2 Days 26 – 63		Stage 3 Days 64 – 79	
	Reactor A	Reactor B	Reactor A	Reactor B	Reactor A	Reactor B
NO_3^- (mg N/L)	5	5	5	5	5	5
ClO_4^- (mg/L)*	0.6	0.6	0.6	0.6	0.6	0.6
SO_4^{2-} (mg/L)	80	80	80	80	80	80
ClO_3^- (mg/L)	0	0	0	5	0	0

*During the first 9 days of Stage 1 the influent concentration was 0.1 mg/L.

3.2.3. Analytical Methods

The influent was sampled every three days and the effluent daily. Samples were filtered with 0.2 µm polyethersulfone (PES) syringe filters before storing at 4°C. The concentrations of NO_3^-, ClO_4^-, ClO_3^- and SO_4^{2-} in the influent and effluent were analyzed using ion chromatography (DIONEX 2500) implemented with a 4 mm AG20 guard column and a 4 mm AS20 analytical column. Sodium hydroxide (NaOH) was used as eluent at 1 mL/min with a gradient program ranging from 5 mM to 55 mM. The detection limit for NO_3^-, ClO_4^-, ClO_3^- and SO_4^{2-} was 0.06 mg N/L, 12 µg/L, 60 µg/L, and 0.6 mg/L respectively. For analyzing the results, the influent for the days between sampling was calculated as the average of the samples.

The pH was measured using a pH meter (Accument® AB250, Fisher Scientific) and dissolved oxygen (DO) in the influent was measured with a DO probe (LOD101, Hach). The O_2 concentration in the effluent was not measured.

3.2.4. Biofilm sampling and DNA extraction

Biofilm samples were collected for microbial community analyses from both reactors at:

i) Day 21, just before adding ClO_3^- to Reactor B (t1)

ii) Day 60, which was 30 days after starting ClO_3^- addition (t2)

iii) Day 77, which was 15 days after stopping ClO_3^- addition (t3)

To sample the biofilm from each reactor, a 2 – 4 cm long section of the coupon membrane was cut, and the remaining section was sealed with a knot to prevent gas leakage. To separate the biofilm from the membrane, the membrane section was placed in a 2 mL centrifuge tube, vortexed at maximum speed for 10 min, and centrifuged it at maximum speed for 10 min. DNA was extracted with UltraClean™ Microbial DNA Isolation kit (MO BIO Laboratories) according to the manufacturer's instructions. The concentrate absorbance was measured with a spectrophotometer (NanoDrop 2000, Thermo Scientific) to ensure sufficient quantity. The extracted DNA was analyzed via 16S rRNA sequencing and quantitative polymerase chain reaction (qPCR) of functional genes involved in nitrate (*nirK* and *nirS*), sulfate (*dsrA*) and (per)chlorate (*pcrA*) reduction pathways. DNA samples were stored at -20°C after extraction.

3.2.5. Statistical analysis

To determine the statistical significance of the effect of chlorate while it was added to Reactor B, the data resulted from Stage 2 (Days 24 to 63) of both Reactors was analyzed with a one-way repeated measured analysis of variance (ANOVA) using the software SPSS Statistics version 25. The data set used from Stage 2 excluded data from the Days 31 to 34 and Days 48 and 49, as at those two times the performance results were affected by the two leaking events.

3.2.6. qPCR analysis

We determined the number of gene copies of *nirK*, *nirS*, *pcrA*, *dsrA* and 16S rRNA using qPCR. The amplification reaction was performed on an Eppendorf Mastercycler ep Realplex thermocycler with a total reaction volume of 20 µL. Each reaction mixture contained 10 µL SYBR *Premix Ex Taq* (Tli RNaseH Plus) (Takara Bio USA, Inc.), 0.2

µL of each primer (100 mM), 1 µL of DNA template (3 – 10 ng/µL) and DNase-free water up to 20 µL. All reactions were done in triplicate, including the negative control (DNA was replaced with DNase-free water). Calibration curves for these five genes were prepared with seven serial dilutions of plasmids containing the genes of interest at concentrations from 10^{-1} to 10^{-7} gene copies/µL. Primers and thermocycler conditions used in this study were described in Ontiveros-Valencia et al. (2013b).

3.2.7. High-throughput sequencing and taxonomic analysis

The microbial community was determined by high-throughput sequencing of regions V4 and V5 of the 16S rRNA, using the primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTYMTTTRAGTTT-3'). Samples were sent to DNA Services Facility at the University of Illinois at Chicago for sequencing with Illumina technology. Taxonomic analysis was performed in QIIME v 1.8, as described previously (Ontiveros-Valencia et al. 2014).

3.3. Results

3.3.1. ClO_3^- and SO_4^{2-} reduction

In both H_2-MBfRs reactors, NO_3^- was reduced to below detection after 5 days (Figure 3.2). ClO_4^- was reduced to approximately 12 µg/L (95%) on Day 9 in Reactor A and on Day 6 in Reactor B. The delay and the lower of perchlorate reduction on Reactor A may be due to a small leaking in the influent tubing during the first 5 days of operation.

On Day 15, the influent supply to both reactors was interrupted for 12 h due to a pump failure, and on Day 17 SO_4^{2-} reduction was detected. SO_4^{2-} removals averaged 71 mg/L and 74 mg/L on Day 22 in Reactors A and B respectively. During this period, ClO_4^- reduction decreased in both reactors, with effluents reaching up to 150 µg/L (77% removal) in Reactor A and 45 µg/L (94% removal) in Reactor B. The greater effect on Reactor A could potentially have resulted from the leak at the beginning of the operation. The reactor behaved similarly for the remaining 50 days of operation.

When ClO_3^- was added to Reactor B on Day 24, it was immediately reduced, reaching 98% reduction (effluent of 0.117 mg/L) in two days (Day 26) and remaining with effluent concentrations of around 100 µg/L during Stage 2, except for two events (leaks and biofilm sloughing). This indicates the microbial community present in the biofilm already had the capability to reduce ClO_3^-, probably due to the PRB. The increase of ClO_3^- reduction during the first four days of addition suggests an enrichment of PRB or CRB. Since the community presumably contained PRB prior to ClO_3^- addition, it is likely that PRB were responsible for the initial reduction, rather than CRB.

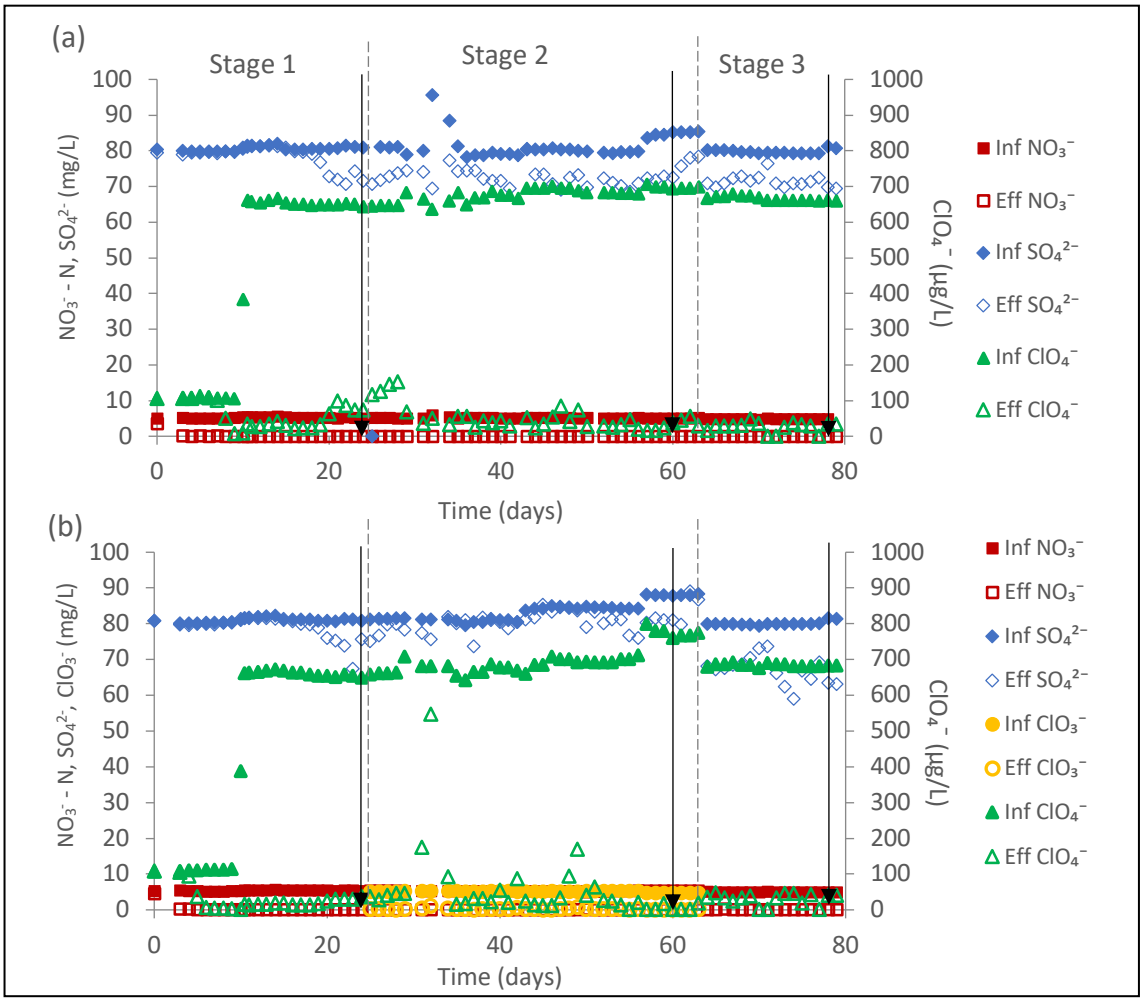

Figure 3.2. Reduction of NO_3^-, SO_4^{2-}, ClO_4^- and ClO_3^-. (a) Reactor A (control). (b) Reactor B (with ClO_3^- addition). In the first stage, both reactors were run without ClO_3^-. From Day 24 to 63, ClO_3^- was added to reactor B. Both reactors then continued to operate for 15 more days. The arrows indicate the days in which the biological samples were taken.

During ClO_3^- addition to Reactor B, SO_4^{2-} reduction diminished to below 5% (80 mg/L in the effluent) and ClO_4^- reduction improved, with an average effluent concentration of 23 μg/L (>96% of reduction). In contrast, the average SO_4^{2-} reduction for Reactor A was 10% (73 mg/L) and ClO_4^- reduction decreased, with an effluent of only 51 μg/L (about 92% reduction). Once ClO_3^- was removed from Reactor B, SO_4^{2-} reduction quickly resumed and reached levels higher than before adding ClO_3^-, and also higher than in Reactor A. There was also a decrease in ClO_4^- removals to below 95%. The ANOVA test results indicate that reduction of SO_4^{2-} and ClO_4^- during Stage 2 are significant (F-ratio = 78.82 and p = 0.0005 for SO_4^{2-}, and F-ratio = 17.21 and p = 0.0005 for ClO_4^-).

From a performance perspective, the addition of ClO_3^- seemed to suppress the activity of SRB. But it apparently did not eliminate SRB, given the fast recovery of SO_4^{2-} reduction after stopping the ClO_3^- feed. ClO_3^- addition enhanced removals of ClO_3^- and ClO_4^-, possibly by outcompeting SRB for H_2 and for space in the biofilm, or possibly because ClO_3^- had a direct inhibitory effect on SRB. The latter seems unlikely, though, since the bulk ClO_3^- concentrations where only 100 μg/L and unlikely to penetrate very deep into the biofilm. ClO_3^- addition stimulated ClO_4^- reduction, probably by increasing the abundance of PRB. The effluent ClO_3^- concentrations were well below the ClO_3^- standard of 0.7 mg/L.

3.3.2. Assessment of the microbial community with qPCR

Biofilm samples were taken from two membranes in each reactor at the end of each stage: before adding ClO_3^- to Reactor B (t1, on day 24), 36 days after ClO_3^- addition to Reactor B (t2, on Day 60) and 14 days after removing ClO_3^- (t3, on Day 77). qPCR was performed to determine the abundance of the genes *dsrA*, *pcrA*, *nirK*, *nirS* and 16S rRNA. *dsrA* is related to SRB metabolism, *pcrA* to PRB, and *nirK* and *nirS* to denitrification metabolism. To obtain relative abundance, the results of the specific genes were normalized to the copies of 16S rRNA (Figure 3.3).

In Stages 1 and 2, before and during ClO_3^- addition to Reactor B (t1 and t2), both reactors had similar proportions of *dsrA* and *nirK*+*nirS* (below 1.5% and 1%, respectively), with *dsrA* being slightly higher than *nirK*+*nirS* (Figure 3.3). However, from

t1 to t2, *dsrA* and *nirK* had a bigger increment in Reactor B than in Reactor A (approximately eight times for *dsrA* and five times for *nirK* for Reactor B, compared to three times and three times for Reactor A), which could indicate a positive effect of ClO_3^- on denitrifying bacteria (DNB) and SRB. Also, from t2 to t3, *nirK*+*nirS* decreased to almost a third part in Reactor A and increased 1.5 times in Reactor B.

In Reactor A, the abundance of *dsrA* increased progressively over the three stages, even though SO_4^{2-} reduction remained around the same. This may be because of growth of SRB may have "pushed" SRB from the biofilm interior towards the exterior, where H_2 may have been depleted. The abundance of *dsrA* also increased in Reactor B during Stage 2, but to a much smaller degree. This suggesting that ClO_3^- addition was unfavorable to SRB.

The abundance of *pcrA* in Reactor A decreased from t1 to t2 and remained similar in t3, but it increased seven times in Reactor B from t1 to t2 and remained similar in t3. This suggests that ClO_3^- addition selected for PRB. Even though a high increase in the gene abundance could correspond to a smaller increase in number of bacteria, the opposite trends of *prcA* abundance in Reactors A and B may also indicate an enrichment of PRB due to the presence of ClO_3^-. The ClO_3^- effect on the microbial community will be discussed in more detail in the Discussion section, together with the 16S rRNA sequencing results.

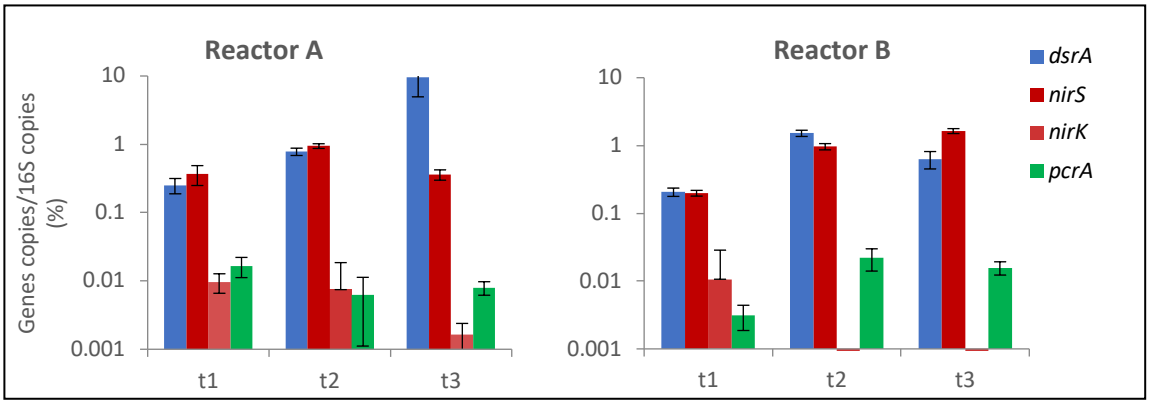

Figure 3.3. Relative abundance of *dsrA*, *nirS* and *nirK* gene copies in Reactors A & B. Genes copies/16S copies (%) at three times: t1: before chlorate addition to Reactor B; t2: 30 days after chlorate addition to Reactor B; and t3: 15 days after stopping chlorate addition to Reactor B. A: Reactor A. B: Reactor B.

The percentage of genes copies of *nirK*, *nirS*, *dsrA* and *pcrA* are very low with pect to the 16S rRNA. The sum of all gene copies of *nirK*, *nirS*, *dsrA* and *pcrA* represented less than 15% of the total gene copies of 16 rRNA. This could be explained by the difference in the number of copies of a particular gene in the different microorganisms present in the microbial community. Bacteria can have 1 to 15 copies of the 16S rRNA gene (Vetrovsky and Baldrian 2013), but, in general, DNB have one gene copy of *nirk* and two gene copies of *nirS*; SRB can have more than one copy of *dsrA* and PRB present 1 copy of *pcrA* (Kondo et al. 2004, Kandeler et al. 2006, Philippot 2006, Nozawa-Inoue et al. 2011). Since the number of gene copies of 16S rRNA depends on the genus, it is not possible to have a real proportion among different genes, but each type of genes is comparable between themselves. Also, the genes related to the metabolism could be underrepresented, and maybe other genes as *narG* and *napA* for DNB, and *dsrB* for SRB could had represented the community better.

3.3.3. Assessment of the microbial community with 16S rRNA sequencing

Sequencing results of 16S rRNA from Reactor A show the evolution of the microbial community in a H_2-MBfR with O_2, NO_3^-, ClO_4^-, and SO_4^{2-} as electron acceptors over 78 days of operation (Figure 3.4a). At the order level, the community was dominated by Rhodocyclales (35%) and Bulkhoderiales (28%) at the end of the first stage (t1). These are very diverse orders, with members capable of denitrification and ClO_4^- reduction (Coates et al. 2001, Zhao et al. 2011, Oren 2014, Willems 2014). Then, the community was enriched with SRB at the second and third stages, represented by the order Desulfovibrionales (Muyzer and Stams 2008). This order was 3% of the microbial community initially, but was 52% at the end.

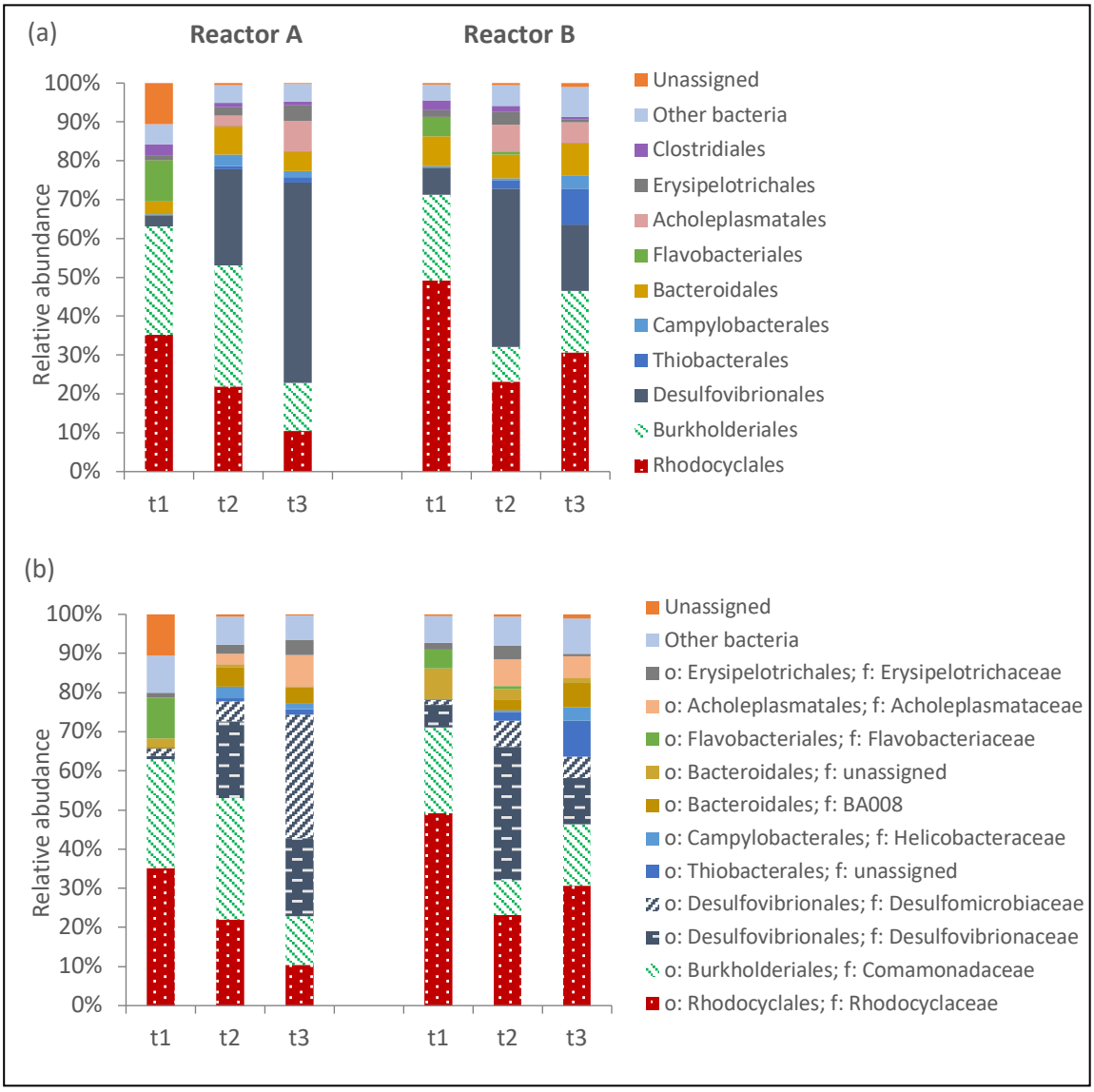

Figure 3.4. Microbial community structure at order and family level in Reactors A and B. (a) Order level; (b) family level. Biological samples were taken at 3 times: i) day 24, before adding chlorate to Reactor B (t1); ii) day 60, after 35 days of chlorate addition (t2); iii) day 78, after 15 days of stopping chlorate addition (t3). The results are an average of two samples at each time in each reactor. The sum of the less abundant phylotypes is classified as "Other bacteria".

As the presence of SRB increased, the phylotypes Rhodocyclales, and Bulkhoderiales decreased from 63% to 22%. At t2 and t3, SOB (Thiobacterales and Campylobacterales) were also present. SOB oxidize H_2S to S^0 or SO_4^{2-} coupled with O_2

or NO_3^- or ClO_4^- (Kersters et al. 2006, Shao et al. 2010, Bomberg et al. 2016). Although SOB only represented 3 – 4% of the total taxonomic breakdown, once SO_4^{2-} was reduced and produced H_2S, these bacteria increased in abundance in the biofilm.

In Reactor B, the community was also dominated by the orders Rhodocyclales and Burkholderiales (71% in total) at the first stage (t1) (Figure 3.4a). After 36 days of ClO_3^- addition to reactor B (t2), Desulfovibrionales increased, from 7% to 41%, and Rhodocyclales and Burkholderiales decreased to 32% in total, even more than in Reactor A. After 14 days without ClO_3^- (t3), Desulfovibrionales decreased to 17% and Rhodocyclales and Burkholderiales increased to 47%. At the third stage of Reactor B, there were also members of SOB (Thiobacterales and Campilobacterales at 12% in total). The higher level of SOB in t3 of reactor B compared to Reactor A, coincides with the higher SO_4^{2-} reduction in Reactor B on day 77, when the microbial community was sampled.

When analyzing the family level (Figure 3.4b), in all the samples from Reactors A and B, SRB were composed by two families: *Desulfomicrobiaceae* and *Desulfovibrionaceae*. In Reactor A, *Desulfovibrionaceae* was the dominant family at t2 (20% versus 5% of *Desulfomicrobiaceae*), but 17 days later (t3) *Desulfomicrobiaceae* became the dominant SRB family (32%). In Reactor B, *Desulfovibrionaceae* was also the dominant family in t2 (34% compared to 7% of *Desulfomicrobiaceae*), but it remained dominant at t3, although it decreased from 34% to 12%.

3.4. Discussion

We have shown NO_3^- and ClO_4^- were reduced to low levels in few days in both reactors. However, ClO_4^- was not removed to below 20 µg/L in average, so it would not meet treatment goals. The reduction of ClO_4^-, and partially NO_3^- reduction, was performed by members of the Rhodocyclales family. This includes bacteria capable of using both compounds as electron acceptors (Coates et al. 2001, Xu et al. 2004), such as *Dechloromonas*, one of the genus present in both Reactors (Figure A1).This explains the selection of Rhodocyclales even with the low concentration of ClO_4^- in the influent. This was suggested by (Nerenberg et al. 2008), who proposed that NO_3^- can serve as a primary

substrate for PRB. *Hydrogenophaga* (from Burkholderiales) was also present, and it is known as a NO_3^- reducer capable of growth on hydrogen (Kersters et al. 2006, Willems A. and Gillis 2015). These two bacteria have also been found in other studies with similar conditions (Nerenberg et al. 2008, Zhao et al. 2011, Ontiveros-Valencia et al. 2014b). SO_4^{2-} reduction was performed by the genus *Desulfovibrio* and *Desulfomicrobium* (Muyzer and Stams 2008).

When SO_4^{2-} reduction started, it became problematic for ClO_4^- reduction in both reactors. As other studies have shown SO_4^{2-} reduction can inhibit ClO_4^- reduction (Ontiveros-Valencia et al. 2013b, Ontiveros-Valencia et al. 2014a). We proposed the addition of ClO_3^- to overcome this effect. By adding an extra electron acceptor to Reactor B, there would be less H_2 availability for the community than in Reactor A, and SRB will be the most affected, due to their slower growth kinetics compared to DNB and PRB/CRB (Thauer et al. 1977, Rittmann and McCarty 2001). Also, since ClO_3^- can be reduced by PRB, it may select for PRB. In fact, once ClO_3^- was added to Reactor B (Stage 2), the reduction rates of SO_4^{2-} were lower than in Reactor A (Figure 3.2), and this continued during most of the second stage. With this, the effluent ClO_4^- averaged 23 μg/L, less than a half than in Reactor A (51 μg/L), and qPCR results indicates *pcrA* increased while ClO_3^- was present (Figure 3.3). This shows ClO_3^- had the desired effect: it decreased SO_4^{2-} reduction and improved ClO_4^- reduction. Nevertheless, this effect does not last long. On Day 53, about 30 days after ClO_3^- addition began, its reduction was slightly higher than previously, allowing SO_4^{2-} reduction to increase. Also, immediately after removing ClO_3^- from the influent (Stage 3), SO_4^{2-} reduction levels doubled. This suggests ClO_3^- concentration in the bulk must be kept above 0.1 mg/L approximately in order to maintain SO_4^{2-} reduction at low levels. Note that 0.1 mg/L this is well below the treatment standard for ClO_3^-

As ClO_3^-decreased SO_4^{2-} reduction in Reactor B, we expected a lower abundance of SRB than in Reactor A at the end of the Stage 2 (Day 60). In contrast, 16S rRNA results indicate there is a larger presence of Desulfovibrionales in Reactor B at this stage (41% compared to 25% in Reactor A) (Figure 3.4a) and qPCR results also showed that *dsrA* increased in Reactor B between Stages 1 and 2 (Figure 3.3). As SRB became more

abundant, Rhodocyclales and Burkholderiales decreased to a half of their abundances in Stage 1 (t1).

An explanation for the higher abundance of SRB during ClO_3^- addition to Reactor B could be that reduction of ClO_3^- was coupled with H_2S instead of H_2. With this, ClO_3^- reduction did not compete for H_2, so SRB had same H_2 availability than in Reactor A to reduce SO_4^{2-}. Also, H_2S was oxidized back to SO_4^{2-}, obscuring the SO_4^{2-} reduction by making the overall SO_4^{2-} reduction appears lower (Figure 3.5). At the end of the Stage 2, ClO_3^- reached even lower levels (Figure 3.2), with consequently less H_2S oxidation. This can explain the increasing SO_4^{2-} reduction levels from day 53 (Figure 3.2). Once ClO_3^- was removed (t3), H_2S was no longer oxidized back to SO_4^{2-}, further increasing the SO_4^{2-} reduction levels. However, if ClO_3^- did not depress H_2 availability and SO_4^{2-} was reduced during Stages 2 and 3, in a similar level as in Reactor A, SRB should have been enriched in the same way they did in Reactor A. It is possible in Reactor B denitrification and ClO_4^- reduction were also coupled with H_2S, allowing SO_4^{2-} reduction to be performed 100% with H_2. Instead, in Reactor A, denitrification and ClO_4^- reduction were performed with H_2, reducing the H_2 availability for SO_4^{2-} reduction. Under this scenario, *Desulfomicrobiaceae* could outcompete *Desulfovibrionaceae* by acting as a K-strategist (oligotroph metabolism), being able to grow under low availability of H_2 (Ontiveros-Valencia et al. 2014a), although species of both families have been found to survive under oligotroph conditions regarding carbon availability (Bade 2000). Evidence of H_2S cycling in the H_2-MBfR is shown in Figure A.2

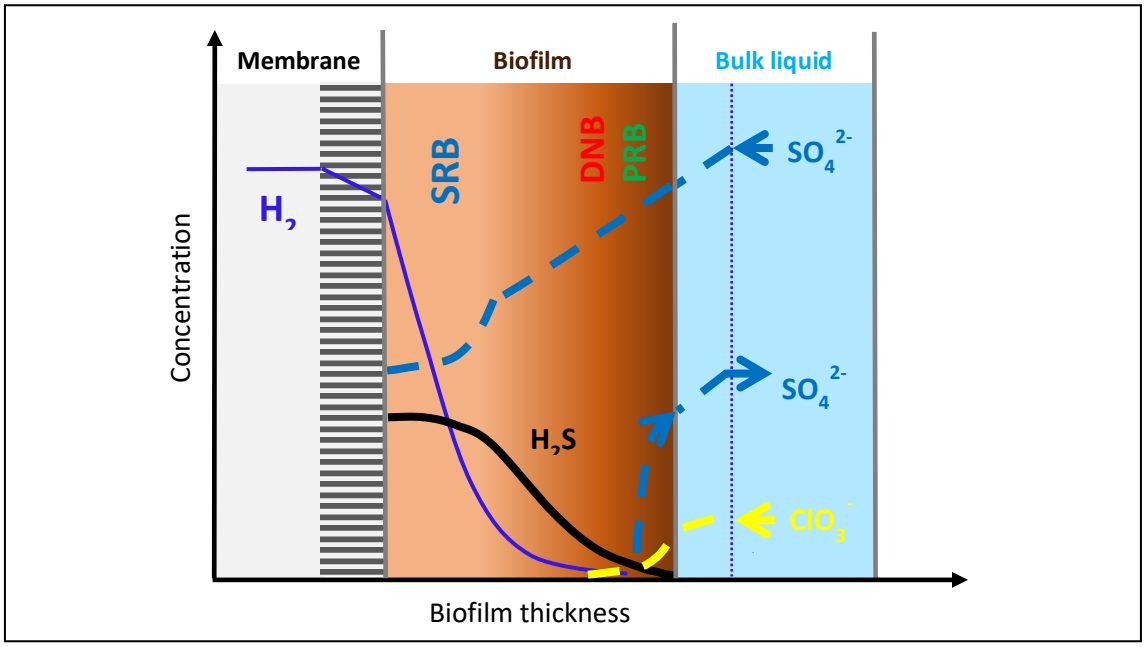

Figure 3.5. Potential sulfate (SO_4^{2-}) cycle in the biofilm. ClO_3^- is reduced with H_2S by perchlorate-reducing bacteria, converting H_2S back to SO_4^{2-}. This makes the overall SO_4^{2-} reduction in the reactor appear to be lower.

Another possible explanation for SRB enrichement in Reactor B, is they were involved in ClO_3^- reduction, specifically members of the family *Desulfovibrionaceae*. (Figure 3.4b). When $ClO_3^-$was present, *Desulfovibrionaceae* may have used it as electron acceptor, increasing its abundance and decreasing the reduction of SO_4^{2-}. On Day 53 (Figure 3.2), when the ClO_3^- concentration decreased to lower levels than before, *Desulfovibrionaceae* needed to start using part of the SO_4^{2-}, explaining the increment in SO_4^{2-} removal from day 55 in Reactor B (Figure 3.2). Once ClO_3^- was removed from the influent to Reactor B, *Desulfovibrionaceae* could only rely on SO_4^{2-} as electron acceptor, increasing its reduction levels to almost the double than when ClO_3^- was present. However, at this point, the abundance of SRB was lower than in t2. The lower abundance of *Desulfovibrionaceae* at t3, indicates that Rhodocyclales and Burkholderiales may outcompete *Desulfovibrionaceae* when they use SO_4^{2-} instead of ClO_3^-.

Although there is no prior evidence that SRB can reduce ClO_3^- or ClO_4^- (Zhao et al. 2013), SRB are very versatile and known to reduce the greatest number of different

terminal electron acceptors (Muyzer and Stams 2008, Barton and Fauque 2009). Several members of the *Desulfovibrionaceae* family can reduce NO_3^- to nitrogen gas and (or) NO_3^- to ammonia in addition to SO_4^{2-}, and, for some, NO_3^- can be the preferred electron acceptor (Marietou 2016). Ontiveros-Valencia et al. (2013a) has found SRB in H_2 biofilms even when there was no SO_4^{2-} reduction. Also, *Desulfovibrio desulfuricans* can use O_2 as electron acceptor when it is present at low concentrations (Ito et al. 2002, Lobo et al. 2007). Besides, bacteria belonging to the order *Desulfovibrionaceae* have been found in ClO_4^- reducing communities, although it has not been shown that they reduced ClO_4^- (Bruce et al. 1999), and there is a reported bacteria that is able to reduce SO_4^{2-} and ClO_3^-, although it does not belong to a SRB order (Stepanyuk V et al. 1992).

Comparing the performance with the microbial community, in some cases they are correlated and in others they are not. For example, the reduction of the different anions in Reactor A was stable from the beginning of Stage 2, but the microbial community evolved with SRB becoming predominant at t3. This is explained by the high levels of SO_4^{2-}, and the very low levels of NO_3^- and ClO_4^- in the bulk liquid (Figure 3.2), which could not continue supporting much growth of DNB and PRB at t3. On the other hand, in Reactor B the SRB abundance is not well correlated with the SO_4^{2-} rates. Immediately after removing ClO_3^- from the influent (Stage 3), SO_4^{2-} reduction levels doubled, but this time SRB abundance was lower, and DNB, PRB and CRB increased. The higher SO_4^{2-} reduction levels at t3 in reactor B compared to Reactor A could be explained by a higher ATPase or DsrA enzymatic activity of *Desulfovibrionaceae* compared to *Desulfomicrobiaceae*. Or, the microbial abundance of SRB might not be completely related to their activity, as the biological samples may contain bacteria that are inactive (dead) but still present in the biofilm. This can be particularly true for SRB, as they might proliferate closer to the membranes, being more protected from detachment. Also, DNB and PRB (Rhodocyclales and Burkholderiales) increased, even when the NO_3^- and ClO_4^-continued being low.

3.5. Conclusions

Even at low influent ClO_4^- concentrations, ClO_4^- was reduced almost completely, but it was affected when SO_4^{2-} reduction increased. Under these conditions, ClO_3^- helped to improve ClO_4^- reduction, although it did not reach 100% of reduction. Also, our results suggest that ClO_3^- addition in Reactor B hindired SO_4^{2-} reduction. It helped selecting for PRB, but at very low relative abundance, and it mainly enriched *Desulfovibrionaceae*. The capability of microorganisms from this family to reduce ClO_3^- needs to be further investigated.

For performance purposes, ClO_3^- could help suppress SO_4^{2-} reduction and help ClO_4^- reduction, but the effect does not last for very long after ClO_3^- is removed from the reactor.

In summary, ClO_3^- addition is a potentially effective means to enhance ClO_4^- reduction by enhancing the abundance of PRB and suppressing SRB. Further research is needed to confirm the mechanisms. Also, other modes of ClO_3^- addition may merit study, such as the intermittent "offline" addition of high concentrations of ClO_3^- result in stronger and longer lasting effects on the microbial community.

CHAPTER 4

Effect of chlorate on the microbial community of a single membrane denitrifying H_2-MBfR

4.1. Introduction

The study presented here is continuation of the work from Chapter 3, were we explored the effect of ClO_3^- on ClO_4^- and SO_4^{2-} reduction. The objective of that study was to improve ClO_4^- reduction when it is present at low concentrations, by selecting PRB. In that study, the permanent addition of ClO_3^- for 35 days helped to enhance ClO_4^- reduction and to decrease SO_4^{2-} reduction, but the effect on the microbial community was not completely conclusive. The enrichment of PRB seemed to be very low, and SRB were more abundant during the addition of ClO_3^- compared to the control reactor, without addition of ClO_3^-.

The work in Chapter 3 was performed in a reactor with a total of 34 hollow fiber membranes, with a total membrane area of 70.3 cm^2. This provided enough biofilm to substantially reduce the influent of O_2, NO_3^-, and ClO_4^-, as well as part of the influent SO_4^{2-}. When ClO_3^- was added, it was also reduced after a few days to below 0.2 mg/L, remaining around that level for more than 20 days. As a result of the low bulk ClO_3^- concentration, the selective pressure effect of ClO_3^- might have been weak, explaining its small effect on the relative abundance of PRB. Also, the membranes were potted together at the ends, forming a bundle. In this configuration, the membranes may have clumped together, with the most active parts the outer membranes. As the biofilm grows thicker, it is likely that advective flow from the bulk to the interior of the bundle is increasingly restricted. Thus, the interior could have favored the proliferation of SRB, as the H_2 and

SO_4^{2-} concentrations would be high, while the concentrations of NO_3^-, ClO_3^-, and ClO_4^- would be very low. Bacteria growing in the inner parts of the biofilm would also be more protected from detachment (Figures 1.6 and 3.5).

To better assess the effects of ClO_3^- on the selection for PRB in a mixed microbial community, we used a new type of H_2-MBfR that allowed better control of the bulk concentrations of SO_4^{2-}, NO_3^-, ClO_4^-, and ClO_3^-. For this, only two single membranes, with a total length of 70 cm, were used to provide a biofilm support area of 6.2 cm^2. This is less than 10% of the membrane area in the previous study. Since the influent concentrations and flows were the same as the previous study, the substrate loadings were around ten times higher. This means the difference between influent and effluent concentrations would be much smaller. By choosing the influent concentrations to mimic the desired bulk environment, it would be possible to see how the microbial community responds, and without concern about membranes bundling or clumping together.

With the above system, we studied the microbial community when the bulk ClO_3^- concentration was much higher than that of NO_3^- and ClO_4^-. Under these conditions, we could more clearly see if ClO_3^- can exert a meaningful selective pressure for PRB.

4.2. Materials and Methods

4.2.1. Reactors set up and culture medium

The experiments were developed in two H_2-MBfRs composed of two glass tubes joined together with Norprene tubing and connectors. Each tube contained a single membrane (Mitsubishi MHF200TL), one considered as the main membrane and the other was used to take biological samples at the first stage (coupon membrane). In both reactors, the membranes had a total length of 70 cm and were connected to the H_2 line in one extreme and open at the other end. The “coupon membrane” was fitted in a small tubing at the open end. After taking the biofilm sample, the membrane was fitted back into the small tubing, remaining with an open end.

To prevent excessive gas venting, a solenoid valve connected to a time (ChronTrol) opened the H_2 inlet for 30 s every 29.5 min (Perez-Calleja et al. 2017). The

H_2 pressure was set to 1 psi for the whole experiment, except that by the end of the experiments the controlling valve was not working well, and the pressure increased to 6 psi.

The reactor characteristics are summarized in Table 4.1 and a diagram of the reactors is presented in Figure 4.1.

Table 4.1. Reactors characteristics and operational conditions

Total membrane area	6.2 cm^2
Total volume	44 mL
H_2 pressure	1 psi
Influent flow	1 mL/min
Recirculation flow	90 mL/min
HRT	44 min
Temperature	Room temperature (~22°C)

The influent media contained (g/L): 0.434 Na_2HPO_4, 0.128 KH_2PO_4, 0.026 $MgSO_4 \times 7H_2O$, 0.14 $MgCl_2 \times 6H_2O$, 0.2 $NaHCO_3$, 0.001 $FeSO_4 \times 7H_2O$, 0.001 $CaCl_2$ and 1 mL of trace minerals solution. The trace minerals solution was composed of (mg/L): 100 $ZnSO_4 \times 7H_2O$, 30 $MnCl_2 \times 4H_2O$, 300 H_3BO_3, 200 $CoCl_2 \times 6H_2$, 10 $CuCl_2 \times 2H_2O$, $NiCl_2 \times 6H_2O$, 30 $Na_2MoO_4 \times 2H_2O$ and 30 Na_2SeO_3. The pH of the media was adjusted to 7.5.

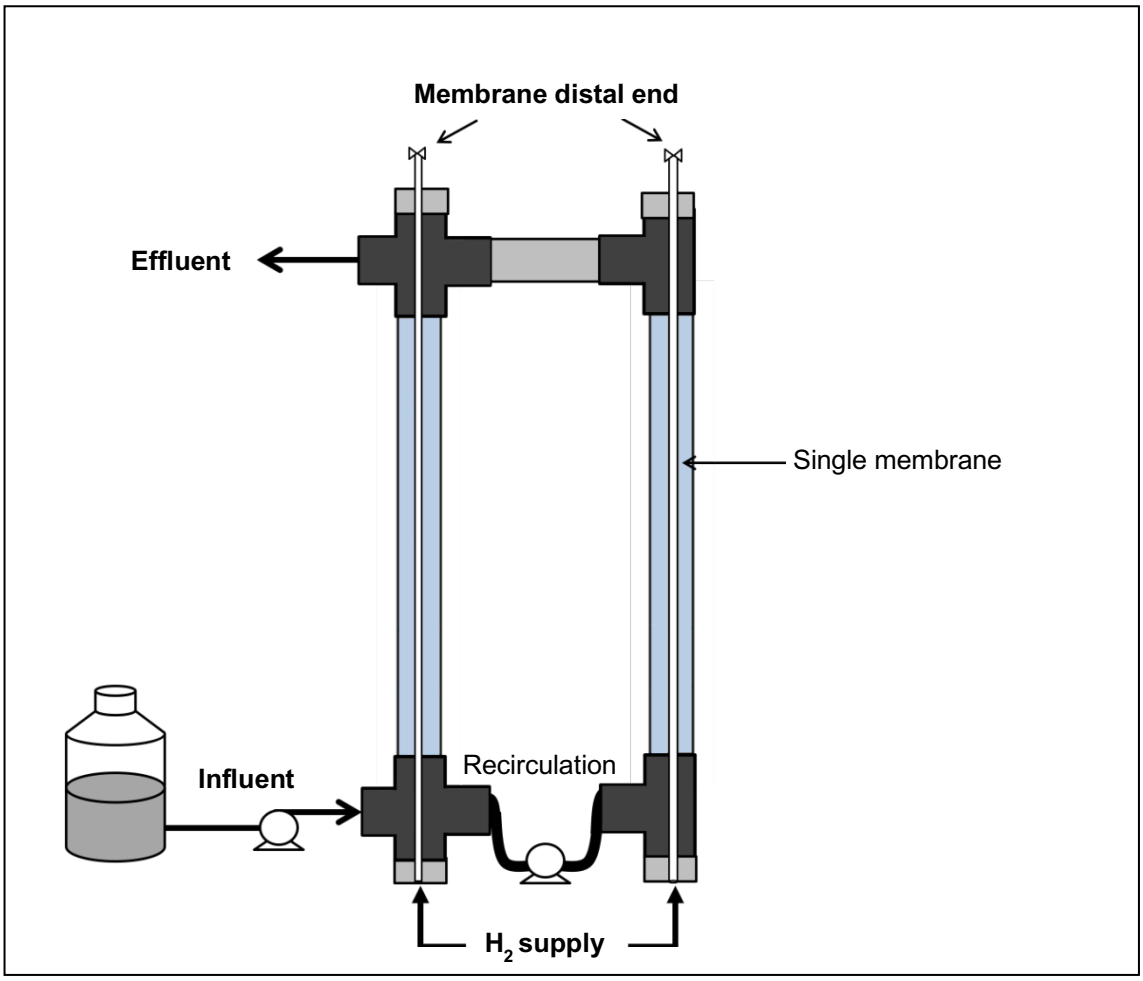

Figure 4.1. Experimental H_2-based membrane biofilm reactor (H_2-MBfR). The reactor was composed of two glass tubes and had a single membrane in each one. Each membrane was open on the distal end to prevent gas depletion.

4.2.2. Startup and stage experiments

Both reactors were inoculated with 1 mL of the same activated sludge used in Chapter 3, and kept in recirculation mode for 16 h before starting a continuous flow. The operational conditions are described in Table 4.2. The recirculation flow rate was 90 times faster than the influent, allowing the reactor to perform as completely mixed.

Table 4.2. Influent concentrations at each stage of Reactors 1 and 2.

Conditions	Stage 1 Days 0 – 22		Stage 2 Days 22 – 36	
	Reactor 1	Reactor 2	Reactor 1	Reactor 2
NO_3^- (mg N/L)	0.1	0.1	0.1	0.1
ClO_4^- (mg/L)	0.04	0.04	0.04	0.04
SO_4^{2-} (mg/L)	10	10	10	10
ClO_3^- (mg/L)	0	0	0	5

The reactors were operated in two stages as described in Table 4.2. In the first stage, Reactors 1 (control) and 2 (experimental) were supplied with an influent of 0.04 mg/L ClO_4^-, 0.1 mg N/L NO_3^-, and 10 mg/L SO_4^{2-} for 22 days. In the second stage, 5 mg/L ClO_3^- was added continuously to Reactor 2 for 14 days. Due to the small membrane area, O_2 in the influent at the equilibrium with air (~8.4 mg/L) would had inhibited the reduction of NO_3^-, ClO_4^- and ClO_3^-. Therefore, the influent medium of both reactors was purged with N_2 gas for 30 min. to remove O_2.

4.2.3. Analytical Methods

We sampled the influent every three days and the effluent daily. Samples were analyzed for NO_3^-, ClO_4^-, ClO_3^- as described in Section 3.3.3. The detection limits for NO_3^-, ClO_3^- and SO_4^{2-} were 0.015 mg N/L, 50 µg/L, and 0.1 mg/L respectively. ClO_4^- was not detected below 50 µg/L.

The pH dissolved O_2 (DO) in the influent were also measured as described in Section 2.4.

4.2.4. Biofilm sampling, DNA extraction, high-throughput sequencing, and taxonomic analysis

We collected biofilm samples for microbial community analyses from both reactors at:

i) Day 22, just before adding ClO_3^- to Reactor 2 (t1)

ii) Day 36, which was after 14 days of ClO_3^- addition (t2)

To sample the biofilm at first stage from each reactor, we cut a 21 – 23 cm long section of the coupon membrane and maintained the membrane with open end. To ensure the sample from the second stage would be representative of the evolution of the biofilm since the beginning, including the ClO_3^- addition, the second sample corresponded to the whole main membrane (~35 cm). Then the DNA extraction and 16S rRNA sequencing were performed as described in sections 3.3.4 and 3.3.6.

4.3. Results

4.3.1. ClO_3^- decreased SO_4^{2-} reduction

Despite of the low influent concentration of NO_3^- to Reactors 1 and 2, it was reduced to even lower levels in less than 10 days (Figure 4.2). Before ClO_3^- addition to Reactor 2 (Stage1), both reactors averaged an overall NO_3^- concentration of 0.04 mg N/L (65% removal). However, until Days 8 – 9, the effluent NO_3^- was 0.06 – 0.08 mg N/L in both reactors, and then it was reduced to 0.02 mg N/L. This effluent concentration was lower than the effluent concentrations in Reactors A and B (0.06 mg N/L) described in Chapter 3.

SO_4^{2-} reduction was always near zero in both H_2-MBfRs during both stages, although by the end of Stage 2 in Reactor 1 (Days 32-36) SO_4^{2-} reduction increased to near 5% (11 mg/L), whereas in Reactor 2 it continued to be essentially zero. From Day 25, Reactor 1 received an influent NO_3^- concentration slightly higher than Reactor 2 (Figure 4.2). Despite this, the effluent levels were similar (~0.05 mg N/L). Thus, the reduction in Reactor 1 was greater than in Reactor 2. Consequently, the increased SO_4^{2-} reduction in Reactor 1 is probably not due to a difference in the NO_3^- concentration, but to more H_2 availability in Reactor 1 compared to Reactor 2, as it did not receive an extra electron acceptor. It also may have resulted from the possible toxic effect of ClO_3^- on SRB.

When ClO_3^- was added to Reactor 2 (Stage 2), it was reduced to 1.5 mg/L within one day, although the average was around 2.4 mg/L in the first week of its addition. Since the influent ClO_4^- concentration was already very low, the immediate reduction of ClO_3^- indicates NO_3^- already selected for a substantial amount of PRB. This will be further discussed in the next section. From Day 33 to 36, ClO_3^- reduction improved, with the effluent dropping to 0.4 mg/L. This probably occurred because the H_2 pressure also increased in that period, from 1 to 6 psi. This fact also coincides with the period of higher SO_4^{2-} reduction in Reactor 1. Other studies have shown that controlling H_2 pressure is key for the performance of these reactors (Ontiveros-Valencia et al. 2018).

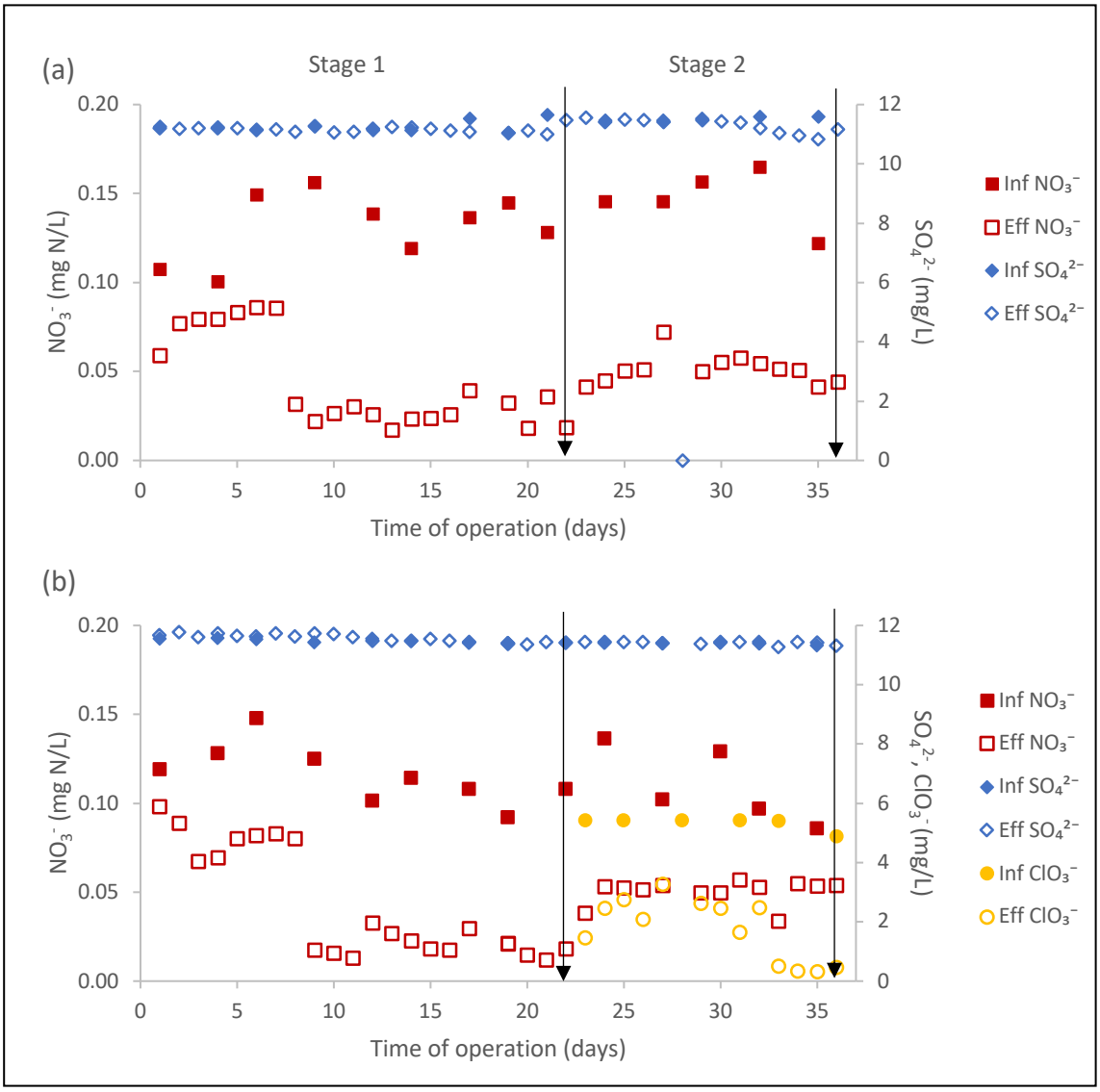

Figure 4.2. Reduction of NO_3^-, SO_4^{2-}, and ClO_3^-. (a) Reactor 1 (control). (b) Reactor 2 (with ClO_3^- addition). In the first stage, both reactors were run without ClO_3^-. From Day 22 to 36, ClO_3^- was added to reactor 2. The arrows indicate the days in which the biological samples were taken. From Day 33, H_2 pressure increased from 1 to 6 psi.

Although NO_3^- reduction decreased at Stage 2 in both Reactors, probably due to the large amount of membrane removed for biofilm sampling before Stage 2, it seems that ClO_3^- affected NO_3^- reduction. At this Stage, NO_3^- reduction averaged 68% in Reactor 1, while it averaged 60% in Reactor 2. Moreover, once ClO_3^- reduction improved to 90%, NO_3^- reduction dropped to 40%. This probably indicates ClO_3^- was the preferred electron acceptor, over NO_3^-. This will be discussed in the microbial community analyses.

Although ClO_4^- was added at 40 µg/L, it was not detected in the influent or effluent samples. This could be due to problems with the ion chromatography method: a relatively high baseline and a very large peak occurring immediately before ClO_4^-. This peak corresponded to phosphate, which is a medium constituent present at very high concentrations. The lack of information about the evolution of ClO_4^- in the reactors makes it difficult to evaluate the effect of ClO_3^- on ClO_4^- reduction. However, ClO_3^- seemed to inhibit SO_4^{2-} when the H_2 availability increased. The performance results reveal the strategy of ClO_3^- addition could be successful, particularly for inhibiting SO_4^{2-}.

4.3.2. Assessment of the microbial community with 16S rRNA sequencing

The sequencing results for Reactors 1 and 2 are shown in Figure 4.3 at the order level. Biofilms from Reactors 1 and 2 had 80% – 90% heterotrophic and autotrophic phylotypes that include members as DNB and PRB. The dominant DNB and PRB orders were Rhodocylales, Bulkholderiales and Rhizobiales. They represented 65% relative abundance in Reactor 1 and 70% in Reactor 2. These three orders were also present in the study of Chapters 3, and also have been detected in other studies reducing NO_3^- and ClO_4^- (Nerenberg et al. 2008, Zhao et al. 2011, Ontiveros-Valencia et al. 2014a, Ontiveros-Valencia et al. 2014b) and in wastewater treatment processes related to denitrification (Lu et al. 2014, Tian et al. 2015, Zielińska et al. 2016). However, Rhizobiales, mainly represented by the family *Xanthobacteraceae*, (Figure A.3) proliferated much more under the conditions given in this study, which include no O_2 and a very low NO_3^- in the influent. Species from this family are aerobic or facultative aerobes, which may indicate the low concentration of NO_3^- could had enriched them (Oren 2014).

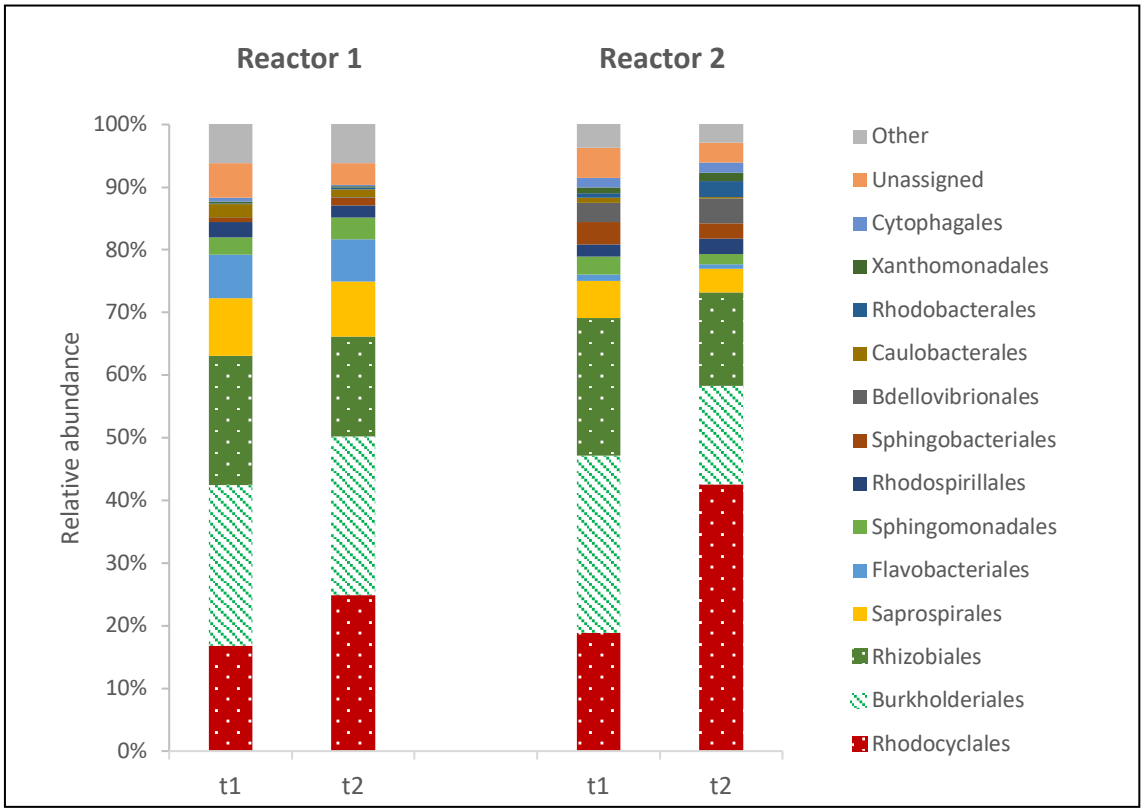

Figure 4.3. Microbial community structure at order level in Reactors 1 and 2. Biological samples were taken at 2 times: i) day 22, before adding chlorate to Reactor B (t1); ii) day 34, after 14 days of chlorate addition (t2). In each Reactor, the results at t2 are an average of two samples. The sum of the less abundant phylotypes is classified as "Other bacteria".

As can be observed in Figure 4.3, both reactors had similar abundance of Rhodocyclales by the end of Stage 1 (17% – 19%). However, after two weeks of ClO_3^- addition to Reactor 2 in Stage 2 (t2), the order Rhodocyclales doubled their abundance compared to Stage 1 (t1). In Reactor 1, it only increased 50%. Moreover, *Dechloromonas*, one of the two genera within the order Rhodocyclales that were present in the microbial community, increased almost four times at Stage 2 of Reactor 2, while it only increased 1.5 times in Reactor 1. As it has been described before, most *Dechloromonas* species are capable of NO_3^- and ClO_4^- reduction, suggesting it was the main organism responsible for ClO_3^- reduction. In addition, it seems that ClO_3^- addition slightly enriched for Rhodobacterales (Figure 4.3), changing from 1% in Stage 1 to 3% in Stage 2. This order

only presented the genus *Rhobodacter*. Several studies have shown the proliferation of Rhodobacterales in denitrifying cultures (Kraft et al. 2014, Zhang et al. 2016), although Roldán et al. (1994) reported species from the genus *Rhodobacter* capable of ClO_3^- reduction. The microbial community distribution in both reactors at Stage 2, shows ClO_3^- addition, for two weeks, can enrich for PRB, but they are not exclusively ClO_4^- reducers.

Even though Bulkholderiales, and particularly members of the family *Comamonadaceae* (the only family from Bulkholderiales present here), have been abundant in environments where NO_3^- and ClO_4^- are reduced (Conneely 2011, Zhao et al. 2011, Ontiveros-Valencia et al. 2013a), they were outcompeted by *Dechloromonas* (Rhodocyclales) during ClO_3^- addition (Stage 2) to Reactor 2. The competition between them could be due to a higher affinity of *Dechloromonas* to ClO_3^- or faster growth using it as electron acceptor. This would also explain the higher ClO_3^- reduction, and the inhibition of NO_3^- reduction, when H_2 loading was higher from Day 32 (Figure 4.2).

Other denitrifying bacteria (mainly heterotrophs) were not affected by the addition of ClO_3^-, indicating they may play a relevant role in the community, such as degrading organic matter. Both Reactors present members from the orders Saprospirales, Sphingobacteriales, and Sphingomonadales; all known to degrade complex organic compounds (García-Romero et al. 2016, Whitman et al. 2016, Szabó et al. 2017). A study showed soluble microorganism products (SMP) and extracellular polymeric substances (EPS) favored the enrichment of Saprospirales and Sphingobacteriales (Zhou et al. 2019). Also, *Chitinophagaceae* (Saprospirales), is a denitrifyer that can degrade chitin, an organic compound (Torrentó et al. 2011, Zhong et al. 2017), and also has been enriched when NO_3^- and methanol are present (Waki et al. 2009, Zhong et al. 2017). Sphingomonadales are relevant in degrading aromatic compounds, Sphingobacteriales can use organic matter, and several members of these two orders are also able of denitrification (Liu et al. 2008, Notomista et al. 2011, García-Romero et al. 2016, Whitman et al. 2016, Szabó et al. 2017). These hydrolytic bacteria differ from the ones developed in the previous studies described in this thesis. There, Bacteroidales was the prevalent the order. Ontiveros-Valencia et al. (2013a) showed this order may stablish a synergistic relationship with SRB, which agrees with high abundance of this group in

those reactors. The proliferation of Saprospirales, Sphingobacteriales, and Sphingomonadales over Bacteroidales in this study could be related to the absence of SRB, or also to the low concentrations of electron acceptors. Despite this, it is not clear why these organisms are present, as there should be no electron donor limitation and the biofilm is very thin. Probably there are other synergistic relationships that we are not aware of.

There are other differences with the results from Chapter 3. At the phylum level, the microbial community was dominated by Proteobacteria (α, β, and δ), accounting for 70% to 80% in both Reactors, followed by the phyla Bacteroidetes, which represented about 17% and 10% in Reactors 1 and 2 respectively. In contrast to the study detailed in Chapter 3, these reactors did not present the phylums Tenericutes nor Firmicutes. Although they are known to have members able to perform denitrification (Keith et al. 1982, Lee et al. 2013, Engelbrektson et al. 2014), their absence in this study could be related to the low concentration of NO_3^-, indicating they may have a copiotrophic metabolism.

Despite the similarities in both H_2-MBfRs with respect to the main orders (Rhodocyclales, Burkholderiales and Rhizobiales), there are some differences between them regarding the rest the community. For example, Flavobateriales, represented only by *Chryseobacterium* is capable of denitrification (Bernardet et al. 2002, Kundu et al. 2014), was significantly enriched (~7%) in Reactor 1 during the whole time it was operated, but not in Reactor 2 (1%). Another example is Bdellovibrionales, which members are known to digest bacteria (Kersters et al. 2006, Dashiff et al. 2011). They were present at 3% – 4% in Reactor 2 at Stages 1 and 2, but they were absent in Reactor 1. These differences are not attributable to ClO_3^- addition, since they are stable at both stages. Also, both reactors were operated under the same conditions. However, there may be some factors not taken into account that could be responsible of the differences. There could have been differences in the H_2 delivery. Sometimes, the system used to keep the "coupon membrane" open after taking the biological sample was clogged with water. This could have lowered the H_2 flow.

One concern with the strategy of ClO_3^- addition is that it could enrich for CRB, which are not able to reduce ClO_4^-. Even though the operational time was not long enough to obtain a steady state microbial community, ClO_3^- addition enriched for PRB, and no members of γ-proteobacteria (the phylum that contains most known CRB) were present. It may be that the presence of PRB prior to ClO_3^- addition could favor PRB. On the other hand, the presence of PRB may be ensured when NO_3^- is present, as it already selected for *Dechloromonas*, a common PRB.

4.3.3. Chlorate addition as a mean to enrich PRB

As a strategy to enrich PRB, ClO_3^- addition to H_2-MBfRs worked well in this study. One particularity of this study is the very small area of the membrane compared to the high concentration of ClO_3^-, and in relation to other similar studies (Nerenberg et al. 2008, Zhao et al. 2011, Zhao et al. 2013, Ontiveros-Valencia et al. 2013b, Ontiveros-Valencia et al. 2014a). This approach tries to overcome some of the difficulties found in the study in which a bundle of membranes, with a larger area, was used (Chapter 3).

The use of a bundle of membranes allows excessively growth of biomass, which entails problems such as a decrease in the reduction activities in the external part of the biofilm, due to a limitation of H_2. In the inner part of the biofilm, limitations of NO_3^-, ClO_3^- and ClO_4^- may occur, but not SO_4^{2-}. As SO_4^{2-} is generally present at much higher concentrations than the other electron acceptors, it more readily diffuses through the biofilm and reaches the membrane surface, where maximum H_2 concentrations are found. This provides perfect conditions for SRB to grow, and also gives them more protection from detachment (Martin et al., 2015). On the other hand, the abundant microbial community was able to reduce ClO_3^- to very low levels (0.3 mg/L) after only a few days, even given the small selective pressure from ClO_4^-. This could have masked the potential effect of ClO_3^- on the microbial community. Furthermore, once ClO_3^- concentration was less than 0.1 mg/L, SO_4^{2-} reduction took place.

By using a single membrane, the formation of a thick biofilms, or clumping of MBfR membranes, is prevented, reducing the likelihood of SRB proliferation. Also, these reactors can represent a "real" H_2-MBfR with multiple "single membranes" (membranes separate from each other) at steady state, as we are using influent concentrations closer to

the effluent concentrations obtain in others H_2-MBfR studies. With this, we were able to keep ClO_3^- concentration in the bulk close to 2 mg/L and observe the shifts in the microbial community. However, despite the small membrane area, an increment in H_2 loading allowed ClO_3^- to be reduced to lower levels. If this situation continued, the problems observed previously could occur, so it is important to maintain the H_2 loadings at reasonable levels. Also, in this configuration SO_4^{2-} reduction was not detected until the increment in the pressure of H_2, but ClO_3^- was able to inhibit it.

4.4. Conclusions

In this study, it was shown that ClO_3^- can enrich putative PRB, such as *Dechloromonas*, which doubled in abundance during ClO_3^- addition. *Dechloromonas* has been shown to be commonly found in other NO_3^- and ClO_4^- reducing H_2-MBfRs. This study clarified the role of ClO_3^- in selecting for PRB, which was not evident in the results from Chapter 3.

Also, for the period tested, ClO_3^- was able to inhibit SO_4^{2-} reduction. However, in this case, we could not determine whether ClO_3^- influenced ClO_4^- reduction or if it had an effect over the SRB community. Nevertheless, the higher presence of *Dechloromonas* could provide an improvement in ClO_4^- reduction, as ClO_3^- reduction was favored over NO_3^- reduction.

The results also showed that the H_2 pressure is a very sensitive parameter, which can strongly vary the reduction level of each compound, in spite the small membrane area and the thin biofilm. This agrees with results from other studies.

Although it should be further tested, the results indicate this strategy could be promising in enriching PRB and particularly in helping to keep SO_4^{2-} reduction at low levels.

CHAPTER 5

Chlorite as an indirect electron acceptor and its effect on perchlorate reduction

5.1. Introduction

Chlorite (ClO_2^-) is an intermediate on the perchlorate (ClO_4^-) reduction pathway, and also a strong oxidant that is toxic to bacteria (van Wijk et al. 1998). As discussed previously, perchlorate-reducing bacteria (PRB) and chlorate (ClO_3^-) reducing bacteria (CRB) have a specialized enzyme, the chlorite dismutase (Cld), that transforms ClO_2^- by dismutation into O_2 and Cl^-. The enzyme has extremely high activities, and therefore ClO_2^- does not accumulate measurably during perchlorate (ClO_4^-) or chlorate (ClO_3^-) reduction. The transformation serves two purposes: it protects PRB and CRB from ClO_2^- toxicity, and it provides them with O_2, a highly energetic electron acceptor. PRB and CRB, together with phototrophs, are the only microorganisms capable of producing O_2 as a result of their metabolic pathways (DuBois and Ojha 2015). While the Cld is present in PRB and CRB, and some other organisms such as nitrite-oxidizing bacteria, for brevity we just refer to PRB in this chapter.

ClO_2^- can be formed as a disinfection by-product, and by ion exchange with clay minerals in environments rich in iron-magnesium (Cao et al. 2018). When present in the environment, ClO_2^- can provide PRB with an O_2 source, conferring them with an important metabolic advantage in acceptor-limited environments. If ClO_2^- concentrations are high and the PRB are present at low concentrations, then ClO_2^- have a toxic effect, even on PRB. In this case, O_2 also will be produced much faster than it can be consumed by PRB, resulting in O_2 release to the surrounding environment. In this case, the O_2 may benefit non-PRB in the nearby environment, as well as PRB. However, if the ClO_2^- concentration is low, the O_2 produced may only be available to the PRB intracellularly and not be released to the environment. In this case, it would just benefit the PRB.

The ability of Cld to produce O_2 has drawn the attention of scientists. A variety of applications, for solving environmental to industrial and health problems, have been proposed and proven (Weelink et al. 2008, Wang and Coates 2017). Several of them exploit the fact that the O_2 produced from ClO_2^- dismutation by PRB or CRB can be released to the extracellular environment providing O_2 to strictly aerobic microorganism in an anaerobic environment. This mechanism has been proposed for bioremediation of xenobiotic compounds, such as benzene and toluene among others. In this case, PRB or CRB supplied with ClO_2^- produce O_2 that is used by strictly aerobic organisms to oxidize the contaminant (Figure 5.1).

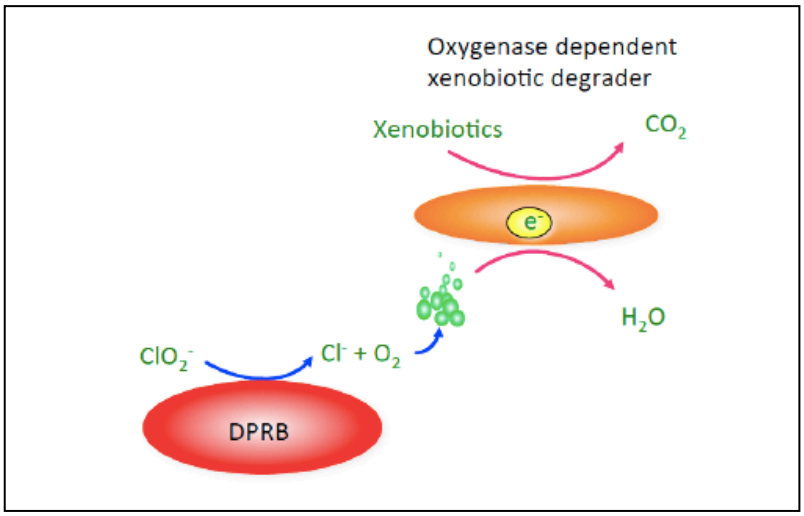

Figure 5.1. Scheme of symbiosis between strictly aerobic bacteria and DPRB (or PRB). O_2 produced by DPRB is used by an aerobic bacteria in an anoxic environment (Wang and Coates 2017).

In the previous example, the O_2 produced from ClO_2^- was needed by the aerobic bacteria to perform the degradation of the xenobiotics. However, the production of O_2 could also be detrimental for microorganisms near PRB or CRB. In the H_2-MBfR presented in Chapter 3, undesired SO_4^{2-} reduction occurred, due to the proliferation of SRB. Most of SRB are inhibited by O_2, thus in this situation the production of O_2 within

the biofilm could help to inhibit SRB. Thus, the addition of ClO_2^- to a perchlorate-reducing H_2-MBfR could diminish SO_4^{2-} reduction.

Purified Cld and cell extracts have been extensively studied and characterized (van Ginkel et al. 1996, Stenklo et al. 2001) and few studies have tested ClO_2^- disproportionation in pure cultures (Rikken et al. 1996, Xu and Logan 2003). Only one study determined the Cld activity in mixed culture of activated sludge (AS) from a ClO_4^- reducing bioreactor and the effect of inhibitors of Cld (Nadaraja et al. 2013). Here, we tested Cld activity and O_2 consumption rates in a mixed PRB or CRB enrichments, the use of ClO_2^- as an indirect electron acceptor in those enrichments, and its effect as a potential analog to ClO_4^- to enrich for PRB and to avoid SO_4^{2-} reduction in a H_2-MBfR.

5.2. Materials and methods

5.2.1. Growth medium for batch enrichments

The growth medium contained (g/L): 0.434 Na_2HPO_4, 0.147 $NaH_2PO_4 \times 2H_2O$, 0.165 $MgCl_2 \times 6H_2O$, 0.2 $NaHCO_3$, 1 mL of trace minerals solution, 1 mL of Ca-Fe solution and 1 mL of N-S source solution. The trace minerals solution was composed of (mg/L): 100 $ZnSO_4 \times 7H_2O$, 30 $MnCl_2 \times 4H_2O$, 300 H_3BO_3, 200 $CoCl_2 \times 6H_2O$, 10 $CuCl_2 \times 2H_2O$, 10 $NiCl_2 \times 6H_2O$, 30 $Na_2MoO_4 \times 2H_2O$, and 30 Na_2SeO_3. The Ca-Fe solution contained (g/L): 1 $FeSO_4 \times 7H_2O$, 1 $CaCl_2 \times 2H_2O$ and 1 EDTA, and the N-S source solution had 0.2 mg/L $(NH_4)_2SO_4$. Acetate was added in excess at 2.72 g/L $NaAc \times 3H_2O$ (20 mM). Either ClO_3^- or ClO_4^- were added as the electron acceptor at1 g/L and 1.2 g/L (12 mM), to grow CRB or PBR respectively.

The medium was deoxygenated by purging it for 10 min with N_2 gas. The pH of the media was adjusted to 7.5.

5.2.2. ClO_3^- or ClO_4^- reducing enrichments

Batch growth experiments with ClO_4^- or ClO_3^- as electron acceptors were carried out to enrich for PRB or CRB, respectively. Cultures were performed in 150 mL serum bottles containing 63 mL of sterile anoxic growth medium inoculated with 7 mL of AS from the South Bend Wastewater Treatment Plant, in South Bend, Indiana, USA, (10%

v/v) to a final optical density at 600 nm (OD_{600}) of approximately 0.01. Also, two negative controls were conducted. One with electron acceptors, but no bacteria, to ensure no contamination occurred; and another with bacteria, but without electron acceptors, to confirm there was no O_2 supporting bacterial growth. The bottles were placed in a shaker at 30 °C and growth was monitored through measurements of OD_{600}.

The enrichments were subcultured under the same conditions, inoculated with 10% v/v to a final OD_{600} of 0.01. These subcultures were used for ClO_2^- dismutation tests and served as inoculum for subsequent enrichments growing indirectly with ClO_2^-.

5.2.3. Chlorite dismutation and O_2 consumption tests

The capability of the PRB and CRB enrichments to dismutate ClO_2^- and consume the produced O_2 was tested by addition of different concentrations of ClO_2^-. Twenty mL of a grown PRB or CRB enrichment with an OD_{600} of 0.04– 0.06 were placed in a 50 mL cylindrical test tube 15 cm long and 2.5 cm in diameter, and purged with N_2 gas for 10 min. The height/diameter ratio was chosen to lower the interaction between the culture liquid and the aerobic atmosphere. Then, ClO_2^- was added from a fresh stock solution to the 20 mL of PRB and CRB enrichments at final concentrations of 5, 15, 30, 50 and 100 mg/L. O_2 production was continuously monitored using a Clark-type oxygen microsensor (Unisense A/S, Denmark) with a 10 mm tip diameter, which tightly fitted into the test tube. The calibration curve was performed following instructions from the manufacturer. After ClO_2^- was fully transformed into O_2, we kept continued monitoring the O_2 consumption of the culture.

The production rates of O_2 (transformation of ClO_2^-) and consumption rates of O_2 were calculated at each addition from the slope of the linear parts of the curves. Appendix 5 has an example on how this rate was calculated. One unit of Cld activity (U) was defined as one µg of O_2 produced per min and were normalized to cell dry weight (DW). The same unit of activity (U) was used for the production rates of O_2. The dry weight of each enrichment was determined using 0.2 µm PES filters (Supor®-200, Pall Corporation). The filters were pre-weighed, then used to filter 20 – 40 mL of the enrichments, and finally were dried at 104 ºC for 6 h.

To verify that the changes in O_2 production/consumption were due to microbial activity and not because of O_2 transfer with the atmosphere, two control experiments were carried out. The control for O_2 consumption consisted on medium saturated with air and no bacteria nor ClO_2^-. The control for O_2 production consisted on a grown enrichment with excess of acetate purged with N_2 gas. O_2 concentrations were monitored for several hours with an O_2 microsensor.

5.2.4. Measurement of indirect growth on chlorite

We performed fed-batch growth experiments with the addition of 20 mg/L of ClO_2^- every 4 – 12 h to evaluate growth on O_2 produced by ClO_2^- dismutation. To reduce O_2 loss to the headspace we used 25 mL vials filled with 21.6 mL of media and 2.4 mL of CRB or PRB enrichment. Autoclaved media was added to each vial, then they were inoculated and sparged with N_2 gas for 10 min under sterile conditions. To ensure bacteria were growing on O_2 produced from ClO_2^-, we included a negative control inoculated with a mixture of CRB and PRB, 50% and 50% v/v respectively (final OD_{600} of 0.05), but no ClO_2^-. Another negative control with no bacteria and ClO_2^- was also performed to verify sterile conditions. Also, culture inoculated with the same AS mentioned previously was performed to confirm ClO_2^- dismutation was specific to CRB and PRB enrichments. The CRB and PRB cultures were performed in duplicate and the ClO_2^- stock solution was prepared daily. Growth was evaluated through measurement of OD_{600} and the initial OD_{600} in the different cultures ranged from 0 to 0.1.

5.2.5. Chlorite addition to a perchlorate reducing H_2-MBfR

For this study, we continued operating the same reactors described in Chapter 3, except in this case the influent medium was anoxic and in the experimental reactor (Reactor B) we tested the effect of ClO_2^- addition instead of ClO_3^-. To make the influent anoxic, the influent medium was sparged for 30 min with N_2 gas. After removing ClO_3^- from the influent, we allowed Reactor B to continue under same conditions as Reactor A for 4 weeks. The culture media was described in Section 3.2.1 in Chapter 3, with the differences mentioned above.

Inoculation, recirculation and operational conditions in both reactors were the same as described in section 2.2 of Chapter 3. We also operated the reactors in three phases, as described in Table 5.1, with Day 0 as the day ClO_3^- was removed from the influent of Reactor B. In the first phase, Reactors A (control) and B (experimental) were supplied with an influent with 8.4 mg/L O_2, 0.6 mg/L ClO_4^-, 5 mg N/L NO_3^- and 80 mg/L SO_4^{2-}, for 25 days. In the second phase, both influents were made anoxic and we continuously added ClO_2^- (5 mg/L) to Reactor B for 62 days. In the last phase, we ran both reactors without ClO_2^- for 28 days.

Table 5.1. Influent concentrations at each stage of Reactors A and B.

Conditions	Stage 1 Days 0 – 25		Stage 2 Days 26 – 87		Stage 3 Days 88 – 111	
	Reactor A	Reactor B	Reactor A	Reactor B	Reactor A	Reactor B
O_2 (mg/L)	8.4	8.4	0	0	0	0
NO_3^- (mg N/L)	5	5	5	5	5	5
ClO_4^- (mg/L)	0.6	0.6	0.6	0.6	0.6	0.6
SO_4^{2-} (mg/L)	79	79	79	79	79	79
ClO_2^- (mg/L)	0	0	0	5	0	0

5.2.6. Statistical analysis

The statistical significance of the addition of ClO_2^- to Reactor B during Stage 2 was analyzed as described in Section 3.2.5. In this case all the data from both Reactors during Stage 2 was used for the analysis.

5.2.7. Analytical methods and microbial community analysis

The influent was sampled every three days and the effluent sampled daily, with some exceptions. Samples were treated and analyzed with ion chromatography for NO_3^-, ClO_4^-, and SO_4^{2-} as described in Section 2.4, but ClO_2^- was also measured. The detection limit for ClO_2^- was 0.05 mg/L. For analyzing the results, the influent between sampling was calculated as the average of the measured influent samples.

To analyze the microbial community, biofilm samples were collected from both reactors at:

i) Day 14, which was 12 days before adding ClO_2^- to Reactor B (t1)
ii) Day 87, which was 62 days after starting ClO_2^- addition (t2)
iii) Day 111 which was 24 days after stopping ClO_2^- addition (t3)

The biofilm sampling method, DNA extraction, DNA absorbance, storage and high-throughput sequencing of regions V4 and V5 of the 16S rRNA were performed as described in sections 3.3.4 and 3.3.6.

5.3. Results and discussion

5.3.1. O_2 production and consumption tests

ClO_2^- was added to PRB and CRB enrichments to determine Cld activity through O_2 production rates (Figure 5.2). The results from negative controls are shown in Figure A.4. ClO_2^- was quickly dismutated to O_2 after adding it to CRB and PRB cultures. The O_2 concentration during ClO_2^- dismutation was monitored, and the production rates were calculated from the slope of the linear part of these curves. In CRB enrichments, Cld activity increased when ClO_2^- concentrations were increased from 5 mg/L to 30 mg/L. The maximum Cld activity was 57 U/mg DW, observed at 30 mg/L of ClO_2^-. At higher ClO_2^- concentrations the activity of Cld started to decrease, reaching 31 U/mg DW when ClO_2^- concentration was 100 mg/L. Cld activity rates from the PRB enrichments followed similar behavior to CRB enrichments, but the changes were very small, and the activity of Cld remained close to 20 U/mg DW at almost all ClO_2^- concentrations. At 100 mg/L of ClO_2^-, Cld activity was 16 U/mg DW. This would indicate ClO_2^- can enrich for bacteria with faster metabolism.

The values of Cld activity determined in this study were similar to rates of PRB and CRB cell suspensions grown aerobically on ClO_3^- (Xu and Logan 2003). However, they are much lower than Cld activity reported for cell suspensions of the strain KJ from the genus *Dechlorosoma* growing anaerobically. KJ had activities that were 20 to 60 times higher. Furthermore, the rates increased with increasing ClO_2^- concentrations of up to saturation at 100 mg/L and then remained constant. In our study, the rates decreased after

the maximum peak was reached. Nadaraja et al. (2013) also measured Cld activity in whole cells in activated sludge from a reactor reducing ClO_4^- and the rates were also 20 to 60 times higher than the rates determined here.

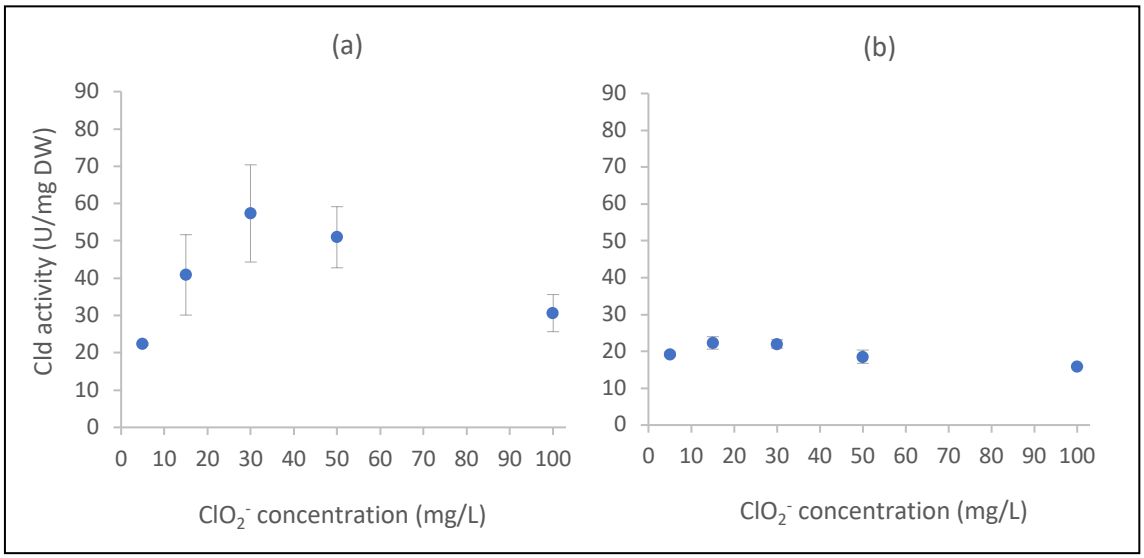

Figure 5.2. Cld activity for CRB and PRB enrichments. ClO_2^- was added at different concentrations to (a) CRB enrichments and (b) PRB enrichments. Error bars show one standard deviation.

The difference in the rates could be partially explained by a difference in the methods used. In this study, the media contained electron donor, so it was possible to consume O_2 concurrently with production. With this, the measured rates are actually a balance between production and consumption. In Xu and Logan (2003) and Nadaraja et al. (2013), they washed the cells to remove residual donor, so their numbers reflect only the O_2 production rate. Also, here the measurements of O_2 production were carried out every 3 s until ClO_2^- was completely transformed, which took 2 to 20 minutes depending on the amount of ClO_2^- added. Xu and Logan (2003) only measured the dismutation rates every 3 – 5 s for 1 minute and Nadaraja et al. (2013) measured it every 10 s for 2 min. In several cases we observed the rate was higher the first minute. This could account for a 50-100% difference in our Cld activity values, although it would not explain the 60-fold

differences. Other factors that can influence the Cld activity are the pH and the type of bacteria (Xu and Logan 2003). Our experiments were conducted at pH 7.5, but Xu and Logan (2003) showed that Cld activity was 75% of the activity obtained at pH 7.0. Also, there is a wide range of Cld activity depending on the strains and the conditions the experiments were performed (Xu and Logan 2003). In this case, the genera and strains in the enrichments are unknown and may correspond to bacteria with lower Cld activities.

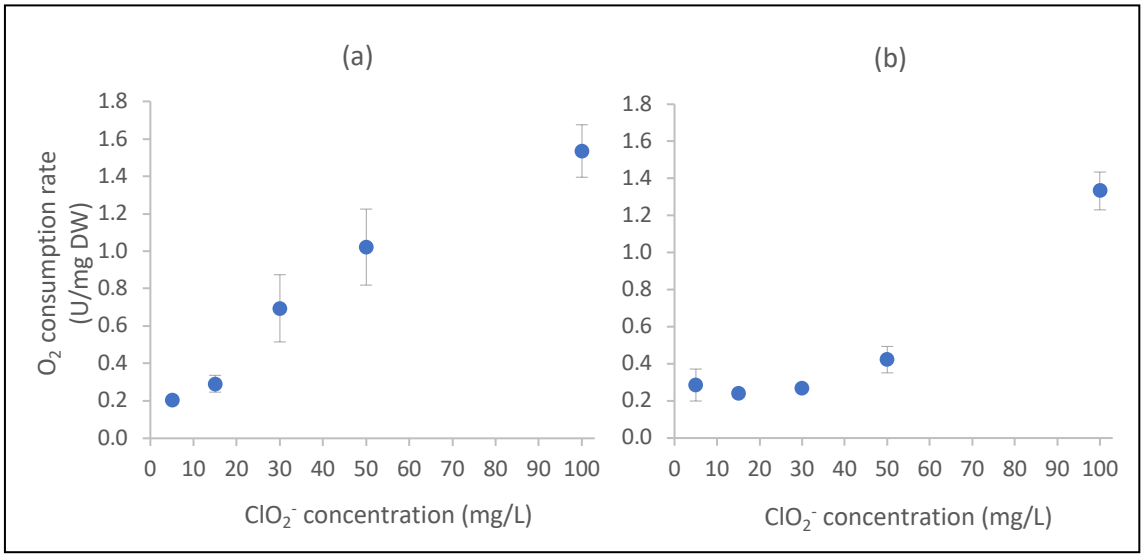

Figure 5.3. Oxygen consumption rates after ClO_2^- additions to CRB and PRB enrichments. After ClO_2^- addition and dismutation to O_2, the consumption rate of O_2 was monitored in (a) CRB enrichments and (b) PRB enrichments. Error bars show one standard deviation.

In order to obtain O_2 consumption rates, the O_2 concentration was measured continuously after ClO_2^- was dismutated in each experiment and the rates were calculated as described before. In the experiments with CRB enrichments the rates of O_2 consumption increased steadily with increasing ClO_2^- concentrations with a maximum O_2 of 1.5 U/mg DW (Figure 5.3). However, in the PRB enrichments the O_2 consumption rates remained at a similar level for the different concentrations of ClO_2^- and only showed a significant increment when the ClO_2^- concentration was 100 mg/L, with a consumption rate similar to the CRB enrichment (1.3 U/mg DW). It can be observed that the

consumption rates of O_2 are much slower than the production rates of O_2, indicating ClO_2^- could be added intermittently to culture and serve as an indirect electron acceptor for growth of PRB or CRB.

5.3.2. ClO_2^- as indirect electron acceptor supporting growth

The ability of bacteria to grow on O_2 produced from ClO_2^- disproportionation was tested in batch experiments. Cultures inoculated with PRB and CRB enrichments showed an increment in OD_{600} after sequential additions of 20 mg/L of ClO_2^- (Figure 5.4). Some lag phases for the PRB cultures (from 24 to 36 h and from 44 to 60 h) may have occurred because the initial ClO_2^- added was not enough to substantially increase the OD_{600}, but after adding more ClO_2^- they continued growing. The OD_{600} obtained with ClO_2^- addition was similar, and in some cases higher, to the OD_{600} reached when bacteria grew with ClO_4^- or ClO_3^-, consistent with the fact that O_2 is a more favorable electron acceptor (Table 1.1).

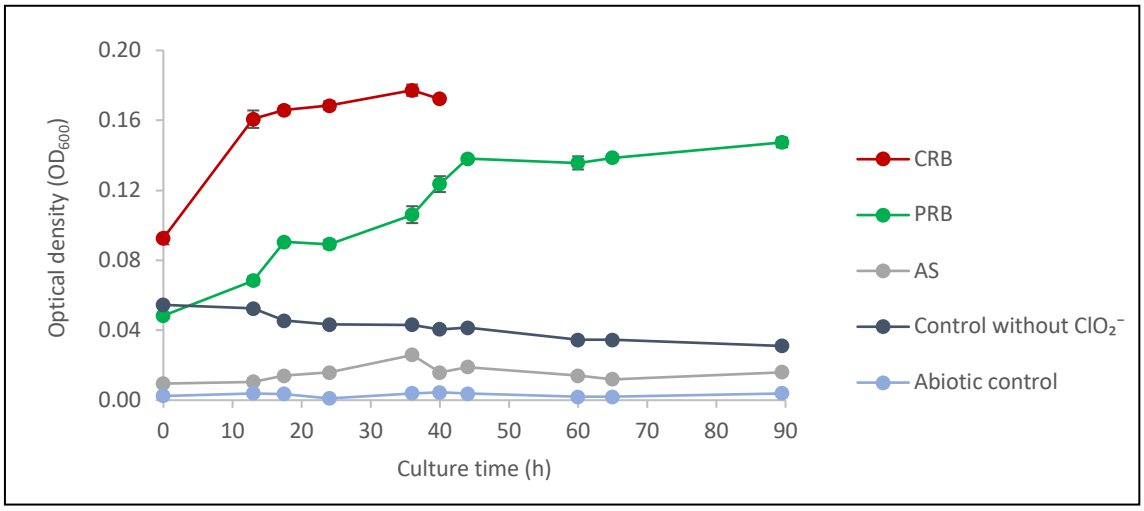

Figure 5.4. Growth curves of PRB and CRB cultures with ClO_2^- as indirect electron acceptor. ClO_2^- was added to culture media inoculated with 10% v/v PRB or CRB. PRB: enrichment growing with perchlorate. CRB: enrichment growing with chlorate. AS: activated sludge. One standard deviation for CRB and PRB enrichments is shown in error bars.

Negative control tests with bacteria but no ClO_2^- did not grow, nor did the culture inoculated with AS. Cultures inoculated with CRB grew faster than cultures inoculated with PRB, which agrees with the results obtained from the activity rates. This may indicate enrichments with ClO_3^- selected for faster bacteria than the cultures using ClO_4^- as an electron acceptor. From these experiments, we confirmed ClO_2^- can serve as an indirect electron acceptor and support bacterial growth for PRB and CRB. However, it is not clear whether the growth was attributed to PRB, CRB, or common heterotrophic bacteria that could have being enriched over PRB or CRB.

5.3.3. Effect of ClO_2^- on perchlorate reduction in a H_2-MBfR

Reactors A and B were the continuation of the Reactors A and B described in Chapter 3, and ClO_2^- was added to Reactor B four weeks after ClO_3^- was removed from the feed for Reactor B. During these four weeks (Stage 1) both reactors were operated with O_2 in the influent. Once ClO_2^- was added the influents were made anoxic. In both reactors, NO_3^- reduction continued stable below 0.06 mg N/L, except for a couple of events that will be discussed later in this section (Figure 5.5). Also, ClO_2^- was fully transformed immediately after it was added to Reactor B, with no ClO_2^- in the effluent. ClO_2^- is a very reactive compound, making hard to determine if it was dismutated by PRB or CRB, or if it reacted chemically with bacteria, since its highly reactive (Bomberg et al. 2016, Atashgahi et al. 2018).

In Reactor A, ClO_4^- reduction was slightly unstable at the end of Stage 1, and its decreasing reduction rates seem to be related to higher SO_4^{2-} reduction rates (Days 19 to 28 in Figure 5.5a), although they are slightly out of phase. The decrease in ClO_4^- reduction is observed after one or two days after SO_4^{2-} reduction increased. For example, on Day 24 the reduction of SO_4^{2-} reduction started to increase, reaching 52 mg/L (36% of reduction) on Day 25 (the lowest value during the operational time of the Reactor) and ClO_4^- reduction decreased dramatically on Day 27 (from 65 μg/L to 300 μg/L). From Day 54, SO_4^{2-} reduction started to be more stable around an average effluent of 73 mg/L. During this period, ClO_4^- reduction increased and averaged 97% reduction (23 μg/L). These results agree with other studies which report a negative effect of SO_4^{2-} reduction on ClO_4^- reduction (Ontiveros-Valencia et al. 2013b, Ontiveros-Valencia et al. 2014a).

However, SO_4^{2-} reduction did not have the same effect on NO_3^- reduction. This indicates NO_3^- is the preferred electron acceptor, as it also has been shown in other studies (Ziv-El and Rittmann 2009, Zhao et al. 2013, Kotlarz et al. 2016, Zhu et al. 2016).

Before adding ClO_2^- to Reactor B (Stage 1), it behaved similarly to Reactor A. The reduction of ClO_4^- was more stable than in Reactor A, but on Days 24 and 25 the SO_4^{2-} reduction also increased (reaching 39 mg/L), and ClO_4^- reduction started to decrease on Day 25 to 44 μg/L (93% reduction) on Day 28. As the decrease in ClO_4^- reduction occurred before ClO_2^- addition, we do not believe they are related. Nevertheless, during ClO_2^- addition to Reactor B (Stage 2), less ClO_4^- was reduced compared to the days before and after ClO_2^- addition. This could be connected to the noticeable SO_4^{2-} reduction (70 mg/L in the effluent) during this stage but, in Stage 1, SO_4^{2-} was reduced to an average of 65 mg/L and ClO_4^- reduction remained completely reduced. Thus, it is possible that ClO_4^- reduction by PRB was slightly inhibited by ClO_2^-, probably due to the O_2 produced. Despite the fact that O_2 produced from 5 mg/L of ClO_2^- are equivalent to only 2.4 mg/L of O_2, and that in Chapter 3 we showed O_2 at 8 mg/L in the influent did not affected PRB or ClO_4^- reduction, this case might be different. In the H_2-MBfRs from Chapter 3, O_2 was supplied from the bulk liquid and it was probably reduced in the outer layer by denitrifying bacteria (DNB), before reaching PRB. In this study, when adding ClO_2^-, it has to first reach PRB, which produce the O_2 that is readily for them to use it, and they consume it before using ClO_4^-. On the other hand, ClO_4^- reduction reached nearly 100% (from Day 81) when ClO_2^- was still present, but SO_4^{2-} reduction rate was the lowest. Neither possibility can be ruled out. However, the statistical analysis ANOVA indicate the effect of ClO_2^-on ClO_4^- and SO_4^{2-} reduction during Stage 2 is not significant ($p > 0.05$).

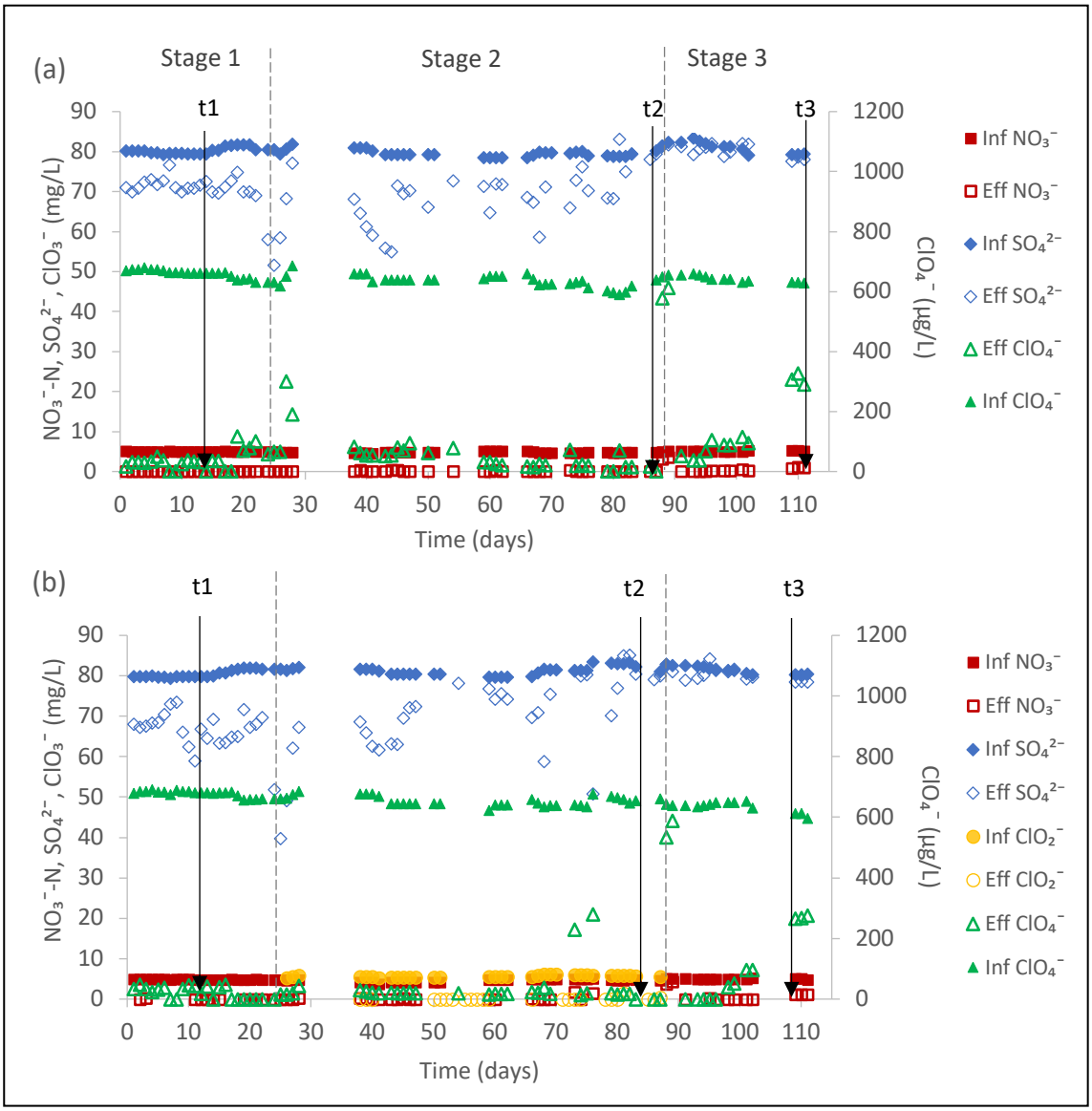

Figure 5.5 Reduction of NO_3^-, SO_4^{2-}, ClO_4^- and ClO_3^-. (a) Reactor A (control). (b) Reactor B (with ClO_2^- addition). In the first stage, both reactors were run without ClO2−. From Day 26 to 87, ClO_2^- was added to reactor B. Both reactors then continued to operate for 24 more days. The arrows indicate the days in which the biological samples were taken.

By the end of the Stage 3 (Day 109), ClO_4^- and NO_3^- reduction rates decreased, and ClO_4^- was the most affected. This cannot be attributable to SO_4^{2-} as its reduction was very low, nor to ClO_2^- as it was absent. An explanation for this can be the increment of

the biofilm thickness. At this point, the reactors had been running for more than five months, and the biofilm was very thick. In another internal study (data not shown), the performance of a H_2-MBfR with same characteristics as Reactor A, showed a significant decrease in NO_3^-, ClO_4^- and SO_4^{2-} reduction after five months of operation, mainly because of excessive biofilm thickness. The thickness was not measured, but in doing some hydraulic tests the biofilm was so thick that it partially blocked the flow. In a biofilm, the compounds in the bulk need to diffuse into the biofilm. Hence, only compounds at higher concentrations can reach the surface of the hollow fiber membrane, where higher concentrations of H_2 are present. It is believed that DNB and PRB/CRB were located on the outer part of the biofilm, and SRB in the inner part. If the biofilm was too thick, the outer part of the biofilm received less H_2, decreasing the reduction of NO_3^- and ClO_4^-.

Finally, there are several outlying points produced by problems in the operational conditions, such as leakage, bubbles in the reactors, problems with pumps, etc. For example, on Day 68, the influent pump had to be stopped for 3 – 4 h. This entailed a slight decrease in ClO_4^- reduction, but a higher increase in SO_4^{2-} reduction. Also, on Day 75 in Reactor B, there was a leak in the influent tubing, which generated a decrease of NO_3^- and ClO_4^- reduction. In a real scenario, these events are likely to occur, and the results show they mainly favor SO_4^{2-} reduction. It is important to take in account these situations and their consequences into account to ensure the desired performance.

5.3.4. Assessment of the microbial community with 16S rRNA sequencing

The microbial community distribution at the three stages is presented in Figure 5.6. Samples for t1 were taken two weeks after we removed ClO_3^- from the influent to Reactor B and two weeks before the reactors were made anoxic. During this time, the influent in both reactors contained 8 – 9 mg/L of O_2. On Day 26, ClO_2^- was added and the influent was made anoxic. Samples from t2 were taken after 62 days of ClO_2^- addition to Reactor B and samples from t3 were taken 24 days after ClO_2^- addition was stopped.

At t1, when the reactors were operated with an oxygenated influent, the communities in each reactor were similar in terms of the main phyla present. In both Reactors, PRB were represented by Rhodocyclales, which was composed mainly by an unclassified genus from the family *Rhodocyclaceae* and, in a minor degree, by the genus

Dechloromonas (also from the family *Rhodocyclaceae*). However, their abundance in Reactor A was only 11%, and in Reactor B was 31%. DNB are represented by Bulkholderiales, with only *Hydrogenophaga* at the genus level. Its presence in Reactors A and B is similar (12 and 16% respectively). Desulfovibrionales is the order representing SRB, with *Desulfomicrobiaceae* as the dominant family in both reactors. There also was a significant difference between Reactors A and B, as they represent 51% in Reactor A and 17% in Reactor B. These differences in PRB and SRB populations do not match the reductions rates of NO_3^-, ClO_4^- and SO_4^{2-} in this period. NO_3^- and ClO_4^- reductions are very similar in both Reactors, and SO_4^{2-} was around the double in Reactor B compared to Reactor A, inversely to the microbial abundance. This could be explained by the presence of inactive bacteria in the biofilm or the two weeks after taking out ClO_3^- was not time enough for the community to evolve to a more stable state.

When shifting the influent from oxygenated to anoxic in Stages 2 and 3, about 60% – 70% of the community remained represented by the same phylotypes in both reactors. In the remaining 30% – 40%, some bacteria were favored over others; furthermore, there are new members that were not present when the influent was aerobic.

After 62 days of ClO_2^- addition to Reactor B during Stage 2 (t2 in Figure 5.6), Rhodocyclales remained stable in Reactor B at 30% compared to Stage 1, what would indicate ClO_2^- did not apply a selective pressure to enrich PRB. Moreover, the abundance of Rhodocyclales in Reactor A reached a similar value (33%). Regarding the shifts in the orders Burkholderiales and Desulfovibrionales from Stages 1 to 2, they both decreased in Reactor A and were mainly replaced by Rhodocyclales. In Reactor B, Burkholderiales decreased and Desulfovibrionales increased. In spite of the changes of the community in each reactor, the abundance levels of these three orders is very similar comparatively between the reactors. In this case, SO_4^{2-} reduction was close to zero and NO_3^- and ClO_4^- reduction rates were high.

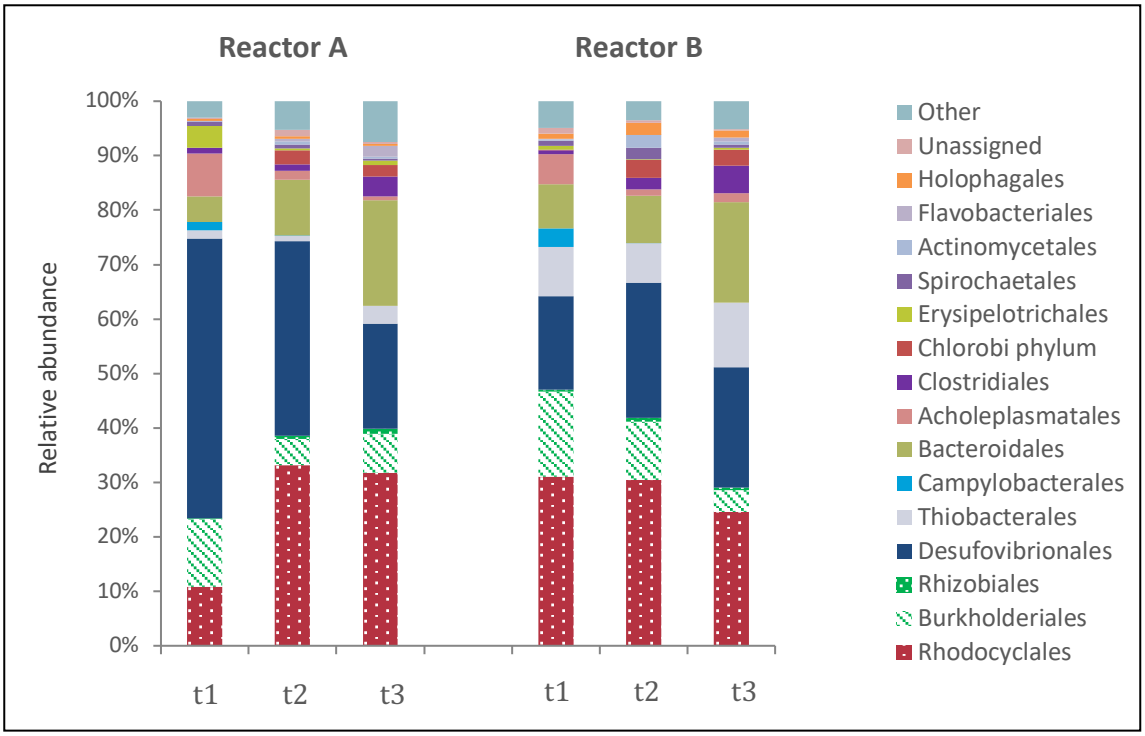

Figure 5.6. Microbial community structure at order level in Reactors A and B. Biological samples were taken at 3 times: i) day 14, two weeks before adding ClO_2^- to Reactor B (t1); ii) day 87, after 62 days of ClO_2^- addition (t2); iii) day 111, after 24 days of stopping ClO_2^- addition (t3). The results are an average of from two samples at each time in each reactor. The sum of the less abundant phylotypes is classified as "Other bacteria".

From Stage 2 to Stage 3, the three main phylotypes Rhodocyclales, Burkholderiales and Desulfovibrionales decreased in Reactor B, with Burkholderiales being the most affected. In Reactor A, Rhodocyclales and Burkholderiales stayed at a similar abundance, but Desulfovibrionales decreased more dramatically. These changes are compensated by an increment in the abundance of community that were present in smaller proportion in the previous two stages. At the end of Stage 3, the SO_4^{2-} reduction was reaching zero, explaining the lower abundance of SRB. Even though the abundance of PRB was important, ClO_4^- started to be affected and no longer reduced to low levels (only 50% of reduction). This may be due to the increase in biofilm thickness, depleting the electron donor in the external layers of the biofilm.

Regarding other orders of bacteria, Bacteroidales became more abundant in both reactors within the three stages and their abundance at each stage is very similar. The presence of Bacteroidales has been related to high SO_4^{2-} reduction rates together with high abundance of SRB, attributed to a collaborative relationship (Ontiveros-Valencia et al. 2013a). However, in this study SO_4^{2-} reduction is decreasing in both reactors, but Bacteroidales still increased. Also, the presence of Thiobacterales was significant in Reactor B, although SO_4^{2-} reduction was lower than in Reactor A. A possible explanation is they were reducing H_2S back to SO_4^{2-} making the overall SO_4^{2-} effluent concentration appear lower.

The anoxic influent in Stages 2 and 3, seems to favor certain bacteria and to inhibit others. In both reactors, the appearance of new phylotypes, not present during the operation with aerobic influent (Stage 1), were observed. These were organisms from the order Clostridiales, and from the phylum Chlorobi and Flavobacterales (only in Reactor A). Clostridiales and Flavobacterales can degrade complex organic matter (Cydzik-Kwiatkowska and Zielińska 2016, Bermingham et al. 2017) and Chlorobi may contain heterotrophic bacteria (Hiras et al. 2016). Clostridiales and Flavobacteriales were present during influent with O_2 in Reactors A and B from Chapter 3, but decreased along the operational time, ending at less than 1%. Here, Clostridiales proliferated to 4% and 5% in Reactors A and B respectively and Flavobacteriales up to 2% in Reactor A. Clostridiales are in general strictly anaerobic, although there are some exceptions (Wells and Wilkins 1996). Chlorobi, green sulfur bacteria, appeared only when the reactors were anoxic. They use H_2S as electron donor, are strict anaerobes, commonly found in anaerobic regions rich in sulfur, and are unable to perform NO_3^- reduction (Hiras et al. 2016, Llorens-Marès et al. 2016). On the other hand, the inhibited bacteria belong to the orders Acholepasmatales, Campylobacterales and Erysipelochitrales. Acholeplasmatales started representing a 6% – 8% of the microbial community, but it ended at 1% – 2% in both reactors. They increased in both reactors A and B described in Chapter 3, but they were outcompeted here, probably due to lack of O_2, even though they are considered as facultative anaerobes and related to NO_3^- reduction (Engelbrektson et al. 2014, Llorens-Marès et al. 2016). Campylobacterales were present at Stage 1 at 2% – 3% in both reactors, but they completely disappeared at

Stages 2 and 3. *Helicobacteraceae* (from Campylobacterales) grows better with H_2 and in microaerophilic environments, thus maybe the absence of O_2 was detrimental them (Kersters et al. 2006, On et al. 2017). Erysipelotrichales were present almost only in Reactor A in Stage 1 (4%) and disappeared in Stages 2 and 3. It includes microaerophilic and anaerobic organisms (Verbarg et al. 2014), indicating probably only microaerophilic members were present here. Besides the absence of O_2, the competition for substrates and space in the biofilm could explain the changes in these members of the microbial community.

The influent without O_2 should not represent a great difference from the case of influent with O_2, since O_2 was reduced to very low levels an only a low concentration available to diffuse into the biofilm. Nevertheless, the absence of a compound compare to a very small concentration can have an effect. For example, if NO_3^- is present at concentrations as low as 0.05 mg/L, SO_4^{2-} reduction can be controlled, but once NO_3^- is taken out, SO_4^{2-} reduction starts to increase immediately (unpublised), which could explain the difference in the microbial communities with and without O_2 in the influent. Despite the absence of O_2, SRB were not favored.

5.3.5. Effect of chlorite effect on the H_2-MBfR

The effect of ClO_2^- in the microbial community seemed to be minor. During Stage 2 in Reactor B, the preponderant community was relatively similar compared to the other two stages. Furthermore, it is hard to relate the small changes in the microbial community to the presence of ClO_2^-. However, there are some groups with low abundance that could have been enriched by ClO_2^-. Actinomycetales, Holophagales and Spirochaetales were only more abundant when ClO_2^- was added to Reactor B (Figure 5.6). Holophagales and Spirochaetales are, in general, strict anaerobes (Dubinina et al. 2011, Fukunaga and Ichikawa 2014). The proliferation of strict anaerobic bacteria while ClO_2^- was added is interesting, as ClO_2^- would be dismutated, producing O_2. Actinomycetales encompass both aerobes and anaerobes (Arensk̈otter et al. 2004, Ghai et al. 2014), so probable they grew using the O_2 produced by ClO_2^- dismutation together with organic matter or sulfur from H_2S oxidation(Conneely 2011, Ghai et al. 2014).

As it was shown in Section 5.3.1 that the O_2 production rates of an enrichment with PRB or CRB are much faster than the consumption rates (Figure 5.2 and Figure 5.3). This would imply that, if ClO_2^- was dismutated in Reactor B, the O_2 produced could have been used by other bacteria. Furthermore, when adding 5 mg/L of ClO_2^- it was possible to measure dissolved O_2, indicating O_2 was released out from the PRB or CRB cells. As ClO_2^- is highly reactive, another alternative is that instead of being dismutated it reacted with some bacteria. However, this depends on which bacteria ClO_2^- reached first. As PRB are believed to be in the outer layers of the biofilm (Ontiveros-Valencia et al. 2013b), and there is a difference in the performance and the microbial communities of Reactors A and B, we speculated that ClO_2^- was dismutated and provided O_2 to the community instead of reacting with organic matter.

5.4. Conclusions

In this study we determined ClO_2^- dismutation rates and O_2 consumption rates in *in vivo* enrichments growing anaerobically with ClO_4^- or ClO_3^-. Cld activities in CRB enrichments were up to three times higher than in PRB enrichment and the consumption rates of O_2 were, in general, also higher in CRB cultures than in PRB cultures. This indicates ClO_3^- enriches for bacteria with faster metabolism. The Cld rates obtained in this study were lower than previously reported rates for an activated sludge from a ClO_4^--reducing reactor, and also for the isolated culture of *Dechlorosomas* growing anaerobically. However, the rates were similar to the activity of this genus when growing aerobically.

In spite of this, we showed ClO_2^- can serve as an indirect electron acceptor and support growth of PRB or CRB through the production of O_2. Yet, the addition of ClO_2^- to a H_2-MBfR had only a slight effect on the performance and on the microbial community. The effect of ClO_2^- on ClO_4^- reduction could not be determined, but it seemed to control SO_4^{2-} reduction.

CHAPTER 6
Conclusions and future work

6.1. Conclusions

The Atacama Desert in Chile is one of the few major natural sources of perchlorate (ClO_4^-). Despite the health concerns from ClO_4^- exposure, there is very limited research in Chile. A systematic assessment of the health risks of the population is an important need. Conversely, ClO_4^- contamination in the Atacama Desert presents opportunities to study novel microorganisms with special features that may help to improve current treatments for perchlorate.

From the treatment perspective, the hydrogen-based membrane biofilm reactor (H_2-MBfR) is a suitable alternative to remediate ClO_4^-, as it can reduce it below the recommended treatment levels. However, it presents several challenges when other electron acceptors are present. In this study we explored the addition of two compounds to enhance selection for perchlorate-reducing bacteria (PRB) and help to diminish sulfate (SO_4^{2-}) reduction: chlorate (ClO_3^-) and chlorite (ClO_2^-). ClO_2^- is transformed into O_2, and may help select for PRB. Besides, we showed the O_2 produced can be released to the environment and be used as electron acceptor.

The results of this research suggest the addition of ClO_3^- and ClO_2^- can be a plausible strategy to improve perchlorate reduction, but the concentrations need to be carefully considered in order to exert a significant effect. When the membrane area of the H_2-MBfR was high (abundant H_2 availability), the community was able to reduce or transform the added compounds very quickly, resulting in low concentrations in the bulk at steady state. These low concentrations exerted a relatively minor effect on ClO_4^- and SO_4^{2-}, although ClO_3^- had a more noticeable effect than ClO_2^-. When the membrane area was

smaller, allowing a higher bulk ClO_3^- concentration, the effect of ClO_3^- addition was stronger, particularly in the microbial community. As is has previously stated in other studies, the H_2 loading is a very sensitive parameter that can strongly vary the reduction level of each compound, particularly increasing SO_4^{2-} reduction, which in some cases affected ClO_4^- reduction.

6.2. Future work

6.1.1. Chlorate addition to enrich PRB and inhibit SRB

The study on the H_2-MBfRs showed the microbial community interactions can be very complicated. ClO_3^- had an effect on PRB, DNB and SRB and may have influenced their interactions, but they are not clear yet. Particularly, it seems important to study the relationship between SRB (*Desulfomicrobiaceae* and *Desulfovibrionaceae)* and ClO_3^-.

Taking microbial community samples at a greater frequency than used here could help understand better the changes in the microbial community over time. Also, microsensors profiling of H_2S and H_2 will give more accurate information about their concentration and profiles within the biofilm and the main bundle. Here we only assumed H_2S production based on SO_4^{2-} reduction, but as it can be reoxidized back to SO_4^{2-}, we might be missing H_2S production in different layers of the biofilm.

In the case of a single membrane, the reactors could be operated for longer time to ensure the microbial community was at steady state. Particularly, monitor the development of SRB and SO_4^{2-} reduction. Also, it is very important to establish the ClO_3^- concentrations needed to perform the desired effect. Ideally ClO_4^- could be measured to determine if ClO_3^- improves its reduction. Later, it would be interesting to explore the effect of intermittent supply of ClO_3^-.

6.1.2. Effects of chlorite addition on selection for PRB

To further test the enrichment of PRB or CRB with the addition of ClO_2^-, we propose operating a continuous reactor with suspended growth or in biofilm growth and evaluate the microbial community compared to a reactor in which only O_2 was provided as the electron acceptor. The inoculum would be a mixed community, like AS, and

bacteria from an enriched culture with ClO_3^- and/or ClO_4^- in order to have members capable of ClO_2^- dismutation. The microbial community can be determined by both qPCR and 16S sequencing. With qPCR it should be possible to determine if bacteria with the genes *pcrA* or *clr* are enriched.

In the practical application to a H_2-MBfR, the dismutation of ClO_2^- within the biofilm could be evaluated using O_2 microsensors to test if it is being transformed into O_2 or if it is reacting with organic matter abiotically. Different concentrations of ClO_2^- can be tested to determine if a higher concentration would have a bigger impact.

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APPENDICES

1. Microbial community at the genus level from Reactors A and B of Chapter 3.

Figure A.1 shows the microbial community at the genus level of Reactors A and B from Chapter 3. At each time (t1, t2 and t3) samples were taken in duplicate.

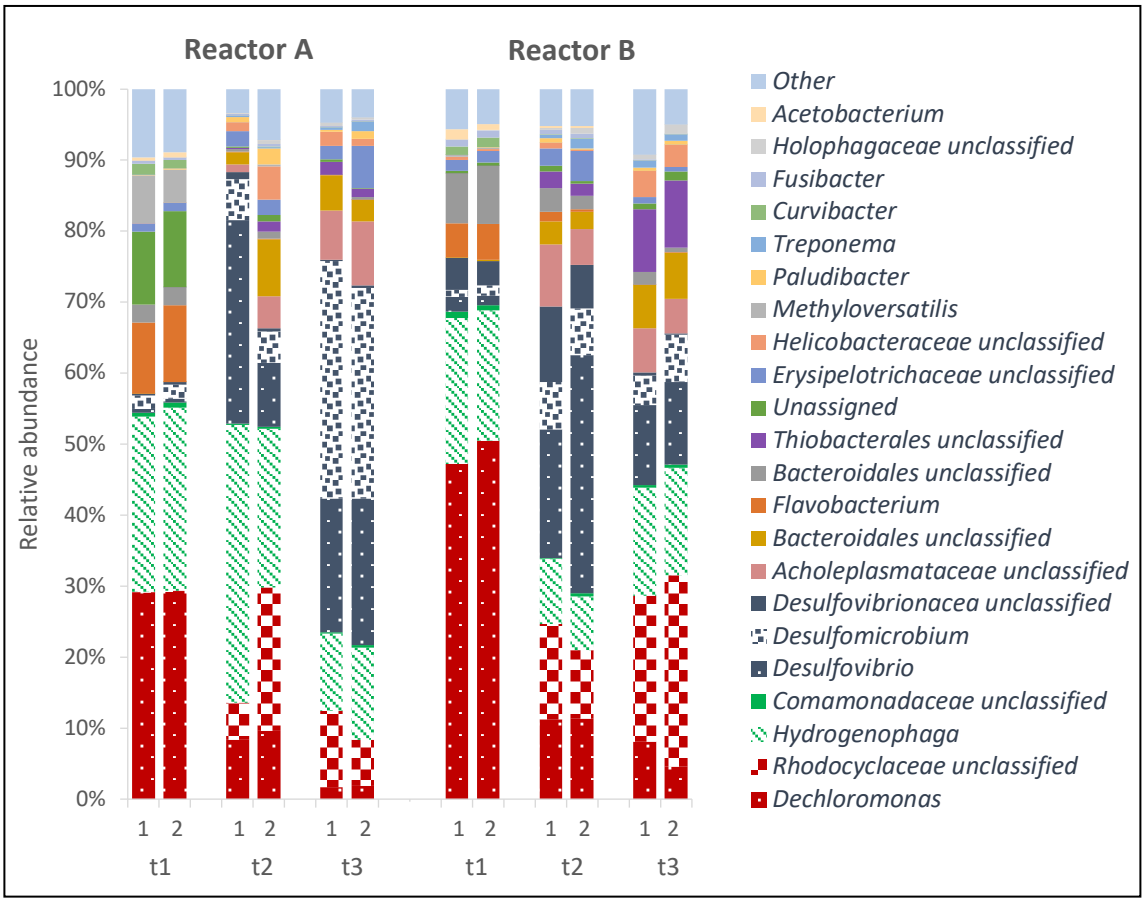

Figure A.1. Microbial community structure at the genus level in Reactors A and B. Biological samples were taken at 3 times: i) day 24, before adding chlorate to Reactor B (t1); ii) day 60, after 35 days of chlorate addition (t2); iii) day 78, after 15 days of stopping chlorate addition (t3). At each time samples were taken in duplicate (1 and 2). The sum of the less abundant phylotypes is classified as “Other bacteria”.

2. SO_4^{2-} cycling in the H_2-MBfR

H_2S was measured through the main bundle in a reactor with the same conditions as Reactor A in Chapter 3 (Figure A.2). An H_2S microsensor (Unisense A/S, Denmark) was used to perform the profiles. The tip of the microsensor started measuring H_2S at the bulk liquid in one side of the bundle (Bundle thickness is 0 µm) and it moved through the bundle to reach the opposite side of the bulk liquid (Bundle thickness is 6000 µm). H_2S is accumulated in the center of the bundle and it diffuses to the exterior while it is consumed. There is a zero concentration of H_2S in the bulk liquid. This indicates H_2S has been oxidized back to either S^0 or SO_4^{2-}.

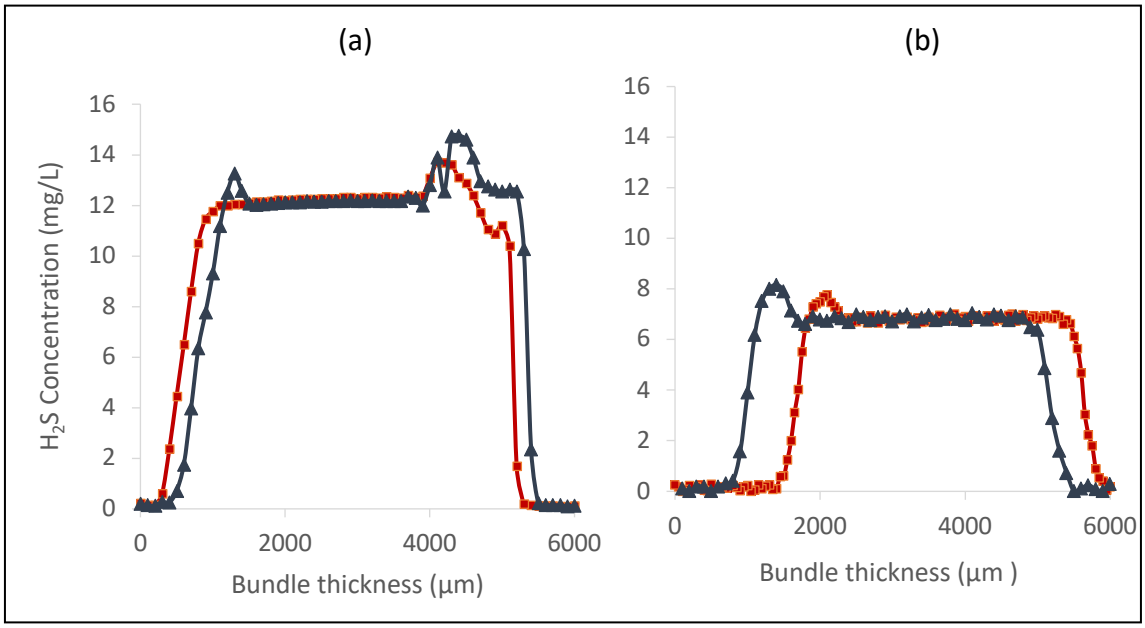

Figure A.2. H_2S concentration along the main bundle. H_2S was measured within the main bunlde in duplicate at two positions: (a) Close to the H_2 inlet; (b) Close to the end of the membranes.

3. Microbial community at the genus level from Reactors 1 and 2 of Chapter 4.

Figure A.3 shows the microbial community at the genus level of Reactors A and B from Chapter 3. At t2 samples were taken in duplicate.

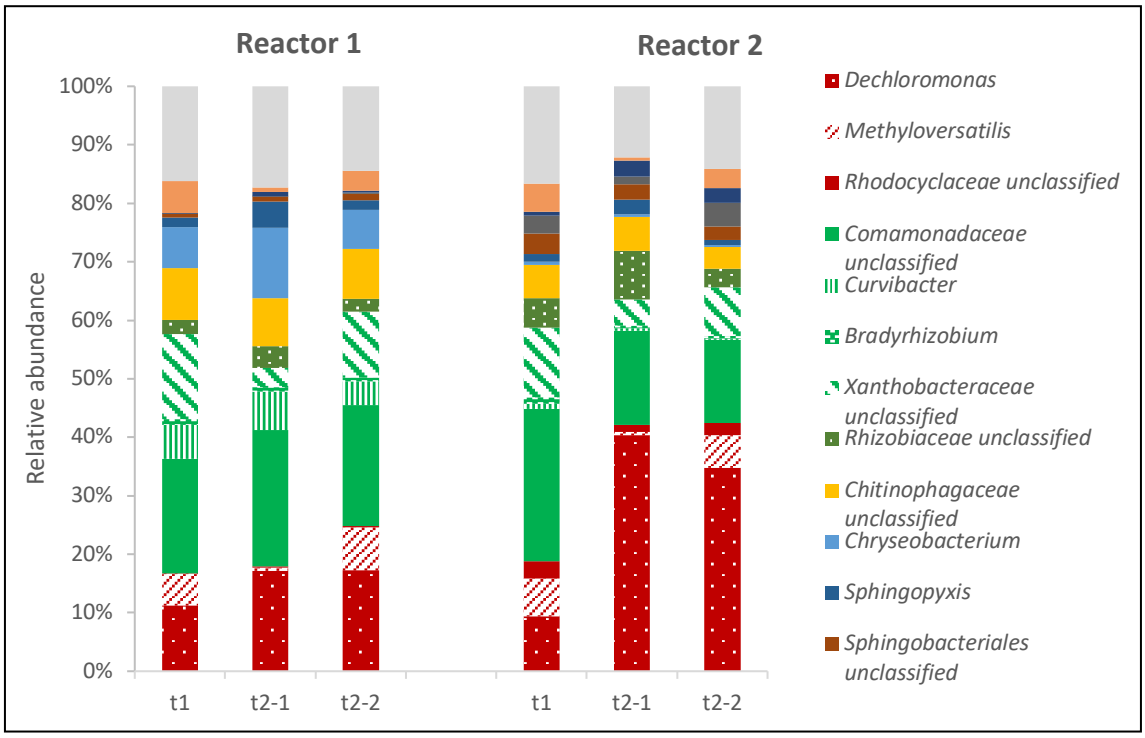

Figure A.3. Microbial community structure at genus level in Reactors 1 and 2. Biological samples were taken at 2 times: i) day 22, before adding chlorate to Reactor B (t1); ii) day 34, after 14 days of chlorate addition (t2). At t2 samples were taken in duplicate (t2-1 and t2-2). The sum of the less abundant phylotypes is classified as "Other bacteria".

4. Chlorite dismutation and O_2 consumption tests

The negative control to determine if O_2 was lost from the medium to the atmosphere is shown in Figure A.4a, and the negative control to determine if O_2 could be incorporated from the atmosphere to the experimental reaction is shown in Figure A.4b.

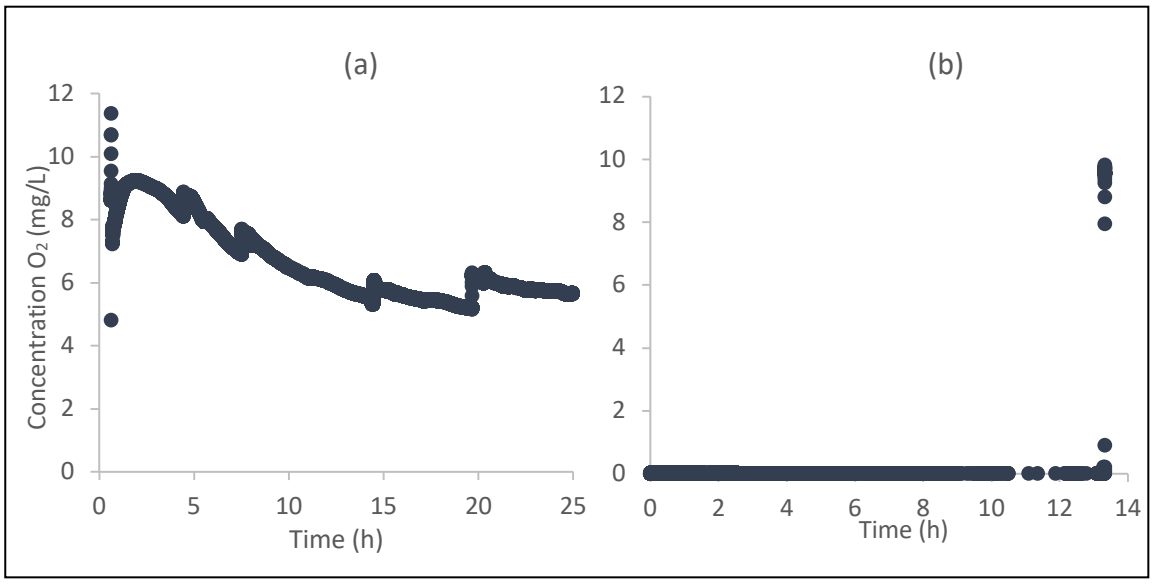

Figure A.4. Control experiments for O_2 production and consumption rates. (a) Negative control to determine O_2 losses from the medium to the atmosphere. (b) Negative control to determine if O_2 could be incorporated from the atmosphere to the experimental reaction.

5. Calculation of O_2 production and consumption rates

The O_2 production and consumption rates were calculated from the curves obtained by measuring O_2 concentration after adding ClO_2^-. Figure A.5 shows one of the O_2 concentration curves obtained in these experiments. Right after ClO_2^- was added O_2 started to be produced, reaching a maximum value. Then O_2 starts to be consumed by the bacteria in the culture and O_2 concentration starts to decrease. The O_2 consumption and production curves were split and a trendline was added. With this it was possible to calculate the O_2 production and consumption rates using the slopes of the equations from each trendline.

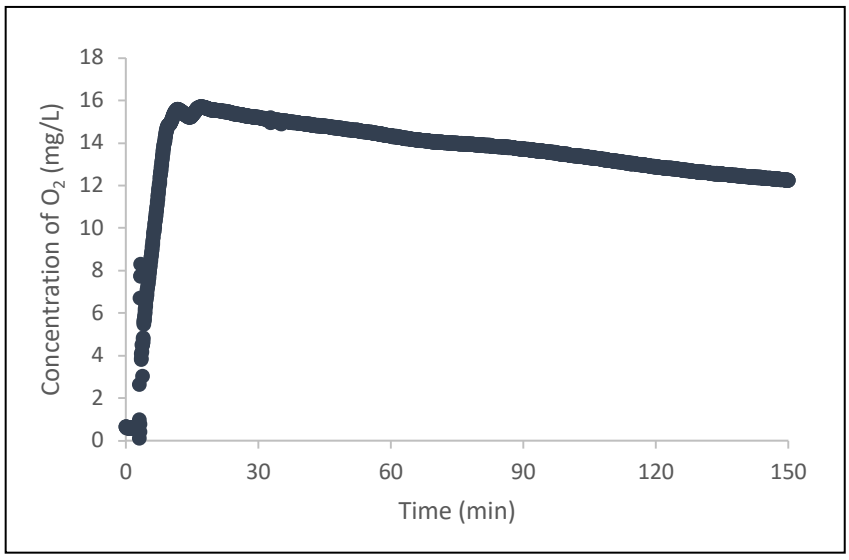

Figure A.5. O_2 production and consumption after addition of ClO_2^-. Cultures growing on ClO_4^- or ClO_3^- were used to determine the O_2 production and consumption rate after addition of ClO_2^-. O_2 concentration was monitored with an oxygen microsensor.

With the trendline, a linear equation was obtained (1). The slope "*m*" in this case is in units of $\left(\frac{\text{mg } O_2}{L-\text{min}}\right)$. This value "*m*" was then multiplied by the total volume of reaction, as shown in (2). In all the experiments the volume was 20 mL. Then it was normalized by the dry weight of biomass (*x*) in the reaction volume (3). After this the production rate was obtained (4). The calculation of the consumption rate followed the same procedure, although the slope was obtained from the decreasing part of the curve.

(1) $y = mx + n$

(2) $O_2\ production\ rate = m\ \left(\frac{\text{mg}\ O_2}{L-\text{min}}\right) \cdot 0.02\ L = 0.02\ m\ \left(\frac{\text{mg}\ O_2}{\text{min}}\right)$

(3) $O_2\ production\ rate = \frac{0.02\ m\ \left(\frac{\text{mg}\ O_2}{\text{min}}\right)}{x\ mg\ DW} = 0.02\ \frac{m}{x}\ \left(\frac{\text{mg}\ O_2}{min-\ mg\ DW}\right)$

(4) $O_2\ production\ rate = 0.02\ \frac{m}{x}\ \left(\frac{\text{mg}\ O_2}{min-\ mg\ DW}\right)$

6. Microbial community at the genus level from Reactors A and B of Chapter 5.

Figure A.6 shows the microbial community at the genus level of Reactors A and B from Chapter 3. At each time (t1, t2 and t3) samples were taken in duplicate.

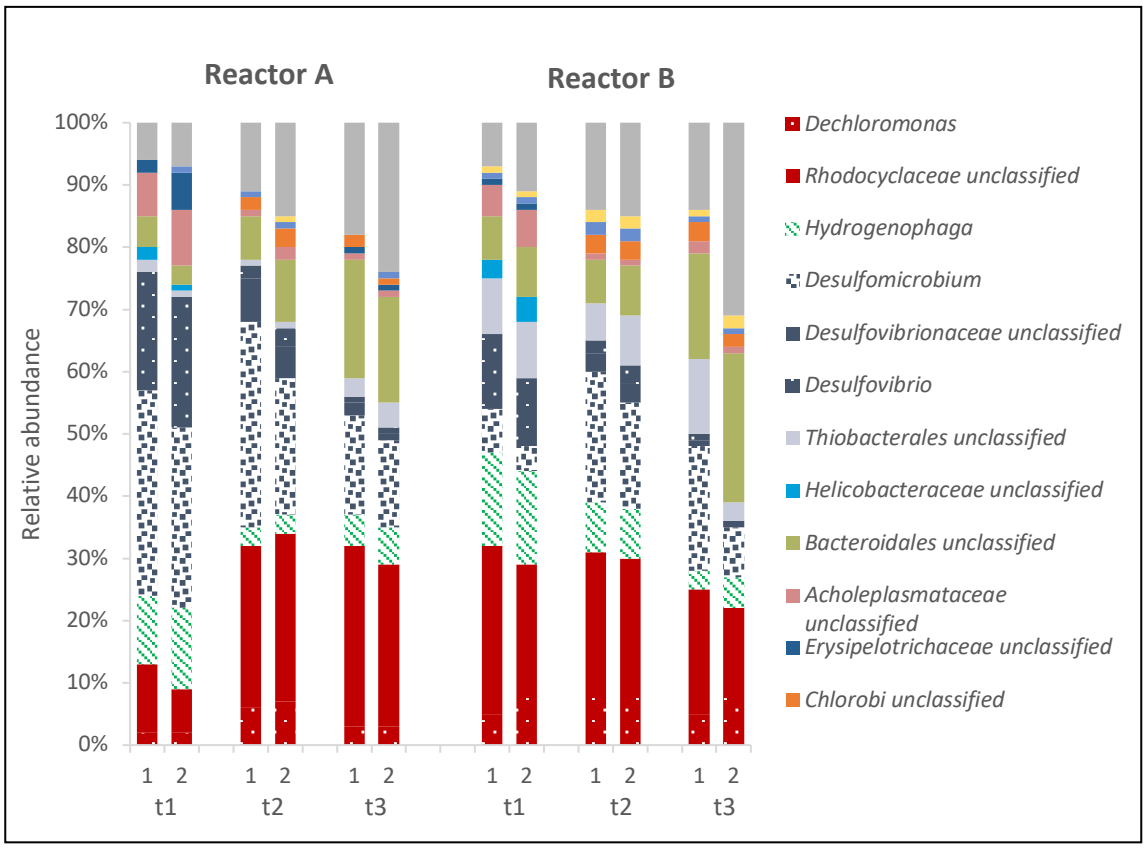

Figure A.6. Microbial community structure at genus level in Reactors A and B. Biological samples were taken at 3 times: i) day 14, two weeks before adding chlorite to Reactor B (t1); ii) day 87, after 62 days of chlorite addition (t2); iii) day 111, after 24 days of stopping chlorite addition (t3). Samples were taken in duplicate (1 and 2) at each time in both reactors. The sum of the less abundant phylotypes is classified as "Other bacteria".