Manganese removal from groundwater in non-bioaugmented pumice biofilters under tropical conditions

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Abstract

The presence of manganese (Mn) in drinking water systems causes aesthetic, operational, and health problems. Biofiltration is a cost-effective technology, that does not require the use of chemical reagents and is easy to operate. However, an important drawback is the long ripening time required for virgin media to reach effective Mn removal. Besides, Mn-biofiltration has not been proven in the tropics and little is known about the occurrence of Mn-oxidizing bacteria (MOB) in these zones. No previous studies have also evaluated the use of pumice for Mn removal in biofilters. Pumice has great potential for Mn-biofiltration because it is a low-cost, porous and low-density medium. In this context, this thesis aimed to obtain new insight into the efficacy of the start-up of non-bioaugmented pumice biofilters for Mn removal using tropical groundwater. For this purpose, an exploratory study was conducted to investigate the presence of MOB in groundwater wells with elevated concentrations of Mn (> 0.3 mg/L). A comparative study using virgin pumice, silica sand, and anthracite filter media was performed at the bench scale to evaluate the use of virgin pumice as an alternative filter medium for Mn removal. In addition, pilot-scale experiments were performed to assess the effect of the regimen flow (flow-through and recirculating) on the start-up of pumice filter columns.

It was found that culturable MOB isolated from raw water samples belong to several bacterial phyla, but a clear domain of *Proteobacteria* phylum (*Alpha*- and *Gammaproteobacteria*) was detected in the biofilter media. At the genus level, the bacterial community was not significantly influenced by the filter material; however, different closest related species colonized selectively the filter media. *Pseudoxanthomonas* sp., not reported in Mn biofiltration before, showed high Mn oxidation activities and was abundant in all the filter media evaluated, being good candidates for inoculums. The Mn oxides ($MnO_{x(s)}$) coated in the ripened media exhibited characteristics typical for birnessite of biological origin, confirming that MOB played an important role in the start-up of non-bioaugmented biofilters. The start-up in bench-scale columns was completed in approximately 80 days. Pumice stone exhibited a similar performance compared to sand and anthracite. Afterward, the start-up in non-bioaugmented pilot-scale pumice biofilters was reduced to only 8 and 23 days in the flow-through and recirculating regime,

respectively. These exceptional rapid start-up were reached due to favorable feed water characteristics (temperatures ~22-24 °C, pH > 7.5, dissolved oxygen (DO) > 6 mg/L and redox potential (Eh) ~300- 400 mV). Moreover, the following appropriate operational strategies were applied: initial filtration velocity of 2 m/h and an empty bed contact time (EBCT) of 21 min; once the Mn removal reached 90% the velocity was increased in steps of about 1 m/h until reaching a final filtration velocity of 5 m/h (~ EBCT of 8.4 min). Fe-loading was prevented using a prefilter (Fe concentrations in the influent <0.10 mg/L). Recirculating regime consumed less feed water during the start-up (~ 50%) than using a flow-through regime, making it a suitable strategy. Besides, both the flow-through and the recirculating flow regime, required a similar total Mn-loading of 0.11 $kg \cdot Mn \cdot m^{-2}$ which was considered a limiting factor. It seems that the intermittent Mn-loading in the recirculation regime influenced the MOB population established at the top of biofilters during the start-up (10^6 $CFUg^{-1}$ vs 10^3 $CFUg^{-1}$ in the flow-through and recirculating regime, respectively).

In addition, the long-term performance of full-scale biofilters for Mn removal was investigated in a converted physicochemical plant located in Argentina. The system effectively removed up to 88% of Mn; besides, 95% of Mn concentrations in treated water reached compliance with the local drinking water criterion (Mn<0.10 mg/L) during 10 years of operation without the use of chemical reagents. Hence, conversion to biofiltration is a suitable strategy, and was proven that it can result in a stable and efficient treatment through the years.

The results presented in this thesis show that non-bioaugmented pumice biofilters are a suitable and effective treatment solution for Mn removal from groundwater under tropical conditions. Groundwater temperature, a speed-up stage, Fe-pretreatment, and a recirculating flow regime, seem to be appropriate to shorten the ripening time of virgin pumice with less consumption of feed water. This conclusion underlines the potential contribution of Mn-biofiltration to the sustainable development goals (SDG) for the adoption of sustainable water management. Furthermore, this research can help to develop the interest of water supply companies in tropical and developing countries that mainly work with chemical treatments for Mn removal.

Resumen

La presencia de manganeso (Mn) en los sistemas de distribución de agua potable puede causar problemas operativos, de aceptabilidad y riesgos a la salud. La biofiltración es una tecnología costo-efectiva, fácil de operar y no requiere del uso de reactivos químicos. Sin embargo, su principal limitación es el tiempo de maduración requerido por el medio virgen para alcanzar una remoción efectiva de Mn. Además, la remoción biológica de Mn aún no ha sido implementada en el trópico y poco se sabe de la ocurrencia de las bacterias oxidantes de Mn (BOM) en estas zonas. Tampoco hay estudios previos que hayan evaluado el uso de la piedra pómez como medio filtrante en biofiltros de Mn. La piedra pómez tiene un gran potencial para la biofiltración ya que es un medio filtrante de bajo costo, poroso y de baja densidad. En este contexto, esta tesis tuvo como objetivo obtener nuevo conocimiento en la eficacia de la puesta en marcha de biofiltros de piedra pómez no-bioaumentados para la remoción de Mn utilizando aguas subterráneas tropicales. Para ello, se realizó un estudio exploratorio para investigar la presencia de BOM en pozos de agua subterránea con concentraciones elevadas de Mn (> 0,3 mg/L). Se realizó un estudio comparativo a escala de laboratorio utilizando piedra pómez virgen, arena sílice y antracita para evaluar el uso de piedra pómez virgen como medio filtrante alternativo para la remoción de Mn. Además, se realizaron experimentos a escala piloto para evaluar el efecto del régimen de flujo (flujo continuo y recirculación) en la puesta en marcha de columnas de filtración utilizando piedra pómez.

Se encontró que las BOM aisladas en el agua cruda son filogenéticamente diversas, no obstante, se detectó un claro dominio del filo *Proteobacteria* (*alfa* y *gamma*) en los medios filtrantes. A nivel de género, la comunidad bacteriana no se vio significativamente influenciada por el medio filtrante; sin embargo, distintas especies colonizaron selectivamente el medio filtrante. *Pseudoxanthomonas* sp., no reportada anteriormente en biofiltración de Mn, mostró una alta capacidad de oxidación de Mn y se encontró de forma abundante en todos los medios filtrantes evaluados, siendo así una buena candidata como inóculo. Los óxidos de Mn identificados en los filtros maduros mostraron características típicas de la birnessita de origen biológico, mostrando así que las BOM desempeñaron un rol importante en la puesta en marcha de los biofiltros no-bioaumentados. El periodo de

maduración de las columnas de filtración a escala de laboratorio se completó en aproximadamente 80 días. La piedra pómez mostró un desempeño similar a la arena y la antracita. Posteriormente, el periodo de maduración de las columnas de filtración a escala piloto se logró reducir a 8 y 23 días en flujo continuo y recirculado, respectivamente. Estos tiempos de maduración excepcionales se pudieron conseguir debido a las características favorables en la calidad de agua de entrada a los filtros (temperaturas ~22-24 °C, pH > 7.5, oxígeno disuelto > 6 mg/L y un potencial redox ~300- 400 mV). Además, se aplicaron las siguientes estrategias operativas: se adoptó una velocidad de filtración inicial de 2 m/h con un tiempo de contacto de 21 min; una vez la eficiencia de remoción de Mn alcanzó el 90%, se aplicaron incrementos de velocidad de aproximadamente 1 m/h hasta alcanzar una velocidad de filtración final de 5 m/h (tiempo de contacto de 8.4 min). La carga de hierro (Fe) se evitó mediante un prefiltro (concentraciones de Fe en el afluente < 0.10 mg/L). La estrategia de recirculación se consideró adecuada ya que consumió un 50% menos de agua comparada con el sistema continuo. Ambos regímenes requirieron una carga de Mn similar de aproximadamente 0.11 $kg \cdot Mn \cdot m^{-2}$, considerada como un factor limitante. Aparentemente, la carga intermitente de Mn durante la recirculación influenció las concentraciones de BOM que colonizaron la parte superior de los filtros (10^6 $CFUg^{-1}$ vs 10^3 $CFUg^{-1}$ en flujo continuo y recirculado, respectivamente).

Además, se investigó el desempeño en un largo periodo de tiempo, de filtros biológicos a escala real para la remoción de Mn en una planta ubicada en Argentina, la cual, antiguamente operó con procesos fisicoquímicos y fue convertida a proceso biológico. Durante 10 años de operación, el sistema registró eficiencias de remoción total de Mn cercanas al 88%; además, el 95% de las concentraciones de Mn en el agua tratada estuvieron por debajo del límite máximo permitido por la regulación local (Mn<0.10 mg/L), sin utilizar productos químicos. Por lo tanto, la conversión a biofiltración es una estrategia adecuada y se demostró que puede resultar en un esquema de tratamiento estable y eficiente a lo largo de los años.

Los resultados presentados en esta tesis muestran que los biofiltros no-bioaumentados de piedra pómez constituyen una solución de tratamiento adecuada y eficaz para la eliminación de Mn de agua subterránea en condiciones tropicales. La temperatura del

agua subterránea, la etapa de aceleración de la velocidad, el pretratamiento de Fe y el régimen de flujo recirculante parecen ser apropiados para reducir el tiempo de maduración de la piedra pómez virgen, con un menor consumo de agua durante el proceso. Esta conclusión resalta la contribución potencial de la biofiltración de Mn a los objetivos de desarrollo sostenible (ODS) para la adopción de una gestión sostenible del agua. Además, esta investigación puede ayudar a desarrollar el interés de las empresas de suministro de agua en países tropicales y en desarrollo que trabajan principalmente con tratamientos químicos para la eliminación de Mn.

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Abbreviations

WHO	World Health Organization
Fe	Iron
Mn	Manganese
$MnO_{x(s)}$	Manganese oxide
O_2	Oxygen
MOB	Manganese oxidizing bacteria
CNS	Central nervous system
MCL	Maximum contaminant levels
HTV	Health-threshold value
WTP	Water treatment plant
DO	Dissolved oxygen
ORP	Oxidation–reduction potential
SDG	Sustainable Development Goals
ROS	Reactive oxygen species
DW	Drinking water
DWS	Drinking water systems
XRD	X-ray Diffraction
SEM	Electron microscopy
OTUs	Operational taxonomic units
ATP	Adenosine triphosphate
RLUs	Relative light units
MOCA	Manganese oxide-coated anthracite
MOCS	Manganese oxide-coated sand
EBCT	Empty bed contact time
IQR	Interquartile range
URF	Up-flow roughing filter
RSF	Rapid sand filter

Chapter 1

General introduction

Overexposure to manganese (Mn) in drinking water systems (DWS) can lead to adverse health effects associated mainly with neurotoxicity effects; being infants and children the most susceptible population (WHO, 2021). Besides, Mn deposits cause aesthetic and operational problems (Brandhuber, 2013). Despite that physicochemical treatments can be very effective for Mn removal, the use of chemical reagents increases the operation-maintenance costs, the complexity of the treatment process, and the risk associated with the formation of harmful oxidation by-products (Bruins et al., 2015b). Biofiltration is a cost-effective technology that does not require the use of chemical reagents and is easy to operate representing lower operation and maintenance costs (Cai et al., 2015; Pacini et al., 2014). However, an important drawback of the implementation of biofiltration is the long ripening time required by the virgin media to reach effective Mn removal (Bruins et al., 2017b). Commonly, it can take between one and four months (Bruins et al., 2015). Consequently, the production of drinking water (DW) is delayed. In the meantime, water utilities may waste large amounts of water, affecting even the operational costs and the sustainability of the process (Bruins, 2016).

Bioaugmented methods can be used to enhance the effectiveness of the inoculation and acclimatization process of the manganese oxidizing bacteria (MOB) into the biofilters. Practical solutions involve the use of well-established biological material (e.g. backwash sludge or matured media) obtained from other active Mn-biofilters (Cai et al., 2015; Cheng, 2016; Štembal et al., 2004; Zeng et al., 2010). Another method comprises use as inoculum, a concentrated source of MOB strains developed in the laboratory (Piazza et al., 2019). Nevertheless, the implementation of these bioaugmented methods is difficult in areas where little is known about biofiltration and there is no practical experience. Therefore, there is still an urgent need to investigate

on criteria design and optimal operational conditions required to accelerate the ripening time for Mn removal of virgin media using only inherent inoculation (referred here as non-bioaugmented biofilters). Some known operational strategies could be implemented such as temporarily operating non-bioaugmented biofilters at a low filtration velocity to enhance the MOB attachment (Donlan, 2002) and/or reduce the Fe-loading and subsequent backwashings (Bruins et al., 2017a). In addition, a novel approach could be applying inherent inoculation in recirculating flow regime. It has been demonstrated to be effective during the start-up for the degradation of organic compounds of non-bioaugmented bench-scale biofilters (Börnick et al., 2001; Worch et al., 2002). For Mn removal, recirculating flow regime has been used only with bioaugmented methods (*e.g.* to recirculate supernatant water containing sediment backwash sludge). However, the influence of the inherent inoculation using recirculating flow regime on the ripening time of virgin media for Mn removal has not been reported. This approach seems promising because it would reduce both water consumption and the negative effect of Fe-loading during the start-up of non-bioaugmented biofilters.

On the other hand, biofiltration has been used for decades in some countries in Europe (Ramsay et al., 2018). Practical experience and important studies regarding the start-up (Štembal et al., 2004), including two recent fundamental investigations can be found in the literature (Breda, 2019; Bruins, 2016). In North America, extensive research has been done over the last 15 years on design, operation, monitoring, optimization, and conversion of biofilters (Brown, 2020). Other important studies have been conducted in China regarding start-up methods (Cai et al., 2015; Cheng, 2016; Zeng et al., 2010; Zeng et al., 2019). Occurrence of MOB, inoculation methods, criteria design, practical experiences, and conversion strategies have been also investigated in Argentina (Ciancio et al., 2020; Pacini et al., 2005; Pacini et al., 2014; Piazza et al., 2019). However, it should be notice that the application of Mn biofilters has been studied mainly in temperate regions (with approximately groundwater temperatures between 3 and 18°C) and using bioaugmented methods. Very little is known about the application of non-bioaugmented biofilters for Mn removal from groundwater under

tropical conditions. Knowledge needs to be gained on whether particular natural environmental conditions (*e.g* warm groundwater temperatures) present in tropical zones, could favor the biological Mn^{2+} oxidation process (*e.g*. reducing the ripening time of virgin media) or if it may impact the dominance of any particular MOB not yet reported. It seems that water temperatures below 15°C affect the Mn capacity uptake in biofilters. MOB growth and biological Mn oxidation can be inhibited at water temperatures below 14°C (Berbenni et al., 2000). Besides, long start-up periods (Cai et al., 2014) and decreased Mn removal in biofilters have been observed when water temperatures drop below 15°C (Evans et al., 2021b).

In addition, considering that financial resources are limited in developing countries, the application of biofiltration for Mn removal should also consider the use of low-cost and available filter media. Despite that the use of pumice in rapid filters for DW production has received increased attention (Çifçi & Meriç, 2015). No previous studies evaluating the use of pumice for Mn removal in biofilters was found in the literature. Pumice is a low-cost, porous, and low-density medium that produces less pressure drop than sand and has a higher specific surface area (Farizoglu et al., 2003). In that sense, the evaluation of the use of pumice during the start-up of non-bioaugmented biofilters for Mn removal is needed. In Costa Rica, pumice stone is cheap and easy to find (Acuña-Piedra et al., 2016).

Finally, another option for the implementation of biofiltration for Mn removal could be the conversion of existing physicochemical plants to biofiltration. In the literature, there is useful information regarding the proper planning and evaluation of the converted biofilters during the early stages of operation (Brown, 2020; Lauderdale et al., 2016; Mouchet, 1992; Pacini et al., 2014). In addition, long-term monitoring is essential to evaluate its performance under variations in the influent water quality (*e.g*. in Mn concentrations and seasonal groundwater temperature), and operational or hydraulics changes over time. However, little is known about the operational performance of converted WTP for Mn removal over a long-term period (>2 years). This information could be of help in planning.

Therefore, knowledge needs to be gained on:

- The isolation and characterization of autochthonous tropical-groundwater MOB.
- The start-up of non-bioaugmented biofilters for Mn removal under tropical conditions.
- The suitability of the use of pumice stone for biotic Mn removal.
- The influence of the flow regime (flow-through and recirculating) during the start-up of the non-bioaugmented biofilters for Mn removal.
- Long-term performance of Mn removal in biofilters from a converted physicochemical plant.

1.1 Approach

The main goal of this thesis is to obtain new insight into the efficacy of the start-up of Mn removal of non-bioaugmented pumice biofilters using tropical groundwater. This research can help to develop the interest of water supply companies in tropical and developing countries that mainly work with chemical treatments for manganese removal. Biofiltration could contribute to the sustainable development goals (SDG) that call for the adoption of sustainable practices in water management.

With a focus on the defined knowledge gaps, the following specific research objectives were defined:

- To identify and characterize naturally occurring, autochthonous MOB in tropical groundwater.
- To investigate, at the bench scale, the start-up period of non-bioaugmented biofilters for Mn removal.
- To evaluate the use of virgin pumice as an alternative filter medium for Mn removal in non-bioaugmented biofilters.
- To study the influence of the flow regime (flow-through and recirculating) during the start-up for Mn removal of non-bioaugmented pumice biofilters.
- To investigate the long-term feasibility of biological filters for Fe and Mn removal in a converted physicochemical plant

1.2 Thesis outline

This thesis has been organized into seven chapters. The current chapter includes the defined knowledge gaps that form the basis for the specific research objectives, developed in the following chapters of this thesis. These knowledge gaps overlap one or more chapters as illustrated in Figure 1-1.

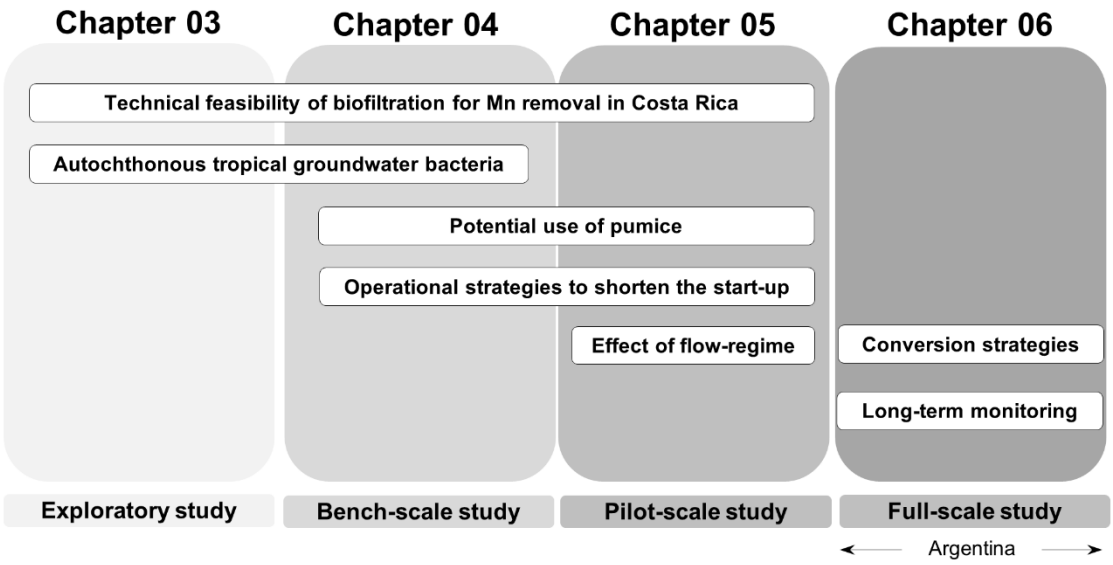


Figure 1-1. Synthesis between chapters and the formulated knowledge gaps

Chapter 2 gives a systematic overview of Mn occurrence in groundwater, the aesthetic, operational, and health problems associated with its presence in DWS, regulatory standards, and control strategies used in DWS. It also includes a theoretical framework regarding the biotic-abiotic oxidation of Mn^{2+} and removal mechanisms. This chapter also provides a detailed explanation of the start-up process of a biofilter for Mn removal, the occurrence of MOB in groundwater sources and biofilters, bacteria removal mechanisms, and factors that may affect biotic Mn (II) oxidation. Finally, as an important tool, the techniques applicable to the detection and characterization of MOB in the laboratory is provided.

Chapter 3 covers the isolation and characterization of manganese oxidizing bacteria (MOB) from groundwater wells in Costa Rica. It includes:

- An inventory of the occurrence of Mn (in function of location, pH, and water temperatures) from 40 groundwater wells with elevated Mn-Fe concentrations.
- An exploratory study to detect the presence of MOB in seven selected Mn-groundwater wells.
- In-vitro characterization of isolated MOB.

Chapter 4 presents the results of a bench-scale biofiltration experiment using tropical groundwater at 22 °C. The study was carried out to investigate the start-up period of the non-bioaugmented biofilters. The use of virgin pumice as an alternative filter medium for Mn removal was evaluated by a comparative study using virgin pumice, silica sand, and anthracite. Results also include the characterization of $MnO_{x(s)}$ on the ripened filter medium coating and the presence and growth of culturable MOB in each biofilter media.

Chapter 5 presents the results of a pilot-scale study conducted using groundwater from a well located in Cartago, Costa Rica. This study aimed to investigate the influence of the regime of operation on the ripening time of virgin pumice media using flow-through and recirculating non-bioaugmented gravity filter columns, with low Fe-loading and increasing filtration velocities (speed-up stage).

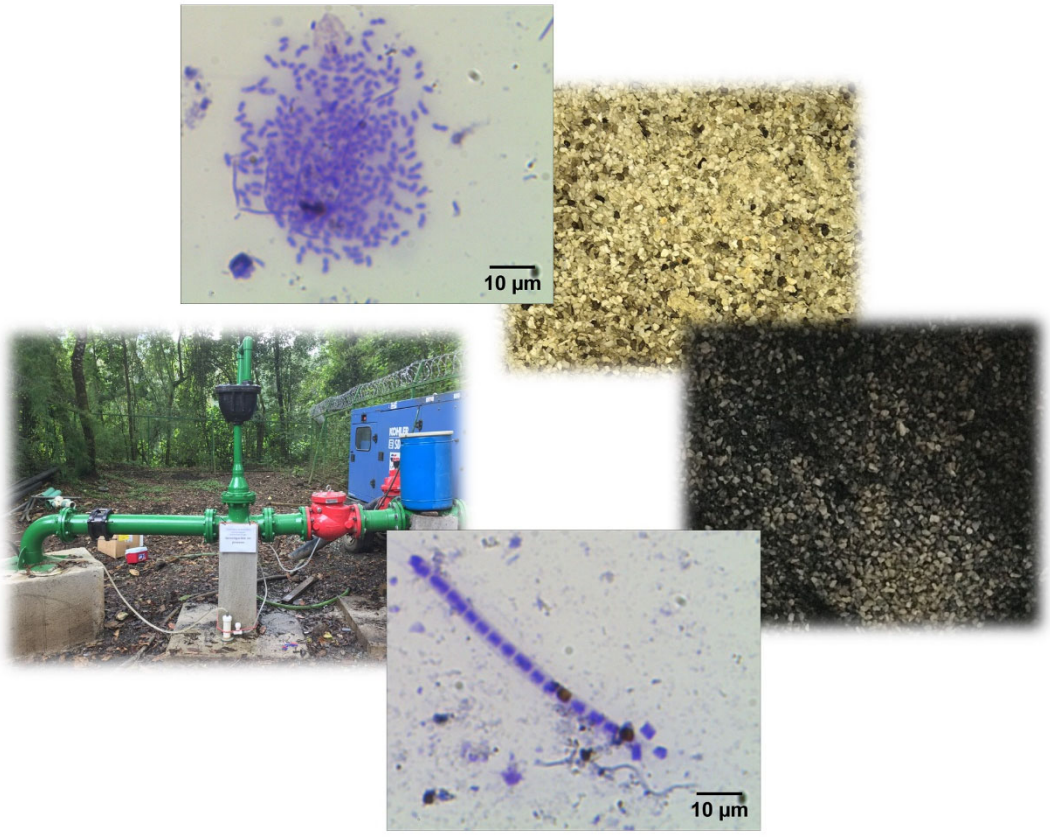
Chapter 6 provides an analysis of monitoring data from November 2011 until April 2021 from the converted physicochemical plant Las Toscas located in Santa Fe, Argentina. This study aimed to demonstrate the long-term feasibility of biological filters. Statistical comparisons were also included to determine seasonal variations in Fe and Mn concentrations at the influents and effluents of the biofiltration units.

Chapter 7 summarizes the main conclusions of this research and gives recommendations for practice and further research on the implementation of biofiltration for Mn removal using non-bioaugmented biofilters.

Chapter 2

State-of-the-Art

Graphical abstract



2.1 Drinking water production in Costa Rica

Costa Rica is a developing country, located in Central America with a land area of 51.100 km^2 and a population of 5.1 million people (INEC, 2021), where 98.1% are connected to a drinking water system (DWS) and 93.5% have access to potable water (Mora & Portuguez, 2021). In total, there are 2.623 DWS and 5674 drinking-water (DW) sources administrated by public services (AyA, Municipalities, and ESPH) and community-based entities. Figure 2-1 shows the percentages of WDS operated, the associated population served (regarding the 5.1 million people) and the groundwater-well sources administrated by each DW entity. It should be notice that approximately 2161 (82%) WDS in Costa Rica are administrated by the community-based entities that provide DW to approximately 30% of the total population in Costa Rica. Besides, from the total of groundwater sources, 1339 (23.6%) are groundwater wells and roughly 900 (67.2%) of these sources are operated by community-based entities.

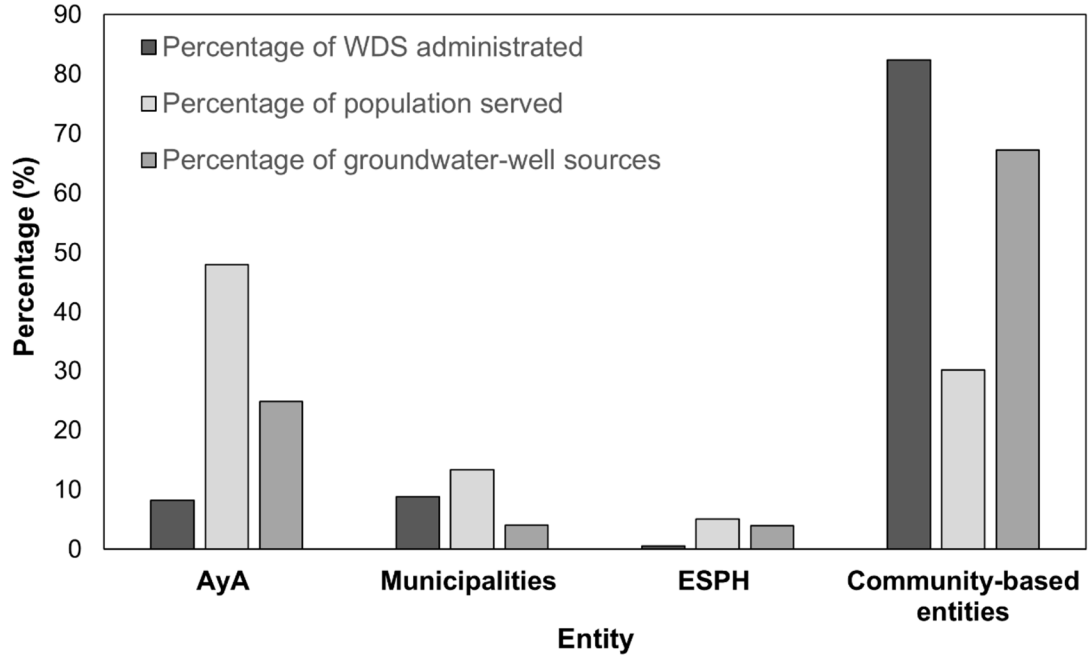


Figure 2-1. Percentages of water distribution systems (WDS), population served, and groundwater-well sources administrated by drinking water entities in Costa Rica.

The Mn control strategies in Costa Rica involve source water management (*e.g*. blend water to achieve an acceptable concentration level, or close wells) and water treatment using commonly chemical pre-oxidation followed by rapid filtration (with commercial $MnO_{x(s)}$ coated media). Biofiltration for Mn removal has not been yet implemented.

2.2 Manganese occurrence

Manganese (Mn) is the second most common heavy metal in the earth's crust and occurs mainly at three different oxidation states of II^+, III^+ IV^+ in natural systems (Tebo et al., 2004). Particularly, Mn^{2+} is the most reduced, stable, and naturally occurring state (Kohl & Medlar, 2006). Mn^{2+} is initially released into the environment through the natural weathering of Mn-containing rocks and sediments (Tebo et al., 2005; USEPA, 2003a). Hence, it is commonly found as dissolved Mn^{2+} in groundwater sources where anaerobic or low oxidation conditions exist (Post et al., 2011). Subsequently, soluble Mn^{2+} is oxidized, resulting in a wide variety of Mn(III), Mn(IV), or Mn(III,IV) oxide/hydroxide minerals ($MnO_{x(s)}$) in most geological systems (Post, 1999). These $MnO_{x(s)}$ are involved in several environmental redox and sorption reactions, influencing even the distribution of other elements (Tebo et al., 2005). The redox cycling of Mn is highly influenced by the pH and oxygen (O_2) concentrations of the local aqueous environment (Tebo et al., 2004). The widespread occurrence of manganese-oxidizing and -reducing bacteria in natural environments suggests that they play an important role in the cycling of Mn, acting as a catalyst in the redox reactions (Hansel & Learman, 2016). In addition to water and soils, Mn occurs naturally in air and food at low levels (Kohl & Medlar, 2006), being food the greatest source of Mn exposure for the general population (USEPA, 2004; WHO, 2021). Mn is also an essential trace nutrient for all living organisms (Hansel & Learman, 2016) and acts as a component of several proteins and enzymes involved in different biological processes, such as bone formation, metabolism (macronutrient), antioxidation system (free radical defense) and brain (neurotransmitter synthesis and ammonia clearance) (Erikson & Aschner, 2019; Silva et al., 2013; WHO, 2021). It is estimated that adults will consume between 2 and

6 mg of Mn per day, whereas intake from drinking water (DW) could be substantially lower (one or more orders of magnitude) (WHO, 2021).

2.3 Manganese health effects

Overexposure to Mn can lead to neurotoxicity effects (Erikson & Aschner, 2019; Leonhard et al., 2019). In adults, health concerns have been mainly associated with occupational inhalation (*e.g*. mining, welding, battery manufacturing, and steel production) (ATSDR, 2012; Santamaria et al., 2007). Besides, it has been hypothesized that there should be no adverse health effects at low Mn exposures given that Mn is an essential nutrient at trace levels (Santamaria, 2008). Nevertheless, recent epidemiological studies have also reported neurological effects in adults and children exposed to lower and high levels of Mn in DW distribution systems (WHO, 2021). The central nervous system (CNS) is the primary target of Mn toxicity (WHO, 2021), being the brain particularly susceptible to excess Mn (Bjørklund et al., 2017; Santamaria, 2008). The effect of Mn in the brain is influenced by the route of exposure (ingestion or inhalation) and the magnitude of accumulation (Erikson & Aschner, 2019). When oral exposure occurs, homeostatic mechanisms in the human body control levels of Mn retained (through adsorption from the gastrointestinal tract) and excreted (through fecal elimination) (WHO, 2021). However, infants and children are more susceptible to neurotoxic effects than adults because both their central nervous system and their Mn homeostasis are not fully developed (Ljung & Vahter, 2007). In drinking water systems (DWS), several child neurodevelopment adverse effects have been associated with mean Mn concentrations as low as 0.02 mg/L up to approximately 1.4 mg/L (Liu et al., 2020).

2.4 Manganese in drinking water systems

The presence of Mn in DWS also causes aesthetic and operational problems. Mn^{2+} is undetectable for customers by taste or sight, even at very higher concentrations (>75 mg/L) (Sain et al., 2014). This threshold value is well above concentrations at which Mn commonly occurs in DWS. For example, the 95th percentile total Mn concentration in groundwater sources typically ranges from ~0.40 mg/L up to 1.20 mg/L (Breda, 2019; Kohl & Medlar, 2006), although maximum Mn concentrations as high as 4.5 mg/L and 5.6 mg/L have also been reported (Kohl & Medlar, 2006; USEPA, 2003b). Nevertheless, Mn usually does not remain as soluble Mn^{2+} due to the prevalent oxidizing conditions in water distribution systems (Kohl & Medlar, 2006). When Mn^{2+} enters the DWS, it is oxidized and precipitated into the pipe walls, storage tanks, and premise plumbing through chemical (*e.g*. by chlorine residual) or biological means (Hill & Lemieux, 2022; Postawa & Hayes, 2013). In fact, in DWS with Mn concentrations as low as 0.02 mg/L, Mn deposition can take place after years of operation (Hill & Lemieux, 2022). It also occurs in all types of pipes commonly used in distribution systems, such as polyvinyl chloride (PVC) and iron materials (Cerrato et al., 2006; Postawa & Hayes, 2013).

Mn deposition can be also incorporated into other physicochemical processes that take place through DWS such as corrosion (Cerrato et al., 2006). This phenomenon provokes operational problems associated with scaling, sediments, water pressure reduction, elevated energy requirements, and increments in operational costs associated mainly with components replacement (e.g. pipes, pumps, valves, meters), extensive cleanups, and treatment (Brandhuber, 2013). Mn release to bulk water can also take place in the distribution systems due to the shear forces produced by water velocities, flow regime fluctuations (Postawa & Hayes, 2013; Sly et al., 1990) and even, by the presence of Mn- reducing microorganisms in the chlorinated water (Cerrato et al., 2010). Oxidation of Mn^{2+} also produces colloidal small black clumps which do not settle (Kohl & Medlar, 2006). Contrary to soluble Mn^{2+}, $MnO_{x(s)}$ can be visually detected at low concentrations as 0.005 mg/L (Sain et al., 2014). This variety of factors may

cause discolored water (usually black in appearance), turbidity, and undesirable black color in clothes, fixtures, and laundry (Kohl & Medlar, 2006). Several water utilities have reported Mn-related customer complaints above 0.02 mg/L (Kohl & Medlar, 2006).

2.5 Regulatory background

Originally, the World Health Organization (WHO) set an Mn health-based value of 0.50 mg/L and an acceptability threshold value of 0.10 mg/L in DW (WHO, 1958; WHO, 1984). In the third edition of the guidelines for DW quality (published in 2004), the health-based value was lowered to 0.40 mg/L (WHO, 2004). At the same time, the US Environmental Protection Agency (USEPA) set a non-regulatory health advisory value of 0.3 mg/L (USEPA, 2004). These guideline values were calculated for adults. As shown in Table 2.1, several countries in America have adopted mainly the threshold values set by WHO, with exception of Argentina, the United States, and Canada which established more rigorous aesthetic limits (below 0.05 mg/L). Recently, WHO set a provisional health-based guideline value for total Mn of 0.08 mg/L, intended to protect both bottle-fed infants and adults (WHO, 2022). Besides, WHO alerts that Mn concentrations above 0.02 mg/l in DW may cause customer complaints about discolored water and staining (WHO, 2022). Historically, Mn has been removed primarily from groundwater due to the acceptability problems because the health-based value of 0.4 mg/L was well about concentrations at which Mn causes problems in DW. However, the current reassessment by WHO also considers adverse health effects in the most susceptible subpopulation exposed to lower Mn concentrations in DW (WHO, 2021).

Table 2.1. Mn threshold values stipulated in local regulations for DW

Country	Maximum concentration (mg/L)			Reference
	AL	HV	NS	
Canada	0.02	0.12	-	(Health Canada, 2019)
United States	0.05	0.30	-	(USEPA, 2004)
Mexico	-	-	0.15	(NOM-127-SSA1-2021, 2021)
Guatemala	0.10	0.40	-	(NTG-29001, 2013)
Honduras	0.10	0.50	-	(Acuerdo No.084, 1995)
El Salvador	0.10	0.50	-	(NSO-13.07.01:08, 2009)
Nicaragua	-	-	0.50	(NTON-05.007-98, 2000)
Costa Rica	0.10	0.50	-	(Decreto Ejecutivo No.41499-S, 2019)
Panama	-	-	0.10	(Resolución No.122, 2021)
Colombia	-	-	0.10	(Resolución No.2115, 2007)
Ecuador	-	-	0.40	(NTE-INEN 1.108:2011, 2011)
Peru	-	-	0.40	(Decreto No.031-2010-SA, 2010)
Bolivia	0.10	-	-	(NB-512, 2016)
Uruguay	0.10	0.40	-	(UNIT 833:2008, 2010)
Argentina	-	-	0.05	(Ley No.26.221, 2007)
Brazil	0.10	-	0.40	(Portaria GM/MS-No.888, 2021)
Chile			0.10	(NCh409/1.Of2005, 2006)

AL: aesthetic limits, HV: health-based value, NS: not specified.

2.6 Manganese removal by traditional or physicochemical methods

Mn control strategies in DWS include both water management (e.g source water blending) and treatment (Tobiason et al., 2016). Typically, Mn removal from groundwater involves oxidizing Mn^{2+} to particulate $MnO_{x(s)}$ to be subsequently removed by filtration (Postawa & Hayes, 2013). However, unlike iron (Fe), abiotic homogenous Mn^{2+} oxidation by oxygen is very slow at a pH below 9 (Stumm & Morgan, 1996). Hence, at commonly pH values occurring in groundwaters (pH 6-8), Mn^{2+} cannot be removed by simple aeration-precipitation (Katsoyiannis & Zouboulis, 2004). Therefore, strategies such as pH adjustment combined with the use of chlorine or strong oxidants (e.g, chlorine dioxide, ozone, or potassium permanganate) are commonly used to

oxidize soluble Mn^{2+} (Zhang et al., 2021). More important than the homogenous reaction, abiotic Mn^{2+} oxidation is also accompanied by a heterogeneous autocatalytic reaction in presence of the formed $MnO_{x(s)}$ in DW treatment units (Katsoyiannis & Zouboulis, 2004; Stumm & Morgan, 1996). This autocatalytic reaction might be visualized as follows (*Eq.1.1*):

$$-\frac{d[Mn^{2+}]}{dt} = k_1[Mn^{2+}] + k_2[Mn^{2+}][MnO_{x(s)}] \quad (1.1)$$

Where: k_1, k_2 are the homogenous and heterogeneous rate constants, respectively. The term $[Mn^{2+}]$ represents the soluble Mn^{2+} concentration (mol/L), and $[MnO_{x(s)}]$ refers to the Mn oxide concentration (mol/L). When the Mn^{2+} is oxidized from bulk water, the formed $MnO_{x(s)}$ is deposited gradually at the surface of the virgin filter media (*e.g*. sand and anthracite). Subsequently, the $MnO_{x(s)}$ adsorb more soluble Mn^{2+} (Tobiason et al., 2008). Besides, the $MnO_{x(s)}$ surface acts as a catalyst for the oxidation of the adsorbed Mn^{2+}, even with oxidants like oxygen and chlorine, resulting in new sorption sites and coating $MnO_{x(s)}$ surface (Tobiason et al., 2008; Vries et al., 2017). Consequently, the $MnO_{x(s)}$ coating gradually changes the physical properties of the media surface, presenting typically a dark black color. Importantly, this phenomenon does not highly affect other media characteristics (*e.g*. size, shape, density) or filter parameters such as media depth and porosity (Hargette & Knocke, 2001). Despite that the $MnO_{x(s)}$ can remove the Mn^{2+} in absence of an oxidant, the filter media should be regenerated using an oxidant when the adsorption capacity is exhausted (Tobiason et al., 2016). Thus, a common treatment approach for Mn removal combines prechlorination with manganese oxide-coated filter media (Cooley & Knocke, 2016). Intermittent regeneration cycles with permanganate in the backwash are also used to regenerate the filter media (Tobiason et al., 2016). To obtain more rapidly $MnO_{x(s)}$ coated filter media, water utilities use natural occurring Mn minerals such as *Pyrolusite,* or different types of commercial manganese dioxide-coated media that are available commercially for Mn removal (Civardi & Tompeck, 2015; Tobiason et al., 2016).

Regardless that physicochemical treatments can be very effective for Mn removal, they have some disadvantages, especially in developing countries such as Costa Rica, where are resource-limited water supply systems operating. In this context, the technical and financial feasibility of physicochemical treatments for Mn removal needs to be carefully examined. An appropriate selection of a treatment system should also consider the sustainability, complexity of the process, operation flexibility, maintenance requirements, location of service technicians, among others (Civardi & Tompeck, 2015). For instance, the use of chemical reagents and commercial adsorbents increases operation and maintenance costs. Continuous or intermittent regeneration with chemical oxidants may also increase the complexity of the process. Besides, the use of strong oxidants should be minimized in WTP because of the risks associated with the usage, handling, and storage of these chemicals (Bruins et al., 2015). Particularly, the use of chemical reagents such as chlorine before filtration can lead to the potential formation of harmful oxidation by-products due to the reaction with organic precursors present in the source water (Tak & Vellanki, 2018).

2.7 Manganese biofiltration and start-up

Another alternative to remove Mn^{2+} from groundwater is biofiltration consisting of aeration followed by rapid filtration. During the start-up, biofilters can be continuously inoculated receiving aerated raw water containing autochthonous manganese-oxidizing bacteria (MOB). This “natural” process is referred in the literature as “inherent inoculation” (Ramsay et al., 2018). The start-up period in a biofilter is defined as the time required to meet compliance with a DW criterion or when effective Mn removal (>90%) is reached. During this time, the surface of the virgin filter media provides support for the attachment of MOB, facilitating the biological Mn^{2+} oxidation. Subsequently, bacteria develop a complex biofilm structure, composed mainly of microbial cells and extracellular polymeric substances (EPS), providing an optimal environment for bacteria growth (Donlan, 2002). While abiotic homogenous Mn^{2+} oxidation by oxygen is negligible at pH values occurring in groundwaters (pH 6-8), biological Mn^{2+} oxidation usually requires pH above 7.5, dissolved oxygen (DO) greater

than 5 mg/L and a redox potential (Eh) of about 300 and 400 mV (Mouchet, 1992). Similar to abiotic Mn^{2+} oxidation, the biogenic $MnO_{x(s)}$ gradually coated the filter media promoting the heterogeneous autocatalytic reaction described by *Eq.1.1*. Consequently, Mn removal in ripened biofilters involves both biological and physicochemical processes (Breda et al., 2019b). Importantly, biofiltration does not require any chemical oxidant and the amorphous formed precipitates are more compact than the formed precipitates during abiotic oxidation, resulting in a higher filter retention capacity and consequently, in long filter runs (Civardi & Tompeck, 2015). Since the early 1990s, the advantages and limitations of using biofiltration instead of the physicochemical treatment process for Fe and Mn removal have been discussed and investigated. At this time, fundamental theoretical framework, operational experience, and strategies for the conversion of existing WTPs from physicochemical to biological processes, relevant to the application of this technology, were provided in fundamental review articles (Bouwer & Crowe, 1988; Mouchet, 1992). Compared to physicochemical systems, biofiltration no requires the use of chemical reagents and is easy to operate, representing lower operational and maintenance costs. Thus, biofiltration based on aeration and rapid filtration is considered a cost-effective and environmentally friendly treatment technology (Bruins et al., 2015a). However, some limitations should be considered, especially when combined with simultaneous biological removal with other contaminants (*e.g.* Fe and ammonium) (Tekerlekopoulou et al., 2013). Besides, as was previously mentioned, the main drawback of Mn-biofiltration is the long ripening time required by the virgin media to reach effective Mn removal (Bruins et al., 2017b).

2.7.1 Mechanisms involved in the biological oxidation of Mn^{2+}

Some studies have addressed the question of whether MOB can use Mn^{2+} as an energy source (Brouwers et al., 2000). However, due to the difficulty of obtaining no unequivocal scientific evidence about MOB species capable of autotrophic growth on Mn^{2+}, it is presumed that MOB oxidize Mn^{2+} mainly as a protective mechanism rather than to derive energy (Brouwers et al., 2000; Tebo et al., 2005). For example, in aerobic

environments, it is suggested that MOB bioaccumulates the Mn^{2+} as a defense mechanism against harmful reactive oxygen species (ROS) such as superoxide and peroxide (Brouwers et al., 2000). The Mn^{2+} ion is likely transported to the inner membrane (where ROS are produced as by-products of respiration) (Tebo et al., 2005). The intracellular Mn^{2+} acts as an antioxidant (in analogy with catalase, peroxidase and superoxide dismutase) during enzymatic detoxification (Horsburgh et al., 2002). It Is also known that MOB can catalyze the oxidation of Mn(II) to Mn(III/IV) directly through enzymatic pathways which involve the mediation of proteins or protein-polysaccharides complex (Brouwers et al., 1999). For example, studies with phylogenetically distinct model-MOB have found similarities with the genes responsible for encoding Multicopper oxidase (MCO) enzymes, apparently involved in the Mn^{2+} bio-oxidation (Brouwers et al., 2000; Zhao et al., 2015). MOCs are a family of enzymes that use copper ions as cofactors to oxidize various substrates with the concomitant reduction of oxygen to water (Brouwers et al., 2000). Specifically, electrons from the substrate are transferred to molecular oxygen via the copper atoms present in the enzyme (Barboza et al., 2016). Importantly, not always the biological oxidation of Mn^{2+} is mediated by these enzymes. Some specific MOB (e.g. *Stenotrophomonas* and *Lysinibacillus* genera) can remove the Mn^{2+} by an indirect-nonenzymatic pathway (Barboza et al., 2015). In these cases, the pH and redox conditions are modified because of the metabolism and/or bacteria growth (Barboza et al., 2016; Nealson et al., 1988). In all cases, the oxidized Mn is deposited gradually as an Mn-oxide ($MnO_{x(s)}$) coating bacteria cells (Mouchet, 1992). Subsequently, the $MnO_{x(s)}$ can act as an electron acceptor, oxidizing organic carbon compounds that can be used as substrates for microbial growth and protecting the MOB against harmful environmental conditions such as UV radiation, predation, viral attack, or heavy metal toxicity (Tebo et al., 2005).

2.7.2 Factors affecting the bioremediation efficiency of the microorganisms

The term “bioremediation” is referred here as the use of biological systems for removing both organic and inorganic contaminants (Barboza et al., 2016). According to (Jacob et al., 2018), the main factors affecting heavy metal bioremediation (including Mn removal) are:

- Cell aggregation due to a higher biomass concentration. The reduction of the intracellular distance decreases the heavy metal capacity uptake.
- Lower temperatures would affect mobility, metabolic activity, and growth rates. In general, optimal temperatures are between 20 and 40° (mesophilic conditions). Further increments may reduce metabolic activity (reducing protein synthesis). Extreme temperatures (low or high) kill the microorganisms.
- Alteration of the optimal pH can affect microbial growth, enzymatic activity, and metal adsorption capacities. Besides, the speciation and mobility of the heavy metal are determined by the redox potential and pH of the environment.
- Elevated levels of the metal ion concentration may be toxic for the microorganism, affecting the bacteria's growth and enzymatic activity. Besides, it may cause the inhibition of metabolic functions and damage to the genetic material.
- A lack of bioavailable nutrients such as carbon, nitrogen, and phosphorus affects microbial growth, metabolism, and synthesis of necessary enzymes.

Particularly, MOB require, a pH > 7.5, dissolved oxygen (DO) > 5 mg L−1, and a redox potential (Eh) of about 300 and 400 mV to oxidize the Mn^{2+} in the biofilters (Mouchet, 1992).

2.8 Detection and characterization of MOB in groundwater sources and drinking water biofilters

The first application of an aeration-filtration unit was on Fe removal and was reported in Halle (Germany) in 1868, however, this process was firstly implemented in a full-scale WTP until 1874 in Charlottenburg (Germany) (Baker, 1948; Weston & Johnson, 1909). The earliest WTP for Mn removal was built in Zutphen (Netherlands) in 1889 and consisted of aeration followed by double filtration (Baker, 1948). Particularly, the aeration step was included to oxidize soluble Mn^{2+} to be subsequently removed by filtration. Initially, the treatment methods for Mn removal were similar to Fe, however, it was rapidly noticed that oxygen alone will not cause the precipitation of soluble Mn^{2+} in a practical time (Weston & Johnson, 1909). One of the first experiences of the application of Mn-biofilters in a Water Treatment Plant (WTP) was documented in Dresden, Germany in 1913 (Zapffe, 1933). However, the process did not include an aeration step, and the results were not completely satisfactory. Subsequently, it was noticed that these bacteria require oxygen (Armstrong, 1924). Around 1927, the relevance for Mn removal of pre-aeration and keeping the MOB present in the filter units (e.g. avoiding backwashing with chlorinated water) was also reported. At the same time, the detrimental effect of the Fe-loading in the Mn removal process was also warned. However, not until the 1980s, when the implementation of biological Mn-Fe removal from groundwater using aerated-filtration systems, and consequently, investigations on the detection and characterization of MOB received increasing attention (Bouwer & Crowe, 1988; Mouchet, 1992; Peitchev & Semov, 1988; Seppanen, 1992; Viswanathan & Boettcher, 1991; Vuorinen et al., 1988).

Leptothrix ochracea, a well-known MOB (Katsoyiannis & Zouboulis, 2004), was discovered in 1843 by Friedrich Kützing (Zapffe, 1933), being one of the first MOB detected. After 1870, several studies on the physiology, morphology, and metabolism of various functional Fe-Mn oxidizing bacteria were conducted. In 1892, Hans Molisch detected specks of Mn around bacterial cells (Zapffe, 1933). In 1913, it was known that

bacteria can catalyze the oxidation of Mn^{2+} to insoluble $MnO_{x(s)}$ (Beijerinck, 1913; Nealson & Tebo, 1980). Another well-known MOB in DWS, *Crenothrix polyspora* (Cai et al., 2015) (also referred as *Crenothrix manganifera*) was discovery in 1870 by Ferdinand Cohn but was firstly associated with problems caused by Fe-precipitates in wells and pipes of groundwater-supply systems (Jackson, 1902). Afterward, *Crenothrix polyspora was* well-recognized as a MOB that played an important role in Mn removal (Weston, 1931). Interestingly, Jackson (1902) provided some of the first microphotographs (Figure 2-2) of isolated sheathed bacteria linked with Mn deposition in DWS (Jackson, 1902). According to the author, the Mn precipitates were removed from the culture media by acid, and bacteria were stained with a Loeffler solution to capture a clear image.

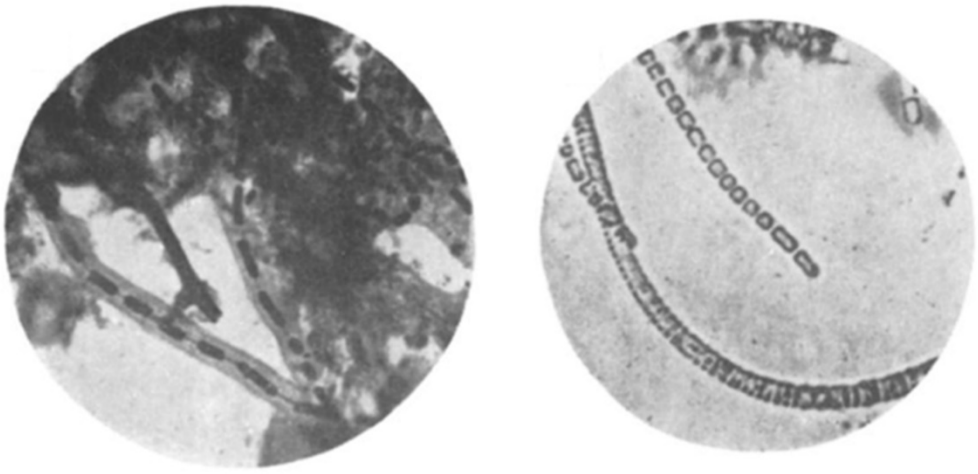


Figure 2-2. Microphotographs of sheathed bacteria, in appearance *Crenothrix*
Adapted from (Jackson, 1902)

Up to now, several methods have been used for the isolation and characterization of MOB in drinking water biofilters. The following sections describe some of these methods, that can be potentially used for preliminary studies, implementation, and monitoring of biofilters for Mn removal.

2.8.1 Surface attachment collection method (flow cell)

The term "*flow cell*" is referred here as a surface attachment collection method for microscopic examination. Providing an abiotic surface for bacteria attachment (e.g. glass or steel coupons), diverse flow cell systems have been widely used in the laboratory for in-vitro cultivation and evaluation of bacterial biofilms under hydrodynamic conditions of flow (Crusz et al., 2012; Wolfaardt et al., 1994). Particularly, the Standard Method 9240 (APHA et al., 2017) provides a practical flow cell procedure for collecting Fe-bacteria in-situ using a flow cell device consisting of an enclosed chamber made of plastic, containing a sterilized glass microscope slide holder with removable screw caps (Figure 2-3). A valve controls the water flow (under the limit of 1 L/min approximately). Slides from flow cell samples can be examined by microscope following the Standard Method D932−20 (ASTM, 2020). This method covers the determination of Fe-bacteria such as *Siderocapsa*, *Gallionella*, *Sphaerotilus*, *Crenothrix*, *Leptothrix*, and *Clonothrix*. It should be notice that, except for *Gallionella,* MOB populations include the major groups of species reported for Fe-bacteria (Mouchet, 1992). This method has been effectively used in previous studies on Mn-Fe biofiltration (Pacini et al., 2005; Pacini et al., 2014). Importantly, the flow cell can be easily connected by tubing to a sampling point (e.g., in wells, influent/effluent of biofilters) or can be simply submerged (*e.g.*, in the supernatant water, raw water reservoirs). Figure 2-4 provides an example of microphotographs of bacteria[1] that were examined by the method D932−20. The flow cell samples were obtained from a full-scale biofilter for Mn and Fe removal. Rod-shaped bacteria shown in Figure 2-4(a) are characteristic of the well-known MOB *Pseudomonas putida* (Wu et al., 2011) and *Bacillus sp.* (Jiang et al., 2010), or some other species such as *Aeromonas hydrophila* (Zhang et al., 2019). As is shown in Figure 2-4 (b and c), filamentous bacteria (e.g.*Leptothrix* and *Crenothrix* species) are easy to identify under the microscope because of their characteristic sheathed structure. The same occurs with the stalked iron bacteria (in appearance Gallionella) shown in Figure 2-4(d). The flow cell method

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provides useful information about the probable occurrence of Mn-Fe oxidizing bacteria in groundwater systems. However, the microphotograph alone does not provide conclusive evidence about some functional MOB. Thus, further examination is required. In that sense, the isolation of MOB strains on culture plates can be employed to confirm the information provided by microscopy (APHA et al., 2017).

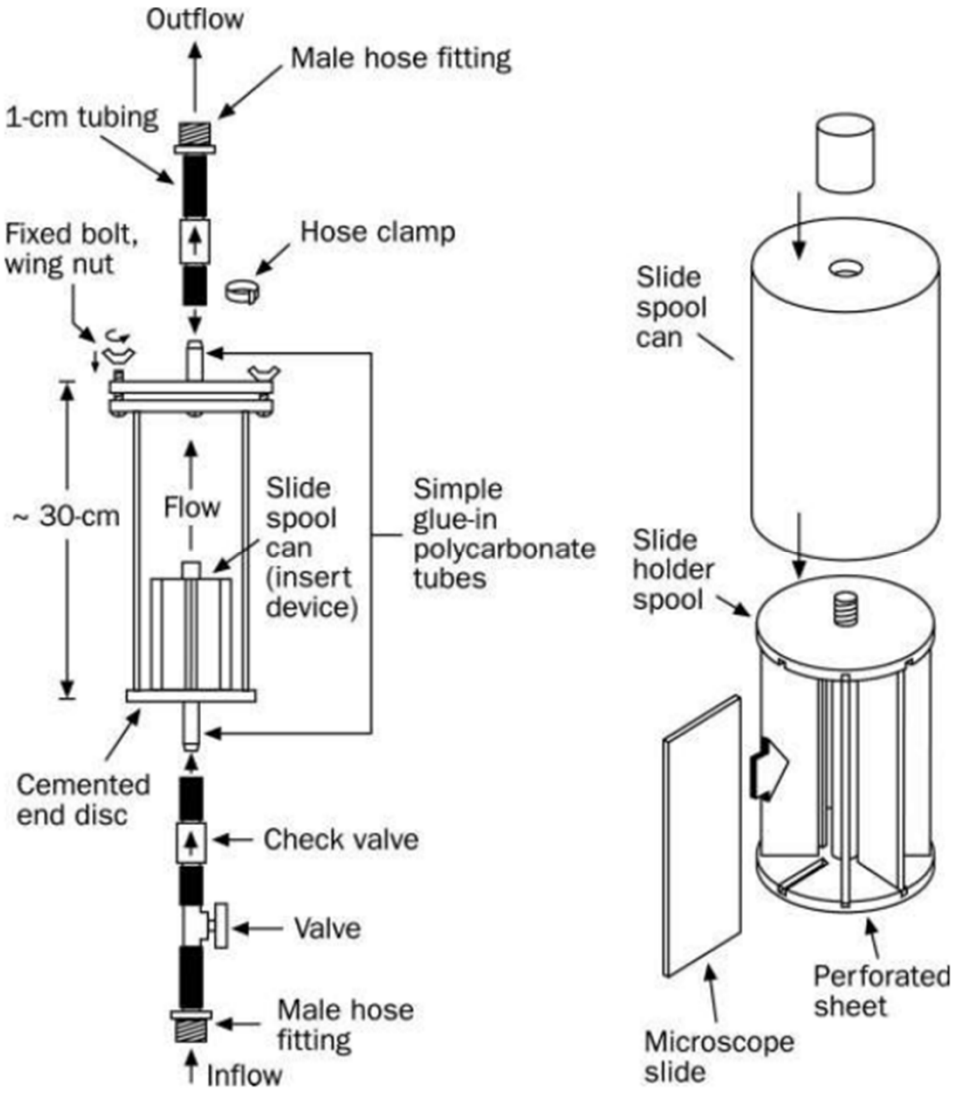


Figure 2-3 Diagram of the flow cell device to collect bacteria for microscopic examination according to the Standard Method 9240.
Adapted from (APHA et al., 2017).

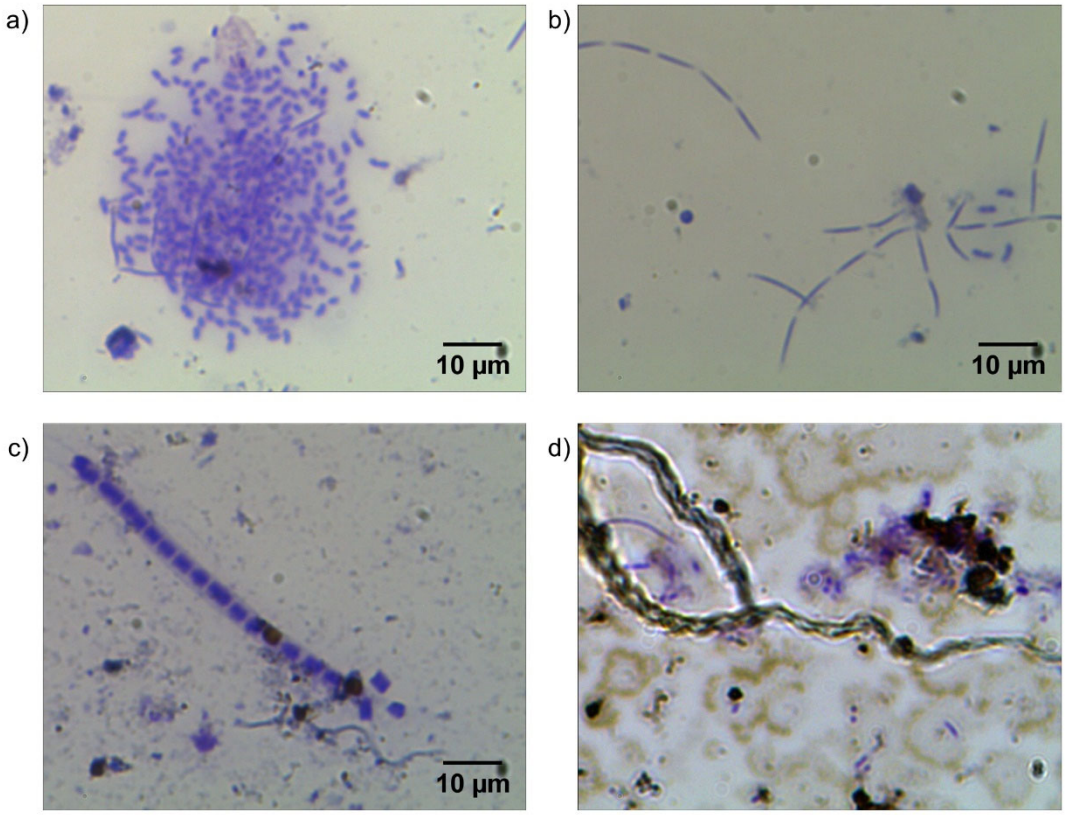


Figure 2-4. Microphotographs of a) rod-shaped (b-c) sheathed, and d) stalked bacteria (in appearance Gallionella) from flow cell samples.

2.8.2 Enriched culture media for MOB detection

The first MOB characterization studies used commonly culture media consisting of a mixture of agar and gelatine containing ferrous and manganous sulfates, and some other ingredients such as beef extract medium, peptone, and a low concentration of proteins (Jackson, 1902; Pringsheim, 1949). Up to now, several (liquid or solid) culture media have been effectively used for MOB cultivation from samples collected of Mn-biofilters in DWS. Some examples are presented in Appendix 8.1. MOB media commonly contain a source of Mn, carbon, nitrogen, vitamins, amino acids, and mineral salts required for bacteria growth, and a buffer near neutrality to avoid indirect Mn(II) oxidation due to pH changes (Nealson, 2006). The agar acts as the solidifying agent

(when required). Despite that culturing has allowed the discovery of essential information on biological Mn removal, there are some limitations. Recent studies have demonstrated that some MOB strains can grow and/or oxidize Mn(II) only in a selective culture media (Piazza et al., 2019). Consequently, the diversity of microbial communities may be underestimated (Jo et al., 2016).

2.8.3 LeucoBerbelin blue assay

For many years, it has been known that direct quantitative estimation of MOB on the Mn-agar medium can be performed by detecting black or brown colonies caused by the $MnO_{x(s)}$ deposited around or near the cells (Pringsheim, 1949). Up to now, it constitutes commonly the rule for the determination of viable cell numbers of MOB by plate counts (Granger et al., 2014). However, similar to the flow cell results, this characteristic does not essentially provide conclusive evidence about the presence of MOB (*e.g.* other bacteria may produce dark pigments) (Nealson, 2006). In that sense, an important complementary method was proposed by Krumbein & Altmann (1973) using the non-toxic reagent leukoberbelin blue (LBB) as a redox indicator to confirm qualitatively the presence of $MnOx_{(s)}$ in a culture medium. Specifically, the LBB reagent is oxidized upon interaction with Mn(III/IV) oxides, changing from colorless to a blue form (Nealson, 2006). Recently, Piazza et al. (2019), provided an illustrative explanation using a comparison of representative plate counts (see Figure 2-5).

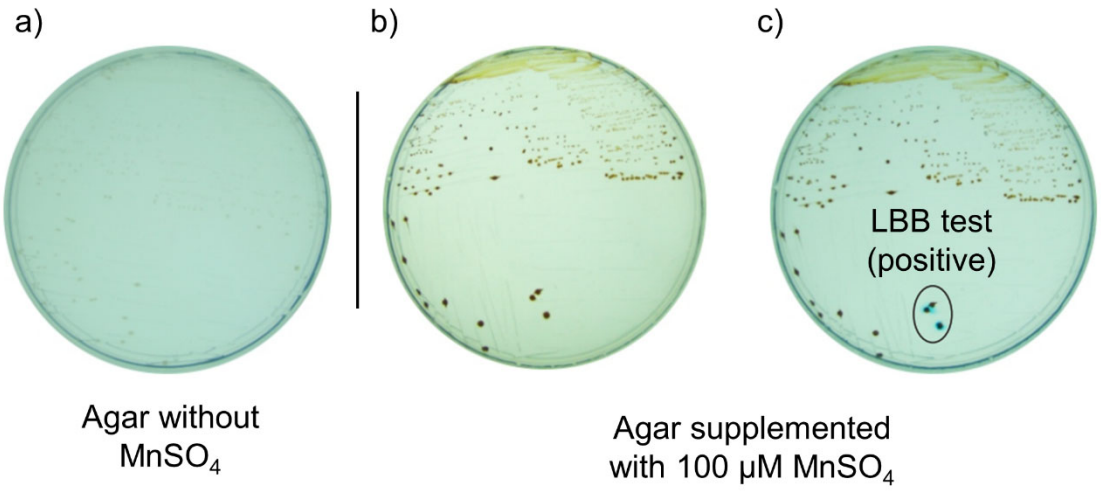


Figure 2-5. Representative plates using (a) agar without $MnSO_4$, and (b) agar supplemented with $MnSO_4$, showing (c) positive MOB strains to LBB test.
Adapted from (Piazza et al., 2019)

According to Piazza et al. (2019), the left plate in Figure 2-5 (a) corresponds to a solid agar without a source of Mn as an ingredient, hence, the colonies remained whitish. Whereas Figure 2-5 (b and c) corresponds to the same solid agar supplemented with $MnSO_4$, thus, MOB were easily identified by the detection of the dark color of the colonies. Finally, Figure 2-5 (c) showed how LBB staining resulted in positive, where the blue color confirmed the presence of the $MnO_{x(s)}$. Similar to the LBB, the benzidinium reagent has also been employed as a redox indicator (Nealson & Tebo, 1980). However, compared with LBB, this reagent is associated with disadvantages such as the benzidinium-reaction with the $MnO_{x(s)}$ can be interfered by iron oxides (causing false positives), and the colonies must be replicated because the reagent is toxic and bactericidal (Nealson, 2006).

2.8.4 Mn(II) oxidation capacity determination

The relative Mn(II) oxidation capacity of the isolated MOB can be determined either by qualitative or quantitative methods. The latter case involves colorimetric assays (adding LBB reagent) or analysis by atomic absorption spectroscopy. They can be summarized as follows:

- ***Qualitative method:*** two drops of the LBB reagent are added to the colonies in solid media. The relative oxidation capacity of Mn(II) is reported according to the level of intensities of the blue coloration in the positive samples as follows: (+): weak; (++): light; (+++): intense; (++++): very intense.

- ***Colorimetric method*****:** bacteria Mn-suspension is firstly mixed with the LBB reagent to quantify the $MnO_{x(s)}$ produced. Subsequently, the samples are stored in the dark and centrifugated to remove cells. Finally, the supernatant absorbance is determined at 620 nm using standard curves with $KMnO_4$ (~1 mM MnO_2 corresponds to 0.4 mM $KMnO_4$) (Piazza et al., 2019; Zhang et al., 2019).

- ***Via atomic absorption spectroscopy*****:** Mn oxidation can also be quantified by measuring the concentration of residual Mn(II) from the growth medium (Nealson, 2006). Specifically, isolated colonies are inoculated in a liquid medium with an initial manganese concentration determined. Afterward, aliquots of the culture collected at different times are centrifuged and stored or filtered to separate suspended $MnO_{x(s)}$ species. Finally, the Mn(II) is determined by atomic absorption spectroscopy.

In addition, biotic oxidizing conditions in biofilters differ from laboratory conditions. In natural environments, diverse microorganisms might be responsible for biotic Mn(II) oxidation (Yang et al., 2013).

2.8.5 Biofilm formation capacity

Bacteria attachment to the filter media in biofilters is accompanied by the production of extracellular polymeric substances (EPS) to form a biofilm (Keithley & Kirisits, 2018), an aggregation of microbial cells enclosed with a matrix composed mainly of polysaccharides (Donlan, 2002). Biofilms enhance the immobilization of the MOB into the biofilters and act as a defense mechanism against harmful toxic substances (Li et al., 2016). However, it should be also monitored because may cause bio-clogging of biofilters (Xia et al., 2016). The biofilm formation capacity of the strains can be determined as follows:

- **By crystal violet staining (qualitative test):** the strains are cultured in a liquid medium and incubated statically at room temperature for 7 days. Samples (~10-15 mL) are dispensed into sterile polystyrene Petri dishes and incubated for 24, 48, 72, and 96 h at room temperature. Afterward, the bacterial suspension is gently discarded from the Petri dish with distilled water. Cells adhered to the plate are stained with crystal violet (CV) and incubated for 20 min. The CV is gently discarded with distilled water. Subsequently, cells are extracted with ethanol and incubated again for 5 min. Finally, the extracted samples are centrifugated and the absorbance is determined at 540 nm using ethanol as blank (Beukes & Schmidt, 2012).

- **By quantification of polysaccharides of extracellular polymeric substances:** the inoculum is prepared in liquid culture media with about 3g of filter media (as support media for surface attachment). After incubation, 2g of media are mixed with an extraction buffer (10 mM Tris, 10 mM EDTA, 2.5% NaCl, pH 8) to extract the EPS from the filter media (Keithley & Kirisits, 2018). Subsequently, samples are stirred and centrifugated. Total solids (TS) of the precipitated (including the filter media) are also quantified. Finally, polysaccharides or proteins in the extractant are measured. Particularly, polysaccharides can be measured by the phenol–sulfuric acid method, mixing the samples with phenol and H_2SO4 and reading the absorbance at 490 nm using standard curves with glucose (results are reported as

mg of glucose per g of ST). Proteins can be measured using commercial kits (Keithley & Kirisits, 2018).

- **ATP bioluminescence:** it is an easy and rapid direct method to quantify viable biomass attached or suspended cells (Wilson et al., 2017). ATP analysis is highly used to confirm the growth of biomass in biofilters (Gude et al., 2018; Velten et al., 2011). The term “ATP” is referred to Adenosine triphosphate, a nucleoside triphosphate that acts as the primary energy source in all organisms (Wilson et al., 2017). Inoculated culture media (or grain media) is first exposed to a buffer to release the biofilm. Subsequently, the cells are suspended and lysed (by physical, chemical, or enzymatic processes) to release intracellular ATP from cells. The sample is added to a solution that contains the enzyme luciferase (responsible for light production in fireflies). This complex reacts with the ATP and emits light. The intensity of the emitted light is measured in a luminometer as relative light units (RLU) (Pharand et al., 2014; Wilson et al., 2017). Finally, RLU values are converted to ATP concentrations using determined calculations (included for example in available commercial kits).

2.8.6 PCR-amplified 16S-rRNA gene sequencing

PCR amplification and gene sequencing (Wilson et al., 1990) can be effectively used to characterize microorganisms. DNA from cultured bacterial cells is commonly extracted using commercial DNA isolation kits (Pani et al., 2017). Interestingly, DNA can also be extracted directly from filter media (kit for soil) and water samples collected in biofilters (Breda et al., 2019; Piazza et al., 2019). This practice has been recently used to investigate the diversity, relative taxa abundance (at phylum or genus levels), and distribution of microorganisms in groundwater rapid filters (Gülay et al., 2016).

The gene most used for bacterial identification is 16S-rRNA, a ~1500 bp base pair gene directly involved in bacteria ribosomal function (Petti, 2007). Since the 1970s, it was known that 16S-rRNA gene is highly conserved across phylogenetic lines (Woese

et al., 1975). Hence, the nucleotide base fragments or sequences of the 16S-rRNA gene serve as “signatures” for different bacteria species (Petti, 2007). 16S-rRNA gene can be cloned using a polymerase chain reaction (PCR) (Yanagihara et al., 2011). Specifically, PCR is an enzymatic process that combines temperature cycles, one pair of oligonucleotides (forward “F” and reverse “R” primers) to achieve selective amplification of the target sequence, and a DNA-polymerase enzyme that catalyzes the synthesis of new DNA strands of interest (Kadri, 2019). Due to bacteria may have single or multiple copies of the 16S-rRNA gene (even, with copy variants), PCR replicates tens of billions of copies to improve the sensitivity of the DNA amplification (Petti, 2007). Importantly, PCR amplification for the identification of MOB is commonly carried out as follows:

- With the full-length 16S rRNA gene using the universal bacterial primers ***27F*** (5′-AGAGTTTGATCCTGGCTCAG-3′) and ***1492R*** (5′-GGTTACCTTGTTACGACTT-3′) (Pani et al., 2017; Therdkiattikul et al., 2020; Yang et al., 2013; Zhang et al., 2019),
- With a specific 16S rRNA fragment using the primers ***341F*** (5’-CCTACGGGNGGCWGCAG) and ***805R*** (5’-GACTACHVGGGTATCTAATCC) (Breda et al., 2019; Piazza et al., 2019).

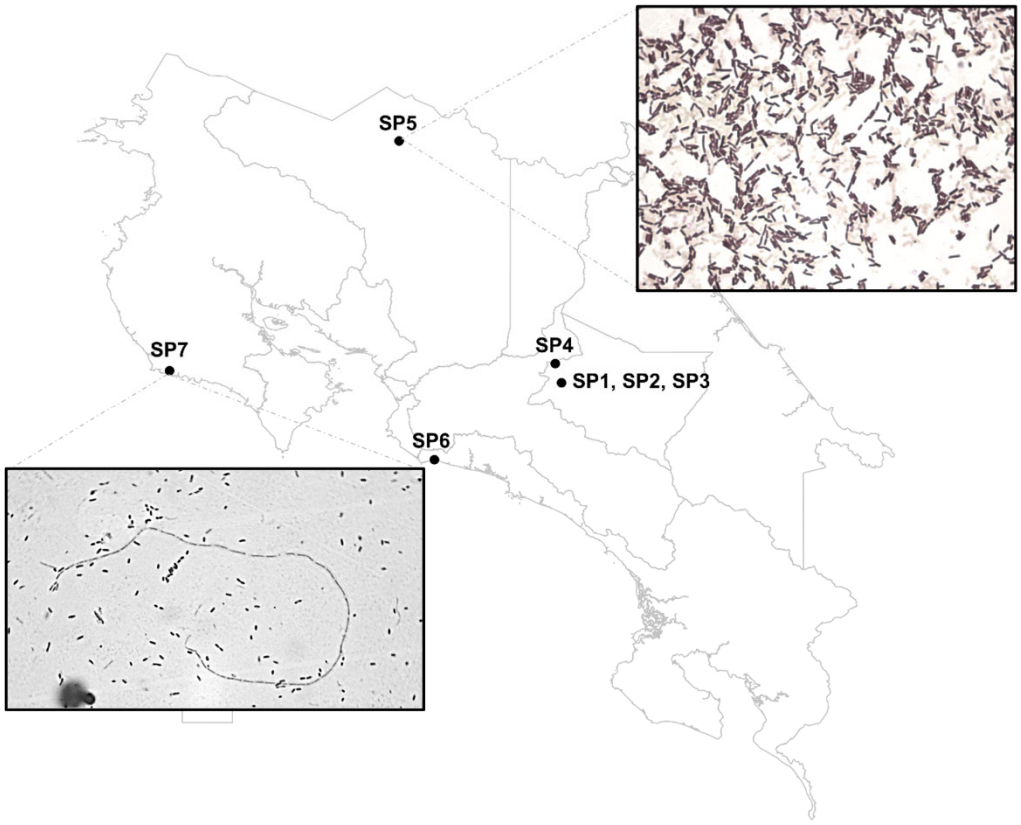
Finally, sequencing is performed with a genetic analyzer followed by a sequence homology analysis comparing the results with biological sequence information obtained from comprehensive reference library databases. The most cited is the public database GenBank (Benson et al., 2013). Reads with more than 97% sequence similarity are commonly accepted for bacteria identified by the 16S-rRNA gene (Petti, 2007) and have commonly quantified as an operational taxonomic unit (OTU) (Cai et al., 2015; Piazza et al., 2019). Compared with traditional culture methods, *PCR-amplified 16S-rRNA gene sequencing* provides much more accurate information about the identity and characterization of MOB. However, this molecular method may be cost prohibitive for resource-limited water supply entities because require complex execution, and advanced equipment and can only be performed in specialized laboratories.

Chapter 3

Isolation and characterization of autochthonous MOB from groundwater wells in Costa Rica

Exploratory and in-vitro studies

Graphical abstract



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Abstract

Little is known about the occurrence of manganese-oxidizing bacteria (MOB) in tropical waters. Therefore, an exploratory study was conducted to investigate the presence of MOB in groundwater wells. For this purpose, raw water samples were collected from 7 groundwater wells. Flow cells were also installed for microscopic examination. Bacteria were grown in Mn-medium and the Mn(II) oxidation activity was verified by the LBB test. In-vitro studies with seven selected autochthonous culturable MOB were also conducted for further characterization (PCR-amplified 16S-rRNA gene sequencing, Mn(II) oxidation, and biofilm formation capacity tests). Microphotographs revealed a great number of rod-shaped bacteria and a low presence of sheathed bacteria and stalked bacteria. LBB test confirmed the presence of functional MOB in all groundwater wells. Furthermore, the potential, at the in vitro level, of pumice stone combined with autochthonous *Gammaproteobacteria (Stenotrophomonas* and *Aeromonas spp.)* for biological Mn removal from groundwater in Costa Rica was revealed.

Keywords: tropical groundwater, Mn-occurrence, manganese-oxidizing bacteria (MOB), in-vitro study.

3.1 Introduction

Manganese-oxidizing bacteria (MOB) are widely distributed in soils, sediments, and aquatic environments. Many phylogenetically diverse manganese-oxidizing bacteria (MOB) have been found capable of oxidizing Mn(II) to Mn(III/IV), including *Firmicutes*, *Actinobacteria*, and the *Alpha(α)*, *Beta(β)* and *Gamma(γ) Proteobacteria* (Tebo et al., 2004). MOB are known to catalyze the Mn oxidation and the subsequent biomineralization by direct and indirect mechanisms.

Autochthonous bacteria play an important role in the performance of DW biofilters (Velten et al., 2011). Some MOB species related to Mn removal in biofilters include the *Leptothrix*, *Crenothrix*, *Hyphomicrobium*, *Siderocapsa*, *Siderocystis*, *Metallogenium, Arthrobacter, and Pseudomonas* genera (Mouchet, 1992; Yang et al., 2014). Other species such as *Sphingomonas, Flavobacterium, and Acinetobacter*, associated mainly with Mn deposits and mining, have been also reported in DW biofilters (Cai et al., 2015). Particularly, three model MOB have been extensively studied in the laboratory: *Leptothrix discophora*, *Pseudomonas putida*, and *Bacillus sp*. (Brouwers et al., 1999; Corstjens et al., 1997; Francis et al., 2002; Hope & Bott, 2004; Jiang et al., 2010; Tebo et al., 2004). *Leptothrix,* which is indigenous from groundwater, has a characteristic structure of filamentous sheath and is commonly found in DW Mn-biofilters (Yang et al., 2014). *Pseudomonas spp*. have been found in ripened filter media and backwash water from Mn-biofilters in a full-scale groundwater treatment plant in Belgium (Bruins et al., 2017). Similarly, abundant *Pseudomonas spp*. were found in an analysis of the bacterial communities present in two full-scale biofilters for Mn removal from groundwater in Argentina (Piazza et al., 2019). *Bacillus spp*. is capable of both oxidizing and reducing Mn and have been found in DWDS (Cerrato et al., 2010).

However, the presence of MOB in DW have been extensively examined, little has been reported about the identification and characterization of autochthonous MOB isolated from tropical groundwater. Some recent in-vitro assays using water samples collected

from an Mn mine in Brazil reported the presence of MOB including *Stenotrophomonas*, *Bacillus*, *Lysinibacillus,* and *Serratia marcescens* genera (Barboza et al., 2015; Barboza et al., 2017). In addition, *Bacillus pumilus* capable of oxidizing Mn^{2+} was isolated from a groundwater source in Mexico (Rivera et al., 2017). This study aimed to investigate the presence of autochthonous MOB in groundwater wells in Costa Rica with elevated concentrations of Mn.

3.2 Materials and methods

Data collected from 120 water-quality analysis reports from 40 groundwater wells, were gathered with the collaboration of the National Water Laboratory (LNA, by their Spanish acronym). The focus of the inventory was on the wells with elevated concentrations of Fe and Mn (period 2012-2017). Data collected also included the groundwater temperature and pH. Data analysis was performed in R (R Core Team, 2021). Summary descriptive statistics and boxplot graphics were done using *stat.desc* (library *pastecs*) and the R graphics package, respectively.

Subsequently, an exploratory study was conducted to investigate the presence of MOB in the groundwater wells. For this purpose, flow cells were installed, and raw water samples were collected from seven groundwater wells located in five different zones in Costa Rica (see Figure 3-1). Figure 3-2 shows the modified flow cell apparatus, used to collect the autochthonous bacteria from wells. Microscope slides were exposed to raw water for about 7 days at approximately 2 L/h. In addition, water samples were filtered using sterile 0.22 µm filters (500 mL per filter). Each filter was placed in Petri dishes with R2A agar modified with 17 mg/L $MnSO_4$ and incubated for 5 days at room temperature (<28 °C). After an incubation period of 20 days, two drops of the LBB reagent (0.04% w/v dissolved in 45 mM acetic acid) were added to the colonies to determine Mn oxidation activity.

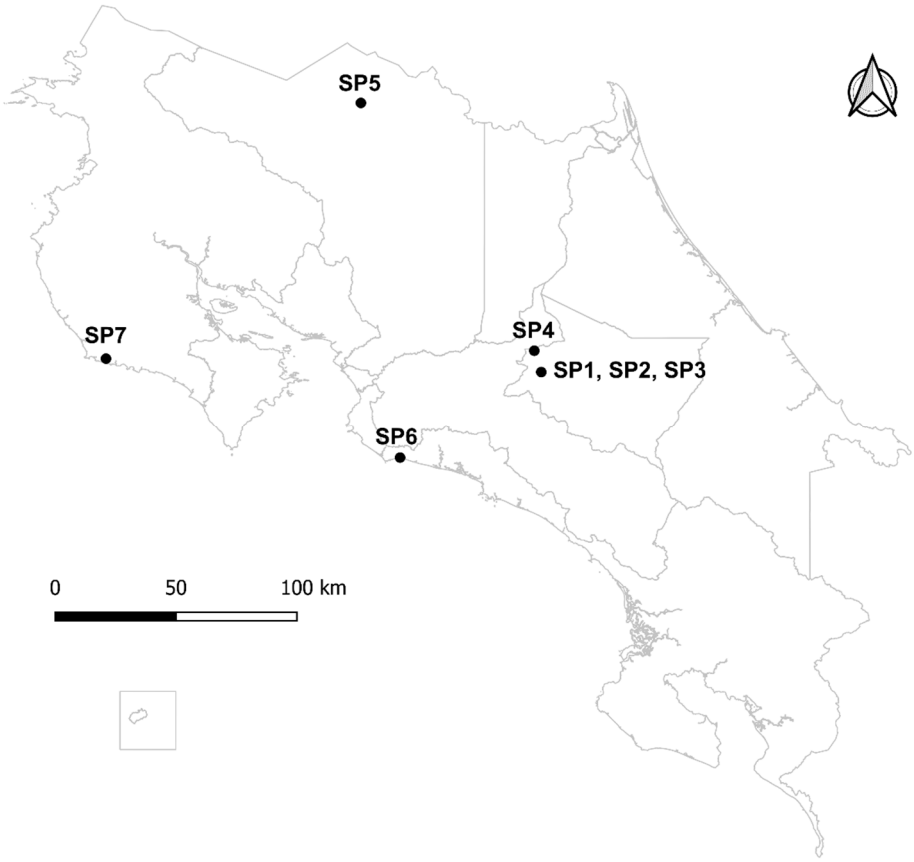


Figure 3-1. Sampling points (SP) installed in groundwater wells to allow water samplings and collection of bacteria by flow cell.

Figure 3-2. Flow cell device to collect bacteria from groundwater systems for microscopic examination.

Finally, an in-vitro study was conducted with seven strains isolated from two of the groundwater wells SP1 and SP2 (see Figure 3-1), which resulted positive for the LBB test. The closest related species were identified by 16S rRNA gene sequencing (see Table 2-3) using the universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The Mn(II) oxidation capacity of the isolated strains was evaluated quantitatively by measuring the residual concentration of Mn(II) in the liquid medium culture broth. Each strain was inoculated in PYM medium (**¡Error! No se encuentra el origen de la referencia.**) with the following modifications: 2 g/L peptone, 0.5 g/L yeast extract, 10 mM HEPES, and 0.1 mM $MnSO_4 \cdot H2O$ (initial concentration of 5.9 mg/L). Aliquots (2 mL) of the inoculated culture media were collected on days 0, 2, 4, 7, 10, and 14. Each aliquot was centrifuged at 4000 rpm for 15 min and filtered through a 0.45 µm cellulose nitrate filter and acidified with 10% nitric acid. The Mn (II) concentration was determined by atomic absorption spectroscopy using AAnalyst800 atomic absorption equipment (Perkin Elmer, Waltham, USA). Finally, the biofilm formation capacity of the strains was determined by crystal violet staining and by quantification of polysaccharides of extracellular polymeric substances (according to section 2.8.5). In the latter case, pumice stone was investigated as support media for MOB attachment. Further details can be found in (Calderón-Tovar et al., 2020).

3.3 Results and discussion

3.3.1 Occurrence and distribution of elevated Mn and Fe concentrations in groundwater wells.

According to the inventory, about 91% of the registered Mn concentrations were greater than the provisional health-based guideline value of 0.08 mg/L, established recently by WHO to protect both bottle-fed infants and adults (WHO, 2022). Besides, around 87% of the Mn concentrations exceeded the aesthetic limit (AL) of 0.10 mg/L stipulated in the local regulation (Decreto Ejecutivo No.41499-S, 2019). The groundwater quality variation in terms of the 95th percentile concentrations of Fe-Mn, the pH, and water temperature is shown in Figure 3-3. The 95th percentile of Mn concentrations was around 0.83 mg/L which is whiting the 95th percentile typically reported in groundwater sources that range from ~0.40 mg/L up to 1.20 mg/L (Breda, 2019; Kohl & Medlar, 2006). The highest Mn concentration registered in the inventory was 2.70 mg/L. This Mn contamination level is in the middle of the maximum Mn concentrations of 4.5 mg/L and 5.6 mg/L that have been previously reported for groundwater sources (Kohl & Medlar, 2006; USEPA, 2003). The maximum Fe concentration was 5.74 mg/L. In addition, the average concentrations of Mn and Fe were similar, 0.44 ± 0.31 mg/L and 0.49 ± 0.84 mg/L, respectively. However, compared with Mn, elevated Fe concentrations exhibited a higher variation. The 75th percentile of Mn concentrations was about 0.55 mg/L, whereas the 75th percentile of Fe was around 0.67 mg/L. On the other hand, the average groundwater temperature was 27.6 ± 2.3 °C with a maximum value of 35.6 °C. In contrast, temperate groundwater temperatures commonly reported in studies on the start-up of Mn-biofilters, vary from 3°C (Cai et al., 2014), 9-13 °C (Breda et al., 2019; Ramsay et al., 2018; Štembal et al., 2004; Zeng et al., 2010; Zeng et al., 2019), up to 17°C approximately (Ciancio et al., 2020). Finally, Figure 3-3 also shows that pH values ranged between the commonly pH levels occurring in groundwaters (6-8). The average pH registered was 7.3 ± 0.4.

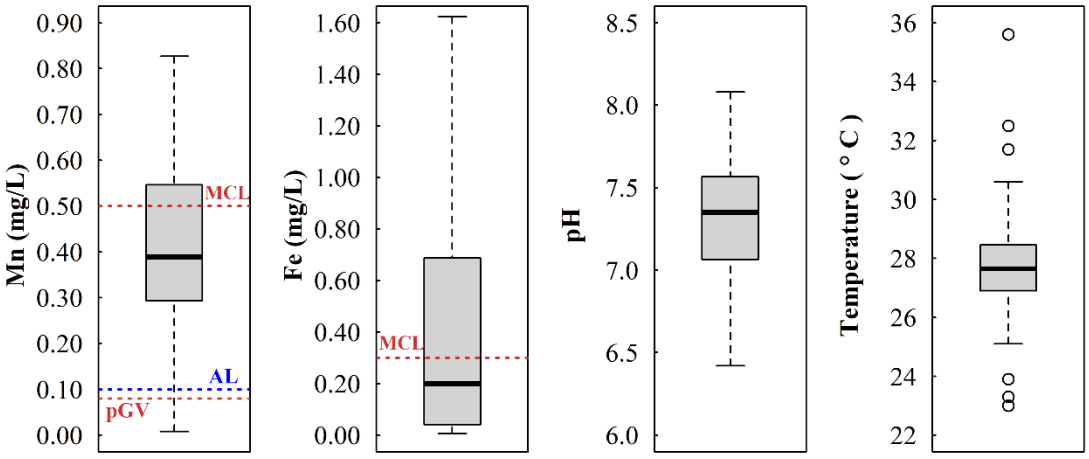


Figure 3-3. Variation of Fe and Mn concentrations (95th percentile), the pH, and water temperature in the selected groundwater wells - Water quality inventory 2012-2017.
pGV: provisional WHO guidelines value; AL: aesthetic limit, MCL: maximum concentration level

Figure 3-4 shows the geographic distribution of the groundwater wells according to the data collected from the inventory. Elevated Mn concentrations (>0.3 mg/L) were found spatially distributed in Costa Rica. In contrast, Fe contamination occurred mainly in the Mideast (Limón province) with Mn. An important finding from this inventory is that the co-occurrence of Mn and Fe happened in about 72.5% of the groundwater wells. The start-up of non-bioaugmented Mn-biofilters is highly influenced by Fe-loading (Bruins et al., 2017). As is shown in Figure 3-4, the main exception was mainly the North-West area (Alajuela and Guanacaste provinces). This evidence suggests that future implementation of biofiltration for Mn removal in Costa Rica may be accompanied by a Fe-pretreatment process.

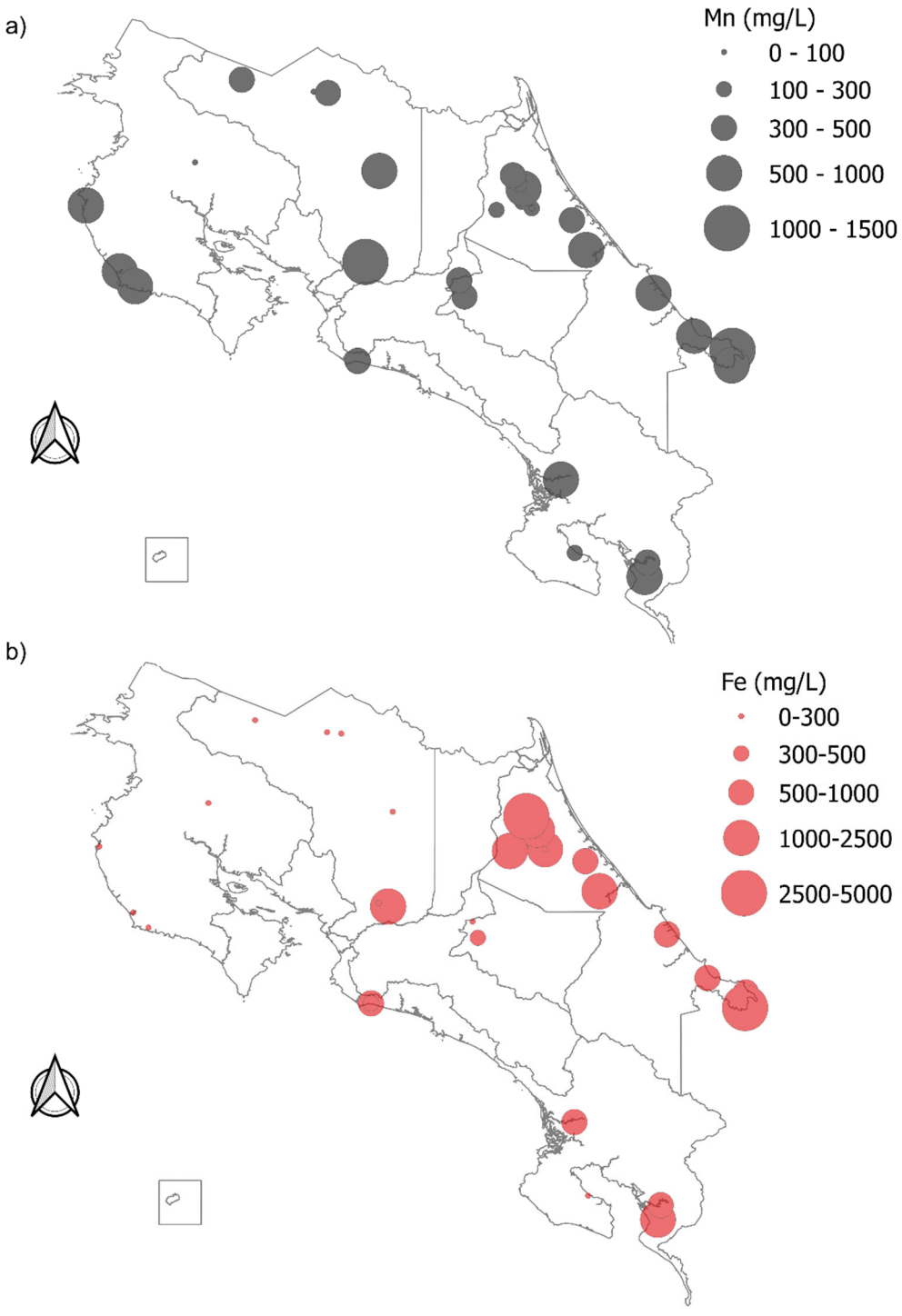


Figure 3-4. Geographic distribution of elevated a) Mn and b) Fe concentrations detected in groundwater-wells for DW production in Costa Rica.

3.3.2 Isolation and characterization of MOB detected in groundwater wells.

Results confirmed the presence of culturable bacteria detected in the water samples that reacted positively to LBB test, confirming the presence of MOB. In addition, microscopic observation[3] of the flow cell samples (with 7 days of exposure to raw water) showed a similar pattern in all sampling points. It was found a great number of rod-shaped bacteria and a low presence of sheathed bacteria and stalked bacteria (in appearance the iron-bacteria *Gallionella*) (see an example in Figure 3-5).

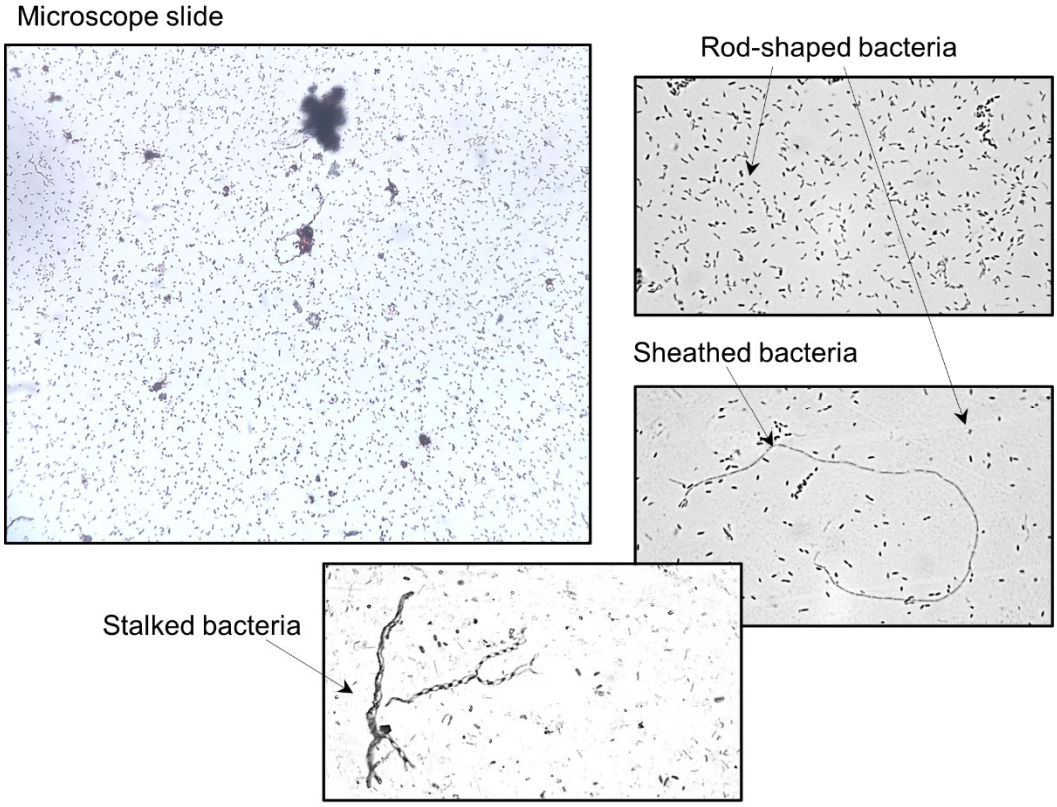


Figure 3-5. Representative microphotographs of bacteria from the flow cell samples collected in the groundwater wells.

[3] By Andrea Quesada, Environmental Protection Research Center (CIPA), School of Chemistry, Instituto Tecnológico de Costa Rica (ITCR)

3.3.3 In-vitro study[4]

In this study, seven strains were selected for further characterization (see Table 3.1).

Table 3.1. Closest related species of MOB isolated from groundwater-wells SP1-SP2.

Sampling point[a]	No. Accession	Closest related species	Similarity (%)	Mn(II) oxidation activity (LBB test)
SP1	***NR 118008***	*Stenotrophomonas pavanii*	98.1	+ +
SP1	***LT906480***	*Stenotrophomonas maltophilia*	99.9	+ +
SP1	***KT998825***	*Aeromonas taiwanensis*	99,0	+
SP1	***LT629770***	*Microbacterium paraoxydans*	96.6	+ + +
SP2	**KC800783**	*Aeromonas hydrophila*	99,0	+ +
SP2	***NR_116584.1***	*Aeromonas sanarellii*	97.3	+
SP2	***MK203000***	*Stenotrophomonas maltophilia*	100%	+

[a] Groundwater characteristics: SP1 (Mn 0.60 mg/L, Fe 0.27 mg/L, T 23.0 °C, pH 7.17), SP2 (Mn 0.22 mg/L, Fe 0.19 mg/L, T 25.4 °C, pH 7.07). ND: not determined.

Genus *Stenotrophomonas* and *Aeromonas* belong to the phylum *Proteobacteria* (*Gammaproteobacteria*)*, whereas Microbacterium* is an Actinobacteria. Importantly, these genera have species that have been reported previously as MOB. *Aeromonas* species were found in a pilot-scale slow sand biofilter for the simultaneous removal of ammonium and manganese from surface lake water (Subari et al., 2018). This genus has been isolated from soil samples (Koo & Park, 2005) and streams impacted by Mn-mining (Zhang et al., 2019). In both cases, *the isolated strains* exhibited a higher Mn(II) oxidation capacity. *Aeromonas spp.* has also shown high efficiency as a nitrification-aerobic denitrification bacteria in wastewater treatments for ammonium removal (Chen et al., 2014). The actinobacterium of the genus *Microbacterium* was isolated from a

[4] Derived from Isis L. Calderón-Tovar-Master's Thesis . Program in Natural Resource Management and Production Technologies, Agroforestry Academic Area, Instituto Tecnológico de Costa Rica, Costa Rica.

drinking water reservoir of a surface water source (Marcus et al., 2017). This genus was also isolated from a consortium of microorganisms in a mixed culture of sewage-activated sludge (SAS) and effectively inoculated in a biological aerated-filter system for the simultaneous removal of ammonium and Mn from water (Abu-Hasan et al., 2012). *Microbacterium sp.* was also reported as MOB in bacterial communities isolated from samples of a powdered activated carbon-amended membrane bioreactor (PAC-MBR) system for the simultaneous removal of Fe, Mn, and ammonia from groundwater (Du et al., 2017). As was previously mentioned, few studies have investigated the ability of indigenous bacteria to oxidize Mn(II) from tropical water sources. Interestingly, MOB of the genus *Stenotrophomonas* detected in this study (Table 3.1) was also isolated and characterized in-vitro using water samples collected from an Mn mine in Brazil (Barboza et al., 2015). Other several autochthonous MOB were isolated from the groundwater-well SP1 during the bench-scale experiments but the description was included in Chapter 4.

In addition, *Aeromonas spp.* (accession numbers KT998825 and KC800783) and *Stenotrophomonas pavanii* (accession number NR 118008) exhibited the highest Mn(II) oxidation capacity, reaching values of 42.5%, 40.1%, and 40.3%, respectively. These values are comparable to the Mn removal efficiencies (42-45%) reported for *Streptomyces spp*. using a similar initial Mn concentration (5 mg/L) (Therdkiattikul et al., 2020). *Bacillus*, *Stenotrophomonas*, and *Lysinibacillus* species isolated from the Mn mine in Brazil reported Mn removal efficiencies of up to ~63%,71% and 83%, respectively, but with an initial Mn concentration of 50 mg/L (Barboza et al., 2015), well above the maximum Mn concentrations at which Mn commonly occurs in groundwater sources, of about 5.6 mg/L (Kohl & Medlar, 2006; USEPA, 2003). Despite that some biological Mn(II) oxidation models assumed that Mn(II) oxidation rate increases with increasing Mn(II) (Zhang et al., 2002), it should be considered with caution. For example, in previous work (Zhao et al., 2018), the Mn(II) oxidation by *Brevibacillus brevis* and *Brevibacillus parabrevis* strains were evaluated using two different initial Mn concentrations of 0.01-1 mM. The results showed that Mn(II) stimulated the growth and Mn(II) oxidation of the *B. parabrevis* strain while causing the inverse effect in the *B.*

brevis strain (under the same experimental conditions). Besides, other factors such as the origin of the strains, bacteria concentration, the type of culture medium, temperature, and duration of the experiment may influence the Mn oxidation activity of MOB in culture media.

Both violet staining and quantification of EPS, confirmed that *Stenotrophomonas* spp. exhibited the highest biofilm formation capacity (approximately between 0.08 and 0.14 mg glucose/g TS. *Aeromonas sp.* registered around 0.05 mg glucose/g TS). In a previous investigation conducted in 11 full-scale drinking-water biofilters with anthracite and granular activated carbon (GAC) as filter media, it was found that EPS polysaccharides ranged from 0.016 to 0.60 mg glucose/g TS (Keithley & Kirisits, 2018).

3.4 Conclusions

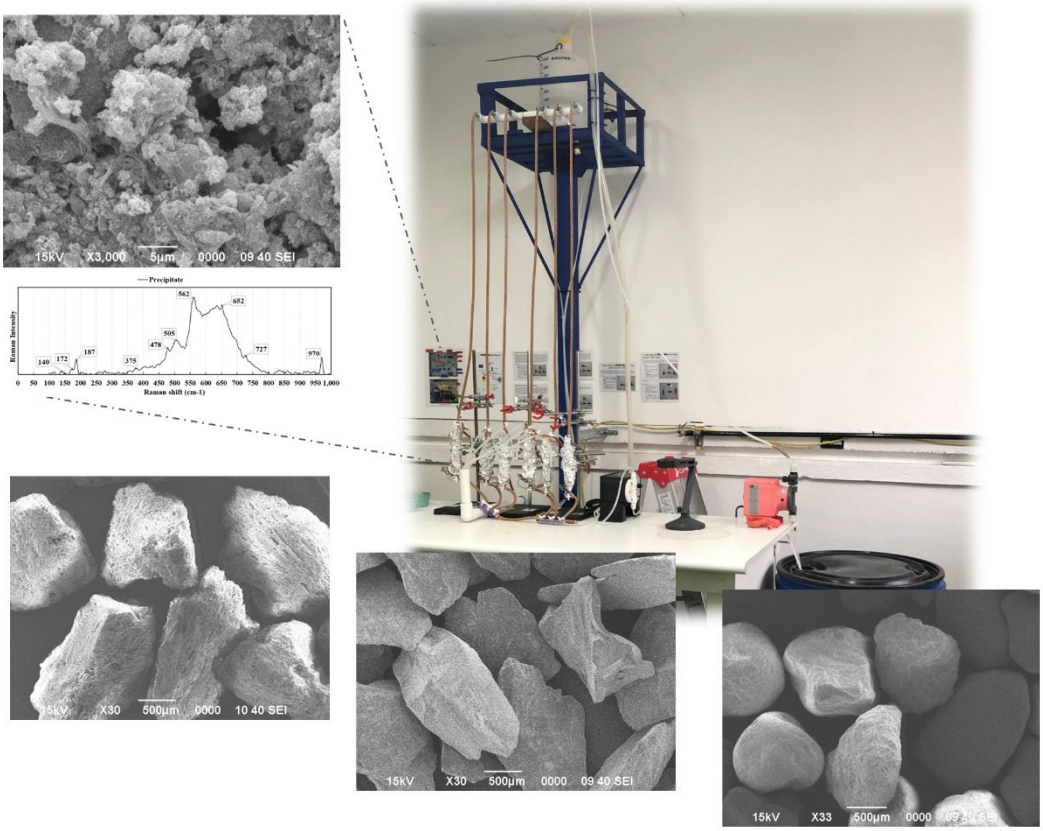
Microscopic observations of the flow cell samples suggested the probable occurrence of Mn-Fe oxidizing bacteria with characteristic structures in groundwater wells. In appearance, rod-shaped bacteria are a dominant specie. LBB test confirmed the presence of culturable MOB in all groundwater wells with elevated Mn concentrations. PCR-amplified 16S-rRNA gene sequencing of isolate MOB reveals genera with known MOB. Besides, the potential, at the in vitro level, of pumice stone combined with autochthonous *Gammaproteobacteria (Stenotrophomonas* and *Aeromonas spp.)* for biological Mn removal from groundwater in Costa Rica was also revealed.

Chapter 4

Start-up of bench-scale biofilters for manganese removal under tropical conditions

A comparative study using virgin pumice, silica sand, and anthracite filter media

Graphical abstract



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Abstract

Biofiltration for Mn removal has not been proven in the tropics. Also, the use of pumice as an alternative filter medium for Mn removal is still poorly known. In this study, a bench-scale biofiltration experiment, using virgin pumice, silica sand, and anthracite, was conducted for 107 days using tropical groundwater at 22 °C. Characterization of manganese oxide ($MnO_{x(s)}$) on filter media was carried out by Raman spectroscopy, X-ray diffraction (XRD), and scanning electron microscopy (SEM). The ability of culturable bacteria to oxidize Mn was verified by the leucoberbelin blue dye assay (LBB). The microbial activity on filter media was studied by ATP analyses. Identification of the MOB was performed by 16S rRNA sequencing. Results showed that the ripening time in each column was similar (~80 days); therefore, the filter media and water temperature do not seem to accelerate the start-up period. The $MnO_{x(s)}$ present on all ripened media was of the birnessite type. The $MnO_{x(s)}$ morphology, influent water parameters, and microbiological activity suggest a start-up period likely assisted by biological oxidation. Therefore, biofiltration for manganese removal is feasible under tropical conditions. The similarity in the performance of pumice with the other media confirmed its suitability for biotic Mn removal. All materials presented similar known MOB at the genus level; however, different closest related species colonized selectively the filter media. Two strains of *Pseudoxanthomonas* sp., a not recognized genus on matured biofilters, look promising as inoculums in pumice and sand. Proper application of biofiltration in the tropics needs operational and bioaugmentation strategies.

Keywords: groundwater, manganese removal, filter media, start-up, manganese-oxidizing bacteria, tropical conditions.

4.1 Introduction

Biofiltration systems based on aeration followed by rapid filtration are commonly used in Europe, North America, and Argentina for the removal of various types of contaminants including iron and manganese from groundwater (Burger, Mercer et al., 2008; Pacini et al., 2014). Compared to physicochemical systems, they are considered a cost-effective option as they are easy to operate and do not require chemical reagents or commercial adsorbents (Bruins, 2016). Despite this, there is still little experience in the application of this technology in tropical regions (Calderón-Tovar et al., 2020; Marsidi et al., 2018)

One of the main drawbacks of manganese (Mn) removal by biofiltration, during groundwater treatment, are the long ripening times of the biofilters. During the start-up period, the virgin filter medium provides the necessary surface for the attachment of the Mn oxidizing bacteria (MOB) present in the raw water, favoring the biological oxidation of Mn (Breda et al., 2016). Specifically, MOB remove Mn by intracellular oxidation (enzymatic action), by extracellular adsorption, or by catalysis under the influence of the biopolymers secreted by the bacteria (Mouchet, 1992). In addition, the Mn oxides ($MnO_{x(s)}$), formed by these processes, gradually cover the filter medium, contributing to the removal of Mn through auto-catalytical adsorption and subsequent oxidation of adsorbed Mn by biological and/or physicochemical means (Bruins et al., 2015b; Štembal et al., 2004). At the end of the start-up period, efficient and stable removal of Mn is expected (Yang et al., 2020). The length of this period is potentially influenced by the type and amount of manganese oxide coated in the filter medium (Bruins et al., 2014b), the quality of the raw water, and some operational or design aspects such as the volumetric loading of iron (Fe), the backwash strategy, and the type of filter medium (Bruins et al., 2017a; Bruins et al., 2015b).

The attachment of bacteria to the filter medium is a physicochemical process that can be influenced by the texture, the properties of the surface, the accessible areas of the

support medium (Wang et al., 1995), and the water temperature (Donlan, 2002). Microbial colonization would increase as the surface roughness of the filter media increases, as shear forces decrease and the surface area increases (Donlan, 2002). However, this bacteria-filter medium interaction is complex since the precipitates that are deposited on the surface of the virgin media will gradually change the physicochemical properties of the surface of the filter medium. In addition, smaller aggregate sizes can provide greater surface area per unit volume which favors the attachment of bacteria (Moore et al., 2001).

Biofiltration has been shown to be effective for Mn removal using various filter media such as silica sand (Li et al., 2005), quartz sand (Bruins et al., 2015a), anthracite, and GAC (Granger et al., 2014) and some other alternative media like polypropylene (Abu-Hasan et al., 2015), lava media (Han et al., 2013), and polystyrene beads (Katsoyiannis & Zouboulis, 2004), among others. The sand and anthracite are the most used, even though they do not exhibit great Mn adsorption capacities (Bruins et al., 2015a), as they have low costs and are commonly available. GAC can contain three to eight times more biomass than sand and anthracite, mainly due to its large surface area (Wang et al., 1995). However, its main disadvantage is investment costs (Marsidi et al., 2018). Additionally, Breda *et al.* (2017) showed that, in specific source water, different filter materials influenced not only the start-up period but also the bacterial community formed, suggesting that the latter can be managed using specific media for a specific type of water. Therefore, it is interesting to evaluate different filter media. One of them is pumice, whose use in rapid filters in both drinking water and wastewater treatment is increasing (Çifçi & Meriç, 2015). Pumice is a low-cost, porous, low-density medium, which, according to Farizoglu *et al.* (2003), produces less pressure drop than sand and has a higher specific surface area for stimulating bacterial growth. However, to our knowledge, no previous studies are evaluated the use of pumice in Mn removing biofilters, either at lab or pilot scale.

Generally, low environmental temperatures (around 7-17°C) decrease the performance of biofilters (Cai et al., 2014; Ciancio et al., 2020). Moreover, slower

ripening has been reported at water temperatures below 15°C (Evans, 2020). Pacini *et al.* (2014) identified a decrease in bacterial growth in sand filters, specifically during the winter period (~8-18 °C). Ciancio *et al.* (2020) confirmed at a laboratory-scale that bacterial diversity in sand filters for Mn removal was influenced by temperature changes and that inoculated sand columns showed the best Mn removal performance under summer conditions (~18-29°C). Cai *et al.* (2014) confirmed that functional oxidizing bacteria could be acclimated in low-temperature groundwater (~3-4°C); however, a long start-up period was required (~240 days) due to the slow growth rate of bacteria. In contrast, an increase in water temperature could benefit the bacteria attachment (Donlan, 2002) and reduced the negative effects of low temperature mentioned before. Therefore, it would be expected that a warm climate and stable temperature during the year would benefit the application of biofiltration in the tropics.

Recently, Calderón *et al.* (2020) reported autochthonous tropical groundwater MOB at the *in vitro* level and their capacity to form biofilms on pumice, suggesting the potential use of biofiltration for Mn removal. However, this technology has not been proven in the tropics and developing countries that mainly work with chemical treatment for Mn removal. Additionally, it is important to evaluate the use of pumice compared with traditional filter media. Hence, the aim of this study was first to investigate, at bench scale, the start-up period of non-bioaugmented biofilters, for Mn removal under tropical groundwater conditions, and second, to evaluate the use of virgin pumice as an alternative filter medium for Mn removal. Therefore, a comparative study at 22.21 ± 1.14 °C using virgin pumice, silica sand, and anthracite filter media was performed at the bench scale to compare the acclimation on each material. Further, characterization of $MnO_{x(s)}$ on the ripened filter medium coating and the presence and growth of culturable MOB were evaluated to identify the start-up mechanism of the manganese removal process at these water temperatures. Moreover, the possible influence of the filter material on the bacterial community was also studied and the potential species to inoculate the respective media were identified.

4.2 Materials and methods

4.2.1 Water source used during the experiment

The raw water was taken from a well that supplies 550 people at the La Hacienda Condominium, Cartago, Costa Rica (9°50'28"N83°58'26"W). The groundwater was collected twice a week in 60-liter plastic containers. During the transfer, the water was saturated with oxygen, generating some expected changes as shown in
Table 4.1. The increase of the dissolved oxygen concentration (OD) promotes changes in the oxidation-reduction potential (ORP) and can remove carbon dioxide contained in the groundwater increasing the pH (Crittenden et al., 2012).

Table 4.1. Quality of groundwater and aerated water used in the experiment

Parameter	Unit	Raw water (n=15)	Laboratory Influent (n=53)
Mn	mg/L	0.57[a] ± 0.04[b]	0.57± 0.06
Fe	mg/L	0.32 ± 0.15	< 0.10[c]
pH	-	7.03 ± 0.11	7.83 ± 0.40
T	°C	22.80 ± 0.48	22.21 ± 1.14
ORP	mV	-35.15 ± 22.50	201.1 ± 55.84
DO	mg/L	0.42 ± 0.11	6.37 ± 0.95
DO	%	5.46 ± 1.40	87.17 ±12.33

a Average; b Standard deviation; c Detection limit.

4.2.2 Experimental set up and monitoring

The experimental unit consisted of a feed tank in which the aerated water from the well was stored (Figure 4-1). Subsequently, the water was pumped to an elevated tank with a constant water level for distribution by gravity to each of six plastic columns packed, in duplicate, with three types of virgin filter media, previously washed and oven-dried at 110 ± 5°C, according to the Standard Method C117-17 (ASTM, 2017b). Each column was 2.8 cm in diameter and had a media height of 10.0 cm. This height was adopted because it has been demonstrated to be appropriate in studying the biological activity and the effectiveness of biofilters for Mn removal at bench scale (Breda et al., 2017; Granger et al., 2014). The flow rate in each column was 5 mL/min and was distributed with a contact time of approximately 12 minutes and at a velocity of 0.75 cm/min.

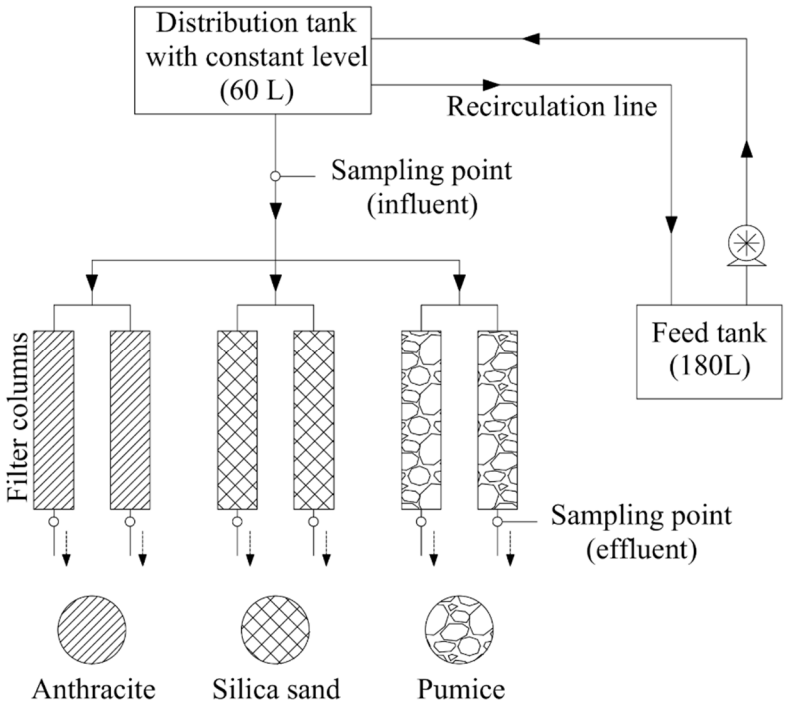


Figure 4-1. Configuration of the experimental unit.

The physical properties of each of the filter media are presented in Table 4.2. The BET surface area was determined by nitrogen adsorption at 77K (Gemini VII 2390p analyzer, Micromeritics). Sieve tests were performed following Standard Method C136/C136M (ASTM, 2019). The specific gravity was measured using the Standard Method C128-15 (ASTM, 2015). The bulk density and medium porosity (voids between particles) were obtained by the Standard Method C29/C29M-17a (ASTM, 2017a). In addition, these parameters were validated using the method described by Happiness (2014). Finally, the average particle size was measured by scanning electron microscopy (SEM TM-3000, company Hitachi High-Tech) according to Song *et al.* (2015).

Table 4.2. Characterization of filter media.

Parameter	Unit	Pumice	Anthracite	Silica Sand
Bulk density	kg/L	0.37 ±0.01	0.78 ±0.01	1.50 ±0.02
Particle density	kg/L	1.02[a] ±0.03[b]	1.56 ±0.03	2.63 ±0.01
Media porosity	%	84 ± 2	43 ± 2	40 ± 2
Grain size (10-90%)	mm	0.88-1.16	0.85 – 1.34	0.81 – 1.31
Uniformity coefficient	[-]	1.20	1.33	1.38
Average particle size	mm	1.03 ±0.10	1.09 ±0.19	1.07 ±0.20
BET surface area	m^2/g	2.72	0.38	0.64

[a] Average; [b] Standard deviation

The start-up period was defined as the time required for the biofilters to achieve Mn removal efficiencies greater than 90% (Bruins et al., 2017a). Under these conditions and granting a few additional weeks of monitoring, the experiment was carried out in the laboratory for 107 days. Backwashing was performed with filtered water when a 20% flow rate reduction was reached, or filters became clogged. According to hydraulics tests performed during the experiment, a backwash strategy of 30 s at a loading rate of 144 mL/min was adopted. In total, backwashing was performed on days 49, 66, 74, 81, 88 and 100 of the experiment.

During the experiment, Mn, and Fe were determined in the inlet and outlet water of the biofilters. The dissolved oxygen (DO), pH, and oxidation-reduction potential (ORP) were monitored in the influent three times a week. Mn and Fe measurements were done using AAnalyst 800 atomic absorption equipment (Perkin Elmer, Waltham, USA) following the Extraction/Air-Acetylene Flame Method 3111-C (APHA et al., 2005). The detection limits for Fe and Mn were 0.10 mg/L and 0.03 mg /L, respectively. The pH, dissolved oxygen, and ORP were determined using Hach HQD30 equipment following the methods recommended by the manufacturer (Hach, USA).

4.2.3 Characterization of the oxides formed ($MnO_{x(s)}$)

At the end of the start-up period, the type of $MnO_{x(s)}$ accumulated on each biofilter was identified using Raman spectroscopy and X-ray Diffraction (XRD). In addition, electronic microscopy (SEM) was used to observe the type of structure of the $MnO_{x(s)}$ and to verify if the $MnO_{x(s)}$ was of biological or physicochemical origin, as explained by Bruins *et al.* (2015a).

4.2.3.1 Raman spectroscopy

An Alpha 300R spectrometer (WITec, Knoxville, USA) was used with the following configuration: objective 100 ×, laser wavelength λ = 532 nm, output power ~0.8 mW, integration time 20 s and grating 1200. Before analysis, the samples were dried at room temperature to avoid fluorescence due to the presence of water as suggested by Bruins *et al.* (2014).

4.2.3.2 X-ray Diffraction (XRD)

XRD analyzes were carried out to confirm whether the structure of the $MnO_{x(s)}$ present on the filter media was crystalline or amorphous. Therefore, a PANalytical diffractometer (Empyrean, Worcestershire, United Kingdom) was used with the following configuration: CuKα radiation: 1.5418 Å, range 6–80˚ 2θ and a step size of

0.013˚ 2θ. Samples of $MnO_{x(s)}$ deposits (without filter media grains) were collected from the top of the pumice and sand columns. Unfortunately, due to the dark color of both the anthracite grains and the $MnO_{x(s)}$ deposits, it was impossible to obtain the latter in the anthracite columns. Pumice and sand deposits were dried at room temperature and pulverized before analysis.

4.2.3.3 Electron microscopy (SEM)

SEM photographs were taken with a JSM 6390 scanning electron microscope (JEOL, Peabody, USA). Before the observation, a chemical fixation process was carried out to preserve the biological samples similar to the study by Zeng *et al*. (2010). A Karnosky (2% glutaraldehyde, 2% paraformaldehyde in 0.5 M phosphate buffer, pH 7.2) fixative was used. The samples were preserved at 4 °C for 48 h. Afterward, each sample was washed in a phosphate buffer (0.05 M, pH 7.2) for 10 min. Dehydration was carried out gradually to avoid drastic cell shrinkage using dilutions with 30%, 50%, 70%, and 100% ethanol in distilled water. Finally, before mounting, they were dried at room temperature. Images of samples with and without gold coating were tested, which were placed on aluminum bases on carbon tape for their observation.

4.2.4 Microbiological analysis

The biofilters were inoculated with the microorganisms present in the raw water. Identification of MOB was carried out by DNA sequencing. At the end of the start-up period, samples of the matured filter media were taken from the top (0-2 cm) and the bottom (8-10 cm) of the columns (to be referred to as “top” and “bottom” respectively) to identify the MOB present in the biofilters. In addition, heterotrophic bacterial counts (CFU/g) were performed together with adenosine triphosphate (ATP) analysis of biofilm medium. The ability of isolated bacterial strains to oxidize Mn was verified using the leucoberbelin blue (LBB) dye assay.

4.2.4.1 Isolation of the MOB present in the filter media

Once the filters were ripened, 10 g of the filter media was taken from the top and the bottom of the columns. The samples were diluted in 90 mL sterile 0.1% (w/v) peptone water isotonic solution. Subsequently, the samples were stomached for 1 min. Serial dilutions were made, up to 10^{-4} and 1 mL of each dilution was plated in duplicate on modified R2A agar with 17 mg/L $MnSO_4$ and incubated for five days at room temperature (<28 ° C). Subsequently, heterotrophic bacteria were counted on days 5 and 20, respectively. The 20-day count corresponded to the MOB strains determined by the LBB method (Section 4.2.4.2).

4.2.4.2 Leucoberbelin blue (LBB) dye assay

After an incubation period of 20 days, two drops of the LBB reagent (0.04% w/v dissolved in 45 mM acetic acid) were added to the colonies. This qualitative method is based on the LBB oxidation reaction with Mn^{+3} or Mn^{+4} producing a blue color (Piazza et al., 2019). A strong blue tone implies the highest oxidation capacity and is assigned "++++", light tones are assigned "+++" or "++" and weak tones "+".

4.2.4.3 Identification of culturable MOB by 16S rRNA sequencing

Genetic material was extracted by the cetyl-trimethyl ammonium bromide (CTAB) method from culturable heterotrophic bacteria positive to the LBB assay. The amplification of the region to be sequenced was performed with the universal primers of the 16S rRNA region, specifically *27F* (5′-AGAGTTTGATCCTGGCTCAG-3′) and *1492R* (5′-GGTTACCTTGTTACGACTT-3′). The results were confirmed by agarose gel electrophoresis (0.8%). Sequencing was performed with the polymerase chain reaction (PCR) product at a concentration of 50 ng/µL by MACROGEN (Korea), according to the Sanger method using a 3730xl Genetic Analyzer from Applied Biosystems. 16S rRNA amplicon data were treated as operational taxonomic units (OTUs) and considered as "closest related" species (Ciancio et al., 2020). To have an insight into

the most dominant related species present on each filter media, read abundance percentages of the culturable MOB based on the OUT counts were determined using the ampvis package (Andersen et al., 2018) in the statistical software R (R Core Team, 2021).

4.2.4.4 Medium biofilm adenosine triphosphate (ATP) analyses.

A deposit and surface analysis kit (DSA™, LuminUltra Technologies, Canada) was used for ATP measurements. According to the manufacturer's instructions, 1 gram of filter medium was transferred to 5 mL of Ultralize™7 and homogenized for two minutes. Contrary to the original method of vortex homogenization, sonication was used in a Branson 1510 ultrasonic cleaner that allowed increasing ATP concentrations by about 8% compared to the recommended method. After 5 min incubation at room temperature, 1 mL of the solution was diluted in a 9 mL UltraLute™ (dilution) tube. Finally, using 100 µL of the sample together with 100 µL of Luminace™ enzyme, the reading was taken on a PhotonMaster™ luminometer as relative light units (RLUs) which were expressed as ATP according to the manufacturer's indications.

4.3 Results and discussion

4.3.1 Start-up period of biofilters

Figure 4-2 shows the average Mn removal efficiencies obtained during the bench-scale experiments. Throughout the start-up period, a similar trend in removal efficiencies was observed in each of the filter media. In the first two weeks, the Mn removal efficiencies were less than 10%. This was expected due to the small adsorption capacity of the virgin materials (*e.g.*, the pumice used in this experiment showed 0.3 mg/g in isotherm data), similar behavior was reported by Bruins *et al.* (2015b) in sand and Mn oxide-coated sand (MOCS). The increase in removal efficiencies was notable from the third week, reaching values greater than 50% after 60 days (~ week 8). Finally, the start-up period was completed in the silica sand and pumice stone columns on day 79 and in the anthracite column on day 84. It is known that the ripening process for Mn removal typically takes between 1-4 months, or even more on non-bioaugmented biofilters (Bruins et al., 2015b; Štembal et al., 2004; Zeng et al., 2010). Our results were in this range and there was a minimal difference between the filter media, which indicates that the pumice behaved similarly to the other materials. Furthermore, the possible advantages of pumice, such as greater surface area and roughness, were not decisive, probably because effective MOB did not grow in all the available surfaces due to the competition with other heterotrophic bacteria present in the filter media (section 4.3.4). Therefore, other parameters such as water quality and filtration velocity might have been more determining factors for ripening.

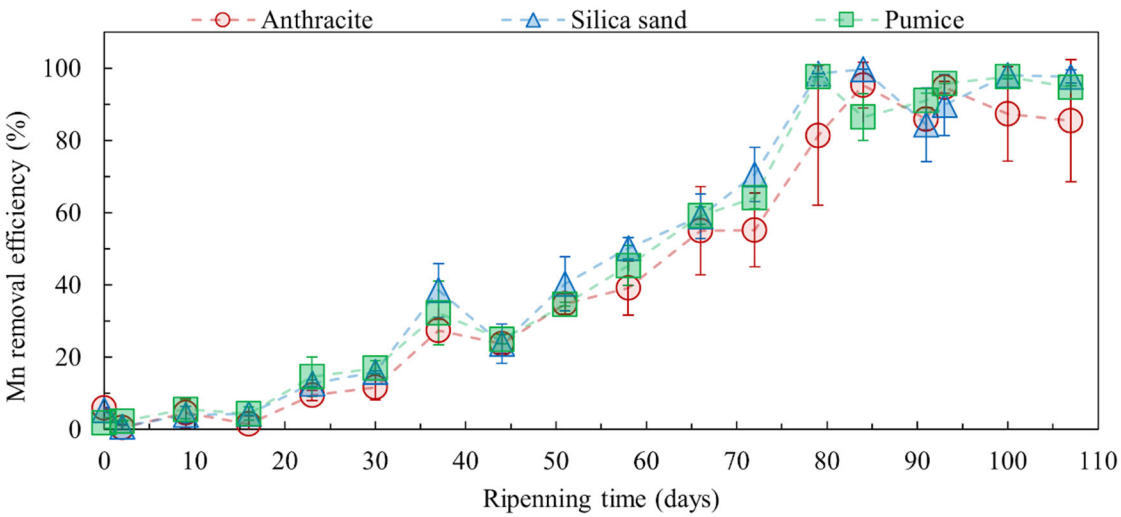


Figure 4-2. Mn removal efficiencies in anthracite, silica sand, and pumice stone columns (error bars represent standard deviations).

During the ripening time of the biofilters, in principle, there were favorable conditions for the biological oxidation of Mn. Mouchet (1992) has mentioned that to oxidize Mn^{2+}, MOB requires pH> 7.5, dissolved oxygen (DO) > 5 mg/L, and a redox potential (Eh) of about 300 and 400 mV. As can be seen in Figure 4-3, most of the time, the experimental conditions of pH and Eh were within this recommended field of action. As shown in Table 4.1, DO, pH, and ORP in the laboratory influent were 6.37 ± 0.95 mg/L, 7.83 ± 0.40, and 201.1 ± 55.84 mV, respectively. Similarly, other studies in temperate zones have reported, at bench scale (Breda et al., 2017; Granger et al., 2014) and pilot-scale (Pacini et al., 2005), effective Mn removal within the DO, pH, and Eh ranges suggested by Mouchet (1992).

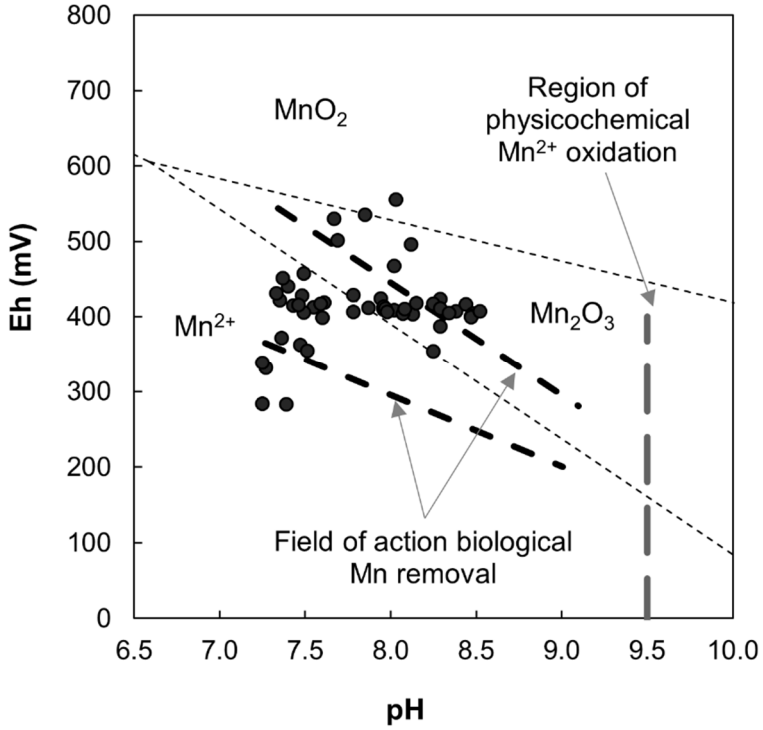


Figure 4-3. Field of action of the MOB in the pH–Eh diagram.
Adapted from Mouchet (1992).

On the other hand, a reduction in the obtained ripening times would have been expected, as the water temperature was around 22.21 ± 1.14 °C. According to Madigan *et al.* (2014), the optimal growth temperature of mesophilic bacteria, which determines the maximum metabolic capacity, is in the range of 20 to 45 °C. In contrast, low-temperature groundwater (~3-18°C) affects the growth rate of functional oxidizing bacteria and subsequently, the start-up period of biofilters (Cai et al., 2014). Moreover, acclimation of new biofilters at water temperature below 15°C has been reported to be slower than at higher temperature (Evans, 2020).

Similar to the study by Ramsay *et al.*(2018), inherent inoculation with native MOB, even from the tropics, seemed to be insufficient to achieve a rapid start-up period during the experiments. Filter media and water temperature evaluated in this study did not substantially accelerate the start-up of biofilters. Therefore, similar to temperate zones,

the implementation of biofiltration in the tropics should consider bioaugmentation strategies to speed up the ripening period. Additionally, an increase in flow velocity or nutrient concentration may also equate to increased bacteria attachment to a more rapid association of cells with the surface filter media (Donlan, 2002).

4.3.2 Characterization of $MnO_{x(s)}$

The Raman spectra of the $MnO_{x(s)}$ deposits obtained in the filter media are shown in Figure 4-4. It is observed in all cases that the principal peaks follow a similar pattern between 497-507, 555-560, and 625-655 cm^{-1}, which means that the spectra obtained in each of the materials were similar. The mentioned pattern is similar to the birnessite spectrum reported by Bruins *et al.* (2014) in samples of aged Mn oxide-coated sand (MOCS) and Mn oxide-coated anthracite (MOCA), obtained from full-scale groundwater biofilters.

The XRD patterns obtained from the $MnO_{x(s)}$ samples (Figure 4-5) show wide and low-intensity peaks, which confirms their amorphous character (Yang et al., 2020). In addition, the three peaks at 26.8° 2θ, 36.9° 2θ and 66.2° 2θ in the XRD patterns coincide with the patterns reported by Cheng *et al.* (2017) associated with Birnessite. In temperate zones, such type of $MnO_{x(s)}$ has been reported as responsible for the auto-catalytic action in ripened rapid filters (Bruins et al., 2014). No XRD spectrum was obtained for $MnO_{x(s)}$ deposits in ripened anthracite because it was not possible to separate them from the similar dark filter medium.

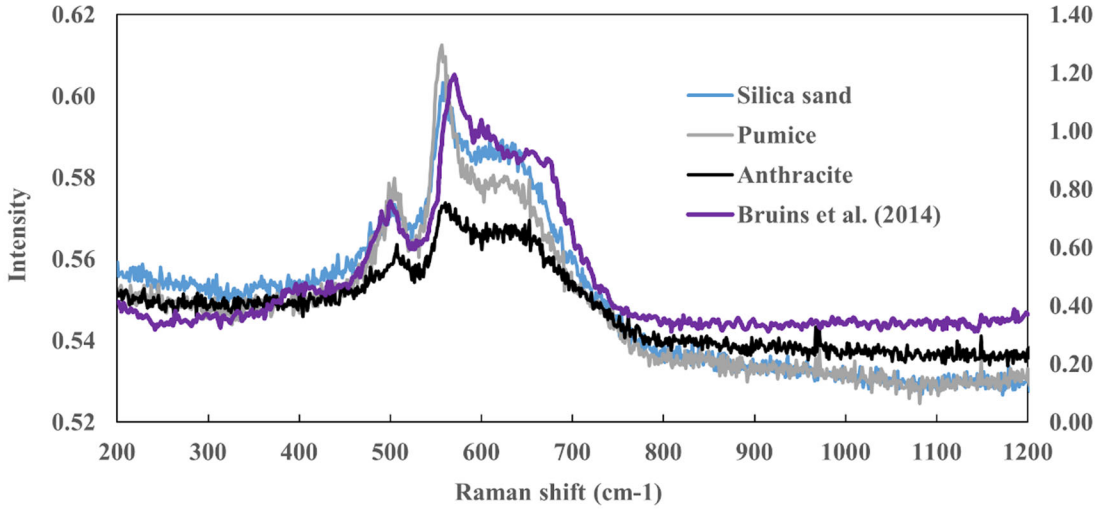


Figure 4-4. Raman spectra (at 532 nm) of $MnO_{x(s)}$ deposits obtained in the ripened materials at 123 days compared to the birnessite reference by Bruins et al. (2014)

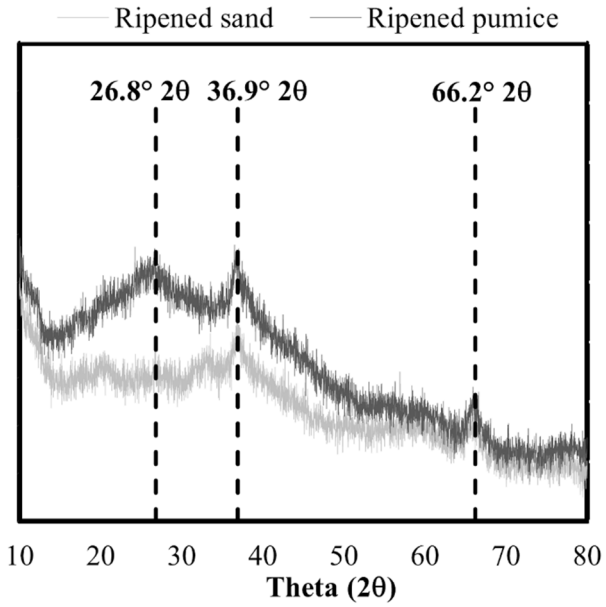


Figure 4-5. XRD patterns of $MnO_{x(s)}$ deposits obtained on the surface of ripened silica sand and ripened pumice.

The morphology of the surface of the materials is shown in Figure 4-6. In the virgin pumice (Figure 4-6.A1 and A2) a porous skeleton structure is observed, related to an

increase in the surface area compared to the virgin anthracite and virgin silica sand (Table 4.2). The virgin anthracite and the virgin silica sand (Figure 4-6.B1, B2, C1 and C2, respectively) are similar to plate structures, with a predominance of smooth regions in the virgin anthracite and a denser, rougher surface with more shallow depressions for the case of virgin silica sand.

On the other hand, SEM micrographs of the $MnO_{x(s)}$, obtained from the samples of ripened pumice, anthracite and silica sand media (Figure 4-6.A3, B3 and C3) show great similarities among them. In all cases, a poor crystalline structure was observed (which confirms the XRD analysis results), with abundant agglomerated structures that form a rough surface with a porous structure that should be favorable for bacterial attachment (Donlan, 2002). Such structure characteristics suggest that probably, in all the materials, the same type of Mn oxide was formed. Furthermore, the same SEM micrographs showed the presence of fluffy plate structures, typical for birnessite of biological origin, as reported by Bruins *et al.* (2015a) during the ripening of virgin sand biofilters at pilot-scale. According to the authors, during the start-up period, this mineral exhibits surface characteristics of biological origin (*e.g.* fluffy plates) and, over time, birnessite of physical-chemical origin takes place. Probably, the $MnO_{x(s)}$, present on all ripened media of this study, were Birnessite of biological origin as reported by Bruins et al. (2015a) in temperate regions with water at a typical temperature of 10-12 °C.

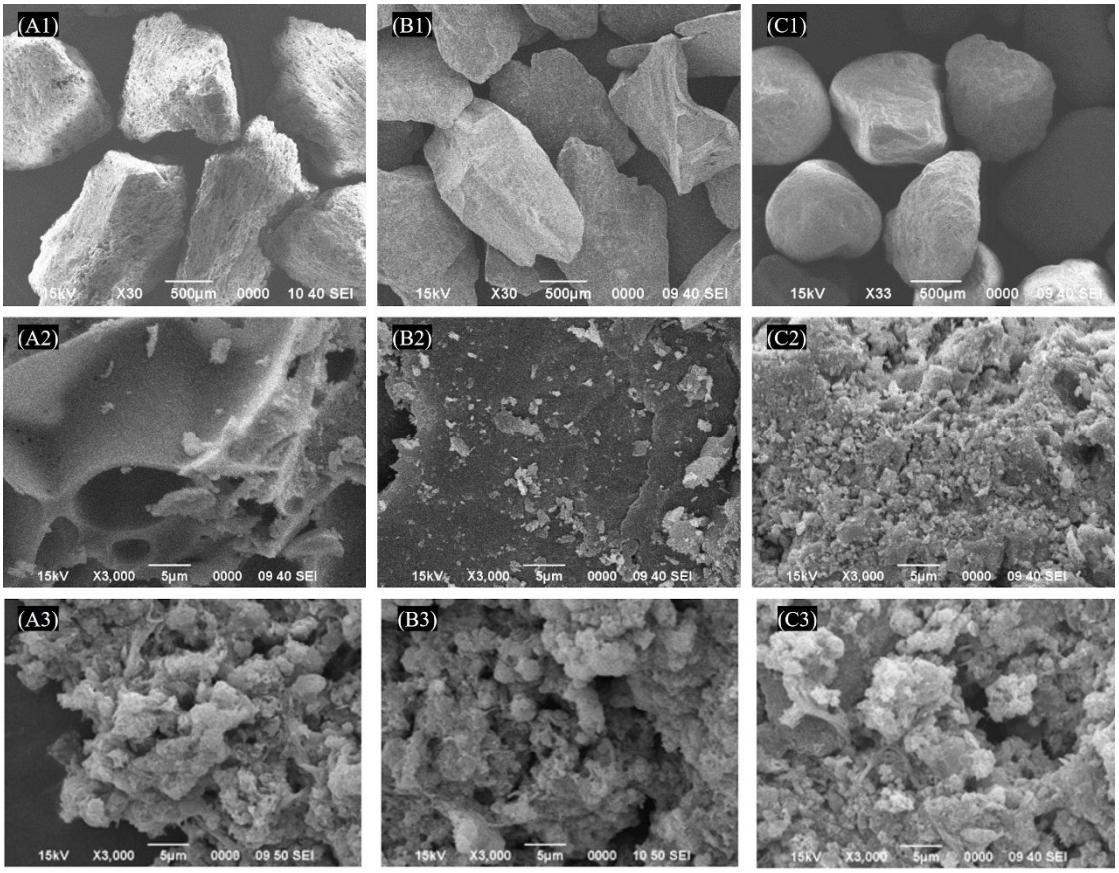


Figure 4-6. Scanning electron microscopy (SEM) images with a magnification of 30× and 3000× of (A) pumice stone (B) anthracite, and (C) silica sand.

4.3.3 Characterization of culturable MOB

Although there is a great variety of MOB that include phyla such as *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* (Piazza et al., 2019), still little is known about the diversity of MOB present in groundwater in tropical countries (Barboza et al., 2015). In the present study, the culturable MOB detected in the water samples belong to the first two phyla and *Proteobacteria* (genus *Stenotrophomonas* and *Aeromonas*) (Table 4.3). In contrast, isolates on the ripened filter media were taxonomically diverse with a clear domain of *Proteobacteria* phylum (*Alpha*- and

Gammaproteobacteria), as also reported in similar studies (Breda et al., 2017; Gülay et al., 2016; Piazza et al., 2019) in temperate climates.

Culturable MOB detected in the raw water samples were of the genus: *Stenotrophomonas*, *Aeromonas*, *Microbacterium*, *Cellulosimicrobium*, and *Staphylococcus* (Table 4.3). The first three genera were already reported by Calderon *et al*. (2020) in the same well water of the present research. Those three genera have also been reported elsewhere: *Stenotrophomonas* in Brazilian mine water (Barboza et al., 2015), *Aeromonas* in simultaneous removal of ammonium and Mn at pilot-scale in slow sand filters (Subari et al., 2018), and *Microbacterium* in water reservoir used for drinking water (Marcus et al., 2017). Cellulosimicrobium sp. is associated with the reduction of Cr(VI) and Cr(III) (Su et al., 2019). To the authors' knowledge, there have not been reports of this species as MOB. According to Das *et al*. (2011), *Staphylococcus* has been identified as a MOB in seawater, but no reports in biofiltration were found.

The bacteria found in all the ripened media include the genera, *Pseudomonas*, *Sphingopyxis*, and *Pseudoxanthomonas*. Additionally, *Sphingomonas* spp. were observed only in pumice. In similar studies with temperate water, Breda *et al*. (2017) reported the dominance of *Nitrospira* in matured quartz (18%), *Novosphingobium* in virgin quartz (35.2%) and calcium carbonate (20.3%), *Sulfuritalea* in polystyrene (83.4%) and *Pseudomonas* in Mn oxide (38.7%). They also detected *Pseudomonas* at 8,2% in mature quartz and less than 1% in virgin quartz and calcium carbonate. Other studies have reported *Pseudomonas* as a MOB in quartz sand biofilters (Bruins et al., 2017b; Yang et al., 2014). *Sphingopyxis* was reported by Breda *et al*. (Breda et al., 2017) in all the filter materials mentioned at a very low percentage (<3%). Piazza et al. (2019) detected this genus in the gravel prefilter and the sand filter in two drinking water treatment plants in Argentina. Cai *et al*. (2015) reported the presence of *Sphingomonas* sp. in bench-scale gravity quartz sand biofilters inoculated with backwashing sludge containing MOB. With regard to *Pseudoxanthomonas* no reports in the literature were found, and further discussion about this genus is presented later.

Table 4.3. MOB isolated from raw water and filter media (influent water temperature 22.21 ± 1.14 °C).

Genus- Phylum (class)	**Closest related species**	**Accession number**	**Similarity (%)**	**(LBB test)**	**Reported genus with known MOB**
Sphingopyxis *Proteobacteria (Alphaproteobacteria)*	*Sphingopyxis soli*	NR-116739.1	97.30	+ +	(Breda et al., 2017)
	Sphingopyxis macrogoltabida	AB675377.1	97.40	+ + + +	
Sphingomonas *Proteobacteria (Alphaproteobacteria)*	*Sphingomonas koreensis*	NR-113868.1	95.50	+ + +	(Cai et al., 2015)
	Sphingomonas colocasiae	NR-159304.1	97.30	+ + +	
Pseudoxanthomonas *Proteobacteria (Gammaproteobacteria)*	*Pseudoxanthomonas mexicana*	KF501482.1	98.79	+ + + +	(Thierry et al., 2004)
	Pseudoxanthomonas japonensis	NR-113972.1	97.00	+ + + +	
Pseudomonas *Proteobacteria (Gammaproteobacteria)*	*Pseudomonas stutzeri*	NR-103934-2	80.00	+ + + +	(Bruins et al., 2017b)
	Pseudomonas alcaligenes	NR-113646	97.60	+ + +	
	Pseudomonas aeruginosa	NR-117678	97.00	+ + +	
		LR130537.1	98.69	+ + + +	
	Pseudomonas oleovorans	LC507444.1	98.70	+	
	Pseudomonas entomophila	CP034337.1	98.93	+ + +	
Stenotrophomonas[a] *Proteobacteria (Gammaproteobacteria)*	*Stenotrophomonas maltophilia*	LR134301.1	99.22	+ + +	(Barboza et al., 2015; Calderón-Tovar et al., 2020)
		CP029773.1	99.59	+ + +	
Aeromonas[a] *Proteobacteria (Gammaproteobacteria)*	*Aeromonas veronii*	MF111930.1	98.92	+	(Koo & Park, 2005)
		MF111973.1	98.59	+ +	
Microbacterium[a] *Actinobacteria*	*Microbacterium sp.*	KX390640.1	98.70	+ + + +	(Marcus et al., 2017)
Cellulosimicrobium[a] *Actinobacteria*	*Cellulosimicrobium cellulans*	HG000003.1	97.78	+	Not reported
	Cellulosimicrobium sp.	MN536509.1	98.89	+ + +	
Staphylococcus[a] *Firmicutes (Bacilli)*	*Staphylococcus warneri*	MK256311.1	87.17	+ + + +	(Palanichamy et al., 2002)
		KX454005.1	97.72	+ +	
	Staphylococcus cohnii	MF111620.1	97.00	+ +	

[a] *Genera found exclusively in water samples.*

Breda *et al*. (2017) found different bacterial compositions between the source water and the biofilm developed in virgin quartz, calcium carbonate, polystyrene and manganese oxide biofilters. The authors suggested that different materials influenced the bacterial composition during the ripening period when using the same raw water. However, in the experiment reported here between the evaluated materials only pumice showed a different genus, *Sphingomonas*. In the present experiment, the characterization of the MOB aimed to detect possible species for future inoculation; therefore, only culturable MOB were analyzed. Knowing that, in an attempt to detect further influence or preference of the bacteria for the type of filter media, culturable MOB read abundance based on the OUTs counts in each ripened filter media was performed, the main results are presented next.

Figure 4-7 compares the read abundance percentage of isolated MOB in each ripened filter medium obtained from rRNA amplicon data treated as operational taxonomic units (OTUs, section 4.2.4.3). Some differences were observed in the diversity of species identified in the ripened pumice compared to those in the ripened anthracite and silica sand. Clearly, pumice stone showed a higher MOB species diversity, probably due to its porous structure and higher surface area than anthracite and sand (Table 4.2). On the other hand, *Sphingopyxis soli* and *Pseudoxanthomonas Mexicana* were exclusively found on ripened anthracite and silica sand. In addition, *Pseudoxanthomonas japonensis* was one of the most abundant closest related species capable of colonizing in the three-filter media, being the most abundant in pumice (42.9%) and also important in anthracite (30%). The *Pseudoxanthomonas Mexicana* was also the most abundant closest related specie in silica sand (36.4%). To our knowledge, the *Pseudoxanthomonas sp*. is not commonly related to Mn^{2+} oxidation on matured biofilter media. This genus was reported as MOB in the biocorrosion of carbon steel inside of pipelines of sewage treatment plants (Ashassi-Sorkhabi et al., 2012). However, in this study under tropical conditions, *Pseudoxanthomonas sp*. seem to play an important role in manganese removal by biofiltration and should be considered as a candidate inoculum for sand and pumice media biofilters. *Pseudomonas aeruginosa* was also detected abundantly in all filter media (Figure 4-7) making it interesting as an inoculum.

It was reported as an excellent model microorganism for the study of biofilms (Park et al., 2011). Yang *et al.* (2014) reported this closest related species in an inoculated pilot quartz sand filter for the simultaneous removal of arsenic, Fe, and Mn from groundwater. About the other species, they do not seem to be significantly different from those reported in other studies in temperate conditions.

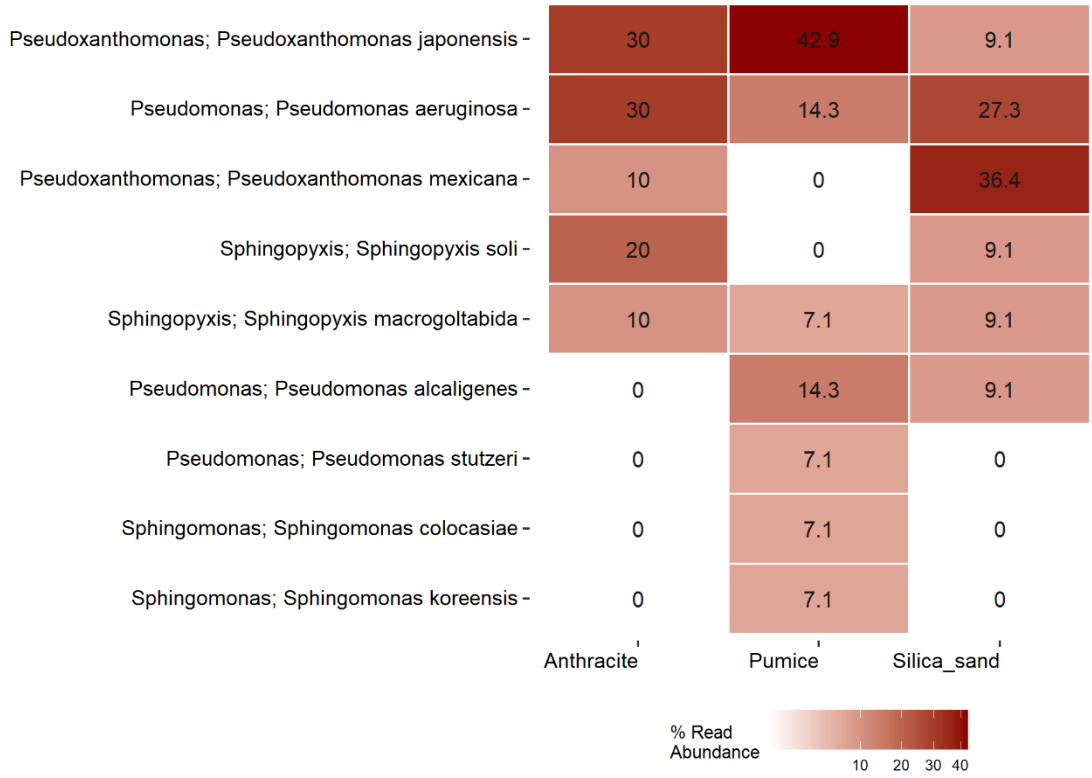


Figure 4-7. Read abundance percentage of the culturable MOB based on the OTU counts in each ripened filter medium

Even though the different filter materials presented no significant difference in the ripening period (Section 4.3.1), similar to the results of Breda *et al.* (2017), they influence the bacterial community developed. Differences in the closest related species on each filter media were caused probably by the biofilm formation capacity and the ability to attach to the surface media. Bacteria that grow more rapidly colonize first and establish a long-term competitive advantage with the others (Donlan, 2002).

4.3.4 Microbiological activity

Heterotrophic plate counts confirm the presence of heterotrophic bacteria in similar amounts in each of the filter media. The total of heterotrophic bacteria on the top of biofilters was in the order of 10^7 CFU/g but only 10^3 CFU/g corresponded at culturable heterotrophic MOB (positive to LBB test). Regarding the bottom of biofilters, the plate counts were around 10^5 CFU/g and no strain positive to LBB test was detected. Also, ATP concentrations, at the top of biofilters, were around 0.49±0.03 µg/g, 0.28±0.06 µg/g y 0.62±0.15 µg/g for ripened anthracite, ripened sand, and ripened pumice, respectively (Figure 4-8). It was found that the ATP values were predominant in the upper part of the columns, which is consistent with the results obtained during the plate counts. Granger *et al*. (2014) reported, in bench-scale bioaugmented biofilters with average water temperatures around 19.2 ± 0.8 °C, similar concentrations of heterotrophic bacteria and MOB of around 10^6-10^7 CFU/g and 10^2-10^3 CFU/g, respectively, and ATP measurements in the range of 0.13-0.32 µg/g on anthracite and 0.28-1.39 µg/g on GAC media.

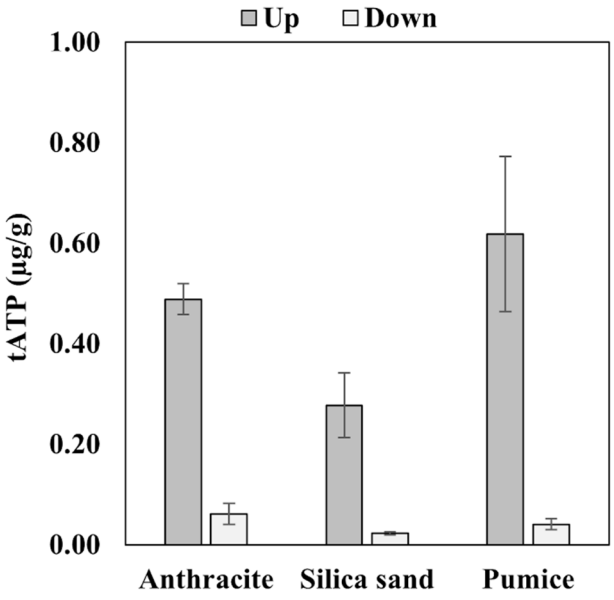


Figure 4-8. Concentrations of ATP obtained from filter medium samples (error bars represent standard deviations).

4.3.5 Biological manganese removal process

Several aspects support that Mn was likely removed by biological activity. First, the influent water pH, OD, and ORP were most of the time in the ranges suggested by Mouchet (1992). Moreover, chemical oxidation was not expected as oxygen was the only oxidant present, and Mn oxidation at pH lower than 9 is very slow (Granger et al., 2014). Secondly, the $MnO_{x(s)}$ deposits formed were similar to the birnessite of biological origin previously reported Bruins et al. (2015a). Thirdly, four genera and nine closest related species capable of oxidizing Mn were detected in the filter media. Lastly, the ATP and levels of culturable heterotrophic MOB are in the same range as those in previous studies (Granger et al., 2014). Interestingly, all of the previous findings are supported by studies in temperate regions, suggesting similar mechanisms for tropical regions at around 22°C.

4.4 Conclusions

Biofiltration for Mn removal is commonly used in temperate regions; however, there are few reports in the tropics. The present research aimed to study the start-up of non-bioaugmented bench-scale biofilters for Mn removal under tropical, Costa Rican, groundwater conditions and to evaluate the use of pumice as an alternative filter medium. Results, with groundwater temperature of 22.21 ± 1.14 °C, demonstrated that biological removal of Mn with non-bioaugmented biofilters is feasible in the tropics, showing similar start-up periods to reported ones in temperate zones, consequently the temperature effect was minimum. Pumice stone performed similarly to sand and anthracite, proving that pumice is a potential material for biological Mn removal. These main results indicate good opportunities for biofiltration in the tropics with any of the materials tested.

Key findings include the following:

- There were only small differences in Mn removal efficiencies between the anthracite, silica sand, and pumice during the ripening period of about 80 days.

- The biological removal mechanism in the experiment was supported by the influent water quality characteristics (pH, ORP and OD), the formation of $MnO_{x(s)}$ similar to birnessite of biological origin, the presence of recognized MOB, and the level of ATP and culturable heterotrophic MOB.

- At the genus level, the bacterial community was not significantly influenced by the filter material, only pumice was specifically influenced by *Sphingomonas* spp. However, different closest related species colonized selectively the filter media. Therefore, a suitable inoculum could be developed depending on the filter media.

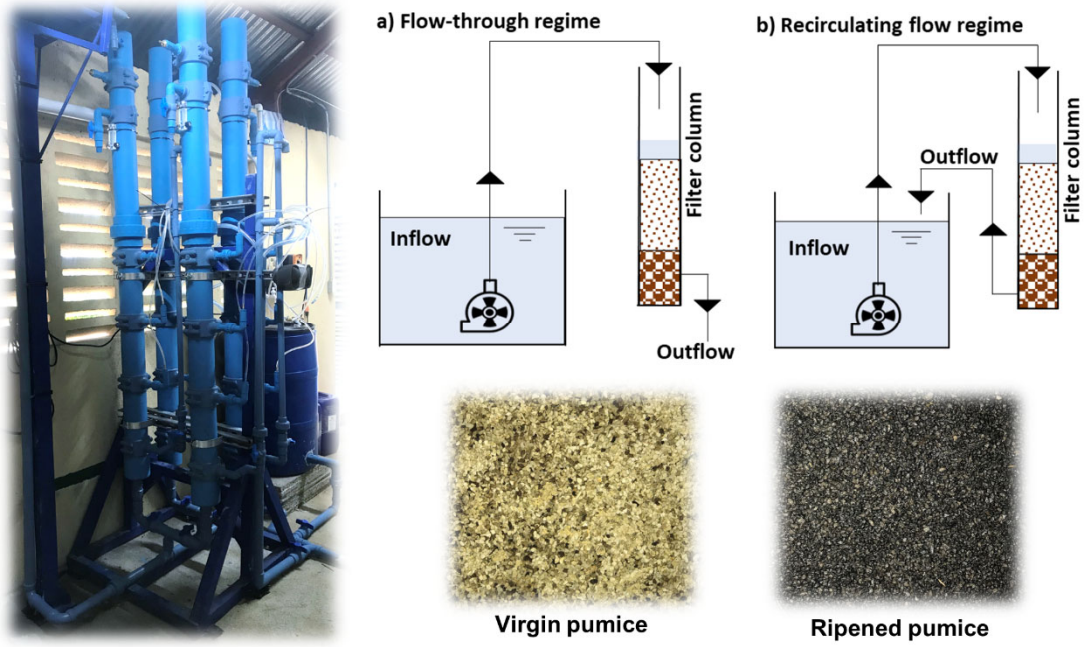
- *Pseudoxanthomonas* sp., not reported in Mn biofiltration before, showed high Mn oxidation activities and was abundant in the three media, being the most important one in pumice and silica sand. Therefore, these strains are good candidates for inoculums in these media.

As a final remark, water supply companies in tropical and developing countries that are planning to implement biological treatment technology for Mn removal in groundwater should consider operational (e.g., filtration velocity) and bioaugmentation strategies to speed up the ripening period of the biofilters.

Chapter 5

Start-up of non-bioaugmented pumice biofilters in flow-through and recirculating flow regime for Mn removal

Graphical abstract



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Abstract

Biofilters are effectively used for drinking water treatment. However, the long ripening time of virgin media for manganese (Mn) removal is a major concern. In this study, the influence of the flow regime on the ripening time of virgin pumice medium was investigated. For this purpose, pilot-scale experiments were performed to compare the start-up of flow-through and recirculating filter columns using inherent inoculation with the same groundwater source. The systems were operated at 2 m/h with gradual flow increments up to 5 m/h and avoiding Fe-loading. Effective Mn removal (>90%) in flow-through and recirculating columns was achieved after eight and 23 days, respectively. Flow-through columns reached compliance with the local drinking water criterion (Mn < 0.1 mg·L-1) at 15 cm filter depth in 11 days. Recirculating filter columns required 32 days to reach compliance at 30 cm depth. The start-up in the recirculation regime resulted in a water consumption reduction of about 50% compared with the flow-through regime. The intermittent provision of the Mn-loading in the recirculating regime impacted the MOB concentration in the pumice stone medium. Both flow regimes required a similar total Mn-loading (0.16 and 0.11 kg·Mn·m^{-2}, respectively), suggesting that Mn-loading was the limiting factor for the ripening of pumice.

Keywords: groundwater, manganese removal, start-up, flow-through, recirculating, pumice media.

5.1 Introduction

Biofiltration is considered a cost-effective and suitable technology for manganese (Mn) removal from groundwater (Marsidi et al., 2018). Compared to conventional physicochemical treatments, a significant advantage of biofiltration is that the use of chemicals is not necessary, representing lower operation and maintenance costs (Cai et al., 2015; Pacini et al., 2014). Mn removal in biofilters involves both biological and physicochemical processes (Breda et al., 2019b; Bruins et al., 2017a). Particularly, Mn removal in a non-coated virgin medium is initiated biologically (Breda et al., 2019b; Bruins et al., 2015a). Mn^{2+} is oxidized by Mn-oxidizing bacteria (MOB) and deposited gradually as Mn oxide ($MnO_{x(s)}$) (Mouchet, 1992). Subsequently, more Mn^{2+} is adsorbed in the $MnO_{x(s)}$ coated filter medium and autocatalytically oxidized (Bruins et al., 2015a; Sahabi et al., 2009). Previous research confirmed that biological Mn^{2+} oxidation occurred mainly at the top of the non-inoculated biofilters during the start-up (Breda et al., 2019b). Finally, the start-up period is considered complete when the biofilter becomes functional in compliance with a drinking water criterion (Breda et al., 2016) or with a high Mn uptake (>90%) (Bruins et al., 2017). However, filter ripening typically continues even when high Mn^{2+} removal efficiencies have already been observed (Breda et al., 2019a; Dangeti et al., 2017).

Bioaugmented methods and inherent inoculation are used to start-up the biofilters. The first ones involve the inoculation of biofilters using a concentrated source of microorganisms, isolated and grown in the laboratory (Ramsay et al., 2018; Zeng et al., 2019), backwash sludge (Cai et al., 2015; Cheng, 2016; Štembal et al., 2004) or matured filter media (Breda et al., 2019b; Bruins et al., 2015b; Zeng et al., 2010), taken from other active Mn biofilters. These methods have been demonstrated to be effective, reporting rapid start-up periods for Mn removal from 2 up to 4 weeks (Breda et al., 2019b; Cai et al., 2015; Štembal et al., 2004; Zeng et al., 2010). The success of these methods relies on the proper selection and acclimatization of the biomass, used as inoculum (Tekerlekopoulou et al., 2013). However, these resources may not be

available, especially in zones where biofiltration technology is just emerging. In such cases, inherent inoculation of non-bioaugmented biofilters by autochthonous (MOB) bacteria present in raw water should be used. However, an important drawback is the long start-up period, required for virgin filter media, to achieve effective Mn removal (Ramsay et al., 2018). Typically, it takes between 1 and 4 months (Bruins et al., 2015b). Bruins *et al*. (2017) affirmed that several factors such as groundwater quality, iron (Fe) loading, type of filter media, and operational parameters (*e.g*. filtration velocity and backwash strategy) seem to influence the start-up of non-bioaugmented biofilters for Mn removal (Bruins et al., 2017a). According to the authors, some strategies could be applied to reduce the ripening time of virgin filter media for Mn removal using inherent inoculation, such as to avoid or previously reduce the presence of other contaminants (*e.g.* Fe) and/or temporarily operating filters at a low filtration velocity.

According to Bruins *et al*. (2015b), the effect of a lower Fe^{2+} concentration in the feed water, combined with appropriate operational conditions (*e.g.* lower filtration velocities and backwash frequency intensity) plays an important role to accelerate the ripening of virgin filter media for Mn removal. In addition, Bruins *et al*. (2017) found that backwashing prolongs the ripening time of a virgin filter, and therefore recommended recirculating part of the filtrate or lowering the filtration velocity to reduce the Fe-loading and subsequent backwashings. Regarding matured filters, Bruins *et at*. (2014a) found from a statistical analysis of 100 drinking water plants, that efficient Mn removal in matured aerated-rapid filters was guaranteed when Fe^{2+} was previously removed or when the Fe-loading per filter run was lower than 2.7 $kgFe \cdot m^{-2}$. Araya-Obando *et al*. (2022) also found that matured rapid sand filters exhibited effective Mn removal during 10 years of operation using an up-flow roughing filter (URG) as Fe pretreatment (removing 68% of the total Fe).

On the other hand, a lower filtration velocity enhances the settling and association of bacterial cells with the media surface (Donlan, 2002). Slow velocities from around 1.5 m/h (Zeng et al., 2010) up to 5 m/h (Breda et al., 2016; Bruins et al., 2017a) are commonly adopted during the start-up, with a constant velocity or gradually increasing

the velocity when the filter effluent reaches the treatment goal (speed-up stage) (Zeng et al., 2019). Other studies have recommended operating the flow-through biofilters at half or one-third of the designed hydraulic load (Li et al., 2005; Ramsay et al., 2018; Štembal et al., 2004). Backwashings with a low flow intensity (<30-35 m/h) (Breda et al., 2016; Bruins et al., 2015b) have been recommended during the start-up to prevent bacteria detachment.

A novel approach, for inherent inoculation, could be to recirculate (between the effluent and influent of biofilter) the flow to improve the effect of inoculation of biofilters. Cheng (2016), in a bioaugmented method, recirculated the supernatant water of the sedimented backwashing sludge through a pilot-scale biofilter for 3 days and repeated it for two more days to improve the effect of inoculation. Then, the filters were operated in flow-through regime at 2 m/h and reached the water quality criteria after 44 days. Subsequently, the velocity was increased to 3, 4 and to 6 m/h. This approach seems promising, as an effective start-up of the degradation of organic compounds in bench-scale (pumice) biofilters by autochthonous microorganisms has been reported (Börnick et al., 2001; Worch et al., 2002). However, to the best of our knowledge, the influence of the recirculating flow regime, using only inherent inoculation, on the ripening time of virgin media for Mn removal, has not been reported.

In previous work with the same groundwater source used in this study, the presence of autochthonous culturable manganese-oxidizing bacteria in raw water was confirmed (Calderón-Tovar et al., 2020). In addition, the feasibility of biofiltration for Mn removal using non-bioaugmented virgin pumice, anthracite and sand media was demonstrated at the bench-scale (Araya-Obando et al., 2021). Although a long start-up period of about 80 days for Mn removal was required for the three materials, it was concluded that pumice has great potential for Mn biofiltration because it is a low-cost, porous, low-density medium and exhibited similar performance compared to sand and anthracite (commonly used in biofiltration). Therefore, to obtain further insight into the efficacy of the start-up of Mn removing of non-bioaugmented biofilters, using groundwater with relatively high temperatures (~23 °C), this study focuses on the influence of the regime

of operation on the ripening time of virgin pumice media. For this purpose, pilot-scale experiments were performed in duplicate to analyze the start-up of flow-through and recirculating gravity filter columns, with low Fe-loading and increasing filtration velocities.

5.2 Materials and methods

5.2.1 Experimental procedure

To study the influence of the flow regime during the start-up for Mn removal of non-bioaugmented biofilters, two experiments were conducted in duplicate. In *experiment 1* (flow-through regime), feed water passed continuously through virgin pumice columns until ripening (Mn removal >90%). In *experiment 2* (recirculating flow regime), feed water was recirculated through biofilter columns until ripening, then the system was switched to a flow-through regime. Both experiments were compared in terms of the start-up period, the Mn^{2+} concentration profiles registered over the depth of filter columns, the Mn-loading, and the water consumption.

5.2.2 Pilot scale setup

A pilot-scale biofilter system (Figure 5-1) was installed at a physical-chemical drinking water plant, located in La Hacienda Condominium, Cartago, Costa Rica (9°50'28"N; 83°58'26"W). Raw water from the well, used for drinking water production, was used to perform the experiments. Because the well was operated intermittently (~12 h/day), a feed raw water tank (T1) of 750 L was installed to continuously provide water to the pilot-scale experiments (24 h/day). During T1 filling, raw water was aerated, changing its redox condition. Besides, a preliminary pilot-scale test indicated that Fe pretreatment was needed (further details in section 5.3.1). Thus, to prevent the effect of Fe-loading on the start-up of the biofilters, a pre-filter using a pressure vessel (Structural FRP, Pentair company) with 60 cm of silica sand (0.8–1.3 mm) filter bed height and 18 cm diameter was installed after T1 (Figure 5-1). The pre-filter was

operated with an empty bed contact time (EBCT) of ~1 min, according to hydraulic tests done before the experiments, to ensure a minimal removal of Mn in the pre-filter. The pre-treated water was pumped to an elevated tank (T2) of 60 L and used as feed water for the experiments. Raw and feed water quality (collected from the groundwater well and at the effluent of T2, respectively) are depicted in Table 5.1. In Costa Rica, maximum Fe and Mn concentration levels (MCL), stipulated in the local regulation, are 0.3 and 0.5 mg·L^{-1}, respectively (Decreto Ejecutivo No.41499-S, 2019). Besides, this regulation sets an acceptability threshold value (AV) for Mn of 0.1 mg·L^{-1}.

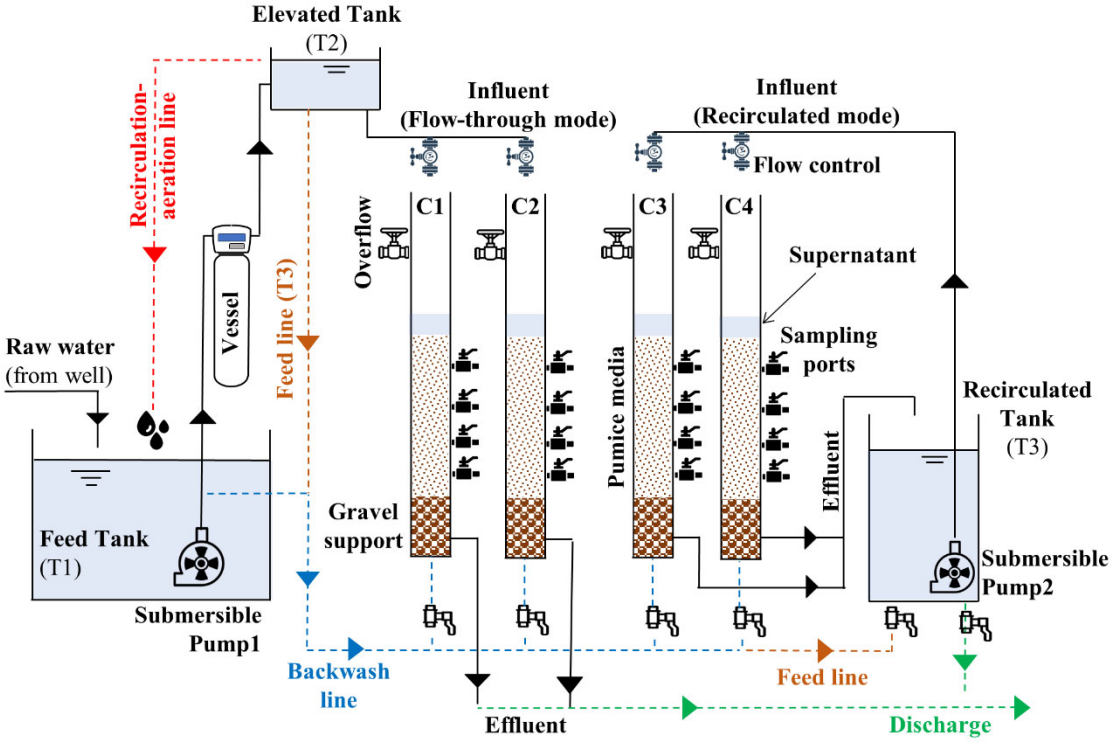


Figure 5-1. Configuration of the pilot-scale unit.

Table 5.1. Chemical composition of the raw and feed water used in the pilot-scale experiments.

Parameter	Unit	Raw water	Feed water
Mn^{2+}	$mg \cdot L^{-1}$	0.55[a] ± 0.05[b]	0.49 ± 0.05
Fe	$mg \cdot L^{-1}$	0.29 ± 0.16	< 0.10[c]
pH	-	7.15 ± 0.23	7.91 ± 0.16
Temperature	°C	22.99 ± 0.56	23.29 ± 0.55
ORP	mV	-21.25 ± 26.15	177 ± 31
DO	$mg \cdot L^{-1}$	0.61 ± 0.35	6.52 ± 0.22

[a] Average, [b] standard deviation, [c] detection limit.

The biofilter columns consisted of four identical PVC columns with a diameter of 10 cm and a filter bed height of 70 cm. Each column was composed of virgin pumice with a grain size between 0.88 and 1.16 mm. To obtain further in-depth information on the start-up of the pilot-scale biofilters, four sampling points were distributed from the top of the media to the bottom at 15, 30, 45, and 70 mm (Figure 5-1). Filter columns were operated as downflow filters. The filtration velocity was controlled in the inlet of the columns (Figure 5-1), using a flowmeter rotameter with valve fit (LZM-6T, Sorekarain company), with a flow range of 100-1000 mL/min. Specifically, two columns were used for the flow-through regime (*experiment 1*) and the other two for the recirculating flow regime (*experiment 2*). In both cases, the start-up period was defined as the ripening time required for the biofilters to achieve Mn removal efficiencies greater than 90% (Bruins et al., 2017a).

5.2.2.1 Flow through regime (experiment 1)

In *experiment 1* feed water from T2 flowed continuously in parallel through column 1 (C1) and column 2 (C2) and the effluents from both columns were discarded. A low filtration velocity of 2 m/h was initially used to promote the attachment of autochthonous bacteria present in the raw water (inherent inoculation). Afterward, a speed-up stage was adopted similar to Zeng *et al.* (2019). Specifically, increments of about 1 m/h were

performed when the Mn removal remains higher than 90%. Once the Mn removal reached 90 % the velocity was increased in steps of about 1 m/h until reaching a final filtration velocity of 5 m/h, keeping the desired removal percentage. In total, the filtration velocities of 2, 3, 4, and 5 m/h were used, resulting in empty bed contact times (EBCT) of 21, 14, 10.5, and 8.4 min, respectively. Besides, an initial supernatant water level of about 2 cm was provided above the filter media, as also used by Gude *et al.* (2018). Backwashing was performed using water from T1 when the supernatant water level rose to 30 cm, using a filter expansion of about 10-20% for ten minutes, similar to the pilot-scale study for Mn removal of Bruins *et al*. (2017a).

5.2.2.2 Recirculating flow regime (experiment 2)

In *experiment 2* (recirculating flow regime), column 3 (C3) and column 4 (C4) were also fed in parallel, but with water from the recirculation tank (T3) (177 L), that was previously filled with feed water from T2. Subsequently, the water from T3 was recirculated at 2 m/h (EBCT of 21 min) through the filter columns until Mn^{2+} concentration in water was not detectable at <0.03 mg L−1. Afterward, feed water in T3 was discarded, repeating this protocol. In practice, feed water was renewed every two to three days. Consequently, feed water in T3 was renewed 9 times in total. Then the columns were switched to flow through regime and the speed-up stage procedure was applied until both columns operated at 5 m/h with an Mn removal higher than 90%. Initial supernatant water level and backwashing procedures were the same as described in *experiment 1*.

5.2.3 Sampling and analytical methods

Raw water and filter column inlet and outlet samples were collected approximately two times a week. Water sampling along the filter bed was conducted weekly. Non-filtered samples were immediately acidified to pH < 2 with nitric acid and stored at 4°C. A preliminary test showed no difference between filtered and non-filtered samples; hence it was assumed that any Mn present was soluble Mn^{2+} (Cooley and Knocke, 2016). Fe

was referred here as total Fe. Mn and Fe concentrations were measured using an Analyst 800 atomic absorption equipment (Perkin Elmer, Waltham, USA), according to the Extraction / Air-Acetylene Flame Method 3111 C (APHA et al., 2005). The detection limits for Fe and Mn were 0.10 $mg \cdot L^{-1}$ and 0.03 $mg \cdot L^{-1}$, respectively. Dissolved oxygen (DO), pH, and oxidation-reduction potential (ORP) measurements were conducted using Hach HQD30 equipment following the methods recommended by the manufacturer (Hach, USA). Isolation of the MOB present in the ripened pumice, plate counts and LBB test analyses were performed according to Araya-Obando *et al*. (2021).

5.2.4 Data analysis

Data analysis was performed in R (R Core Team, 2021). Summary descriptive statistics for all variables were done using *stat.desc* (library *pastecs*). Time series and concentration profiles were done using packages R Graphics.

5.3 Results and discussion

5.3.1 Start-up of filter columns operated in flow-through regime (experiment 1)

Figure 5-2 shows the registered Mn removal efficiencies and the performed filtration velocities during the start-up period of filter columns C1 and C2 operated in a flow-through regime. In eight days, 90% removal of Mn was already reached using filtration velocities of 2 m/h, resulting in a Mn-loading of 0.16 kg·Mn·m^{-2}. Afterward, the filtration velocity was increased up to 3 and 4 m/h on day 8 and 11 of filter operation, respectively. A final filtration velocity of 5 m/h was adopted, which is similar to a typical mid-speed value used in rapid biofilters (Cai et al., 2014). The fact that Mn removal efficacy in the flow-through columns remained constant after eight days of operation, even with the flow increments, indicates effective inoculation of the filters, following Zeng *et al*. (2019).

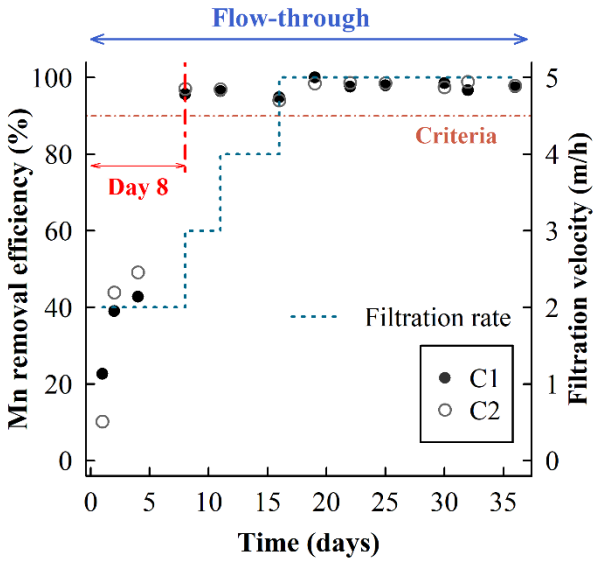


Figure 5-2. Mn^{2+} removal efficiencies and filtration velocities over time in biofilter columns C1 and C2 in flow-through regime.

Blue dotted line: filtration velocity increments. Horizontal dashed line: start-up criterion (>90%).

Figure 5-3 shows the average Mn^{2+} concentration profiles registered over the depth of filter columns C1 and C2, indicating the ripening progress of the pumice media and the most active sections of the biofilters. The profiles show that the flow-through columns reached Mn^{2+} < 0.1 $mg \cdot L^{-1}$ (compliance with the local drinking water criterion) already at 15 cm depth after 11 days of operation. It can therefore be concluded that the ripening of the filters was more rapid than some others reported in the literature (Breda et al., 2019a; Ramsay et al., 2018). For instance, Breda et al. (2019b) studied the start-up of a non-bioaugmented pilot-scale biofilter for Mn removal and reported that virgin sand media required 41 days to achieve Mn^{2+} concentrations below the MCL (0.05 $mg \cdot L^{-1}$). However, the filter columns needed 72 days of filter operation to reach compliance at 30 cm depth.

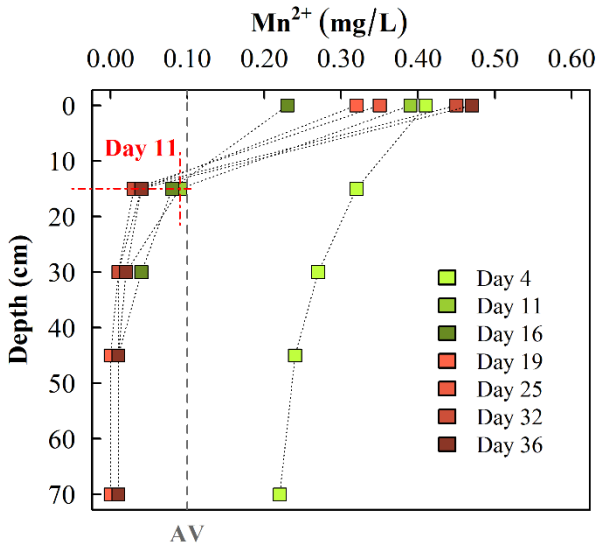


Figure 5-3. Average Mn^{2+} concentration profiles over time in columns C1 and C2 in the flow-through regime.

Vertical dashed line: acceptability threshold value (AV) for Mn stipulated in the local regulation (Decreto Ejecutivo No.41499-S, 2019). Standard deviation of Mn^{2+} concentration lower than 0.03 $mg \cdot L^{-1}$ in all points (error bars not shown).

Figure 5-3 also shows that Mn^{2+} concentration profiles after day 11 remained constant, locating the active zone for effective Mn removal at the top (<15 cm) of the pumice media bed. Inspection of the pumice medium after completion of the experiments

showed dark Mn oxides, mainly deposited in the top 15 cm of the filter bed (see Figure 5-4). Since, non-catalyzed, homogeneous Mn^{2+} oxidation (by oxygen only) is very slow at pH < 9 (Granger et al., 2014) (feed water pH ~7.9, Table 5.1), and biological Mn^{2+} oxidation is supposed to occur in the top of the non-inoculated sand filter during the start-up (Breda et al., 2019b); therefore, it can be concluded that Mn removal by the virgin pumice media was probably dominated by biological processes. This finding is in line with previous studies that have also reported that Mn removal in a non-coated virgin medium was initiated biologically (Breda et al., 2019b; Bruins et al., 2015a).

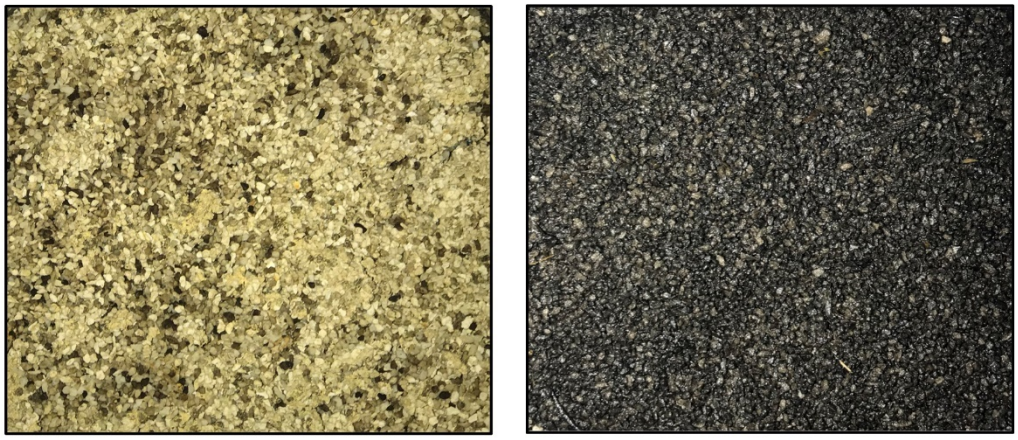


Figure 5-4. Representative photographs of investigated virgin pumice (L) and ripened pumice (R) surfaces at the top of the biofilters.

Virgin filter media typically takes between one and four months to achieve effective Mn removal (Bruins et al., 2015b). Similar to Bruins *et al.* (2015b), the rapid ripening of virgin pumice could be explained by a combination of factors that combined favorable feed water quality and operational conditions. Firstly, the pH and Eh of feed water during the experiments were within the field of action of MOB reported by Mouchet (1992) (Figure 5-5). Besides, as shown in Table 5.1, the average water temperature during experiments was relatively high (around 23.29 ± 0.55 °C). This condition is quite different than the observed in cold winter temperatures (~3–17 °C) where functional oxidizing bacteria typically require a longer start-up to be effectively acclimated (Cai et al., 2014; Ciancio et al., 2020; Evans et al., 2021a; Lauderdale et al., 2016; Pacini et

al., 2014). Secondly, the presence of Fe and the subsequent Fe-loading was avoided using a sand filter as pre-treatment (pressure vessel in Figure 5-1), therefore, backwashing was not required, avoiding detachment of biofilms during operation. In preliminary experiments using the same columns set up and without the sand filter, pumice filter maturation was not reached, even after 130 days of the experiment, probably due to the high Fe loading (average 0.5±0.1 kg·Fe·m^{-2} per filter run) (see supplementary material, section 5.5.1). Previous studies have also found that Fe-loading and more frequent backwashing prolongs the ripening time of virgin media for Mn removal (Bruins et al., 2017a; Bruins et al., 2015b). Finally, the adoption of a low initial filtration velocity of 2 m/h and the applied speed-up stage, suggested by Zeng *et al.* (2019), was appropriate to enhance bacteria attachment. However, in our previous study at a bench-scale using the same feed water and similar temperature, a filtration velocity of 0.45 m/h resulted in 80 days of ripening (Araya-Obando et al., 2021). Thus, it seems that at such lower velocity bacteria attachment depends mainly on cell size and mobility, reducing the association with the material surface (Donlan, 2002).

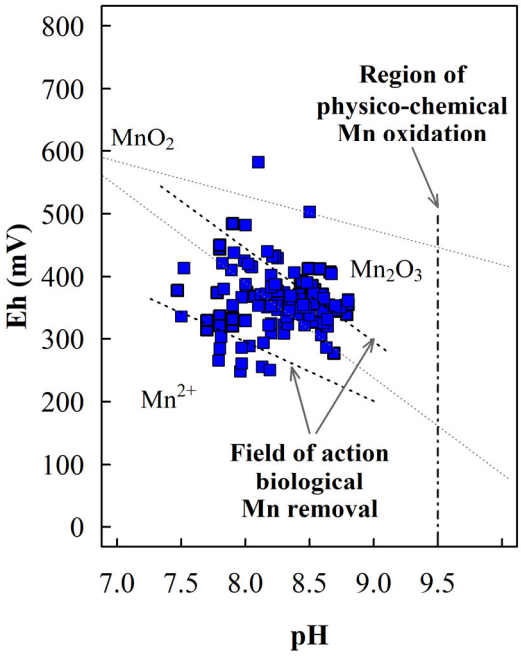


Figure 5-5. Field of action of the MOB in the pH–Eh diagram.
Adapted from Mouchet (1992)

5.3.2 Start-up of filter columns operated in recirculating flow regime (experiment 2)

Figure 5-6 shows the registered Mn removal efficiencies and the filtration velocities performed during the start-up period of filter columns C3 and C4. Inherent inoculation using the recirculating flow regime was started using a constant filtration velocity of 2 m/h to enhance bacteria growth. As can be observed in Figure 5-6, at this filtration velocity the filter columns required a start-up period of 23 days to achieve effective Mn removal (>90%). Afterward, both columns were switched to the flow-through regime on day 33, and only a slightly reduced removal efficiency to approximately 80% during the first two days of filter operation was observed in column C4; but it rapidly recovered above 90%, as observed in column C3 (Figure 5-6). Once the removal efficiencies in both columns remained constant, the filtration velocity was increased to 3, 4, and 5 m/h (on days 40, 47, and 54, respectively), and stable operation was observed (Figure 5-6). From the above it can be concluded that recirculating filter columns required 15 days longer ripening time, compared to the start-up time of the flow-through filter columns, to reach effective removal (>90%). However, compared to earlier studies, the start-up can still be considered rapid, since effective Mn removal with virgin filter media typically takes between one and four months (Bruins et al., 2015b), as was previously mentioned. Similar to the flow-through system, the feed water characteristics (Table 5.1) and the relatively low filtration velocity may have played an important role to accelerate the ripening of virgin pumice.

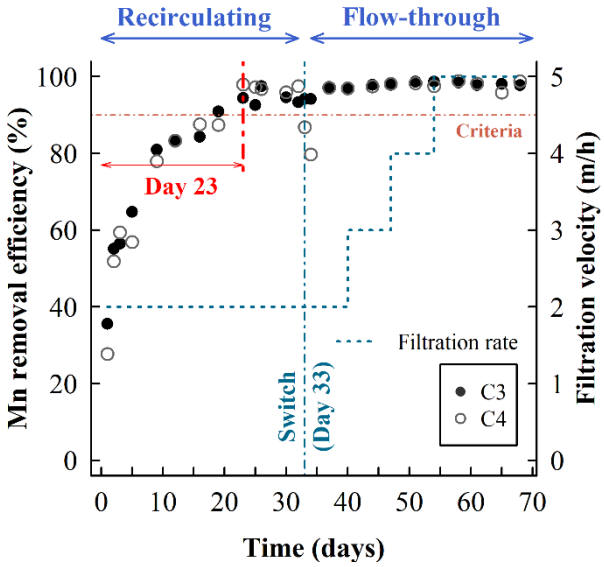


Figure 5-6. Mn^{2+} removal efficiencies and filtration velocities over time in biofilter columns C3 and C4 in recirculating flow regime.

Blue Dotted Line: filtration velocity increments. Horizontal Dashed line: start-up criterion (>90%).

Figure 5-7 shows the average Mn^{2+} concentration profiles registered over the depth of columns C3 and C4, using (a) recirculating and (b) flow-through regimes. As shown in Figure 5-7a, Mn^{2+} < 0.1 $mg \cdot L^{-1}$ (local AV) was reached on day 23 (after start-up). Subsequently, the operation of the filter columns in recirculating flow regime continued for one week (until day 32) to study the development of the ripening of the pumice media. However, after 30 days of operation, effective Mn removal only occurred at a depth of 30 cm (Figure 5-7a). A similar Mn^{2+} concentration profile was reported by Breda *et al*. (2019b) during the start-up of non-inoculated sand filters.

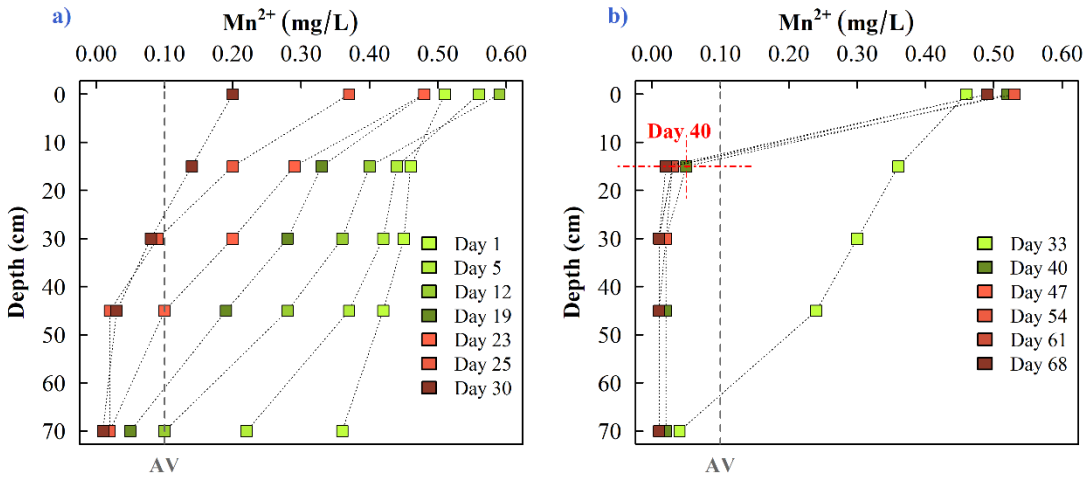


Figure 5-7. Average Mn^{2+} concentration profiles over time in columns C3 and C4 in (a) recirculating-flow and (b) after switching to flow-through regime.

Vertical dashed line: acceptability threshold value (AV) for Mn stipulated in the local regulation (Decreto Ejecutivo No.41499-S, 2019). Standard deviation of Mn^{2+} concentration lower than 0.03 mg·L^{-1} in all points (error bars not shown).

As seen in Figure 5-7b, immediately after filter columns were switched to the flow-through regime (day 33) the active Mn removal zone was in the first 15 cm of filter bed depth. As is shown in Figure 5-6, column C4 also reduced removal efficiencies up to about 80% but recovered the higher Mn capacity uptake in only two days. This finding suggests that the biologically active zone in the filter columns required a short time of acclimatization to the new flow regime conditions (see section 5.3.3). Full ripening, and thus effective Mn removal at 15 cm depth, was finally registered on day 40. Besides, the speed-up stage carried out after day 40 (Figure 5-6), did not affect the Mn^{2+} concentration profiles patterns, indicating that the virgin pumice media was also fully inoculated with the autochthonous groundwater bacteria, following Zeng *et al*. (2019).

5.3.3 Comparison of the start-up of the filter columns in flow-through and recirculating flow regime

During the start-up period in the flow-through regime (Figure 5-2) the Mn^{2+} concentration was always around 0.50 $mg \cdot L^{-1}$. In contrast, in the recirculating experiment, the average initial Mn^{2+} concentration in T3 was around 0.52 $mg \cdot L^{-1}$ but was typically reduced to less than 0.1 $mg \cdot L^{-1}$ in 7-8 hours (see supplementary material, section 5.5.2). The water replacement was conducted two times a week, therefore, during 48-64 h of operation, the recirculated water did not contain Mn^{2+}. Therefore, the flow-through columns, through the experiment, registered a Mn-loading of about 0.16 kg $Mn \cdot m^{-2}$ in total (until day 8 of operation, >90% removal). During the recirculating flow experiment, the columns had a total Mn-loading of 0.11 $kg \cdot Mn \cdot m^{-2}$ to reach the 23 days of ripening time, comparable to the loading of the flow-through columns. The difference between both experiments was mainly associated with the intermittent provision of the Mn-loading in the recirculating regime in contrast with the continuous Mn loading in the flow-through regime. It suggests that the total Mn-loading, in this case, 0.11 $kgMn \cdot m^{-2}$, was a limiting factor during the start-up of the non-bioaugmented Mn biofilters. Furthermore, the microbiological analysis showed that during flow-through experiments MOB count was 1.9 x 10^6 $CFUg^{-1}$, whereas in the recirculating regime the MOB concentration was 4.0 x 10^3 $CFUg^{-1}$ and was reduced to 2.9 x 10^3 after switching to the flow-through regime. It seems that the intermittent Mn loading impacted the MOB population in the recirculated biofilters, and probably slowed down the acclimation time in this system.

Comparing the Mn^{2+} concentration profiles (Figure 5-3 and Figure 5-7), it can be observed that flow-through columns reached Mn^{2+} <0.1 mg/L (compliance with the local drinking water criterion) at 15 cm depth in only 11 day, while recirculating filter columns required 32 days to reach Mn^{2+} < 0.1 mg/L at 30 cm depth. Usually, filter ripening continues even when high Mn removal efficiencies have already been observed (Breda et al., 2019a; Dangeti et al., 2017). Under the present conditions, inherent inoculation using a recirculating flow regime required more time for ripening than a flow-through

regime. However, an advantage of the recirculating system is that it consumed less feed water. During the first eight days of filter operation, the flow-through column registered a water consumption of 3.19 m^3, whereas the recirculating column consumed only 1.59 m^3 in the 23 days of filter operation (see estimations supplementary material, section 5.5.3). It represents a water consumption reduction of approximately 50%, thus making it a suitable strategy for groundwater applications, where spillage of water should be minimized. However, there are still chances to improve the performance of the recirculating system. For example, Worch *et al.* (2002) applied the recirculating approach for the biodegradation of synthetic organic chemicals (SOCs) and found that the adaptation of microorganisms depended on the contact time with the SOCs. Thus, the authors recommended stimulating the microorganisms by maintaining the SOCs concentration in the recirculated water. In that sense, a more frequent refreshment of water in T3 (e.g. 1.5 or 2 days) and/or even supplementing dissolved Mn^{2+} into the recirculated water could help to reduce the ripening time in the recirculating system.

5.4 Conclusions

This study aimed to investigate the influence of the flow regime (flow-through and recirculating) on the ripening time of virgin pumice media in non-bioaugmented biofilters. For this purpose, pilot-scale experiments using the same tropical groundwater (~23 °C) were performed to compare the start-up of flow-through and recirculating filter columns. The flow-through filter columns exhibited an Mn removal above 90% after eight days of filter operation and reached Mn <0.1 mg/L (compliance with the local drinking water criterion), whereas the recirculating filter columns registered a start-up of 23 days. Results suggest that inherent inoculation using recirculating mode was less effective than using a flow-through regime in terms of ripening time. However, an advantage of this operational strategy was that it consumed less feed water during the start-up (approximately 50%) than using a flow-through regime, making it still a suitable strategy for groundwater applications, where spillage of water should be minimized.

The start-up of the non-bioaugmented biofilters in both flow regimes was reached under comparable conditions, using the same feed water and adopting an initial low filtration velocity of 2 m/h. Both the flow-through and the recirculating flow regime required a similar total Mn-loading (0.16 and 0.11 $kg \cdot Mn \cdot m^{-2}$, respectively). In that sense, differences between the start-up periods through both regime flows could be attributed to the intermittent Mn-loading of the recirculating system. It seems that the intermittent Mn-loading impacted the MOB populations formed in the pumice stone medium during the start-up of biofilters operated in recirculating flow regime. It was therefore concluded that the total Mn-loading, in this case, 0.11 $kg \cdot Mn \cdot m^{-2}$, was a limiting factor during the start-up of the non-bioaugmented Mn biofilters.

5.5 Supplementary materials

5.5.1 Preliminary results with Fe-loading

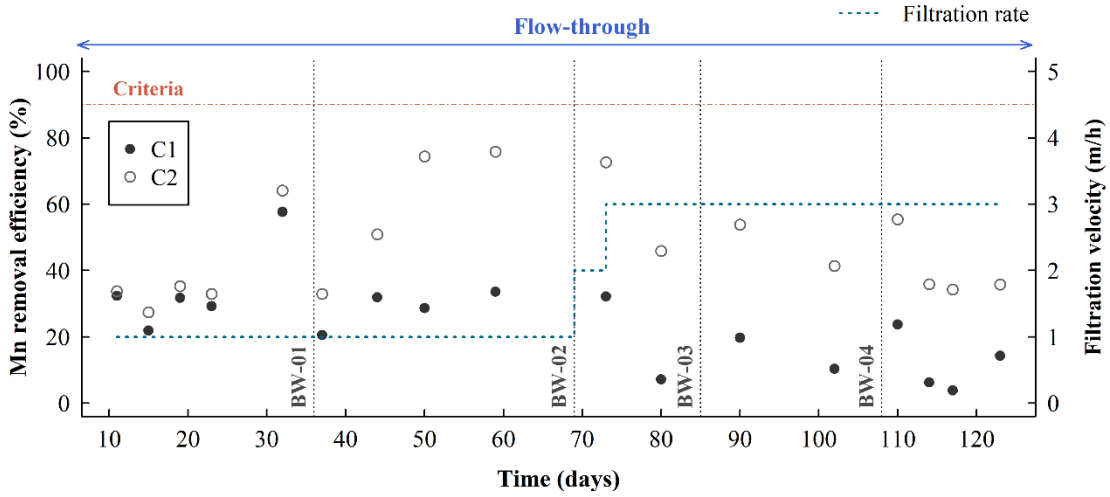


Figure 5-8. Mn removal efficiencies and filtration velocities over time in filter columns C1 and C2 in flow-through regime (average Fe-loading 0.5 ± 0.1 kg·Fe·m^{-2} per filter run).

Blue dotted Line: filtration velocity increments. Horizontal dashed line: start-up criterion (>90%). BW: backwash.

5.5.2 Mn concentrations decay over the time in T3 after filling.

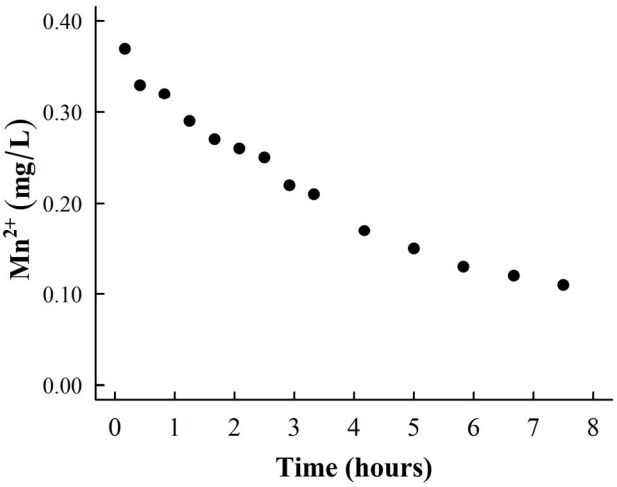


Figure 5-9. Mn^{2+} concentrations decay in T3 registered after filling for recirculating on day 26 of filter operation.

5.5.3 Water consumption estimation during the start-up of flow-through and recirculating filter columns.

Table 5.2. Estimations per filter column of water consumption operating in flow-through regime (*experiment 1)*

Filtration velocity (m/h)	Flow (m^3/h)	Daily water consumption (m^3)	Operation time (h)	Total water consumption (m^3)
2	**1.6 E-02**	**0.38**	**203**	**3.19**[a]
3	2.4 E-02	0.57	72	1.70
4	3.1 E-02	0.75	120	3.77
5	3.9 E-02	0.94	479	18.81

[a] *Start-up (Mn removal efficiencies >90%)*

Table 5.3. Estimations per filter column of water consumption operating in recirculating flow regime at 2 m/h (*experiment 2*)

Filter run	Operation time per filter run (h)	Accumulated water consumption (m^3) [a]
1	24	0.18
2	24	0.35
3	24	0.53
4	48	0.71
5	96	0.89
6	72	1.06
7	96	1.24
8	72	1.42
9	**96**	**1.59**[b]
10	48	1.77
11	24	1.95
12	96	2.12
13	48	2.30
14	24	2.48

[a] *Renewed water volume per filter run = 177 L,* [b] *start-up (Mn removal efficiencies >90%)*

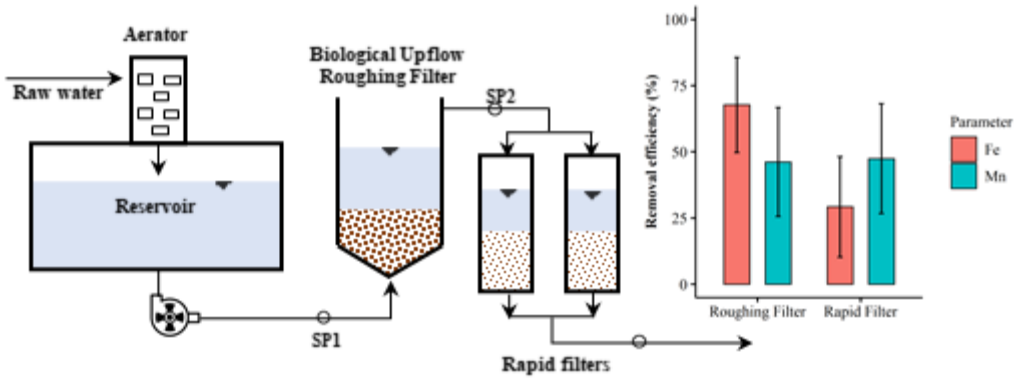
Table 5.4. Comparison of the water consumption required by flow-through and recirculating columns to reach effective Mn removal (<90%)

Id	Regime flow	Total water consumption (m^3)	Difference (%)
Experiment 1	Flow-through	3.19	50.04%
Experiment 2	Recirculating	1.59	

Chapter 6

Long-term monitoring of Mn and Fe removal in biofilters from a converted plant.

Graphical abstract



A main part of this chapter was published as:

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Abstract

Conversion from physicochemical to biological treatment in water treatment plants has received increased attention due to the potential benefits of biofiltration. However, information is limited about the operational performance of converted water treatment plants for iron (Fe) and manganese (Mn) removal over a long-term period. In this study, Fe and Mn removal from biofilters was investigated in a converted plant from November 2011 until April 2021. The plant comprises an aeration unit followed by a modified up-flow roughing filter (URF) and by two rapid sand filters (RSF) in parallel. Data analysis was based on Fe and Mn concentrations collected from 222 water analysis reports. Results showed that 98% of Fe and 95% of Mn concentrations in treated water were below the local regulatory limits of Fe < 0.2 mg/L and Mn<0.1 mg/L. Fe was mainly removed in the URF (68%), and Mn was removed nearly equally by the URF (46%) and the RSF (47%). The combination of the URF with RSF produced total Fe and Mn removal efficiencies of 95% and 88%, respectively. No significant differences between the seasonal variation of Fe and Mn concentrations in the URG and RSF were found. The effectiveness of biofiltration technology without the use of chemical reagents or nutrient substrate additions was demonstrated over a long-term period of monitoring.

Keywords: biofilter performance, converted plant, groundwater, long-term monitoring, iron, manganese.

6.1 Introduction

Removal of iron (Fe) and manganese (Mn) from groundwater using biological filtration is considered suitable and cost-effective (Bruins, 2016). This method has been commonly used in Europe, North America (Ramsay et al., 2018), and Argentina (Pacini et al., 2014). However, little is known about the biological removal of contaminants from water resources in some developing countries (Abu-Hasan et al., 2020). Recent studies have just confirmed the presence of autochthonous culturable manganese-oxidizing bacteria (MOB) and the feasibility of using biofiltration for Mn removal from groundwater sources under tropical conditions (Araya-Obando et al., 2021; Calderón-Tovar et al., 2020). Biological filtration has some advantages over conventional treatment processes. Biofilters have minimal operation and maintenance requirements, do not require chemicals, and may reduce disinfection byproducts (DBP), for example (Evans et al., 2021). Thus, full-scale biofiltration conversion from conventional filtration systems has begun to receive increased attention (Bassett et al., 2019).

The first descriptions of the conversion of about 20 conventional treatment plants in France to biological processes date from the early 1990s (Mouchet, 1992). The strategies used then involved media replacement, pH optimization, changes in the aeration conditions, and eliminating prechlorination or any reagents added at the head of the treatment line, representing an operational cost reduction of about 50–80%. Another study describing the conversion of an existing plant for Fe and Mn removal from physicochemical to biological processes was reported by Pacini *et al.* (2014). That study was carried out in the water treatment plant (WTP) at Las Toscas, Argentina, in October 2011 and included the following modifications: aeration of raw water using a perforated tray aerator with plastic rings, conversion of an existing circular settler in an up-flow roughing filter (URF) and eliminating dosing chemical reagents. Similar to Mouchet (1992) study, operating costs were reduced by 60%. Furthermore, conversion from conventional filtration to biofiltration and removing prechlorination is increasingly

being used in surface water treatment plants throughout the U.S. to enhance the removal of organic and inorganic constituents (Brown, 2020).

Short-term water quality deterioration (*e.g.*, Mn release, turbidity breakthrough) and operational/hydraulic challenges can be present during the start-up of the converted plants (Brown, 2020). Moreover, it is well-known that the long ripening time of virgin filter media to achieve very effective manganese removal is a major concern (Breda et al., 2019b; Bruins, 2016). Therefore, it is critical to have proper planning and evaluation of the biofilter conversion strategies during the early stages of operation to avoid unintended consequences affecting the overall plant (Brown, 2020). Recently, nutrients (phosphorus)/pH and substrate augmentation strategies have been demonstrated to be effective means to improve Mn control during the conversion to biofiltration (Lauderdale et al., 2016).

Once an acclimated biofilter has reached steady-state contaminant removal, many factors can impact its performance, such as variations in influent water quality, water temperatures, operational parameters, and biofouling (Brown, 2020). Thus, long-term monitoring of converted biofilters is essential to evaluate their performance in case of variations in raw water quality and operational and hydraulic changes over time. Additionally, the monitoring can provide important evidence about seasonal impacts, for example, if cold groundwater temperatures have a negative impact on the Fe and Mn removal of full-scale converted biofilters. In general, a noticeable gap exists in the literature about the operational performance of converted water treatment plants for Fe and Mn removal over a long-term period.

This study is a continuation of previous studies conducted in the Las Toscas WTP in Argentina. The operation of the converted plant began on October 31, 2011. As previously mentioned, Pacini *et al*. (2014) first studied the transformation from a physicochemical to a biofiltration process. That study included pilot-scale experiments, details about the design and construction of modifications, and monitoring of the start-up period of biofilters during the first 20 months of the converted plant operation. The

following work performed by Piazza *et al*. (2019) confirmed the presence of several culture Mn-oxidizing bacteria (MOB) with Mn^{2+} oxidation and biofilm formation capacities in matured filter media collected from the biofilters of Las Toscas WTP after seven years of operation. The aim of this study was to demonstrate the long-term feasibility of biological filters for Fe and Mn removal in a converted physicochemical plant. For this purpose, monitoring data from November 2011 until April 2021 from the converted physicochemical plant Las Toscas located in Santa Fe, Argentina, was evaluated. Statistical comparisons were also included to determine seasonal variations in Fe and Mn concentrations at the influents and effluents of the biofiltration units.

6.2 Methods

6.2.1 Description of the converted water treatment plant

Las Toscas WTP is located in Santa Fe, Argentina. The source water is groundwater from 10 wells. The treatment line (Figure 6-1) starts with a perforated tray aerator with plastic rings that was installed during the conversion in 2011, followed by the raw water tank of 250 m^3. Subsequently, the aerated water is pumped to the biological filtration systems composed of an up-flow gravel roughing filter (URF) (old circular settler) and two pressure rapid sand filters (RSF) configured in parallel. The URF is composed of gravel (6–12 mm size) with a filter bed height of 1 m, whereas the RSF are composed of a 1m layer of sand (1.10–2.20 mm size). Finally, the treated water is stored in a reservoir for its subsequent chlorination and distribution. The operational parameters of these filtration units are shown in Table 6.1. Note that total water production is typically reduced from 150 to 100 m^3/h in winter. Subsequently, filtration rates and empty bed contact times (EBCT) are different during winter compared with the other seasons. In addition, a summary of the upgrades, with three main events, in Las Toscas WTP during the period of study (November 2011 until April 2021) is shown in Table 6.2. The reduction of the aerated water reservoir (*event 1*) was made to increase the volume of treated water. Meanwhile, the reasons why *events* 2 and 3 were implemented will be discussed in section 6.3.2.

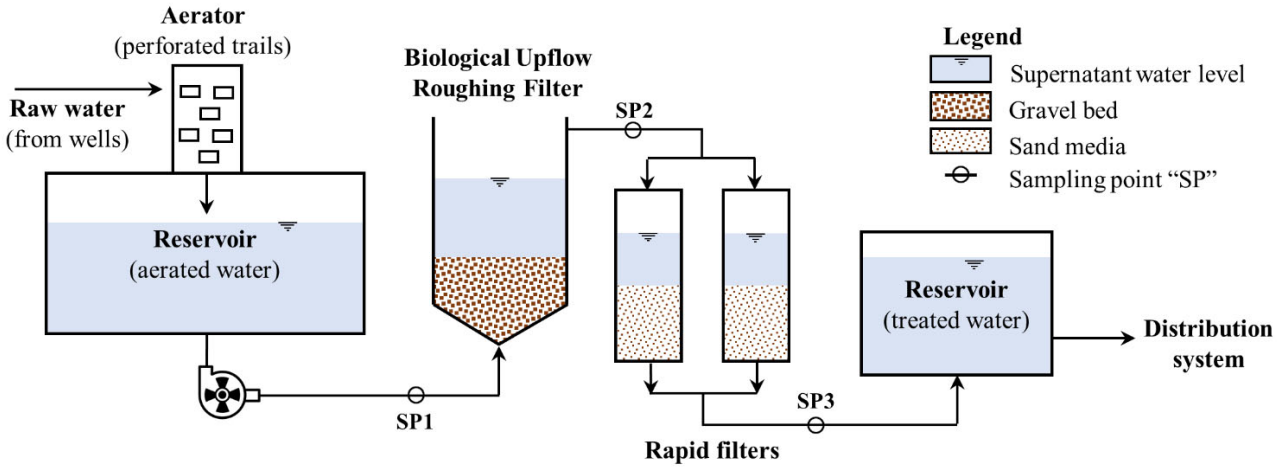

Figure 6-1. Diagram of the water treatment process in Las Toscas WTP, Santa Fe, Argentina.

SP: sampling points.

Table 6.1. Operational parameters of the filtration units of Las Toscas water treatment plant

Id	Parameter	Units	URF		RSF	
			Summer/ Autumn/Spring	Winter	Summer/ Autumn/Spring	Winter
1	Flow per unit	m^3/h	150	100	75	50
2	Filtration rate	m/h	5	4	12	8
3	Empty bed contact time	min	17	11	7	5
4	Backwash criterion	-	head loss	head loss	head loss	head loss
5	Backwash frequency	n/week	~ 1	~ 1	~ 2	~ 2

Table 6.2. Summary of upgrades made in Las Toscas WTP from November 2011 to April 2021.

Id	Date	Description	Reference
start-up	August 2011 to November 2011	Aeration of raw water using a perforated tray aerator with plastic rings, conversion of an existing circular settler to an up-flow roughing filter, and elimination of dosing chemical reagents.	(Pacini et al., 2014)
event 1	November 2013	Reduction of the aerated water reservoir (Figure 6-1) from 250 m^3 to 30 m^3 including an internal wall.	(N. Zanier, personal communication, June 30, 2021)
event 2	April 2017	Removing internal wall on the aerated water reservoir (reverting to 250 m^3).	
event 3	August 2020 to November 2020	URF upgrades: granular filter media were removed for washing and put back, a manifold was installed at the bottom of the filter to distribute influent water uniformly through the granular media, and a compressed air cycle for backwashing was added. The backwash criterion and frequency were the same as described in Table 6.1.	

6.2.2 Sampling and analytical methods

As shown in Figure 6-1, the sampling points considered in this study comprised the water samples collected monthly from the URF influent (*SP1-URF$_i$*), the URF effluent (*SP2-URF$_e$*), and the RSF effluent (*SP3-RSF$_e$*). In addition, the main operational records were reviewed by interviewing key employees of the WTP. Data included turbidity, and total Fe and Mn concentrations collected from 222 water analysis reports from November 2011 to April 2021. Mn and Fe were determined using a DR 2700 spectrophotometer (Hach, Loveland, CO, USA) following the phenanthroline and the 1-(2-Pyridylazo)-2-Naphthol PAN methods, respectively. Turbidity was measured using a 2100P portable turbidimeter (Hach, USA). The manufacturer's instructions were followed in all three tests.

6.2.3 Data analysis

Data analysis was performed in R (R Core Team, 2021). Summary descriptive statistics for all variables were done using *stat.desc* from the *pastecs* library. Time series and boxplot of Fe and Mn concentrations were done using the R graphics package. Cumulative frequency distribution and density plots were made using the *DescTools* package to know the summary of data frequency below maximum contaminant levels (MCL) stipulated in local regulation (EnReSS, 1994) and the distribution of the numeric variables, respectively. In addition, R was used to determine statistically significant differences between the seasonal variation of Fe and Mn concentrations in the URG and RSF. A statistical *p*-value less than 0.05 was considered to be significant. For this purpose, the *Fligner–Killeen* test (*Stats* Package) and *Levene's* test (*DescTools* package) were first performed to check the homogeneity of variance across groups (i.e., seasonal Fe and Mn concentrations). In addition, a test of normality of these data was done with the *Shapiro–Wilk* (n<50) (*Stats* Package) and *Lilliefors (Kolmogorov–Smirnov) normality* tests (n>50) (*Nortest* package). Considering the non-normality test results and that data were generally highly skewed to the right, a nonparametric approach was adopted using the *Kruskal–Wallis rank sum* test (*Stats* Package).

Subsequently, median and interquartile range (IQR) were used for the description of non-normally distributed data (Habibzadeh, 2017). Finally, as the groups had unequal numbers of observations, Dunn's test was used for post hoc pairwise multiple comparisons (*DescTools* package).

6.3 Results and discussion

6.3.1 Water quality reflecting the general performance of Las Toscas WTP

Water temperature in *SP1-URF$_i$* (which represents the aerated water pumped from the reservoir), ranged from around 15 °C (winter) up to 30 °C (summer), and the pH varied between 6.6 and 7.3 and remained within the same range in the filtration units. Pacini *et al*. (2014) reported that the dissolved oxygen concentration in this sampling point fluctuated in a range from 2.6 up to 5.3 mg/L depending on the water level in the reservoir. The authors also reported that the redox potential varied between 230 and 260 mV. Table 6.3 shows the Fe, Mn, and turbidity results for the 10 years of the operation monitoring period. *SP1-URF$_i$* showed a lower variation of Fe and Mn concentrations. Moreover, the median Fe concentration is higher than that of Mn. It can be also seen that a substantial reduction of Fe concentrations occurred between *SP1-URF$_i$* and *SP2-URF$_e$*, indicating that Fe was mainly removed in the URF. Clearly, the URF played a role in pretreatment reducing Fe and Mn concentrations in *SP2-URF$_e$* (which represents the RSF influent), thus facilitating the performance of the RSF. In addition, results in *SP3-RSF$_e$* did not exceed the maximum contaminant levels (MCL) stipulated in the local regulation (EnReSS, 1994). Thus, the converted biological filtration system of Las Toscas WTP effectively removed these parameters during the period of study. Specifically, around 98% and 95% of all Fe and Mn concentrations, respectively, were below the MCL. Similar to the metals, turbidity was partially reduced in the URF and subsequently in the RSF. Moreover, turbidity in the treated water never exceeded the MCL of 2.0 NTU and just 6 % of the data did not meet the recommended value of 0.5 NTU called for in the local regulations (EnReSS, 1994).

Table 6.3. Average values of Fe and Mn concentrations and turbidity in Las Toscas WTP

Parameter	Sampling point			MCL
	SP1-URF_i	SP2-URF_e	SP3-RSF_e	
Fe (mg/L)	1.11[a]±0.45[b]	0.34±0.29	0.03±0.04	0.2
Mn (mg/L)	0.33 ±0.06	0.21 ±0.14	0.03 ±0.03	0.1
Turbidity (NTU)	1.10±1.18	0.53±0.32	0.27±0.13	2.0

[a] median, [b] Interquartile range [IQR]

Figure 6-2 shows a comparison of how efficiently the entire treatment process was working for the two metals. To identify the contribution of each filter, removal efficiencies of Fe and Mn in the URF and RSF were estimated considering the influent as the values at the head of the treatment line (*SP1-URF_i*). As shown in Figure 6-2, around 68% of the Fe was removed in the URF, whereas the remaining 29% was removed in the RSF. In comparison, Mn removal efficiencies were quite similar through the filtration units. About 46% and 47% of Mn were removed in the URF and RSF, respectively. Moreover, the combination of URF with RSF produced total Fe and Mn removal efficiencies of nearly 96% and 88%, respectively. Similar removal percentages have been reported in different double filtration systems. For example, Pacini *et al*. (2005) reported in a pilot system that combined URF with RSF, total removal efficiencies of 94% for Fe and 92% for Mn. In the same study but combining URF with slow sand filtration (SSF), the total removal efficiencies were around 95% for Fe and 88% for Mn. Sánchez & Burbano (2006) reported in a full-scale URF combined with SSF, total removal efficiencies of 92% and 89% for Fe and Mn, respectively. Breda *et al*. (2016) reported that two full-scale rapid filters in series removed all Fe and 90% of the Mn in the first filter after the start-up period. Different mechanisms (physicochemical and biological) may have contributed to Fe and Mn removal in mature biofilters. Piazza *et al*. (2019) found that the URF and RSF in Las Toscas WTP were still biologically active after seven years of operation. According to Mouchet (1992), the biocatalytic process performed by bacteria provokes Fe and Mn biogenic oxides. Subsequently, its

autocatalytic properties contribute to maintaining the functionality of the mature media over time (Breda et al., 2019a; Bruins et al., 2015a).

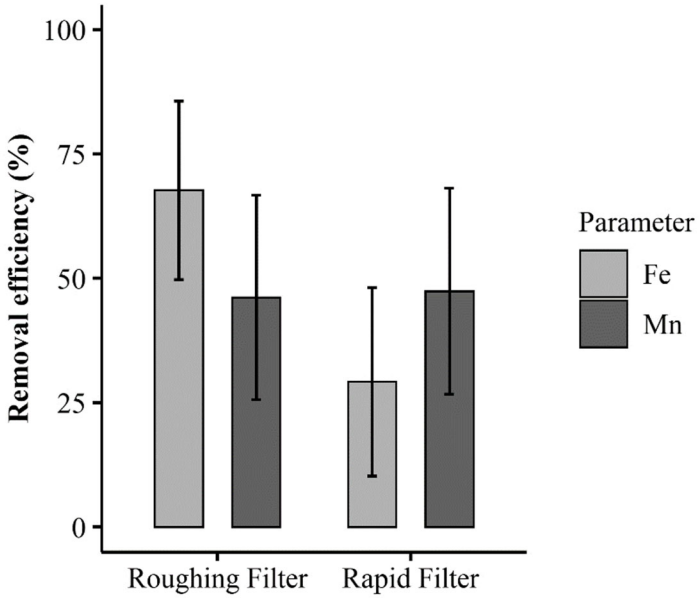

Figure 6-2. Average percentage removal of Fe and Mn in the filtration units of Las Toscas WTP from November 2011 until April 2021.

6.3.2 Biofilters long-term performance

Figure 6-3 shows the variation of Fe and Mn concentrations over time in the sampling points. Vertical dotted lines denote dates of upgrades that were made in the converted water treatment plant during the period of study (see Table 6.2). During the first two years of operation, concentrations detected in *SP1-URF*$_i$ ranged around 0.65-1.5 mg/L and 0.25-0.50 mg/L for Fe and Mn, respectively. Additionally, *SP2-URF*$_e$ reached steady-state conditions for Fe and Mn removal after ~11 months of operation. Concentrations of Fe in treated water (*SP3-RSF*$_e$) were below the MCL 24 h after conversion and Mn required just 15 days to reach the same result. This rapid start-up of RSF was probably due to the original aged coated-sand not being replaced during the conversion (Pacini et al., 2014). Moreover, Mn concentrations in SP3-RSFe did not exceed Mn concentrations (Figure 3b) during the first months of operation. Therefore,

there was no evidence of Mn release from the original Mn-coated filtration media. It is well-known that the removal of a preoxidant can result in the release of Mn^{2+} (Bassett et al., 2019; Brown, 2020).

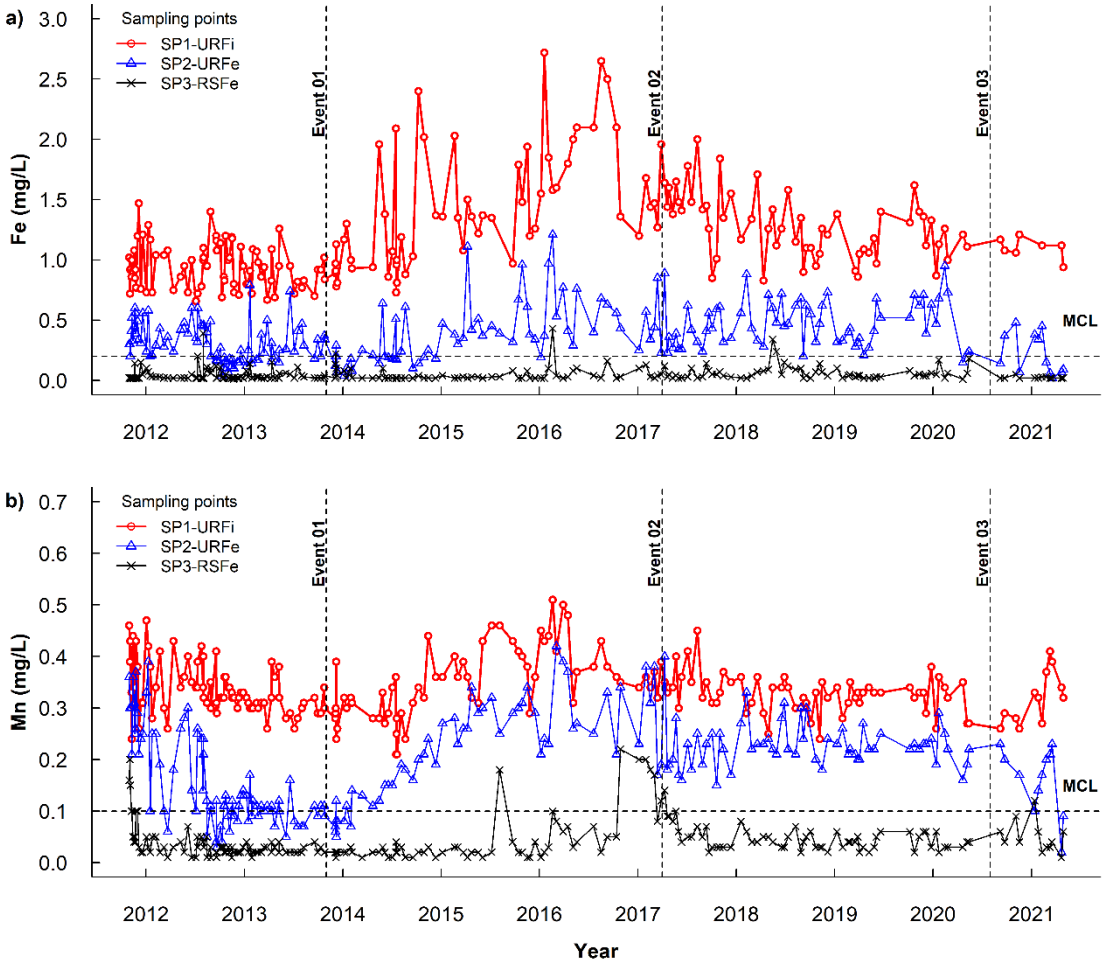

Figure 6-3. Variation of Fe and Mn concentrations at the influent and effluent of the filtration units from November 2011 to April 2021.

After the reduction of the aerated water reservoir from 250 m^3 to 30 m^3 (*event 1*), Fe concentrations in *SP1-URF$_i$* (Figure 6-3a) increased from around 1.0 up to 2.5 mg/L. This result may be explained by the reduction of the hydraulic residence time of the aerated water reservoir by about 88%, with, therefore, more iron sludge accumulating in the tank and, due to inefficient cleaning processes, precipitates passing to the filters.

Similarly, Mn concentrations increased from around 0.3 up to 0.5 mg/L in *SP1-URF$_i$* (Figure 6-3b). Probably, the increase of Mn was less noticeable than Fe because the abiotic oxidation of Mn^{2+} by oxygen is very slow at pH values below 9 (Buamah et al., 2009). Subsequently, the higher Fe loading seems to have a negative effect on Fe and Mn removal in the URF. Notably, Mn concentrations in *SP2-URF$_e$* gradually increased from around 0.1 up to 0.4 mg/L. It has been reported that Fe concentrations in the feed water and Fe loading were found to be inversely proportional to Mn removal (Bruins et al., 2014). Despite this, URF is appropriate as a pretreatment given its high solid-retention capability (Pacini et al., 2005). Thus, this phenomenon did not affect the Fe and Mn concentrations in treated water (*SP3-RSF$_e$*) and the MCL were met. The main exception was of Mn at the end of 2017; however, no explanation was found for this in the historic operational reports.

As was described in Table 6.2, the aerated water reservoir was restored to its original capacity of 250 m^3 in April 2017 (*event 2*). It can be seen from Figure 6-3 that after that, Fe and Mn concentrations in *SP1-URF$_i$* decreased, as was expected. Fe concentrations in *SP2-URF$_e$* (Figure 6-3a) did not show variations from their behavior before *event 2*. Moreover, *SP2-URF$_e$* showed more stable behavior (Figure 6-3b) even though it exhibited less Mn capacity uptake compared with the first two years of operation. The upgraded strategy in 2017 did not consider that excess biomass accumulation and inorganic particles in biofilters provoke long-term fouling of media and filter underdrains (Brown, 2020). Thus, the backwash system was not improved. Backwashing with water alone is a weak cleaning process due to the limited abrasion and collisions between fluidized particles (Amirtharajah, 1993). If the backwashing is ineffective, excessive inorganic particles increase head loss and affect water quality (Amirtharajah, 1993). To overcome these shortcomings, in *event 3*, a new backwashing strategy using a compressed air scour cycle was implemented together with manifold upgrades to improve the fluidization of the filter media. The discharge was reduced by up to 75% after the upgrade. In fact, from Figure 6-3, it can be seen that Fe concentrations in *SP2-URF$_e$* decreased in the subsequent months. Moreover, some values of Mn in *SP2-URF$_e$* were around MCL. Remarkably, treated water in RSF

(*SP3-RSF$_e$*) showed steady-state operation for Fe and Mn removal below MCL during the period of study. Results confirmed the relevance of URF as pretreatment and RSF as a polishing step during the simultaneous removal of Fe and Mn in Las Toscas WTP.

6.3.3 Seasonal effect on Fe and Mn concentrations

The location of Las Toscas WTP results in typical seasonal temperature variations of 21-32 °C (summer), 17-26 °C (autumn), 11-21 °C (winter), and 16-27 °C (spring) (WeatherSpark.com., 2020). According to data collected from 2012, these variations resulted in water temperatures in SP1-URFi of 25-30°C (summer), 22-25°C (autumn), 15-19°C (winter), and 19-26°C (spring).

Figure 6-4 shows seasonal Fe and Mn concentrations at the sampling points. Data include ripening and steady-state conditions of the URF and RSF. Although the groundwater source at Las Toscas WTP was from 10 wells and despite the changes in water temperatures, no seasonal variations of Fe and Mn concentrations were observed in the raw water (before aeration). Similar behavior was shown by URF influent *SP1-URF$_i$*, suggesting a constant performance of the aeration step. In some cases, groundwater sources have shown important seasonal variations in Fe and Mn concentrations (Li et al., 2005). Seasonal long-term variations in Mn concentration are common at surface water sources (Hoyland et al., 2014). Figure 6-4 shows similar median values between seasons, in all sampling points of the influents and effluents of the filters, indicating that the seasonal effect was probably negligible (for the operational parameters shown in Table 6.1). Further statistical tests (section 6.2.3) confirmed no significant differences between the seasons and Fe and Mn concentrations were found. Moreover, it can be seen from Figure 6-4 that the performance of Las Toscas WTP was not affected during winter where the water total production is typically reduced and the operational parameters were different (Table 6.1).

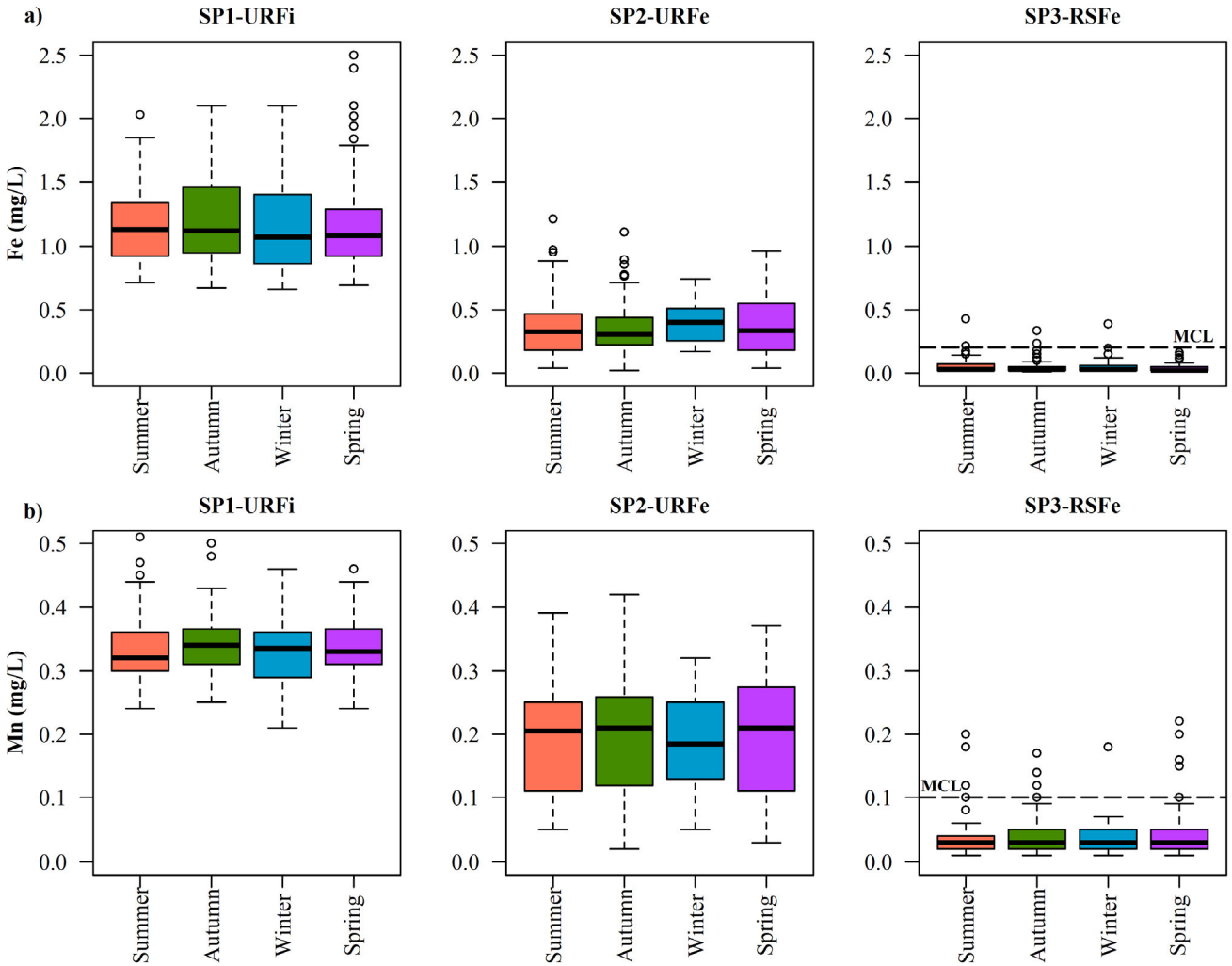

Figure 6-4. Seasonal variation of Fe and Mn concentrations in Las Toscas WTP from November 2011 to April 2021.

Several studies have demonstrated that cold winter temperatures (~3–17°C) cause a long start-up of biofilters (Cai et al., 2014; Ciancio et al., 2020; Evans et al., 2021; Lauderdale et al., 2016; Pacini et al., 2014). Furthermore, Pacini *et al.* (2014) found during the first 20 months of operation of Las Toscas WTP a noticeable decrease in the removal efficiency of Fe and Mn in the URF during winter (~7–17°C). However, evidence from the present report suggested that this effect was gradually dissipated during the years of operation, probably by the development of new bacterial communities in the filter media capable of surviving at winter water temperatures (15°C). In the case of the RSF, seasonal temperature variation did not affect its performance. This is consistent with earlier studies that reported good performance or no seasonal effect on the Mn removal efficiency after the start-up of biofilters. Tekerlekopoulou *et al.* (2012) found that seasonal variations did not affect the performance of full-scale biofilters for ammonium, Fe, and Mn removal. On the other hand, it is known that low-temperature water will change the growth rate of functional oxidizing bacteria (Cai et al., 2014). Previous studies showed that bacteria growth and biological Mn oxidation could be inhibited at water temperatures below 14°C (Berbenni et al., 2000; Ratkowsky et al., 1983). However, in this study, it seems that seasonal water temperature variations (from 25-30°C in summer to 15-19°C during winter) do not affect biofilter performance.

6.4 Conclusions

Fe and Mn full-scale biofiltration conversion from conventional filtration systems has received increased attention due to the potential benefits compared with physicochemical systems. However, few studies have addressed their performance during a long-term period, considering the impact of water quality and seasonal variation in the removal efficiencies. The main goal of this study was to evaluate Fe and Mn removal in the converted Las Toscas water treatment plant (WTP) located in Santa Fe, Argentina, for long-term monitoring (from November 2011 to April 2021). The results showed that WTP conversion, consisting of an up-flow roughing filter (URF)

and two parallel rapid sand filters (RSF), was effective. The converted WTP effectively removed up to 95% and 88% of Fe and Mn from groundwater for 10 years without the use of chemical reagents or nutrients substrate augmentation strategies. Moreover, significant Fe and Mn removal in the URF (68% and 46%, respectively) and subsequent removal in the RSF confirmed the relevance of the URF as pretreatment and the RSF as a final polishing step. Probably, Fe and Mn biogenic oxides and their autocatalytic properties have contributed to maintaining the full functionality of the mature media over time. No significant differences between the seasonal variation of Fe and Mn concentrations in the URG and RSF influents and effluents were found despite the seasonal fluctuation of the water temperature. Most likely, minimal water temperatures (~15°C) did not affect the growth or the Fe-Mn oxidizing capacities of functional bacteria. On the other hand, results emerging from this study indicate that the performance of Las Toscas WTP was mainly influenced by other factors such as the modifications in the aerated water reservoir and the subsequent Fe-loading fluctuations in the feed water to the filters. Finally, this study showed that converting a conventional filtration system to a biological one is stable and efficient through the years and it is, therefore, a suitable strategy.

Chapter 7

Conclusions and recommendations

The main goal of this thesis was to obtain new insight into the efficacy of the start-up of non-bioaugmented biofilters for manganese (Mn) removal using tropical groundwater. In this concluding chapter, the major findings are highlighted, and specific conclusions are made to address the defined knowledge gaps and the specific research objectives stated in *Chapter 1*. Recommendations are also included, aiming to improve the implementation of biofiltration for Mn removal in Costa Rica. The focus of the follow-up research is also given. The major findings obtained in this research have contributed to the formulation of the main conclusion of this thesis:

> The results presented in this thesis show that non-bioaugmented pumice biofilters are a suitable and effective treatment solution for Mn removal from groundwater under tropical conditions. Groundwater temperature, a speed-up stage, Fe-pretreatment and a recirculating flow regime, seem to be appropriate to shorten the ripening time of virgin pumice with a less consumption of feed water.

This conclusion underlines the potential contribution of Mn-biofiltration to the sustainable development goals (SDG) for the adoption of sustainable water management. Furthermore, this research can help to develop the interest of water supply companies in tropical and developing countries that mainly work with chemical treatments for Mn removal.

With a focus on the identified knowledge gaps in *Chapter 1*, the specific conclusions are addressed as follows:

7.1 Isolation and characterization of manganese-oxidizing bacteria (MOB) in tropical groundwater.

In this study, several autochthonous culturable MOB were detected and identified in groundwater wells. Culturable MOB detected in the groundwater samples belong to several bacterial phyla, including *Firmicutes*, *Actinobacteria*, and the *Alpha(α)*, *Beta(β)* and *Gamma(γ) Proteobacteria*. Microscopic observations combined with surface attach methods revealed that, in appearance, rod-shaped bacteria are a dominant specie in the groundwater wells. At the in-vitro level, the potential of *Gammaproteobacteria (Stenotrophomonas* and *Aeromonas spp.)* was proved. Remarkably, bench-scale experiments also showed that MOB prevalent in the biofilter media (pumice stone, anthracite, and silica sand) were taxonomically diverse, but, with a clear domain of *Proteobacteria* phylum (*Alpha*- and *Gammaproteobacteria*). Moreover, at the genus level, the bacterial community was not significantly influenced by the filter material; however, different closest related species colonized selectively the filter media. It was also found that most of MOB identified belong to genera that have known-MOB species. *Pseudoxanthomonas* sp., not reported in Mn biofiltration before, showed high Mn oxidation activities and was abundant in all the filter media evaluated, being also good candidates for inoculums.

Raman spectra, XRD, and scanning electron microscopy (SEM) analyses of the $MnO_{x(s)}$, of all ripened media at bench scale exhibited characteristics for birnessite of biological origin, confirming that MOB played an important role in the start-up of non-bioaugmented biofilters. In addition, several techniques were used effectively for the detection and characterization of MOB, including microscopic examination, in-vitro studies, DNA gene sequencing, and ATP analyses. R2A agar modified with 17 mg/L $MnSO_4$ resulted in effective isolation of MOB during the study.

Therefore, it can be concluded that:

- Functional MOB detected in this research were phylogenetically diverse suggesting that particular tropical environmental conditions did not favor the dominance of any particular MOB.
- Most of the MOB identified has been reported previously. However, the presence of *Pseudoxanthomonas* sp., a promising MOB not reported before in drinking water biofilters, indicates that the diversity of tropical MOB needs further investigation.
- The dominance of *Proteobacteria* phylum (*Alpha*- and *Gammaproteobacteria*). in the filter media showed that this phylum played an important role in biofiltration for Mn removal.
- The fact that some MOB species were selective to the filter media suggests that a suitable inoculum could be developed depending on the filter media.
- Formed biogenic $MnO_{x(S)}$ characterization may bring insights into how acclimated rapidly the MOB in the biofilters. Further research is needed, for example, on creating synthetic birnessite on virgin media in biofilters and assessing its effect on MOB growth and ripened times.
- Fundamental evidence was obtained from diverse microbiological analyses in this thesis but is highlighted that Mn-culturing accompanied by LBB assay provides a rapid and effective low-cost alternative for the detection of functional MOB in groundwater sources. Hence, this method is highly recommended in future studies for planning Mn-biofiltration in Costa Rica.

These findings also contribute to the understanding of functional MOB in tropical groundwater and could be of help in the development of bacterial inoculum applicable to shorten the start-up of Mn-biofilters in future applications.

7.2 Start-up of non-bioaugmented biofilters for Mn removal.

In this study, the start-up of non-bioaugmented biofilters for Mn removal was evaluated both at the lab and pilot-scale. Initially, the start-up period in bench-scale columns was completed in approximately 80 days, quite similar to those previously reported in temperate zones. Afterward, the ripening time of the non-bioaugmented pilot-scale pumice biofilters was reduced to only 11 and 23 days in the flow-through and recirculating columns, respectively. It was found that these exceptional rapid start-up periods registered in non-bioaugmented biofilters were reached due to a combination of factors explained as follows:

- ***Field of action of MOB*:** effective biological Mn removal was reached with aerated water at ~22-24 °C, pH > 7.5, dissolved oxygen (DO) > 6 mg/L and redox potential (Eh) between ~300 and 400 mV. Precisely, it is the field of action of MOB reported in the literature. Despite the reducing conditions prevalent in the groundwater wells, these optimal conditions were achieved easily just with the pre-aeration step and pH adjustment was not necessary.

- ***Water temperature*:** as was expected of tropical zones, the water temperatures during the experiments (~22-24 °C) were relatively high compared to previous studies on Mn-biofiltration in temperate zones. According to the literature, it seems that water temperatures below 15°C typically prolong the start-up period of non-bioaugmented biofilters and may affect both MOB growth and the Mn capacity uptake in matured Mn-biofilters. However, results showed that neither the filter media tested, nor water temperature alone, substantially accelerated the start-up of biofilters. Hence, it was concluded that further operational strategies should be implemented to achieve an effective start-up of non-bioaugmented biofilters in tropical zones. Further studies using warm water temperatures (22-36°C) are recommended.

- ***Fe-pretreatment:*** In this study, Fe-loading of approximately 0.5 kg·Fe·m^{-2} per filter run affected the ripening time of virgin media for Mn removal. Under this condition, pumice filter maturation, at the pilot-scale level, was not reached after 130 days of operation. The rapid start-up periods of 11 and 23 days were reached after the Fe-prefilter was installed, reaching 0.10 mg/L of total Fe in the influent of biofilters. In that sense, it is highly recommended to prevent the Fe-loading during the start-up of non-bioaugmented biofilters for Mn removal.

- ***Initial filtration velocity and speed-up stage:*** results, at the pilot scale, showed that non-bioaugmented biofilters operated with an initial filtration velocity of 2 m/h and an empty bed contact time (EBCT) of 21 minutes resulted in a rapid start-up. According to Zeng *et al.* (2019), following the speed-up stage strategy, once the Mn removal reached 90% the velocity was increased in steps of about 1 m/h (speed-up stage) until reaching a final filtration velocity of 5 m/h (~ EBCT of 8.4 min), which is typically used during the start-up of biofilters. In contrast, during bench scale experiments using a lower velocity ~0,45 m/h and an EBCT of 12 min, the maturation time reached approximately 80 days. Hence, the filtration velocity of 2 m/h seems to be appropriate to enhance the association of the MOB with the filter media during the start-up. Moreover, the speed-up stage strategy permitted to reach typical filtration velocities.

7.3 Influence of the flow regime during the start-up

A novel approach, for inherent inoculation, was also evaluated in this thesis. The influence of the recirculating flow regime, using only inherent inoculation, on the ripening time of virgin media for Mn removal, was not reported before. Effective Mn removal (>90%) in the flow-through regime was completed in 8 days whereas recirculating required 23 days. Despite that recirculating regime resulted less effective in terms of ripening time, it consumed less feed water during the start-up (approximately 50%) than using a flow-through regime, making it a suitable strategy.

Due to the feed water in recirculating regime was renewed every two to three days, it resulted in an intermittent Mn-loading compared with the flow-through regime. Interestingly, both the flow-through and the recirculating flow regime required a similar total Mn-loading (0.16 and 0.11 $kg \cdot Mn \cdot m^{-2}$, respectively) during the start-up. Therefore, it was concluded that the total Mn-loading, in this case, 0.11 $kg \cdot Mn \cdot m^{-2}$, was a limiting factor. It seems that the intermittent Mn-loading in the recirculation regime influence even the MOB population established at the top of biofilters during the initial stage of maturation of the filter (10^6 $CFUg^{-1}$ vs 10^3 $CFUg^{-1}$ in the flow-through and recirculating regime, respectively). This finding could be of help to accelerate the start-up of non-bioaugmented biofilters. Probably, more frequent refreshment of water (e.g. 1.5 or 2 days) and/or supplementing dissolved Mn^{2+} into the recirculated water could help to reduce the ripening time in the recirculating system. Besides, further characterizations of the $MnO_{x(s)}$ deposits and bacteria diversity during the start-up, may provide additional insight into the role of the Mn-loading as a limiting factor during the start-up. Overall, further investigations should be conducted on Mn-loading as a limiting factor, which has received little attention in the literature.

7.4 Virgin pumice as an alternative filter medium for Mn removal.

At the in-vitro level, pumice resulted effective in broth culture media. Such results were confirmed at bench-scale were pumice exhibited similar performance to sand and anthracite (commonly used in biofiltration). The possible advantages of pumice compared with these traditional media, such as greater surface area and roughness, were not decisive to shorten the start-up, probably because functional MOB did not grow on all the available surfaces due to the competition with other heterotrophic bacteria present in the filter media. Additionally, pumice is a porous, low-density medium, that produces less pressure drop than sand and subsequently, less backwashing. Therefore, it can be concluded that pumice stone can be effectively used

in non-bioaugmented biofilters for Mn removal. An advantage, in Costa Rica and many countries, is that pumice stone with these characteristics is cheap and easy to find.

7.5 Long-term feasibility of biological filters for Fe and Mn removal in a converted WTP

Another option for the implementation of biofiltration for Mn removal in developing countries could be the conversion of existing physicochemical plants to biofiltration. In that sense, it was important to show that such conversion is possible and that it is effective over a long-term period (>2 years) and could be of help for planning. Furthermore, confidence in the use of biofiltration needs to be gained, especially in zones such as Costa Rica where the Mn-removal has been performed only by well-established physicochemical methods. This study evaluated the performance and seasonal variations of biological filters for Fe and Mn removal in a converted physicochemical plant located in Santa Fe, Argentina, for about ten years of operation. The converted WTP effectively removed up to 95% and 88% of Fe and Mn from groundwater during the period of study without the use of chemical reagents or nutrient substrate augmentation strategies. Besides, 98% of Fe and 95% of Mn concentrations in treated water were below the local regulatory limits of Fe < 0.2 mg/L and Mn<0.1 mg/L. No significant differences between the seasonal variation were found probably due to water temperatures higher than ~15 °C, the lower value that could affect the Mn capacity uptake in matured Mn-biofilters. Interestingly, as in the case of the experiments in Costa Rica, Fe-pretreatment was important during the simultaneous removal of Fe and Mn. Besides, it behaved as a protective mechanism against high Fe variations in influent water quality. Therefore, Fe-pretreatment cannot be omitted from planning the future implementation of biofiltration in a new site. Taking that into account, it was concluded that the conversion of existing physicochemical plants to biofiltration is a suitable strategy, stable and efficient through the years.

Appendices

8.1 Enriched culture media for MOB detection

Table 8.1. Representative culture media used for isolation of MOB from Mn-biofilters.

Id	Ingredients (Per liter of deionized water)	References
A medium	0.2 g $MnSO_4 \cdot H_2O$, 0.8 g peptone, 0.2 g yeast extract, 0.1 g $CaCl_2$, 0.1 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2g $NaNO_3$, 0.1 g $(NH_4)_2CO_3$, pH 6.8–7.2	(Li et al., 2005)
(Modified)[a] MSVP medium	17mg $MnSO_4$,[a] 10 mM HEPES buffer, 0.06 g $CaCl_2 \cdot 7H_2O$, 0.02 g KH_2PO_4, 0.06 g $MgSO_4 \cdot 7H_20$, 0.03 g Na_2HPO_4, 0.24 g $(NH_4)_2SO_4$, pH 7.0 plus filter sterilized sodium pyruvate solution (1.0 g) and vitamin solution[b] (0.025 ml)	(Emerson et al., 1989) [a] (Burger, Krentz et al., 2008) [b](Staley, 1968)
(Modified)[c] R2A medium	17mg $MnSO_4$,[c] 0.5 g yeast extract, 0.5 g proteose peptone, 0.5 g casamino acids, 0.5 g glucose, 0.5 g soluble starch, 0.3 g K_2HPO_4, 0.05 g $MgSO_4$*$7H_2O$, 0.3 g sodium pyruvate, 15 g agar. pH 7.2	(Reasoner & Geldreich, 1985) [c](Burger et al., 2008; Granger et al., 2014)

(continues)

Table 2.2 *(Continued)*

Id	Ingredients (Per liter of deionized water)	References
Mn-oxidation medium	0.15 g $MnSO_4 \cdot H2O$ (0.001 g $FeSO_4*7H_2O$), 10 mM HEPES buffer, 2 g peptone, 0.5 g yeast extract (15 g agar) pH 7.0 -7.5	(Hoyland et al., 2014; Piazza et al., 2019; Stein et al., 2001)
Modified PYCM medium (MPM)	0.2 g $MnSO_4 \cdot H2O$ (0.8 g $FeC_6H_5O_7NH_4OH$), 0.5 g peptone, 0.2 g yeast extract, 0.1 g $CaCl_2$, 0.1 g K_2HPO_4, 0.3 g glucose, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2g $NaNO_3$, 0.1 g $(NH_4)_2CO_3$, pH 7.0	(Li et al., 2016)
MnOB medium	10 mM $MnCl_2$, 10 mM HEPES buffer, 0.5 g peptone from casein, 0.5 g peptone from soybean, 0.1 g glucose, 0.1 g soluble starch, 0.5 g meat extract, 0.5 g yeast extract, (2% agar)	(Breda et al., 2017)
PC Medium	0.2 g $MnSO_4 \cdot H2O$ 0.5 g yeast extract (2% agar)	(Piazza et al., 2019; Tyler & Marshall, 1967)
PYM-medium	0.5 g $MnSO_4 \cdot H_2O$ 10 mM HEPES buffer, 1 g peptone, 0.1 g yeast extract (15 mM NaN_3)	(Vandenabeele et al., 1992)

(continues)

Table 2.2 *(Continued)*

Id	Ingredients (Per liter of deionized water)	References
Lept medium	3.7 μM $FeSO_4 \cdot 7H_2O$, 10 mM HEPES buffer, 0.5 g yeast extract, 0.5 g casamino acids, 5 mM glucose, 0.48 mM $CaCl_2$, 0.83 mM $MgSO_4$, pH 7.5 0.15 μM $ZnSO_4$, 0.08 μM $CoCl_2$, 0.06 μM Na_2MoO_4	(Boogerd & de Vrind, 1987; Piazza et al., 2019)

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