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DOI
10.4233/uuid:d73c2d06-7152-4cbc-b864-931b112836ca

Publication date
2022

Document Version
Final published version

Citation (APA)
Bicudo, B. (2022). *Municipal effluent disinfection by Iron Electrocoagulation*. [Dissertation (TU Delft), Delft University of Technology]. https://doi.org/10.4233/uuid:d73c2d06-7152-4cbc-b864-931b112836ca

Municipal effluent disinfection by Iron Electrocoagulation

Bruno Bicudo Pérez

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DISSERTATION

For the purpose of obtaining the degree of doctor
at Delft University of Technology
by the authority of the Rector Magnificus, Prof.dr.ir. T.H.J.J van der Hagen,
chair of the Board for Doctorates
to be defended publicly on
Monday 12 December 2022, at 15:00 o'clock

by

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The research presented in this thesis was performed at the Sanitary Engineering Section, Department of Water Management, Faculty of Civil Engineering, Delft University of Technology, The Netherlands. The research is part of the LOTUS^{HR} project, and was financially supported by NWO TTW, grant N^{o}15424.

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Acknowledgements

The book in your hands is the culmination of almost five years of intense learning and laboratory work, and yet it falls short to depict either. Numerous people helped me write it in a way or another, and this chapter is a tribute to them.

As tradition dictates, the first acknowledgements are to my supervisors, who five years ago gave me their vote of confidence and the opportunity to pursue this challenging PhD. **Prof. dr. ir. Doris van Halem**, thank you for your continuous guidance and support, and for making me the researcher that I am today. **Prof. dr. Gertjan Medema**, my gratitude to you for shining my way the beacon of knowledge you possess and making me an even more curious person in a field that was unknown to me before. Finally, I would like to thank **Dr. Giuliana Ferrero** for helping me steer during my first year, where the waters were the roughest.

I would also like to show my gratitude to the people involved in the LOTUS^{HR} programme with whom I shared endless committee meetings but also enjoyable dinners, clubs, planes, trains, buses and even TV broadcasts in India. **Ralph, Merle, Steef**, **Theo**, you are some of the most fantastic people I have ever encountered, and I will always cherish the memories of our times together. To the WaterLab staff, without whom my work would have been impossible; thank you **Armand** for your help and unfading enthusiasm since the very beginning, and thank you too **Patricia**, for working your magic around the ICP-MS so many times in such short notice.

My gratitude also goes to my fellow colleagues, with whom I shared the lab, the office, the coffee corner, birthday parties, weddings, publications, and perhaps one or two beers; **Javier, Carina, Sara, Emiel, Lenno, Magela, Shreya, Mrinal, Lihua, Simon, Erik, and Roos**. I would also like to acknowledge **Suzanne, Bart-Jan, Tiza, Ruby-Jane** and **Guido**, whom I had the privilege to assist during their MSc and/or internships, and who in turn helped me discover a taste for teaching I didn't know I had.

I would also like to acknowledge **Anto, Rafa, Yanny, Stefan and Claudia,** and the rest of the **ICAA** staff, the people who cheer me up outside of office hours and that are more vital to me than they will ever know. Thank you guys! To my family abroad, **Berend**, and **Mona**, with whom I can talk for hours about both gardening and the hardships of PhD life, who have been a pillar on my life away from home, and who somehow managed to keep me sane since we first met back in 2013.

Naturally, I would like to send the warmest and tightest of hugs to my parents **Julio** and **Carmen**, who gave me everything and asked for nothing. But most importantly, I owe this PhD to my wife, **Fiorella**, who decided to marry me even after getting to know me, and who believed I could complete it even before I did.

Summary

In a global context of ever increasing population, climate change, and freshwater quality deterioration, water reclamation presents itself as a valid and valuable supplier of the resource. However, sewage and treated effluents remain important sources for waterborne pathogens, including Antibiotic Resistant Bacteria (ARB), a global emerging threat with potential to cripple our health systems and make us vulnerable once again to simple infections. Effluent disinfection thus becomes paramount to ensure suitable microbiological water quality and reuse applicability, although other water quality parameters critical for reuse applications (i.e. solid content, nutrients and organic matter) should also be addressed. Technologies developed for drinking water disinfection, such as chlorination, UV-irradiation, or ozone, usually fail to satisfy reclamation standards across all microbial groups, are not designed for nutrient or solid removal, and are known sources of hazardous disinfection by-products (DBPs). This research looks into a rather unknown water treatment technology, Iron Electrocoagulation (Fe-EC), as a suitable candidate for municipal effluent reclamation, based on its inactivation efficacy of a wide range of microorganisms, nutrient and solid removal, and absence of DBPs. The disinfection of ARB by Fe-EC and other processes is also described in detail, especially due to the shortage of literature regarding their inactivation, their general absence in water quality standards, and the health risk they pose to users. Significant efforts were made as to understand if indeed ARB are more resistant than other faecal indicator bacteria (FIB) when it comes to wastewater treatment, and if disinfection of both groups can be correlated.

Just like FIB, ARB are present in the human gut and subsequently in our sewage streams. In order to understand the correlation between FIB and ARB while undergoing water treatment processes, raw and treated effluent samples from a municipal WWTP were screened for several months. Faecal indicators *E. coli* and Enterococci were selected as antibiotic sensitive bacteria (ASB), and resistant strains of public health concern of each one, namely Extended Spectrum β-Lactamase-producing E. coli and Vancomycin Resistant Enterococci (ESBL-*E. coli* and VRE respectively), were chosen as ARB indicators. ARB were present in sewage all year round and in stable concentrations, and their numbers bore direct relation to those of their sensitive analogues. Effluent samples were also subjected to diverse disinfection and decay processes to further study ARB response following disinfection and discharge into water bodies. In these experiments, a novel approach to tertiary treatment was included, with the application of Iron Electrocoagulation (Fe-EC), a technology not usually considered for disinfection purposes. Overall, ARB behaved notably similarly to ASB in all scenarios, meaning the latter can be used for inferring water treatment systems' ARB removal efficacy and their presence in sewage, secondary effluents, disinfected effluents and possibly in the receiving water bodies. From a regulatory perspective this result is highly valuable, as FIB are already used by most drinking water, wastewater, surface water and reuse water quality standards and regulatory bodies for microbial safety, while no such numerical standard contemplating ARB or antibiotic resistance genes exists anywhere to this date, partially due to lack of consensus on what a suitable indicator could be. Given its stable behaviour, the ratio between ASB and ARB described in this dissertation could become a suitable candidate for evaluating resistance in surface water or evaluate resistance spread by wastewater discharge, even on a regulatory level. The measurement of this "resistance ratio" by culture methods, as described throughout this dissertation, does not imply significant changes in the methodology already in place for FIB measurements, requires meagre investments, can be performed by operators after short training, and allows for viable resistant bacteria to be enumerated directly. It is also independent of state of the art molecular methods (qPCR, metagenomics, etc.), which can be off-limits to most water utilities and regulatory bodies in the developing world due to lack of resources, equipment, or trained personnel.

The potential of Fe-EC for effluent disinfection was further investigated by expanding the microbial indicators to include viruses (somatic coliphages) and protozoan surrogates (*C. perfringens* spores). Disinfection increased with increasing Fe-dosage, with removal values between 2-4$\log_{10}$ for all indicators when 400 C/L were applied. Bacteria were the most effectively removed group, followed by spores and viruses respectively, achieving disinfection comparable to conventional methods such as chlorine or ozone. In addition to disinfection, Fe-EC offered notable PO_4^{3-} removal (>99%) and considerable COD abatement (≈30-50%), added benefits over traditional disinfection methods, with a cost estimate below 0.08 €/m^3. Although results for microbial attenuation were promising, the mechanisms behind disinfection required further description, as some credit its success to floc entrapment, and others to the production of Fenton-type intermediates, consequence of the Fe^{2+} oxidation. To discriminate the Fe-EC inactivation mechanisms, specifically regarding the role of Reactive Oxygen Species (ROS), a series of disinfection experiments were conducted under the presence and absence of the Fenton inhibitor TEMPOL, to quantify inactivation by Fenton-type disinfectants, using *E. coli* and coliphage ØX174 as indicators. Total *E. coli* inactivation was shown to be proportional to the total amount of oxidized Fe^{2+}, and the rate of inactivation proportional to the rate of Fe^{2+} oxidation. *E. coli* inactivation bears direct relation with Fe dosage, and follows a Chick-Watson-like

behaviour in which current intensity acts as the disinfectant's concentration surrogate. Phage ØX174 total inactivation showed some dependence on the total dosed Fe^{2+}, yet bore no relation to its oxidation rate. ROS exposure was the main bacterial inactivation route following Fe-EC, but other inactivation processes such as Fe^{2+} exposure can also be pertinent for virus inactivation. Inactivation of *E. coli* and somatic coliphage ØX174 by ROS also showed pH dependency, with higher inactivation under acidic conditions as a consequence of higher production of the more toxic hydroxyl ($^{\bullet}OH$) radicals. Water acidification however, should not be considered as the tool of choice for enhancing Fe-EC inactivation, as this could slow down or directly prevent Fe^{2+} oxidation and subsequent ROS formation. Fe-EC can be advantageous for disinfection when effluents are of a slightly acidic nature, yet boosting disinfection via Fe-EC should resort to enhancing the ROS production, for example by the addition of H_2O_2, which increases the ROS/Fe ratio, yet without influencing the type of produced ROS.

In addition to inactivation by ROS, flocculation was found to be of critical importance to obtain high log removal value by Fe-EC. It was determined that without proper flocculation, microbe-laden microscopic flocs settle poorly, leading to relatively low log reduction of both phage ØX174 and *E. coli*. Flocculation experiments showed a strong correlation between orthokinetic-like flocculation, floc sedimentation, and microbe removal, demonstrating that floc entrapment, together with ROS inactivation, constitute the major disinfection mechanisms following Fe-EC for both bacterial and viral indicators. The interaction of both processes however is antagonistic, as the suppression of one will not necessarily affect the outcome in terms of disinfection: failure to flocculate will still produce disinfection provided Fe^{2+} oxidation (and ROS production) is achieved, while ROS quenching will not impact the outcome significantly as long as proper flocculation and sedimentation are in place. However, having both processes occurring simultaneously provides an extra safety barrier, making it more fail-proof. This extra safety barrier is absent in conventional chemical coagulation, being perhaps the main advantage of the Fe-EC process. Municipal applications making use of Fe-EC should incorporate a flocculation stage either during or immediately following the application of electric current, as this greatly improves the quality of the formed flocs and enhances their sedimentation speed, with a subsequent decrease in microbial and solid content in the treated effluents.

Fe-EC offers a high potential for adoption in the municipal sector, not only for wastewater treatment but also for drinking water production. This dissertation provides detailed insight into the fundamentals of Fe-EC and its attractiveness as an affordable and robust water reclamation technology, not only due to its intrinsic double barrier and high effectiveness against a broad range of microorganisms (*E. coli*, ESBL-*E. coli*, Enterococci, VRE, somatic coliphages, *C. perfringens* spores), but also due to its capacity for nutrient, solids, organic micro pollutants and heavy metal removal, and the addition of ROS as an extra safety barrier for disinfection. It features the first mathematical description of ROS inactivation facilitated by ferrous iron oxidation during Fe-EC, demonstrating that it is possible to directly correlate electric parameters with disinfection. It also offers an interesting look into the behavior of ARB during wastewater treatment, and its indistinguishable behavior from that of FIB, rendering the latter outstanding proxies for culture-based antibiotic resistance screening.

Samenvatting

Met een wereldwijd groeiende bevolking, klimaatverandering en verslechtering van de waterkwaliteit wordt hergebruik van afvalwater en solide en waardevol alternatief. Rioolwater en gezuiverd afvalwater zijn echter belangrijke bronnen voor aquatische ziekteverwekkers, waaronder antibiotica resistente bacteriën (ARB). Dit is een wereldwijde bedreiging die onze gezondheidsstelsels kan verlammen en ons opnieuw kwetsbaar kan maken voor eenvoudige infecties. Desinfectie van afvalwater is dus van het grootste belang om een geschikte microbiologische waterkwaliteit en toepasbaarheid van hergebruik te kunnen garanderen. Daarnaast moeten andere waterkwaliteitsparameters die cruciaal zijn voor hergebruiktoepassingen (d.w.z. zwevende stof, nutriënten en opgeloste verontreinigingen) ook worden aangepakt. Technologieën die zijn ontwikkeld voor de desinfectie van drinkwater, zoals chlorering, UV-straling of ozon, voldoen meestal niet aan de norm voor hergebruik voor alle microbiële groepen. Bovendien zijn deze technologieën zijn niet ontworpen voor het verwijderen van nutriënten of zwevende stoffen en kunnen tevens een bron zijn van gevaarlijke desinfectiebijproducten (DBP's). Dit onderzoek kijkt naar een vrij onbekende waterbehandelingstechnologie genaamd ijzerelektrocoagulatie (Fe-EC), als een geschikte kandidaat voor de terugwinning van gemeentelijk afvalwater. Fe-EC heeft de potentie om een breed scala aan micro-organismen te verwijderen, maar ook nutriënten en zwevende stoffen, zonder productie van n DBP's. De desinfectie van ARB door Fe-EC en door andere processen wordt ook in detail beschreven vanwege het gebrek aan literatuur over hun inactivering, hun algemene afwezigheid in waterkwaliteitsnormen en het gezondheidsrisico dat ze vormen voor gebruikers. Er zijn aanzienlijke inspanningen geleverd om te begrijpen of ARB inderdaad resistenter zijn dan andere fecale indicatorbacteriën (FIB) als het gaat om afvalwaterzuivering en of de desinfectie van beide groepen kan worden gecorreleerd.

Net als FIB zijn ARB aanwezig in de menselijke darmen en komen vervolgens in onze afvalwaterstromen. Om de correlatie tussen FIB en ARB te begrijpen tijdens de waterbehandelingsprocessen, werden gedurende enkele maanden verse en behandelde effluent monsters, van een gemeentelijke afvalwaterzuivering, gescreend. Fecale indicatoren *E. coli* en Enterococci werden geselecteerd als antibioticagevoelige bacteriën (ASB). Resistente stammen met een belang voor de volksgezondheid

Extended Spectrum β-Lactamase-producing *E. coli* en Vancomycin Resistant Enterococci (respectievelijk ESBL-*E. coli* en VRE) werden gekozen als ARB-indicatoren. ARB waren het hele jaar door aanwezig in rioolwater in stabiele concentraties en hun aantal hield rechtstreeks verband met die van hun gevoelige analogen. Afvalwatermonsters werden onderworpen aan diverse desinfectie- en afbraakprocessen om de ARB-reactie na desinfectie en lozing in waterlichamen verder te bestuderen. In deze experimenten werd ijzerelektrocoagulatie (Fe-EC) als een nieuwe benadering van tertiaire behandeling toegepast. Deze technologie wordt gewoonlijk niet overwogen voor desinfectiedoeleinden. Over het algemeen gedroeg ARB zich in alle scenario's op dezelfde manier als ASB, wat betekent dat deze geschikt zijn voor het voorspellen van ARB-verwijdering in waterbehandelingssystemen en hun aanwezigheid in rioolwater, secundaire effluenten, gedesinfecteerde effluenten en mogelijk in de ontvangende waterlichamen. Dit resultaat is zeer waardevol voor regelgeving, aangezien FIB al wordt gebruikt voor de meeste kwaliteitsnormen voor drinkwater, afvalwater, oppervlaktewater en water hergebruik door instanties voor microbiële veiligheid. Tot op heden bestaat nergens een dergelijke numerieke norm voor ARB of antibiotica resistente genen, deels door een gebrek aan consensus over wat een geschikte indicator zou kunnen zijn. Gezien het stabiele gedrag, zou de in dit proefschrift beschreven verhouding tussen ASB en ARB, een geschikte parameter kunnen worden voor het evalueren van resistentie in oppervlaktewater of het evalueren van resistentieverspreiding door afvalwaterlozing. Zelfs op het niveau van regelgeving. De meting van deze "weerstandsverhouding" door kweekmethoden, zoals beschreven in dit proefschrift, impliceert geen significante veranderingen in de reeds bestaande methodologie voor FIB-metingen. Het vereist weinig investeringen en kan worden uitgevoerd door operators na een korte training en maakt het mogelijk om levensvatbare resistente bacteriën direct te kunnen tellen. Het is ook onafhankelijk van de modernste moleculaire methoden (qPCR, metagenomica, enz.) die vaak niet beschikbaar zijn voor waterbedrijven en instanties in ontwikkelingslanden door een gebrek aan middelen, apparatuur of opgeleid personeel.

Het potentieel van Fe-EC voor de desinfectie van afvalwater werd verder onderzocht door de microbiële indicatoren uit te breiden met virussen (somatische colifagen) en protozoaire surrogaten (*C. perfringens*-sporen). Desinfectie nam toe met toenemende Fe-dosering, met verwijderingswaarden tussen 2-4 $\log_{10}$ voor alle indicatoren wanneer 400 C/L werd toegepast. Bacteriën werden het meest effectief verwijderd, gevolgd door respectievelijk sporen en virussen. Een desinfectie niveau dat werd bereikt was vergelijkbaar met conventionele methoden zoals chloor of ozon. Naast desinfectie bood Fe-EC een opmerkelijke PO_4^{3-} verwijdering (>99%) en een aanzienlijke CZV-reductie (≈30-50%). Extra voordelen ten opzichte van traditionele desinfectiemethoden, met een kostenraming van minder dan 0.08 €/m^3.

Hoewel de resultaten voor microbiële vermindering veelbelovend waren, moesten de mechanismen achter desinfectie nader worden beschreven. Aangezien sommigen het succes ervan toeschrijven aan het insluiting in vlokken en anderen aan de productie van tussenproducten van het Fenton-type, als gevolg van de Fe^{2+}-oxidatie. Om de Fe-EC-inactiveringsmechanismen te onderscheiden, specifiek met betrekking tot de rol van Reactive Oxygen Species (ROS) werd een reeks desinfectie-experimenten uitgevoerd onder de aanwezigheid en afwezigheid van de Fenton-remmer TEMPOL. Om inactivatie door Fenton-type desinfectiemiddelen te kunnen kwantificeren werden *E. coli* en coliphage ØX174 gebruikt als indicatoren. De totale inactivatie van *E. coli* bleek evenredig te zijn met de totale hoeveelheid geoxideerd Fe^{2+} en de snelheid van inactivatie was evenredig met de snelheid van Fe^{2+}-oxidatie. Inactivering van *E. coli* houdt rechtstreeks verband met de Fe-dosering en volgt een Chick-Watson-achtig gedrag waarbij de stroomintensiteit fungeert als surrogaat voor de desinfectant concentratie. De totale inactivatie van faag ØX174 vertoonde enige afhankelijkheid van de totale gedoseerde Fe^{2+}, maar had geen verband met zijn oxidatiesnelheid. Blootstelling aan ROS was de belangrijkste bacteriële inactivatieroute na Fe-EC, maar andere inactivatieprocessen zoals Fe^{2+}-blootstelling kunnen ook relevant zijn voor virus inactivatie. Inactivatie van *E. coli* en somatische colifaag ØX174 door ROS toonde ook pH-afhankelijkheid, met hogere inactivatie onder zure omstandigheden als gevolg van hogere productie van de meer giftige hydroxyl ($^{\bullet}OH$) radicalen. Water met lage zuurgraad moet echter niet worden beschouwd als het instrument bij uitstek voor het verbeteren van Fe-EC-inactivatie, omdat dit de oxidatie van Fe^{2+} en de daaropvolgende ROS-vorming zou kunnen vertragen of zelfs voorkomen. Fe-EC kan voordelig zijn voor desinfectie wanneer effluenten een licht zuur karakter hebben. Het stimuleren van desinfectie via Fe-EC zou moeten focussen op het verhogen van de ROS-productie door bijvoorbeeld de toevoeging van H_2O_2, wat de ROS/Fe-verhouding verhoogt, zonder de geproduceerde ROS te Beïnvloeden.

Naast inactivatie door ROS, bleek ook uitvlokking van cruciaal belang te zijn om een hoge log verwijderingsgraad door Fe-EC te verkrijgen. Er werd vastgesteld dat met microben beladen microscopische vlokken slecht bezinken zonder goede uitvlokking. Dit leidt tot een relatief lage log-reductie van zowel faag ØX174 als *E. coli*. Uitvlokkingsexperimenten toonden een sterke correlatie tussen orthokinetisch-achtige uitvlokking, vloksedimentatie en verwijdering van microben. Dit toont aan dat vlokinsluiting samen met ROS-inactivering de belangrijkste desinfectiemechanismen vormen bij Fe-EC voor zowel bacteriële als virale indicatoren. De interactie van beide processen is echter antagonistisch, aangezien de onderdrukking van één proces niet noodzakelijk de uitkomst van desinfectie zal beïnvloeden: het niet uitvlokken zal nog steeds desinfectie produceren op voorwaarde dat Fe^{2+}-oxidatie (en ROS-productie)

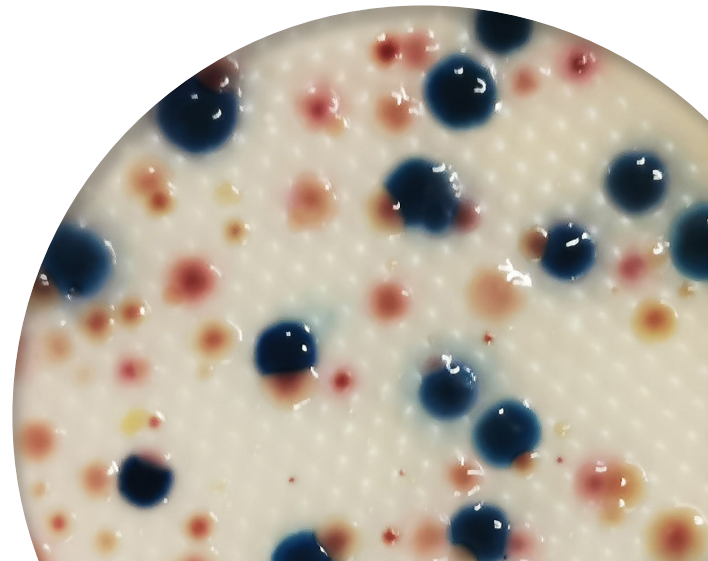
wordt bereikt, terwijl ROS-quenching geen significante invloed heeft op het resultaat zolang de juiste flocculatie en sedimentatie aanwezig zijn. Het gelijktijdig laten plaatsvinden van beide processen zorgt echter voor een extra veiligheidsbarrière, waardoor het een kleinere kans tot falen heeft. Deze extra veiligheidsbarrière ontbreekt bij conventionele chemische coagulatie, wat misschien wel het belangrijkste voordeel is van het Fe-EC-proces. Gemeentelijke toepassingen die gebruik maken van Fe-EC dienen een uitvlokkingsfase in te bouwen, hetzij tijdens of onmiddellijk na toepassen van de elektrische lading. Aangezien dit de kwaliteit van de gevormde vlokken aanzienlijk verbetert en hun sedimentatiesnelheid verhoogt wat een afname aan microben en zwevende stof in het behandelde afvalwater tot gevolg heeft.

Fe-EC laat een groot potentieel zien voor de adoptie in de gemeentelijke sector en niet alleen voor de behandeling van afvalwater. Het kan namelijk ook worden gebruikt voor de productie van drinkwater. Dit proefschrift geeft gedetailleerd inzicht in de fundamenten van Fe-EC en zijn aantrekkelijkheid als betaalbare en robuuste waterterugwinningstechnologie. Dit is niet alleen vanwege de intrinsieke dubbele barrière en hoge effectiviteit tegen een breed scala aan micro-organismen (*E. coli*, ESBL-*E. coli*, Enterococci, VRE, somatische colifagen, *C. perfringens* sporen), maar ook door zijn capaciteit voor nutriënten, vaste stoffen, organische microverontreinigingen en zware metalen verwijdering en de toevoeging van ROS als extra veiligheidsbarrière voor desinfectie. Het bevat de eerste wiskundige beschrijving van ROS-inactivatie die mogelijk wordt gemaakt door -ijzeroxidatie tijdens Fe-EC. Dit toont aan dat het mogelijk is om elektrische parameters direct te correleren met desinfectie. Het biedt ook een interessant inzicht in het gedrag van ARB tijdens het zuiveren van afvalwater en dat het gedrag niet te onderscheiden is van dat van FIB. Hierdoor zijn de laatst genoemden uitstekende proxies voor op kweek gebaseerde screening van antibioticaresistentie.

1

CHAPTER 1

Introduction



1.1 Water Scarcity & Reuse

Fresh water in sufficient quality and quantity is a key component to all aspects of human life and sustainable development. This is stated in the sixth Sustainable Development Goal (SDG 6) from the United Nations General Assembly, titled "C*lean water and sanitation for all",* one of seventeen goals designed to serve as a "blueprint to achieve a better and more sustainable future for all" (UN, 2018). Still, two thirds of the world's population experience severe water scarcity at least during one month every year (Mekonnen & Hoekstra, 2016), while 1.42 billion live in high or extremely high water-vulnerable areas (UNICEF 2021). The forecast for the coming years does not look encouraging either: global demand for water has seen a steady 1% yearly increase over the last decades, trend expected to continue in the foreseeable future (UN-Water, 2021). Climate change also makes the picture more complex, as arid regions become drier and humid regions undergo more frequent extreme rainfalls. The largest increase on fresh water demand is expected to come from the domestic and industrial sectors, though agriculture is expected to remain the largest consumer globally (FAO, 2020; Winpenny et al., 2010).

Although water scarcity is usually addressed from a quantitative standpoint, dwindling water quality is also a major aspect of water scarcity, as even when available, contaminated water sources are unfit for a multiplicity of applications (Pereira et al., 2009). Agricultural run-off, discharge of raw or partially treated sewage, and industrial effluents reaching both surface and groundwater sources are major contributors to freshwater pollution, specially throughout the developing world (Calapez et al., 2019). In times of water shortage, water reuse is often seen as a solution with great potential, particularly in water-stressed regions where droughts can be extreme.

Water reuse can be simply defined as the act of using municipal wastewater (raw or treated) for any purpose, such as construction, irrigation, firefighting, landscaping or even drinking (Angelakis et al., 2018). Besides from being the largest freshwater consumer, agriculture is also the largest reclaimed water consumer, accounting for over 20 million irrigated hectares in 2010 (Winpenny et al., 2010) (10% of the world's croplands), most of which happens in Asia (Howell, 2001).

Despite the obvious advantages of water reuse, reclaimed water may harbour a wide range of pathogens, including viruses, bacteria, protozoa and helminth eggs, capable of inflicting high disease burdens, or even death, to the exposed users. Reclaimed water also contains highly variable concentrations of Antibiotic Resistant Bacteria (ARB), a relatively novel menace which "*threatens the very core of modern medicine*", according to the World Health Organization (WHO, 2015). Hence, the careful management of these municipal effluents becomes paramount from a public safety standpoint.

1.2 Waterborne pathogens

Microorganisms capable of producing sickness, disability or death to humans or animals, which can be transmitted by water contaminated with faecal matter are referred to as "waterborne pathogens". These use a variety of routes to infect a human being: they can enter the intestinal tract by ingestion, access the circulatory system by penetrating the skin, cause external infections by contact, or affect the respiratory tract by inhalation of contaminated water droplets or other bodily fluids. Waterborne pathogens are very diverse, and can be found in the form of viruses, bacteria, protozoa and parasitic worms (Bridle, 2014).

As shown in table 1-1, amongst the most common exposure routes for waterborne diseases are the ingestion of contaminated food or water, and the faecal-oral transmission due to poor handwashing. Diarrhoea, abdominal pain, fever and vomiting are in turn the most common symptoms. According to the Centers for Disease Control and Prevention (CDC, 2013), diarrhoeal diseases are the 2nd leading cause of death in infants worldwide, killing over 800.000 children on a yearly basis, a significant fraction of which can be prevented by improving access to water and sewage disposal.

Enteric pathogens are normally found in sewage, and their concentration will usually reflect the general conditions of water supply, sanitation and hygiene of the population producing it (Cui et al., 2019). Although wastewater treatment processes can usually eliminate waterborne pathogens to a certain extent, it is safe to assume that treated municipal effluents are never devoid of human pathogens. Hence, certain reuse applications demand additional water treatment aimed at disinfection, in order to minimize the health risks to its users (Carré et al., 2018; Scott et al., 2003).

1.3 Antimicrobial resistance

1.3.1 Antibiotic Resistant Bacteria

Antibiotics are perhaps the most significant and influential medical breakthrough of the 20th century (Uddin et al., 2021). Discovered by accident in 1928 by Alexander Flemming, Penicillin G became the first non-synthetic antibiotic to be refined and mass produced since 1943, saving countless of lives. However, in 1940, years before its mass production began, Ernst Chain and Edward Abraham reported "unsusceptible" bacteria, which degraded penicillin by the production of enzymes, rendering them resistant to the antibiotic (Abraham & Chain, 1940). Bacteria that have acquired resistance against antibiotics are called Antibiotic Resistant Bacteria (ARB), as opposed to Antibiotic Sensitive Bacteria (ASB) which are vulnerable to them (CDC, 2019).

Resistance to antibiotics is the result of selective pressure and natural selection; when a population of bacteria is exposed to lethal concentrations of an antimicrobial agent (selective pressure), sensitive organisms will die-off, while those capable of resisting will survive (natural selection). The resistant offspring of these organisms will replenish the population (Figure 1-1) (Kolář et al., 2001). Survival in this case, is made possible by the acquisition of specific genes, usually referred to as Antibiotic Resistance Genes (ARG).

Table 1-1 Common waterborne pathogens, main exposure routes and infection symptoms. Pathogen list adapted from Chahal et al., 2016. Data on pathogens extracted from https://www.ecdc.europa.eu and https://www.cdc.gov databases.

		Main exposure routes				Main symptoms									
		Contaminated food & water ingestion	Faecal oral (poor hygene or handwash)	Through skin, contact with contaminated water	Inhaling of cough sneeze droplets	(Bloody) Diarrhea	Abdominal pain	Fever	Vomit /nausea	Flu-like symptoms	Headache/corporal pain	Cough	Pneumonia	Kidney failure	Skin itch or rash
Bacteria	*Salmonella typhi*	●				●	●	●	●						
	Vibrio cholerae	●				●			●						
	E. coli O157:H7	●	●			●	●	●	●						
	Shigella spp.	●	●			●	●	●	●		●			●	
	Campylobacter spp.	●				●	●	●							
Virus	Adenovirus		●		●	●	●	●	●	●		●	●		
	Norovirus	●	●			●	●	●	●						
	Hepatitis A virus	●	●				●	●							
	Rotavirus	●	●			●	●	●	●						
	Enterovirus		●		●		●	●		●	●	●			●
Protozzoa	*Cryptosporidium parvum*	●	●			●									
	Giardia lamblia	●	●			●	●								
	Entamoeba histolytica	●	●			●	●	●							
	Toxoplasma gondii	●	●							●					
Parasitic worms	Schistosomes			●				●			●				●
	Hookworms	●	●				●								
	Roundworms	●	●				●	●							

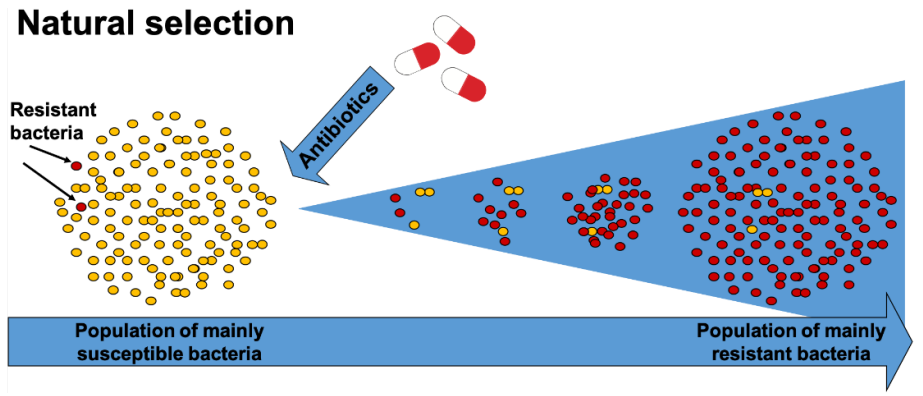


Figure 1-1: Graphical representation of the interaction between antibiotic-driven selective pressure and natural selection leading to the growth of ARB (source: https://www.reactgroup.org).

It is very important to highlight however, that ARB are usually resistant against a specific antibiotic, or against a specific group of antibiotics, prompted by the type of ARG they carry. When bacteria acquire multiple different types of ARG simultaneously encoding for protection against several types of antibiotics, the term Multi Drug Resistant Bacteria (MDRB) or "superbug" is used. Some hardy MDRB have been dubbed as "Extensively Drug-Resistant" or even "Totally Drug-Resistant", due to their elevated resistance to some (or all) of the common antibiotics used against them. Such is the case of the so called "extensively drug-resistant" tuberculosis, first reported in South Africa in 2006. Drug resistant tuberculosis is currently considered a public health "Serious Threat" by the US CDC, together with Vancomycin-resistant Enterococci and ESBL-producing Enterobacteriaceae (CDC, 2019), both of which will be later addressed in this dissertation.

When a person or animal acquires an infection with ARB, treatment becomes difficult as the infection will resist the antibiotic and continue to expand, causing prolonged sickness, disability or death. It was estimated that in 2019 alone, 4.95 million people died as a consequence of ARB-related complications, 1.27 million of which were direct casualties of the ARB-infection (*GLASS*, 2021). The ARB death toll is only expected to rise in the coming decades, with some studies even suggesting that by 2050, ARB will kill 10 million people annually, even surpassing cancer (O'Neill, 2016).

1.3.2 Wastewater treatment plants as hotspots for Antimicrobial Resistance

ARB are abundant in sewage, as it collects faeces from large populations which in many cases can be infected by them, either symptomatically or asymptomatically. Together with faeces and bacteria, urban sewage carries antibiotics and their metabolites (Guo et al., 2017; Manaia et al., 2018; Michael et al., 2013), usually excreted by their users

through urine and faeces, and in some cases simply disposed-off in the toilet after their expiry date. These antibiotics make their way into wastewater treatment plants (WWTP), where they can interact with the bacteria present in large concentrations, and exert selective pressure, thus promoting resistance. Several researchers have tried with relative success to link the type of resistance in incoming ARB to the types of antibiotics used by the population (Pärnänen et al., 2019; Wang et al., 2020). However, it is clear that the concentrations of ARB in the sewage bears relation with the types and amounts of antibiotics used by the general public, with ARB/ARG concentration being highest where less restrictions exist on antibiotic purchase (Fugère. & Keen., 2018).

Besides from their potential role in promoting bacterial resistance within their facilities, conventional WWTP are generally not efficient in removing the antibiotics present in the sewage (Christou et al., 2017; Michael et al., 2013; Mutiyar & Mittal, 2014). Hence, ARB together with non-removed antibiotics are frequently detected in the effluents that municipal WWTP discharge into the environment, for which numerous researchers have labelled these facilities "antimicrobial resistance hotspots" (Guo et al., 2017; Michael et al., 2013; Rizzo et al., 2013). However, this label has been contested by several other researchers who did not observe any particular enrichment of ARB or any measurable negative impact in the receiving water bodies downstream (Leclercq et al., 2013; Nimonkar et al., 2019; Osińska et al., 2016; Varela et al., 2013).

1.4 Water reclamation and the need for disinfection

Water reclamation schemes make use of treated municipal effluents for a multiplicity of applications, i.e. crop irrigation, landscaping, fountains, firefighting, fish farming, dust abatement, potable reuse, etc. (Bruvold et al., 1981; Rose et al., 1996; Saidan et al., 2020; Voulvoulis, 2018), in which users may come in close contact with the reclaimed effluents. When these effluents are insufficiently treated or inadequately handled, the pathogenic organisms they contain can come in contact and find their way into a new human host. Hence, limiting the exposure risk and minimizing the pathogen load in each application is paramount for the scheme's success. This does not mean that reclaimed water requires to be sterile, for example, human pathogen concentration is irrelevant if the water is reused for timber-tree drip irrigation. However, if the irrigation of trees is performed with sprinklers, the staff carrying out the irrigation might be exposed to contact, droplet inhalation or even accidental ingestion. Conversely, direct potable reuse will require extensive treatment and disinfection of the municipal effluents to make it fit for human consumption, as the exposure of the costumers to the reclaimed water carries 100% certainty of ingestion and other forms of contact.

The way in which the required microbiological quality of a reuse water for a specific application is determined, is obtained through a risk analysis called Quantitative Microbial Risk Assessment (QMRA). With this analysis, the user's exposure to a pathogen is quantified (i.e. amount of water ingested during bathing, or the amount of time exposed to an aerosol), and the probability of infection is calculated by using pathogen-specific dose-response models (Drechsel et al., 2010; Owens et al., 2020; Zhiteneva et al., 2020). In simple terms, QMRA determines if the microbial quality of a specific water source is acceptable for a given reuse, based on how and how often people interact with it, and a probability of infection (risk) that is considered acceptable. This means that applications in which the contact between users and pathogens is unlikely (limited and infrequent) will demand less-stringent regulations in microbiological terms. For example, in the European Union, regulation (EU) 2020/741 determines the characteristics reclaimed water must fulfil to be suitable for irrigation, depending on the type of exposure the crop has to it. Edible crops in contact with reclaimed water (i.e. strawberries, onions, tomatoes, etc.) have to meet stringent microbiological requirements, while higher concentrations of *E. coli* are allowed for crops that are not to be consumed (i.e. linen, sugar beet, lavender, etc.) and thus requiring less water treatment (Table 1-2).

Table 1-2 *E. coli* maximum allowed concentrations in reclaimed water for irrigation of different crop types (from edible raw, to inedible), and suggested level of treatment, according to (EU) 2020/741 regulation on minimum standards for water reuse.

Crop type	Irrigation type	*E. coli* (cfu/100ml)	Indicative treatment
- crops eaten raw, crop in contact with reuse water	All methods	< 10	2ry treatment, filtration, disinfection
- crops eaten raw with no contact with reuse water	All methods	< 100	2ry treatment, disinfection
- process food crops - non-edible crops - crop for foraging animals	Drip irrigation	< 1.000	
Industrial and energy production crops	All methods	< 10.000	

In case after 2ry treatment municipal effluents have microbial concentrations that renders them unfit for several applications, effluent disinfection becomes necessary when this water is needed for more high-end purposes. Large scale municipal WWTP in which disinfection is implemented, normally use UV irradiation, ozonation or chlorination as the most common disinfection processes (Henze et al., 2008).

1.5 Conventional wastewater disinfection

Water disinfection is the destruction, inactivation or removal of waterborne pathogens from the water, reducing or eliminating the risk of infection to users. Contrary to popular conception, disinfection is not limited to adding biocidal agents to the water, such as chlorine gas or other chlorine-based compounds. In fact, some of the most modern wastewater treatment and reclamation technologies (i.e. ultrafiltration, reverse osmosis, etc.) effectively disinfect water without the use of any biocides, and can achieve far better results than chlorine (Abou-Elela et al., 2012; Francy et al., 2012). In this section, some of the most common effluent disinfection technologies will be presented, including advantages and disadvantages of each method, e.g., the production of harmful Disinfection By Products (DBPs), costs and effectiveness will be discussed.

The most commonly used effluent disinfectant worldwide is chlorine (Azuma & Hayashi, 2021; Henze et al., 2008; Rizzo et al., 2013). It is a well-established technology, cheaper than other methods such as UV or ozone (except when de-chlorination is performed), and can extend disinfection past the injection point as residual chlorine continues acting downstream. It is a flexible and reliable disinfection method, effective against a wide range of pathogens (USEPA, 1999a). However, the reaction between chlorine and organic matter present in wastewater produces DBPs such as Trihalomethanes and Haloacetic acids, substances which have been linked to cancer and miscarriages (Pulido, 2005; Kerwick et al., 2005). Chlorine is also toxic to aquatic life, for which chlorinated effluents need to be de-chlorinated before environmental discharge. Additionally, certain protozoan cysts (i.e. *Giardia Lamblia*, *Cryptosporidium parvum*), as well as parasitic worm eggs (i.e. *Ascaris lumbricoides*) have been shown to be extremely resistant against the effect of chlorine (USEPA, 1999a,b), for which physical removal by sand filtration remains critical for their elimination.

Ozonation is also a commonly used disinfection method for municipal effluents, process that implies the dissolution of gaseous O_3 into the bulk liquid. Disinfection by O_3 occurs by oxidative damage to microbial cell walls and nucleic acids, by damage to carbon-nitrogen bonds, and by non-specific oxidation by the formed hydroperoxy and hydroxyl radicals (HO_2 and $^{\bullet}OH$ respectively) (Roy et al., 1981; Tyrrell et al., 1995). Ozonation has also been successfully used for organic micro-pollutants (OMP) removal of diverse nature, as well as ARG (Hollender et al., 2009; Pruden, 2014; Snyder et al., 2003). During the ozonation phase, off-gases rich in O_3 can be released into the atmosphere. These are highly irritating and potentially toxic, so their destruction by heat is required in order to preserve the health of the WWTP operators (USEPA, 1999c; Spit et al., 2022). Ozonation is not devoid of DBP production, and while Bromate is the most known by-product (especially in bromide-rich waters),

other toxic compounds such aldehydes and carboxylic acids are frequently formed and discharged in the effluents (USEPA, 1999c; Wang et al., 2022).

Ultraviolet systems are also amongst the most used disinfection technologies for secondary effluents. Disinfection targets the microbial genetic material (DNA/RNA) by dosing it with electromagnetic energy (ultraviolet light 250-270nm wavelength), hindering the organism's capability of reproducing (Bolyard et al., 2019; USEPA, 1999d). As it does not imply the dosage of any biocidal agent, UV disinfection is considered a physical process, which forms no DBPs, and has no toxic effects for humans or the environment downstream. UV light is particularly effective against hardy microorganisms such as viruses, spores and cysts, with required contact times of typically 20-30 seconds (Chahal et al., 2016; Paidalwar & Khedikar, 2016). However, because the process relies on the UV light penetration in the water, it can be hampered by turbidity, suspended solids and dissolved organics (Bolyard et al., 2019; Carré et al., 2018). UV lamps are also prone to fouling, for which periodic cleaning is required. The process is also more costly to implement and operate than other technologies such as chlorine (USEPA, 1999d; Paidalwar & Khedikar, 2016).

Table 1-3 Disinfection performance, advantages and disadvantages of chlorination, ozonation and UV light following municipal effluent treatment. Based on USEPA 1999a,b,c,d and WHO, 2016.

Treatment Process	Pathogen group	Log_{10} removal	Advantages	Disadvantages
Chlorine	Virus	2 (C.T 2-30min.mg/L, 0-10°C, pH 7-9)	Long track-record. Inexpensive and reliable. Provides residual disinfection. Easy to scale up.	Turbidity and chlorine-demanding solutes inhibit the process. De-chlorination may be required. Poor protozoa inactivation (high CT). Issues with taste & odor
	Bacteria	2 (C.T 0.04-0.08 min.mg/L, 5°C, pH 6-7)		
	Protozoa	2 (C.T 25-245 min.mg/L, 0-25°C, pH 7-8)		
Ozone	Virus	2 (C.T 0.006-0.2 min.mg/L)	On-site generation (fewer logistics and handling problems). No microbial regrowth.	Capital & power intensive. No residual disinfection. Off-gases need to be destroyed. Produces DBPs.
	Bacteria	2 (C.T 0.02 min.mg/L)		
	Protozoa	2 (C.T 0.5-40 min.mg/L)		
UV light	Virus	4 (7-186 mJ/cm2)	Chemical-free process. No toxic effect downstream. Contact time 20-30s	Turbidity and dissolved compounds can interfere with disinfection. Tubes prone to fouling. Microbial regrowth possible.
	Bacteria	4 (0.65-230 mJ/cm2)		
	Protozoa	4 (<1-60 mJ/cm2)		

1.6 Iron Electrocoagulation

A green and robust alternative to conventional disinfection is Iron Electrocoagulation (Fe-EC). This technology is typically not considered for disinfection processes, possibly due to its resemblance to chemical coagulation. Successful applications of

Fe-EC also involve nutrient removal (Delaire et al., 2016; Lacasa et al., 2011; Symonds et al., 2015), arsenic removal (Amrose et al., 2013; Delaire et al., 2015; Zodi et al., 2011) turbidity/suspended solid abatement (Cotillas et al., 2014; Merzouk et al., 2009), and degradation of a wide variety of OMPs such as hormones and antibiotics (Maher et al., 2019; Yoosefian et al., 2017). This makes Fe-EC attractive for the treatment of complex secondary waste water effluents. Also, the technology relies on current instead of chemicals, making it also promising for decentralised or off-grid application.

Fe-EC is an electrochemical water treatment process in which Fe^{2+} ions are released into the bulk liquid from a metallic anode following the application of electric current (Jiménez et al., 2012; Lakshmanan et al., 2009; Sasson et al., 2009), subsequently forming insoluble flocs constituted by amorphous $Fe(OH)_2$, or by $Fe(OH)_3$ under the presence of oxygen. These flocs are credited with entrapping suspended pollutants, which can later be removed by either sedimentation or flotation (Figure 1-2).

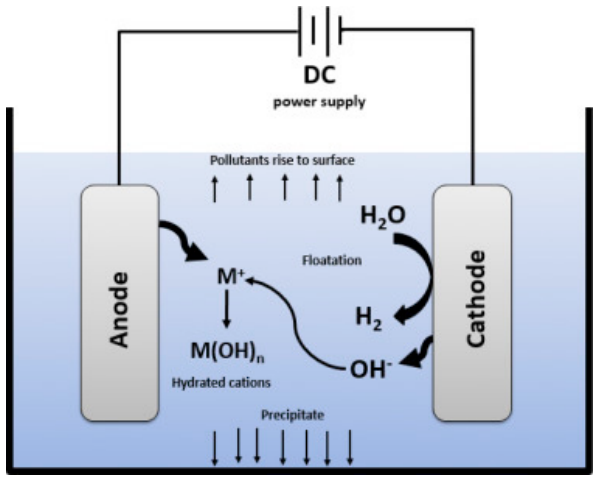


Figure 1-2 Basic electrocoagulation setup and process description (Moussa et al., 2017).

Fe-EC has typically been restricted to industrial applications in heavy industries, such as petrochemical processing, slaughterhouses, paper pulp and dairy industry (Khansorthong & Hunsom, 2009; Kushwaha et al., 2010; Yavuz et al., 2010) where in some cases its capacity for disinfection has been reported (Bazrafshan et al., 2013; Bergmann, 2021). Microbial inactivation and OMP degradation during Fe-EC has also been linked by several researchers to the production of Fenton-like intermediates, usually known as Reactive Oxygen Species (Delaire et al., 2016; Heffron, 2019b; Tanneru & Chellam, 2012). These short lived compounds (Rubio & Cerón, 2021) have a highly

oxidative nature, which allows them to degrade a wide range of organics (including microorganisms) and without DBP generation. In other words, it is a technology with promising traits to be used as tertiary treatment in municipal effluents, as besides from disinfection, it offers added advantages that conventional disinfection methods can seldom deliver, such as clarification and nutrient removal, and no production of DBPs.

Unfortunately, accurate mechanistic descriptions of the (disinfection) processes behind Fe-EC are missing, and there is little understanding on which are the main parameters driving it. Dose-response data on Fe-dosage and disinfection is fragmentary at best, for which performance of Fe-EC when compared to other conventional disinfection methods is largely unknown. Cost estimations of the process are not covered by scientific literature, which seriously hinders the adoption of the technology for being considered on large scale applications.

1.7 Research questions

- Do antibiotic sensitive and resistant bacteria behave differently during wastewater treatment processes (activated sludge, electrocoagulation and chlorination) and subsequent effluent discharge (**Chapter 2**)?
  - Are ASB suitable proxies for ARB during wastewater treatment, disinfection and decay?

- How does Fe-EC perform in disinfecting real secondary effluents (**Chapter 3**)?
  - Is there any difference in response to Fe-EC between virus, bacteria, ARB and protozoan indicators?
  - Which are the main operational parameters driving disinfection?
  - How does Fe-EC compare cost-wise to conventional disinfection techniques?

- What role do Reactive Oxygen Species (ROS) play during Fe-EC disinfection (**Chapter 4**)?
  - How can ROS disinfection be quantified during Fe-EC?
  - Can ROS disinfection kinetics be described mathematically?
  - Do Fe-EC disinfection kinetics differ between viruses and bacteria?

- How do floc sorption/entrapment and ROS inactivation interact during Fe-EC (**Chapter 5**)?
  - Can disinfection by coagulation/sedimentation and ROS be decoupled and independently quantified?
  - What is the role of pH in ROS inactivation and floc entrapment?
  - How can flocculation enhance disinfection in Fe-EC?

1.8 Thesis outline

This thesis investigates the potential of Fe-EC as a disinfection technology, specifically for the treatment of municipal secondary effluents. A significant portion of the work here conducted is focused on the elimination of ARB, whose behavior during electrochemical treatment remains largely unknown.

The study of the behavioral similarities between ASB and ARB during conventional and unconventional wastewater treatment operations and spontaneous decay is discussed in Chapter 2. For this, wastewater samples from a real municipal WWTP (raw sewage and secondary effluent) were collected and subjected to chlorination, Fe-EC and long term decay experiments at different temperatures. The performance of the WWTP itself was also evaluated throughout a 5 month sampling campaign.

Chapter 3 is a detailed prospection of Fe-EC capabilities in terms of disinfection, critical operational parameters and cost estimation. It also elaborates on the differences in disinfection between the various microbial indicators, and on the conflicting results in literature obtained from experiments using real secondary effluents as opposed to those obtained from synthetic water matrices.

In order to describe the specific processes leading to ROS inactivation by Fe-EC, Chapter 4 investigates inactivation in the presence and absence of ROS, by the introduction of a Fenton inhibitor. It also describes the ROS inactivation kinetics promoted by the (semi)Fenton reactions triggered by the aerobic Fe^{2+} oxidation, arriving into a first-of-its-kind mathematical expression linking disinfection to electrical settings.

Chapter 5 looks at the de-coupling of the two main disinfection processes following Fe-EC, namely floc sorption/entrapment and ROS inactivation. The optimization of the floc formation process and disinfection by floc removal, and its link to the hydraulic mixing conditions is described in detail. The ROS disinfection capacity is also described in this chapter, where an explanation to the differences in disinfection is provided based on the different nature of the formed radicals at different pH levels.

CHAPTER 2

Antibiotic resistant ESBL-*E. coli* and VRE mirror the behaviour of faecal indicators *E. coli* and Enterococci during municipal wastewater treatment and discharge

Abstract

Following activated sludge treatment, chlorination, iron electrocoagulation, and spontaneous decay, classic faecal indicators as *Escherichia coli* and intestinal enterococci were investigated as prospective proxies for presence and disinfection of their resistant strains Extended Spectrum Betalactamase- producing (ESBL)-*E. coli* and Vancomycin Resistant Enterococci (VRE), organisms of global public health concern. Activated sludge treatment provided a 2.23 ±0.13 LRV for antibiotic resistant and sensitive bacteria alike, and did not significantly impact the ratios between them in the secondary effluent. Disinfection by electrocoagulation proved more effective against *E. coli* and ESBL-*E. coli* than against Enterococci and VRE, with first order rate constants of 0.066 ±0.003 $l.mg^{-1}$ Fe and 0.039 ±0.001 $l.mg^{-1}$ Fe respectively, though no significant difference was observed between the resistant bacteria and their sensitive counterparts. Free chlorine disinfection (0.45-0.50 mgCl/l) proved slightly more effective against *E. coli* and ESBL-*E. coli* than against Enterococci and VRE, with first order rate constants of 0.365 ±0.006 $l.mgCl^{-1}.min^{-1}$ and 0.320 ±0.008 $l.mgCl^{-1}.min^{-1}$ respectively. Decay experiments at 4, 13 and 24 ^{o}C showed a biphasic behaviour, with faster die-off at the beginning of the experiments, slowing down towards the end, with no relevant difference in decay between either of the indicators, irrespective of them being sensitive or resistant. It is therefore concluded that antibiotic resistant ESBL-*E. coli* and VRE mirror the behaviour of faecal indicators *E. coli* and Enterococci, experiencing the same rates of disinfection/ decay, and maintaining similar ratios between sensitive and resistant populations before and after treatment.

2.1 Introduction

The World Health Organization highlighted in 2015 the dimension of the Antibiotic Resistance (AR) menace, which “threatens the very core of modern medicine”, as few viable replacement drugs are being developed (WHO, 2015). A more recent global survey conducted by the Global Antimicrobial Resistance and Use Surveillance System (GLASS) in 2021 concluded that in 2019 alone, approximately 4.95 million people died due to AR-related complications, of which 1.27 million were a direct consequence of the ARB-infection, surpassing the 2019 death toll of HIV/AIDS and malaria combined. The survey indicates that the highest mortality rates occur in Sub-Saharan Africa and Southern Asia.

Antibiotic resistance is the result of a process by which bacteria acquire resistance against specific antibiotics. Although commonly associated with clinical infections caused by pathogenic organisms, the term Antibiotic Resistant Bacteria (ARB) does not limit itself to pathogens, as it can be observed in a wide range of bacteria, both human (or animal)-related and environmental. Several studies have traced the origin of clinically relevant ARB strains and/or their resistance mechanisms to bacteria living in water or soil in natural environments (Finley et al., 2013; Singer et al., 2016).

AR exists since long before human developed antibiotics, as a part of a never ending microbial warfare by which bacteria outcompete others by naturally producing toxic metabolites, some of which resemble the pharmaceuticals we know today (Larsson & Flach, 2021). Antibiotics exert selective pressure over bacterial populations, killing those that lack the adequate defence mechanisms, thus allowing resistant ones to take over. Even at sub-lethal antibiotic concentrations, ARB tend to outcompete Antibiotic Sensitive Bacteria (ASB) (Gullberg et al., 2011; Hrenovic et al., 2017; Liu et al., 2011). The introduction of antibiotics has greatly accelerated the spread of resistance, promoting the acquisition of Antibiotic Resistance Genes (ARG) by Horizontal Gene Transfer (HGT) and other processes that allow bacteria to acquire these genes from other bacteria, even if these belong to different species (Lamba et al., 2017; Rizzo et al., 2013; Zhang et al., 2016).

Waste water treatment plants (WWTPs) have been flagged as hotspots for AR dissemination (Brown et al., 2006; Czekalski et al., 2012; Díaz-Cruz et al., 2003; Hirsch et al., 1999; Kümmerer, 2009; Rizzo et al., 2013) due to the simultaneous discharge of antibiotics, ARB and ARG into the environment. This is mainly facilitated by ARB and ARG on the incoming faecal matter, high cell densities associated with biological treatment, presence of nutrients and a steady selective pressure caused by low concentrations of incoming antibiotics and their metabolites in domestic sewage

(Guo et al., 2017; Manaia et al., 2018; Michael et al., 2013). As a consequence, treated effluents usually carry high concentrations of human and animal bacteria, many of which harbour ARGs, thus becoming potential vectors for their dissemination into the environment (Berendonk et al., 2015; Manaia, 2017; Pruden, 2014).

Literature is divided on whether municipal WWTP selects for ARB during biological treatment or not. Some authors point to increases in the proportion of ARB in treated effluents (Al-Jassim et al., 2015; Biswal et al., 2014; Łuczkiewicz et al., 2010; Korzeniewska & Harnisz, 2018), while others indicate a decrease of ARB relative abundance after treatment (Nimonkar et al., 2019; Varela et al., 2013; Guardabassi et al., 2002). The effect of the WWTP discharge in the receiving water bodies is also highly controversial, as some studies indicate ARB enrichment of water and sediment populations downstream (Akiyama & Savin, 2010; Leclercq et al., 2013; Osińska et al., 2016; Sidrach-Cardona et al., 2014), others prove inconclusive or without significant variations (Czekalski et al., 2012; Schreiber & Kistemann, 2013; West et al., 2011; Zhang et al., 2015), and a final group describes either simultaneous enrichment of certain ARB populations and decrease of others, or seasonal increase/decrease cycles (Blaak et al., 2014; Koczura et al., 2012; Marti et al., 2014; Zhang et al., 2015).

The efficacy of municipal wastewater treatment is evaluated based on a list of parameters, normally described in national/local guidelines, comprising diverse contaminant groups such as organic content, nutrients, metals and microbiological indicators. Regarding the latter, (sensitive) *Escherichia coli* and intestinal enterococci appear as the most commonly used microbial indicators, together with faecal coliforms (Lin & Ganesh, 2013; Scott et al., 2003). Water quality screening designed for the evaluation of potential faecal contamination on other water uses such as recreational waters and reclaimed water for irrigation or potable reuse, also rely on these faecal indicators to assess suitability of use (Purnell et al., 2020; Rodrigues & Cunha, 2017; Salgot et al., 2006). However, to date, no guideline limiting the presence of ARB and/or ARG in drinking water, wastewater, reuse water, or any other water of municipal concern, mainly due to a lack of consensus on which are adequate AR indicators to measure.

In this chapter, we study the similarities between the classic microbial indicators *E. coli* and intestinal enterococci and specific resistant *ESBL-E. coli and VRE* of concern in municipal effluents, in order to determine whether the former can be used as proxy for elimination of their resistant counterpart through conventional and novel water treatment processes, as well as their natural decay.

2.2 Materials & Methods

2.2.1 Sewage and secondary effluent collection

Grab samples of raw sewage and secondary effluent were collected every two weeks from a large municipal activated sludge wastewater treatment plant (AS-WWTP) located in the southwest of the Netherlands. All samples were taken between the months of November 2020 and March 2021, with twelve sampling events in total. All samples were transported in coolers directly to the laboratory and processed within 6 hours of collection. In order to avoid debris, raw sewage samples were collected immediately after the screens. Samples of secondary effluent were collected from the discharge mains of the secondary settlers.

2.2.2 Disinfection and decay experiments

Experiments evaluating secondary effluent disinfection by Iron Electrocoagulation (Fe-EC) were performed by dosing continuous current into the liquid through two parallel and partially submerged ARMCO iron plates (maximum percent- ages: 0.14% carbon, 0.10% silicium, 0.80% manganese, 0.025% phosphorous, 0.015% sulphur, 0.010% nitrogen, 0.20% copper and 0.080% aluminium). These were connected to a dual 30 V - 3 A TENMA 72-10500 bench DC supply by crocodile clip cables. Electrodes were square-shaped (40 mm X 40 mm), and provided with an thin elongation parallel to one of the sides (40 mm X 5 mm) to act as a dry contact for the clip cables (preventing the crocodile clips' dissolution). Plates were polished with coarse and fine sand paper and rinsed with demineralized water before each experiment. Beakers containing the effluent were fitted with a PTFE coated bars and placed on LABNICO L23 magnetic stirrers for mixing purposes. To maintain oxygen saturation, air was supplied continuously during the application of current using an OASE OxyMax200 air pump. 30L grab sample of secondary effluent was divided in two for duplicate production, and for each half, experiments were performed in 2 l beakers by exposing the samples to a current of 287 mA during varying amounts of time in order to provide an increasing dosage of Fe. Once the desired dosages were achieved, samples were covered and let to settle during 2 h, after which the supernatant was collected for microbial and physical/chemical screening in triplicate.

Experiments evaluating disinfection by chlorine were performed using a 30L grab sample of secondary effluent, divided in two for duplicate testing, and dosed with NaOCl (Sigma-Aldrich, Germany) for disinfection. The dosage of NaOCl (1.25%) was of 0.9 ml/l, which yielded an initial free chlorine value of approximately 0.50 mg/l as determined with the spectrophotometric US-EPA DPD method (HACH, USA), processed on a Spectroquant®NOVA60 spectrophotometer (Merck, Germany). Chlorine demand was measured in the same way throughout the experiment,

simultaneously with the sample extractions once the desired exposure times were achieved, after which Free Chlorine was neutralized by the addition of 5 ml/l of 0.1M $Na_2S_2O_3$ Sodium Thiosulfate solution (Sigma-Aldrich, USA). The disinfected effluent samples were then processed immediately for microbial screening in triplicate.

To simulate the microbial decay of sensitive and resistant bacteria in secondary effluent, 30 l effluent grab samples were divided into 15 l containers and stored in the dark at different temperatures: 4, 13 and 24 ºC. All conditions were tested in duplicate. These were placed on an orbital shaker for 21, 9 and 4 days respectively, during which aliquots were extracted and screened for microbial concentrations in triplicate.

2.2.3 Microbial indicators and culture media

The screening of microbial indicators was based exclusively on culture methods. *E. coli* and Enterococci are two of the most commonly used faecal indicators due to their extensive contact with human beings and their ease of detection and quantification (Al-Jassim et al., 2015; Anfruns-Estrada et al., 2017; Harwood et al., 2005; Noble et al., 2004; Petri et al., 2008; Rosenberg Goldstein et al., 2014). For each of them, resistance against a specific type of antibiotic was selected, namely Betalactams for *E. coli* and Vancomycin for Enterococci, as these ARB are listed under the category of "serious threat" by the US CDC and the ECDC 2019 and 2022 Antibiotic Resistance reports respectively (ECDC, 2022; US CDC, 2019). The four indicator organisms and their respective growth media are indicated in Table 2-1.

Table 2-1 Selected indicators and growth media

Indicator	Growth medium
Escherichia coli (E. coli)	Chromocult® agar medium (ISO 9308-1), Merck Millipore.
Extended Spectrum β Lactamase (**ESBL**) producing ***–E. coli***	ChromID® ESBL agar medium. Biomerieux-Diagnostics (Marcy l'Etoile, France).
Enterococci	Slanetz-Bartley agar medium (ISO 7899), Merck Millipore
Vancomycin Resistant Enterococci **(VRE)**	ChromID® VRE agar medium. Biomerieux-Diagnostics (Marcy l'Etoile, France).

2.3 Results

2.3.1 AS-WWTP

During the 5-month sampling campaign on the AS-WWTP, samples of raw sewage and secondary effluent were collected every two weeks, and the concentrations of *E. coli*, ESBL-*E. coli*, Enterococci and VRE were determined in triplicate in each sample. Within each sample, standard deviation was in almost all cases one order of magnitude lower than the concentration average for the triplicates, indicating the uncertainty in the observed concentrations was low (Figure 2-1). Microbial concentrations in the raw sewage and secondary effluent from the selected municipal WWTP were relatively stable during the sampling period for both the sensitive indicator bacteria as well as the ESBL-*E. coli* and VRE enterococci, with no clear temporal trends.

The activated sludge process and in particular its capability for removal of faecal indicator bacteria, have been extensively studied for decades, with most literature reporting LRV between 1 and 3 for diverse faecal indicators (De Luca et al., 2013; Hata et al., 2013; Fu et al., 2010). The selected WWTP performs as expected, with an average LRV of 2.1 – 2.4 $\log_{10}$, with standard deviations of 0.3-0.4 $\log_{10}$ for all indicators including ESBL-*E. coli* and VRE. This shows that the resistant ESBL-*E. coli* and VRE did not experience any better or worse removal than sensitive *E. coli* or Enterococci during the activated sludge treatment, not being significantly better or worse suited to withstand the process (ANOVA p-value = 0.43), irrespective of their antimicrobial resistance condition.

For the raw sewage, average *E. coli* concentration was $5.5x10^7$ cfu/l, while average ESBL-*E. coli* concentration was determined at $5.1x10^5$ cfu/l, for which the *E. coli*/ESBL-*E. coli* ratio in the sewage was 124 ±27 to 1. Enterococci average concentration in the sewage was $7.3x10^6$ cfu/l, while that of VRE was $3.4x10^5$, meaning that the Enterococci/VRE ratio was in the 25 ±13 to 1 range (Table 2-2). On the secondary effluent however, average *E. coli* concentration was $3.3x10^5$ cfu/l, while average ESBL-*E. coli* concentration was determined at $2.2x10^3$ cfu/l, for which the *E. coli*/ESBL-*E. coli* ratio in the sewage was in the 140 ±36 to 1 order. Enterococci average concentration in the secondary effluent was $5.8x10^4$ cfu/l, while that of VRE was $1.2x10^3$, meaning that the Enterococci/VRE ratio was in the 48 ±28 to 1 order. This means that in the sewage, only 1% of *E. coli* cells were beta-lactam resistant and 5% of the Enterococci cells were Vancomycin resistant, while in the secondary effluent less than 0.7% of *E. coli* cells were beta-lactam resistant and 2% of the Enterococci cells were Vancomycin resistant. For both groups, results show that the resistant bacteria fractions did not increase as a consequence of the activated sludge treatment, whereas in fact a slight (not significant) decrease was observed.

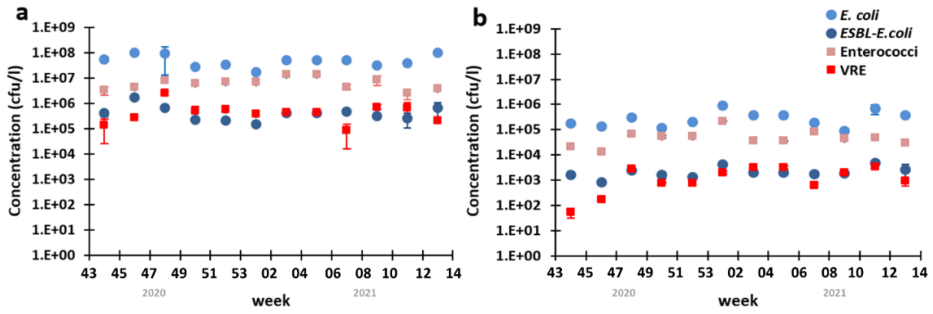


Figure 2-1 Concentrations of *E. coli*, Enterococci, ESBL-*E. coli* and VRE during November 2020-March 2021 sampling campaign in the WWTP's a) raw sewage, and b) secondary effluent. Microbial determination was performed in triplicate. Error bars represent standard deviation.

Table 2-2 Average concentrations and standard deviation of *E. coli*, Enterococci, ESBL-*E. coli* and VRE during the November 2020-March 2021 sampling campaign in the WWTP's raw sewage and secondary effluent, and mean Log removal value (LRV) for each indicator.

	Influent (cfu/l)		Effluent (cfu/l)		LRV	
Indicator	**Average**	**stdev**	**Average**	**stdev**	**Average**	**stdev**
E. coli	$5.5x10^7$	$3.1x10^7$	$3.3x10^5$	$2.5x10^5$	2.2	0.4
ESBL-*E. coli*	$5.1x10^5$	$4.5x10^5$	$2.2\ x10^3$	$1.2\ x10^3$	2.4	0.4
Ratio	124	27	140	36		
Enterococci	$7.3x10^6$	$3.7x10^6$	$5.8x10^4$	$5.3x10^4$	2.1	0.3
VRE	$3.4x10^5$	$3.1x10^5$	$1.7\ x10^3$	$1.2\ x10^3$	2.3	0.4
Ratio	25	13	48	28		

2.3.2 Decay

In this chapter, temperature decay kinetics were studied for real secondary effluent samples under controlled temperature conditions for all indicators, simulating a prospective discharge into a hypothetical receiving water body. Following effluent discharge into aquatic environments, faecal microorganisms generally progress towards non-viability, process usually termed as decay (Korajkic, et al., 2019). Water temperature is a major factor in decay, as it has been show to influence first order decay rate constants on a proportional basis, meaning that decay progresses faster in warmer waters and slows down when the water is colder (Easton et al., 2006; Hellweger et al., 2009; Medema et al., 1997). To determine whether sensitive and resistant *E. coli* and Enterococci show similar survival in receiving water bodies, decay experiments

were performed at different temperatures. Three temperature scenarios were assayed, namely; cold (4°C), mild (13°C) and warm (24°C). For each assayed temperature, experiments were concluded once the concentrations of the resistant strains VRE/ESBL-*E. coli* were below levels that allowed accurate quantification. Results are displayed in Figure 2-2.

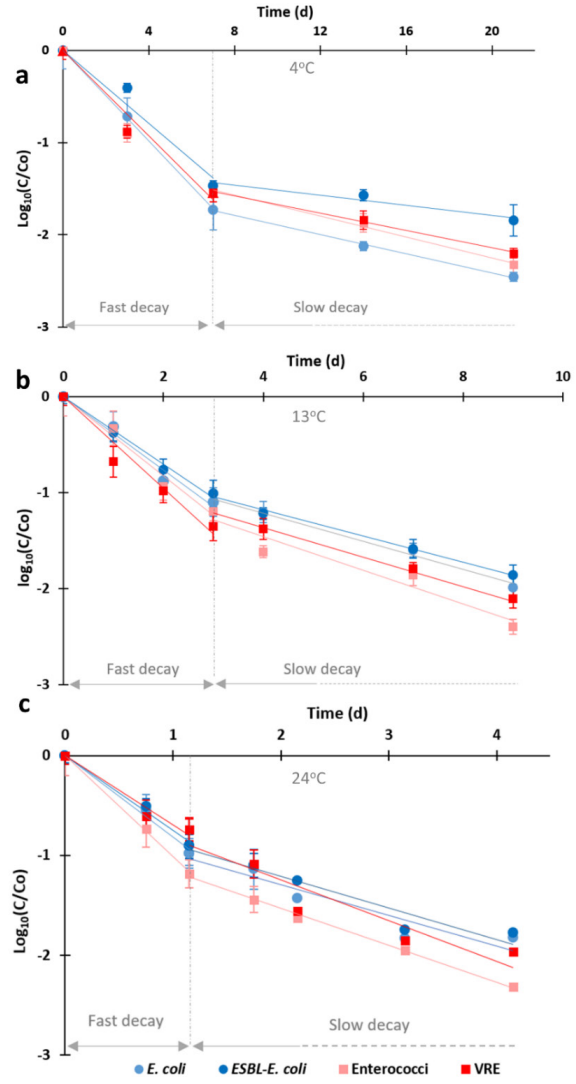


Figure 2-2 Log_{10} data series of the relative microbial concentration (C/C_o) for *E. coli*, ESBL-*E. coli*, Enterococci and VRE on secondary municipal effluent stored at a) 4°C b) 13°C and c) 24°C. Experiments were performed in duplicate. Microbial screening was performed in quadruplicate for T=0, and triplicate for the rest of the samples. Error bars indicate standard deviation.

For the cold scenario (4°C), microbial decay appeared to be biphasic, with a sharper decrease in concentration during the first 7 days. An inflexion can be observed in all the trendlines on day 7, after which a much slower decrease in microbial concentration is observed (Table 2-3). Experiments concluded at 21 days, with all indicators presenting a LRV of ≈2.15 ± 0.35 (Figure 2-2a). For the mild scenario (13°C), similar observations were made, as a biphasic behaviour is displayed for all indicators, presenting an inflection point at day 3. Experiments concluded after 9 days, with all indicators presenting a LRV of ≈2.12 ± 0.27 (Figure 2-2b). For the warm scenario (24°C), biphasic behaviour was also observed, presenting the highest rates of decay of all assayed conditions. The inflection point was determined at approximately 1.15 days, and experiments concluded at 4.15 days when all indicators presented a LRV of ≈2.05 ± 0.28 (Figure 2-2c).

Based on the bi-phasic behaviour of the decay process, and the good linearity observed in each of the phases, it can then be described as follows:

$$log_{10}\left(\frac{C}{C_0}\right) = -k_1.t \quad \text{if } t<t_{inflection} \quad (2\text{-}1)$$

$$log_{10}\left(\frac{C}{C_0}\right) = -k_1.t_{inflection} - k_2.(t - t_{inflection}) \quad \text{if } t> t_{inflection} \quad (2\text{-}2)$$

Where:

C= Bacteria concentration at time t (cfu/l)

C_0=Bacteria concentration at time 0 (cfu/l)

K_1=First order rate constant observed during the fast decay phase (d^{-1})

K_2=First order rate constant observed during the slow decay phase (d^{-1})

t= time since the beginning of the experiments (d)

$t_{inflection}$= timestamp in which a change in decay rate is observed (d)

Table 2-3 Slope values k_1 and k_2 for the linear trendlines of *E. coli*, ESBL-*E. coli*, Enterococci and VRE during the temperature decay experiments.

	k_1 (d^{-1})			k_2 (d^{-1})		
	4°C	13°C	24°C	4°C	13°C	24°C
E. coli	0.25	0.38	0.82	0.05	0.15	0.31
ESBL- *E. coli*	0.20	0.35	0.75	0.04	0.14	0.32
Enterococci	0.23	0.41	1.02	0.06	0.18	0.37
VRE	0.23	0.48	0.70	0.05	0.15	0.41

2.3.3 Disinfection

Municipal effluent disinfection, usually termed tertiary treatment, is increasingly seen as a way of obtaining high quality polished effluents, with low organic and nutrient content, as well as reduced microbiological load (Henze et al., 2008). Chlorination, is the most popular disinfection technology currently applied in WWTPs (Manaia et al., 2018) due to its broad disinfection spectrum, high efficiency, and low O&M costs (Azuma & Hayashi, 2021; How et al., 2017; Nihemaiti et al., 2020; Rizzo et al., 2013), hence selected for secondary effluent disinfection experiments. Secondary effluent samples were exposed to chlorine by applying 0.9 ml/l NaOCl [1.25%], which yielded an initial free chlorine concentration of ≈0.50 mg/l. This concentration remained relatively stable during the duration of the experiment, with a final average concentration of 0.44 mg/l after 16 minutes. A $Log_{10}(C/C_0)$ plot was constructed as a function of the product of free chlorine concentration (mg/l) and exposure time (min), commonly known as CT following the Chick-Watson equation for disinfection. Results are indicated in Figure 2-3.

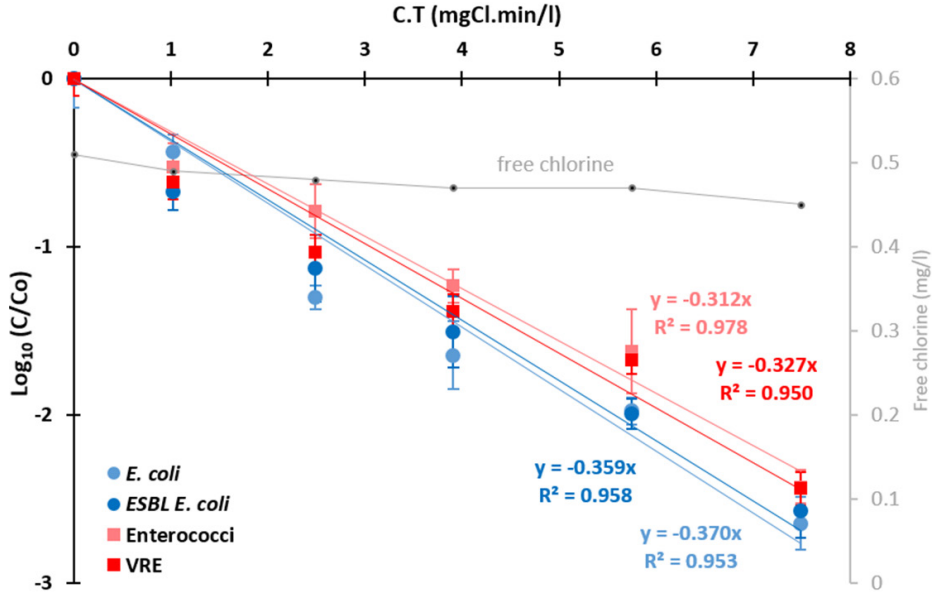


Figure 2-3 Log_{10} data series of the relative microbial concentration (C/C_o) for *E. coli*, ESBL-*E. coli*, Enterococci and VRE following chlorine disinfection in real municipal secondary effluent by the use of NaOCl during 15 minutes. Initial Free Chlorine values were ≈0.50 mg/l. Cumulative C.T. values were calculated based on the length of the time intervals and the measured Free Chlorine value during said interval. Experiments were performed in duplicate. Microbial screening was performed triplicate. Error bars indicate standard deviation.

Chlorine disinfection showed a good linear fit between the $\log_{10}(C/C_0)$ values and CT for all indicators ($R^2 \geq 0.95$), and disinfection data series were described by a first order kinetic process, namely:

$$log_{10}\left(\frac{C}{C_0}\right) = -k.C_{FCl}.t \quad (2\text{-}1)$$

Where:

C = Bacteria concentration at time t (cfu/l)
C_0 = Bacteria concentration at time 0 (cfu/l)
K = First order rate constant ($l.mgCl^{-1}.min^{-1}$)
C_{FCl} = Concentration of free chlorine (mgCl/l)
T = exposure time to disinfectant (min)

The inactivation rate constant (k-value) of *E. coli* was very similar to that of ESBL-*E. coli* (0.370 vs 0.359 $l.mgCl^{-1}.min^{-1}$). Similarly, the inactivation rate of enterococci was very similar to that of VRE (0.312 vs 0.327 $l.mgCl^{-1}.min^{-1}$). The Enterococci/VRE are somewhat more difficult to inactivate with chlorine than *E. coli*/ ESBL-*E. coli*. This distinct behaviour of gram positive and gram negative bacteria has been previously reported, and attributed to differences in bacterial membranes and cell wall structures, as chlorine reacts more aggressively with lipid-rich membranes (Mir et al., 1997).

2.3.4 Coagulation-sedimentation

Coagulation processes have seldom been reported as a mainstream disinfection mechanism, as more conventional technologies like chlorination, UV and ozonation usually take precedence. For this set of experiments, secondary effluent samples were subjected to a Fe electrocoagulation process, conducted by electrolysis with high purity Fe-electrodes, with dosages up to 42.4 mgFe/l. Samples collected at regular intervals were left to settle for 2 h and then screened for the sensitive and resistant *E. coli* and Enterococci. Linear models were used to fit the $Log_{10}(C/Co)$ plots versus Fe dosage (Figure 2-4), as this dose-response linearity for Fe-EC had been suggested in our previous research (Bicudo et al., 2022).

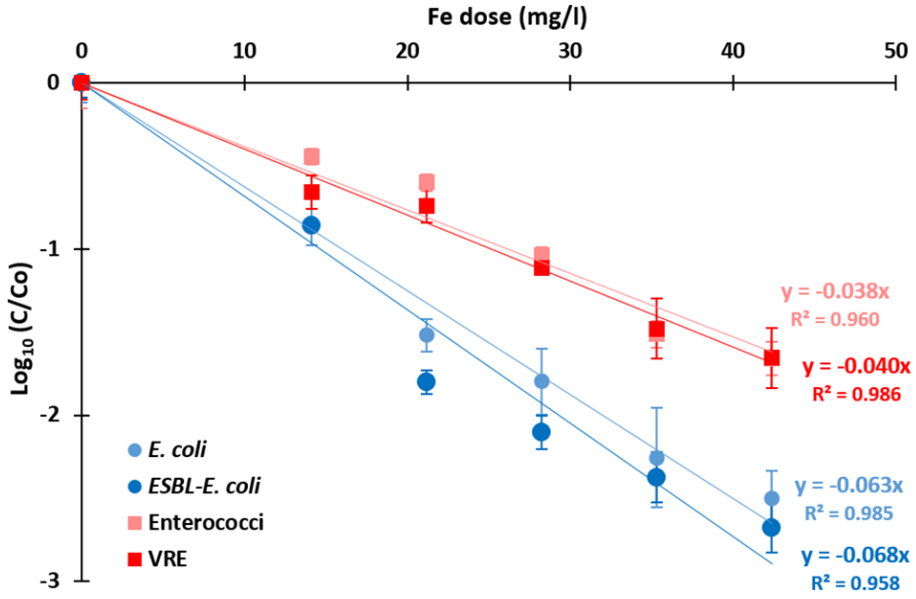


Figure 2-4 Log_{10} data series of the relative microbial concentration (C/C_0) for *E. coli*, ESBL-*E. coli*, Enterococci and VRE on secondary municipal effluent treated with Fe-EC. Fe dosage range was 0.0 - 42.4 mgFe/l. Experiments were performed in duplicate. Microbial screening was performed in quadruplicate for Fe=0 mg/l, and triplicate for the rest of the samples. Error bars indicate standard deviation.

Results show that the LRV for all indicators are directly proportional to the Fe dosage with removal reaching 2-3 $\log_{10}$ for Fe dosages of ≈42.4 mgFe/l, as previously observed by Bicudo et al., 2022. Other sources who had also investigated Fe-EC for secondary effluent disinfection arrived to similar results (2-3 $\log_{10}$ attenuation) using other microbial indicators, such as heterotrophic bacteria, somatic coliphages and *Clostridium perfringens* spores (Anfruns-Estrada et al., 2017; Bicudo et al., 2021). Because of the good linear fits obtained between the $Log_{10}(C/Co)$ and the iron dosage for the selected bacteria ($R^2 > 0.95$), disinfection was described with first order kinetics, namely:

$$\boldsymbol{log_{10}\left(\frac{C}{C_0}\right) = -k.[Fe]} \quad (2\text{-}2)$$

Where:

C= Bacteria concentration at time t (cfu/l)
C_0=Bacteria concentration at time 0 (cfu/l)
k=First order rate constant (l/mg Fe)
[Fe]= Iron dose in the bulk liquid (mg Fe/l)

Figure 2-3 indicates that the slopes (k-values) of all four indictors are clustered in pairs, with Enterococci/VRE having k-values in the 0.039 ±0.001 l/mg Fe range, and *E. coli*/ESBL-*E. coli* having k-values in the 0.066 ±0.003 l/mg Fe. The slope analysis indicates that disinfection of Enterococci was very similar to that of VRE (Δk ± 4.4%), while the same applies for *E. coli* and ESBL-*E. coli* (Δk ± 2.5%). A distinct response was observed between the Enterococci/VRE cluster and the *E. coli*/ESBL-*E coli* cluster, indicating in this case that *E. coli* (sensitive and resistant) bacteria are better removed than Enterococci (sensitive and resistant).

2.4 Discussion

The present chapter is a comprehensive evaluation of the similarities in behavior between *E. coli* and ESBL-*E. coli* and between Enterococci and VRE during conventional and non-conventional wastewater treatment/disinfection processes. Only real municipal sewage and secondary effluents were used for all experiments, as well as culture-based methods for quantification of all indicators. Discussion is geared towards understanding the value of classic microbial indicators as a proxy for both the presence and disinfection of resistant organisms using a simple, yet robust approach, currently lacking in AR literature.

Observations regarding the disinfection by activated sludge were in line with similar research including not only faecal indicators but also ARB (Wang et al., 2020; Turolla et al., 2018; Yuan et al., 2016), with most reported LRV in the 1-3 $\log_{10}$ range. The selected AS-WWTP does also not seem to affect the *E. coli*/ESBL-*E. coli* ratio significantly nor the Enterococci/VRE ratio between influent and effluent, further suggesting that removal of all four microbial indicators is proportional (ANOVA p-value>0.05). Our results, which are exclusively culture-based, show that the studied ARB undergo the same removal process than that of ASB during activated sludge treatment. No enrichment of ARB was observed in the secondary effluent, as the fraction of resistant organisms in the effluent's microbial population was not larger than that of the influent, and as the total concentration of ARB decreased >99% when compared to the incoming sewage.

Following effluent discharge, this study also examined the decay of *E. coli*, ESBL-*E. coli*, Enterococci and VRE in simulated receiving water bodies at different temperatures. Though several other factors have also been identified in microbial decay on fresh and estuarine environments, such as the incidence of sunlight, salinity, presence of heavy metals, or predation (Deller et al., 2006; Gonzalez et al., 1990; Iriberri et al., Noble et al., 2004; 1994; Sinton et al., 2002), temperature is perhaps the most relevant. In all scenarios a clear bi-phasic decline pattern was observed, consistent with previous research on faecal bacteria decay in fresh, estuarine and seawaters (Brouwer et al., 2017; Hijnen et al., 2007; Medema et al., 1997). For all assayed temperatures, die-off was faster at the beginning of the experiments and slowed down towards their end. Microbial decay plots indicated similar decay behaviour across *E. coli*, ESBL-*E. coli*, Enterococci and VRE, both qualitatively and quantitatively, as indicated by similar k-values and inflection times in the decay curves. We observed no relevant differences between the temperature decay kinetics of *E. coli* and Enterococci, and of their resistant strains ESBL-*E. coli* and VRE for any of the assayed temperature conditions. This suggests that classic microbial indicators such as *E. coli* and/or Enterococci are good proxies for tracking the decay of ESBL-*E. coli,* VRE and possibly other ARB from municipal effluents in water bodies. Disinfection with both coagulation and chlorination showed a differential response for *E. coli* and Enterococci.

For Fe-EC the first order rate constant for *E. coli and ESBL-E. coli* (0.066 ±0.003 l/mg Fe) was approximately 60% larger than that of Enterococci and VRE (0.039 ±0.001 l/mg Fe), indicating a higher sensitivity of *E. coli* towards the Fe-EC induced disinfection. Similar observations were obtained with secondary effluent chlorine disinfection (≈0.5 mg/l, room temperature and circumneutral pH), where no significant differences on inactivation first order rate constants existed between *E. coli* and ESBL-*E. coli*, nor between Enterococci and VRE. First order rate constants obtained in this study for the selected resistant strains are not only similar to those obtained for the respective sensitive strains, but also to those obtained by other researchers involving the same sensitive strains in similar temperature and pH conditions (Mwatondo & Silverman., 2021; Tyrrell et al., 1995). This indicates that the behaviour of VRE during Fe-EC and chlorination mirrored that of Enterococci, in the same way that the behaviour of ESBL-*E. coli* mirrored that of *E. coli*, in both cases within a reasonable margin of error in their first order rate constants (<5%). Hence, ESBL-*E. coli* and VRE deserve no further distinction in terms of disinfection than *E. coli* or Enterococci, which are common faecal indicators, as the latter can be used to estimate inactivation of the resistant strains.

2.5 Conclusions

In this study, we subjected *E. coli*, ESBL-*E. coli*, Enterococci and VRE obtained from municipal sewage and secondary effluents to diverse wastewater treatment processes. For all the studied microbial removal/decay processes, results conclusively demonstrated that no difference existed between the disinfection of *E. coli* and ESBL-*E. coli*, nor between Enterococci and VRE, and that the ratios between the sensitive and resistant strain concentrations were not significantly affected by any of the processes (ANOVA p-value>0.05). Activated sludge wastewater treatment offered 2.1 – 2.4 $\log_{10}$

average removal for all indicators, irrespective of them being antibiotic resistant or not. Fe-EC performed better for *E. coli*/ESBL-*E. coli* (log_{10} removal 0.066 ± 0.003 l/mgFe) than for Enterococci/VRE (log_{10} removal 0.039 ± 0.001 l/mgFe), yet still removing sensitive and resistant bacteria in the same proportion. Disinfection by chlorine also proved Enterococci/VRE to be hardier to inactivate than *E. coli*/ESBL-*E. coli* (LRVs of 0.320 ± 0.008 $l.mgCl^{-1}.min^{-1}$ and 0.365 ± 0.006 $l.mgCl^{-1}.min^{-1}$ respectively), yet had no influence on the ratios between same-species sensitive and resistant organisms, also suggesting that neither ESBL-*E. coli* nor VRE fare better than their respective sensitive counterparts. Experiments by spontaneous decay under different temperatures showed that all four indicators present a biphasic behaviour, with decay progressing faster at the beginning and slowing down after a variable amount of time, with no significant difference in behaviour between resistant and sensitive organisms.

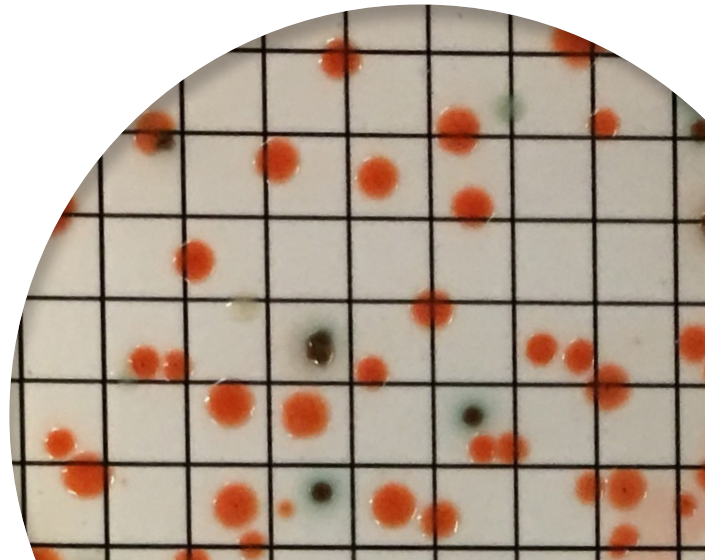
It may be concluded that for all the microbial disinfection/decay processes covered in this chapter (activated sludge, chlorination, Fe-EC and spontaneous decay), our results demonstrated that the microbial reduction profiles, including those of the resistant strains ESBL-*E. coli* and VRE, are in line with sensitive faecal indicators. This means that the resistance status of these two organisms provided them with no competitive advantage over their sensitive counterparts *E. coli* and Enterococci. Logically, these observations are method-specific and should not be lightly extrapolated to treatment operations not covered in this chapter, nor to all ASB/ARB pairs. We do propose that the use of *E. coli* and Enterococci, whose detection and quantification are simple, inexpensive, and low-tech, remain very valuable indicators for estimating disinfection of ESBL *E. coli* and VRE, and for inferring their presence in sewage, secondary effluents, disinfected effluents and possibly in the receiving water bodies over a wide range of temperatures.

3

CHAPTER 3

Low voltage iron electrocoagulation as municipal wastewater tertiary treatment

Published as: Bicudo, B., van Halem, D., Trikannad, S., Ferrero, G., & Medema, G. (2021). Low voltage iron electrocoagulation as a tertiary treatment of municipal wastewater: removal of enteric pathogen indicators and antibiotic-resistant bacteria. *Water Research*, *188*. https://doi.org/10.1016/j.watres.2020.116500



Abstract

In this paper we analyse the feasibility of low voltage iron electrocoagulation as a means of municipal secondary effluent treatment with a focus on removal of microbial indicators, Antibiotic Resistant Bacteria (ARB) and nutrients. A laboratory scale batch unit equipped with iron electrodes was used on synthetic and real secondary effluent from a municipal wastewater treatment plant. Synthetic secondary effluent was separately assayed with spiked *Escherichia coli* WR1 and with bacteriophage ØX174, while real effluent samples were screened before and after treatment for *E. coli*, Extended Spectrum Betalactamase-producing *E. coli*, Enterococci, Vancomycin Resistant Enterococci, *Clostridium perfringens* spores and somatic coliphages. Charge dosage (CD) and charge dosage rate (CDR) were used as the main process control parameters. Experiments with synthetic secondary effluent showed >4$\log_{10}$ and >5$\log_{10}$ removal for phage ØX174 and for *E. coli* WR1, respectively. In real effluents, bacterial indicator removal exceeded 3.5$\log_{10}$, ARB were removed below detection limit (≥2.5$\log_{10}$), virus removal reached 2.3$\log_{10}$ and *C. perfringens* spore removal exceeded 2.5$\log_{10}$. Experiments in both real and synthetic wastewater showed that bacterial removal increased with increasing CD and decreasing CDR. Virus removal increased with increasing CD but was irresponsive to CDR. *C. perfringens* spore removal increased with increasing CD yet reached a removal plateau, being also irresponsive to CDR. Phosphate removal exceeded 99%, while total nitrogen and chemical oxygen demand removal were below 15% and 58%, respectively. Operational cost estimates were made for power and iron plate consumption, and were found to be in the range of 0.01 to 0.24 €/m^3 for the different assayed configurations. In conclusion, low voltage Fe-EC is a promising technology for pathogen reduction of secondary municipal effluents, with $\log_{10}$ removals comparable to those achieved by conventional disinfection methods such as chlorination, UV or ozonation.

3.1 Introduction

At present, one-third of the world's population lives in water-stressed countries and by 2025 the figure is expected to rise to two-thirds (Elimelech, 2006). Discharge of untreated wastewater into the water sources can degrade water quality, increasing the risk to human health and ecosystem degradation. In this context, water reuse is often recognized as a solution with great potential in reducing the gap between availability and demand. Agriculture is currently the largest consumer of reclaimed water, providing an all year round water source with an estimate 200 million farmers worldwide using either raw or treated wastewater for irrigation of over 2000 km^2 (Qadir et al.,2007, Raschid-sally & Jayakody, 2008), constituting roughly 8% of all irrigated land in the planet, most of which happens in Asia (Howell, 2001).

The United Nations agenda for Sustainable Development Goals targets improvement in water quality by reducing pollution, eliminating the discharge of polluted waters, halving the proportion of untreated wastewater and increasing safe water reuse globally (Anfruns-Estrada et al., 2017). Despite reclamation being an attractive concept, municipal wastewater harbours a wide range of enteric pathogens such as virus, bacteria, protozoa, parasitic worms and eggs, and its (re)use calls for careful management of its associated health risks. Such risks depend on the type of water to be recycled, the type and concentration of pathogens, and in particular, the ability of such pathogens to survive treatment, as well as the type of exposure of susceptible community members to such waters. The required level of pathogen reduction in reclaimed water depends on the nature of reuse application and potential for human exposure to water.

In this context, the feasibility of Iron(0) Electrocoagulation (Fe-EC) for microbial attenuation in municipal secondary effluents was explored. Previous studies have demonstrated the effect of Fe-EC in the removal of a wide range of microorganisms from bacteria to viruses in different water matrices, mainly for drinking water applications (Ghernaout et al., 2019, Heffron et al., 2019a, Heffron et al., 2019c, Delaire. 2016,). The application of Fe-EC for the reclamation of secondary municipal effluents provides interesting advantages, since their higher conductivities reduce electrolysis cost, plus residual iron is a lesser concern than for drinking water. In spite of this, only few Fe-EC studies focused on real secondary effluents (Ding et al., 2017, Anfruns-Estrada et al., 2017, Llanos et al., 2014) and to the best of our knowledge, none investigated the removal of Antibiotic Resistant Bacteria (ARB).

The main objective of the present research is to evaluate the performance of low voltage Fe-EC during the treatment of municipal secondary effluents (for reclamation purposes)

in the removal of microbial pathogen indicators (*Escherichia coli*, enterococci, somatic coliphages and *Clostridium perfringens* spores) and Antibiotic Resistant Bacteria (Extended Spectrum Beta Lactamase (ESBL) and Carbapenem Resistant (CRE) -*E. coli* and Vancomycin Resistant Enterococci-VRE). Other parameters of importance such as nutrients, COD, turbidity, colour, pH and residual iron were also analysed (Table 3-5). Charge Dosage (CD) and Charge Dosage Rate (CDR) were selected as the main process-control parameters for the iron dosage, directly linked to the electrolysis time and current intensity, respectively (Ghernaout et al., 2019,Van Genuchten et al., 2017, Delaire et al., 2015, Amrose et al., 2013, Gu et al., 2009). CD is defined as the total electric charge per unit volume applied to a given water sample, while CDR is defined as the speed of application of electric charge (Charge Dosage per unit time). In this way CD [C/l] represents the total dose of current, while CDR [C/l/min] represents the current dosing speed. The influence of CD and CDR in the microbial removal of municipal secondary effluents using Fe-EC is central to this research.

3.2 Materials and Methods

3.2.1 Field sampling and selection of microbial indicators

Prior to the beginning of the EC experiments and in order to collect data on the expected background levels on pathogenic indicator organisms and ARB in secondary effluents, a six month sampling campaign (June-Dec 2018) was conducted in a municipal wastewater treatment plant (WWTP) from the Netherlands. This facility is of the activated sludge type, with primary and secondary treatment and no disinfection. Grab samples from raw sewage and secondary effluent were collected approximately every two weeks and screened for diverse indicators, namely *E. coli*, enterococci, Extended-Spectrum Betalactamase-producing *E. coli* (ESBLE), Carbapenemase-producing-*E. coli*, (CRE) Vancomycin Resistant Enterococci (VRE), *C. perfringens* spores and somatic coliphages. The premise underlying such selection was to cover bacterial, viral and protozoan indicators, as well as ARB. The screening was culture-based, using selective agar medium for each one of the aforementioned indicators. This microbial toolkit is described in further detail in Supporting Information (Table 3-4).

3.2.2 Laboratory setup

Parallel experiments in cylindrical glass beakers (0.5 L for synthetic effluents and 1 L for real effluents) were conducted in the laboratory, as depicted in Figure 3-1. The dual power source was a 30V-3A TENMA 72-10500 bench DC power supply, connected with crocodile clip cables to two S235 steel plates (maximum percentages: 0.14% carbon, 0.10% silicium, 0.80% manganese, 0.025% phosphorous, 0.015% sulphur, 0.010% nitrogen, 0.20% copper and 0.080% aluminium). Dimensions of the steel plates were 6cm x 4cm, of which 4cm x 4cm were submerged (2cm emerging to connect the

clip cables), being polished with coarse and fine sand paper before each use. The plates were mounted in the end of a plastic tube with carved parallel slots ensuring the plates remained parallel and spaced approximately 1cm as described elsewhere (Heffron et al., 2019b, Anfruns-Estrada et al., 2017, Ndjomgoue-Yossa et al., 2015, Merzouk et al., 2009). The beakers were mounted on identical LABNICO L23 magnetic stirrers and fitted with PTFE coated bars for stirring purposes. During each individual experiment, only two process-control parameters were varied, namely CD and CDR, by controlling the electrolysis time and the supplied amperage, respectively. These parameter combinations were selected beforehand for each experiment. Stirring was provided by the magnetic stirrers and the speed was set to 100rpm for all experiments.

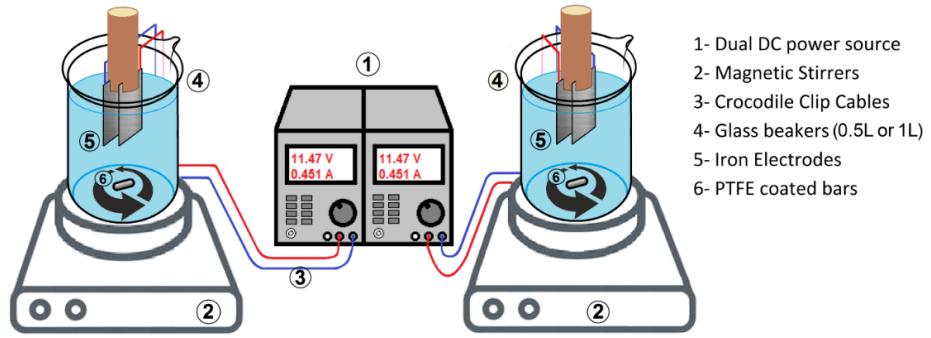


Figure 3-1 Bench scale EC laboratory setup

For each assay, after the current was applied, the iron plates were removed from the beaker, and the latter was covered with aluminium foil to prevent the entry of dust. Particles were left to settle overnight (as reported by Delaire et al., 2016, Delaire et al., 2015), after which the supernatant was carefully harvested with the use of a sterile 50ml serological pipette. Supernatant was collected from the surface until only a 1-2cm layer of water over the sediments was left. The harvesting procedure was conducted carefully, in order not to generate ripples that could disturb the settled particles. The collected supernatant was transitorily deposited in a clean container, and used immediately for microbiological and physical/chemical characterization.

3.2.3 Synthetic and real secondary effluents

The formula for the synthetic secondary effluent was based on the specifications from the Organisation for Economic Co-operation and Development guidelines (OECD, 2001) and then adjusted based on a preceding water sampling campaign at the WWTP. Based on these samples, readily biodegradable COD was replaced by less degradable compounds as expected in secondary effluents: yeast extract was replaced by starch, and peptone was replaced by microcrystaline cellulose, which also provided particulates.

The nitrogen and phosphorous sources (urea and dipotassium phosphate, respectively) were adjusted following the same principle. Sodium chloride was increased in order to provide a conductivity of approximately 1000μS/cm, similar to that of the grab samples collected from the municipal WWTP. Adopting this composition as the baseline, two variants were produced: one medium with higher nutrients than the baseline (HNM), the other with lower (half) the level of nutrients (LNM) (Table 3-1).

Table 3-1 Composition of synthetic effluent (OECD 2001) and synthetic effluent medium with high nutrients (HNM) and low nutrients (LNM).

	Concentration (mg/l)		
Compound	**OECD**	**HNM**	**LNM**
Yeast Extract	22	-	-
Peptone	32	-	-
Starch	-	8	8
Microcrystaline Cellulose	-	5	5
Urea	6	8.6	4.3
Dipotassium Phosphate	28	5.4	2.7
Sodium Chloride	7	60	60
Calcium chloride dihydrate	4	4	4
Magnesium Sulphate Heptahydrate	2	2	2

Two non-pathogenic organisms were used to spike the synthetic effluents, namely *E. coli* WR1 (NCTC 13167) and somatic coliphage ØX174 (ATCC 13706-B1), bacterial and viral indicators respectively. It is worth noting that experiments were conducted either with *E. coli* or with coliphage ØX174, but not both simultaneously to prevent *E. coli* from being infected by the coliphage. *E. coli* WR1 was propagated in TYGB broth for 3h at 37°C to concentrations of approximately $2x10^8$cfu/ml, while phage ØX174 was propagated following the ISO 10705-2_2000 method, to concentrations of approximately $1x10^{12}$pfu/ml. *E. coli* WR1 and ØX174 were dosed into the test liquid at initial concentrations of approximately $1x10^5$(cfu/pfu)/l in order to match the concentrations in the real secondary effluent detected during the sampling campaign.

Experiments involving real secondary effluents were conducted with unaltered grab samples (20-30L) collected downstream from the secondary settlers of the aforementioned WWTP during the months of May-June 2019. All assays on these samples were performed immediately upon arrival in the laboratory during the same day of collection. The complete physical/chemical characteristics of these samples can be observed in SI Table 3-5.

3.2.4 Operational Fe-EC parameters

Fe-EC experiments conducted on the spiked synthetic secondary effluents and real secondary effluents followed the configurations of CD and CDR described in Table 3-2. For synthetic medium, conditions apply for both kinds of effluent (HNM and LNM) and for both spiked indicators (*E. coli* WR1 and somatic coliphage ØX174). All Fe-EC experiments with synthetic effluent were performed in 0.5L beakers equipped with iron electrodes as described in 2.2. Synthetic medium was freshly prepared every day before the assays. Experiments using real secondary effluent were conducted in 1L beakers, using *E. coli*, ESBL-*E. coli*, enterococci, VRE, Somatic coliphages and *C. perfringens* spores as indicators. The experiments described in this section were conducted on four different days, and hence under slightly different real secondary effluent qualities. The characteristics of the real secondary effluent and the scheduling for the different days and microbial groups can be found under SI Tables 3-5 and 3-6. The theoretically dosed iron concentration Fe_{theo} was calculated according to Faraday's law (equation 3-3).

Table 3-2 Operational parameters for synthetic and real secondary effluent assays

Water type	Electrode area (cm^2)	Vol (l)	CD (C/l)	CDR (C/l/min)	Dosed Fe_{theo} (mg/l)
Synthetic secondary effluent	32	0.5	10	5 - 7.2 - 36 -72	2.9
			20	5 - 7.2 - 36 -72	5.8
			50	5 - 7.2 - 36 -72	14.5
			75	5 - 7.2 - 36 -72	21.8
			150	5 - 7.2 - 36 -72	43.9
			200	5 - 7.2 - 36 -72	58.1
Real secondary effluent	32	1	50	7.2	14.5
			100	7.2	29.0
			200	7.2	58.1
			400	7.2	116.1
	40	1	50	36	14.5
			100	36	29.0
			200	36	58.1
			400	36	116.1

3.2.5 Analytical methods

Microbiological screening and quantification was performed either by spread plate method or by membrane filtration according to APHA-Standard Methods for the Examination of Water and Wastewater, 23rd Edition, depending on the expected microbial load of the sample. The specific culture media used for each indicator is

detailed in SI Table 3-4. Screening of somatic coliphages was performed by pour plate technique following ISO 10705-2.

Analysis of ions such as NO_2^-, NO_3^{2-}, NH_4^+, PO_4^{3-} and Cl^- in filtered water samples was carried out with Metrohm 881 basic IC plus and 883 compact IC pro Ion chromatography. For the analysis of total nitrogen, Spectroquant® nitrogen cell test were used, with digestion at 120ºC for 1h, followed by reading in a Spectroquant® NOVA60 photometer (Merck, Germany). Total iron (Fe^{2+}, Fe^{3+}) was measured using Spectroquant® Iron Cell Test (1-50 mgFe/l), read in the aforementioned Spectroquant® NOVA60 (Merck, Germany) photometer. COD analysis was performed using HACH-Lange test kits (LCK314, 15-150mgO_2/l) with 2h digestion at 148ºC and reading in a HACH DR3900 spectrophotometer. Total suspended solids analysis of samples was carried out according to APHA- Standard Methods for the Examination of Water and Wastewater 23rd Edition. Turbidity was measured using Turb 430IR multimeter. Colour was analysed in both unfiltered and filtered samples using UV-VIS spectrophotometer at a 410nm wavelength.

3.2.6 Methodology for Fe-EC cost estimation

Simplified operational cost estimates for each individual Fe-EC experiment were performed considering as inputs the consumed electric power and metallic iron. As for 2019 average energy cost in The Netherlands for a medium size consumer = 0.0679 €/kWh (Eurostat, 2019), and the steel S235 cost = 0.21 €/kg (MEPS International Ltd, 2019).

For a given Fe-EC experiment, in which U is the applied voltage (v), I is the current intensity (mA), t is the treatment time (h) and V is the volume of the cell (m^3), then the consumed power can be estimated as:

$$P\left[\frac{kW.h}{m^3}\right] = \frac{U.I.t}{V} \quad (3\text{-}1)$$

Then, the operational cost for each particular experiment is determined by the amount of consumed power and the amount of dosed iron (described Faraday's equation 3-3), multiplied by their respective unit costs:

$$\text{Cost } (€/m^3) = P\left[\frac{kW.h}{m^3}\right] x\ 0.0679\ €/kWh + m_{Fe}(kg)\ x\ 0.21\ €/kg \quad (3\text{-}2)$$

3.2.7 Data Analysis

Data series of somatic coliphage and *E. coli* attenuation in synthetic effluents was analysed with the statistical test ANOVA (analysis of variance) in order to determine if CDR introduced significant logarithmic removal variations for the different CDs

assayed. In this case, the obtained data was comprised by duplicate microbial sampling in duplicate assays (n=4). For real effluent samples, obtained data was comprised by duplicate microbial samples in single assays (n=2). For microbial indicators presenting removal stagnation, Tukey's (honest significance) range test was used to verify the so called "removal plateau". Faradaic efficiency determination in real effluent experiments was determined by the use of the least square function approximation.

3.3 Results

3.3.1 Microbial indicators in raw sewage and secondary effluent

The average concentrations of target microbial indicators in the WWTP influent were found in the $1x10^5$-$1x10^8$cfu/l (or pfu/l) range, while for the effluent, average values were between $1x10^2$ and $1x10^5$cfu/l (or pfu/l). The observed 2-3log_{10} removal is typical for activate sludge-based treatment systems (Hata et al., 2013, De Luca,et al., 2013, Fu et al., 2010, Tanji et al., 2002, Rose et al., 1996). Concentrations of bacterial indicators (*E. coli* and enterococci) exceeded that of ESBLE and VRE by 2-3log_{10} in both influent and effluent, indicating that ARB were present in lower numbers (Figure 3-2). Also the removal of ESBLE and VRE by secondary treatment was comparable to that of *E. coli* and enterococci. The levels of the selected indicators in the WWTP influent and effluent streams were in-line with published literature (Schmitt et al., 2017, Karon et al., 2016, Harwood et al., 2005, Lodder & De Roda Husman, 2005, Noble et al., 2004, Hot et al., 2003, Cetinkaya, et al., 2000, Lasobras et al., 1999, Gantzer, et al., 1998); therefore, the WWTP was selected as source of real wastewater for further laboratory experiments. Of particular interest were the levels of *E. coli* and somatic coliphages in the effluent (approximately $1x10^5$cfu/l and $1x10^4$pfu/l respectively), as these were used to determine the spike concentration values for *E. coli* WR1 and somatic coliphage ØX174 in the synthetic effluent during Fe-EC experiments (section 2.3).

3.3.2 E. coli WR1 and ØX174 removal from synthetic effluent

Experiments assessing the removal of *E. coli* WR1 are shown in Figure 3-3. For both synthetic water types, removal of *E. coli* WR1 increased gradually with increasing CD. In the experiments using LNM (Figure 3-3a), the effect of CDR in the removal appears negligible, with no clear pattern for microbial attenuation. ANOVA tests conducted for all CDs in LNM using CDR as the independent variable, produced p-values consistently below 0.05 for CDs≥50 C/l, meaning that although for a given CD (≥50 C/l) removal variations seem unimportant in operational terms (≤0.5log), the influence of CDR in removal is statistically present. In the experiments using HNM (Figure 3-3b), similar ANOVA tests were conducted, once again producing for CDs ≥50 C/l, p-values below 0.05, meaning that the influence of CDR in removal is statistically significant, with greater removal values associated to lower CDRs. Obtained *E. coli* removal using HNM was lower than with LNM, reaching 4.9$\log_{10}$, hence suggesting that the presence of higher nutrient concentration negatively affected *E. coli* removal.

Somatic coliphage ØX174 attenuation during LNM experiments (Figure 3-4a) and HNM experiments (Figure 3-4b) displayed in qualitative terms a very similar behavior; removal being <1$\log_{10}$ in the 10–75 C/l range without any significant variation, and levelling off at 150-200 C/l, reaching a plateau of 3.0-4.0$\log_{10}$ removal. ANOVA tests conducted for all CDs in both water matrices using CDR as the independent variable produced for all cases p-values above 0.05, meaning that for somatic coliphages the influence of CDR in removal is statistically insignificant throughout the whole range of dosed charge, irrespective of the considered water matrix. For the aforementioned plateaus in removal observable at 150 C/l and 200 C/l, Tukey tests were performed taking all 16 bars comprising both plateaus, as independent variables. No combination of 2 bars yielded a Tukey-p value <0.05, meaning that all 16 bars constitute the same plateau. Hence, the concentration of nutrients was not found to affect the response of coliphages to the iron dosing speed (CDR), nor to the value of the maximum removal (plateau).

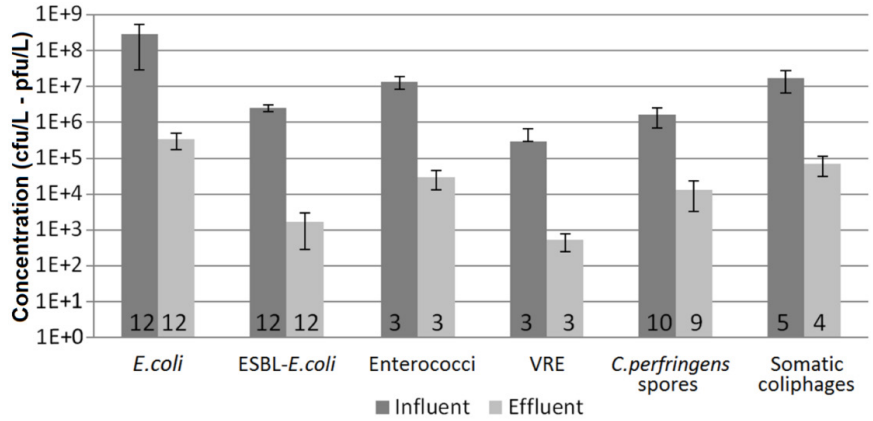


Figure 3-2 Microbial indicators *E. coli*, ESBL-*E. coli*, enterococci, VRE, *C. perfringens* spores and somatic coliphages in raw influent and secondary effluent of a conventional Dutch WWTP. Number of samples analysed for each indicator is noted on the foot of each bar. Error bars represent standard deviation.

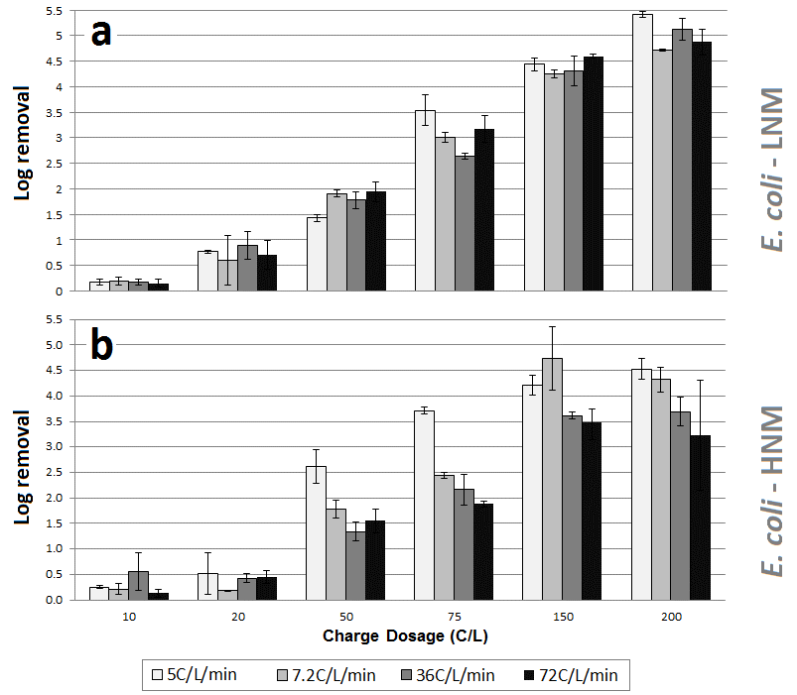


Figure 3-3 *E. coli* WR1 removal during Fe-EC experiments in LNM (a) and HNM (b) with increasing CD (10, 20, 50, 75, 150 and 200 C/l) and CDRs (5, 7.2, 36, 72 C/l/min). Each bar represents the average of four values (duplicate microbial screening in duplicate assays), error bars represent standard deviation.

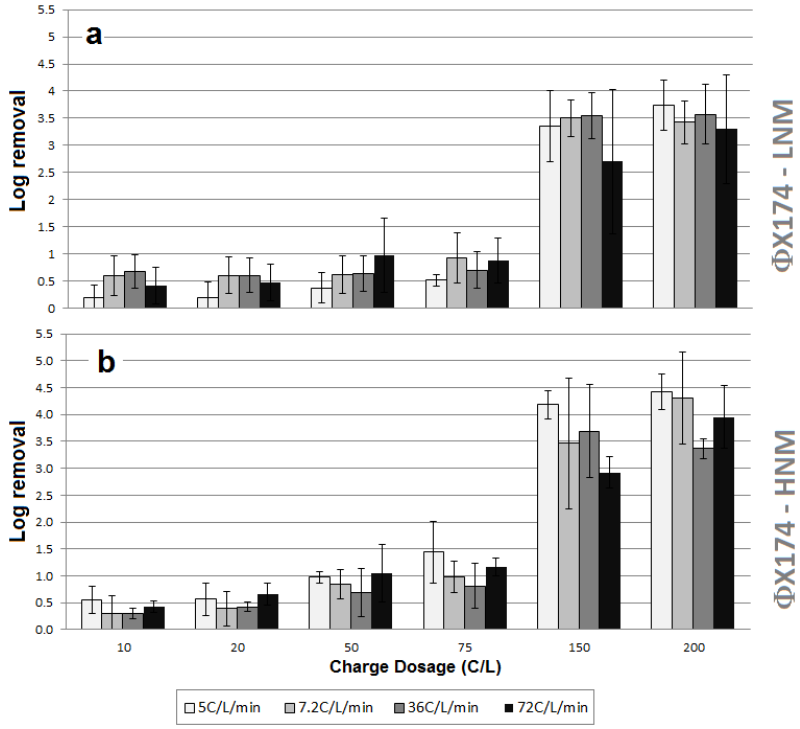


Figure 3-4 Somatic coliphage ØX174 removal during Fe-EC experiments in LNM (a) and HNM (b) with increasing CD (10, 20, 50, 75, 150 and 200 C/l) and CDR (5, 7.2, 36, 72 C/l/min). Each bar represents the average of four values (duplicate microbial screening in duplicate assays), error bars represent standard deviation.

3.3.3 Microbial and physical chemical attenuation in real effluents

Following the experiments using spiked synthetic secondary effluent, real secondary effluent from the WWTP was assayed. Two CDRs (7.2 and 36 C/l/min) in combination with four different CDs (50, 100, 200 and 400 C/l) were assayed. For all experiments here described, a single assay was conducted in which duplicate samples were screened for microbial indicators. The results for *E. coli* and ESBL-*E. coli* are depicted in Figure 3-5. The removal was found to increase with increasing CD, for both assayed CDRs. *E. coli* removal spanned from $0.5\log_{10}$ at CD 50 C/l, to a maximum of $3.7\log_{10}$ at 400 C/l. This is considerably lower than the results obtained for *E. coli* in synthetic effluent, since removals of $5.4\log_{10}$ were achieved using half of the iron dose (200 C/l). CDR showed a significant effect on *E. coli* attenuation, with removal rates of $3.7\log_{10}$ and $2.4\log_{10}$ at 7.2 and 36 C/l/min, respectively (ANOVA p-values between same CDs and either CDR<0.05 for 50, 100 and 200 C/l). A similar behavior was observed during Fe-EC experiments with ESBL-*E. coli*, with increasing removal following increases in CD, and favored by the lower CDR of 7.2 C/l/min over 36 C/l/min. Minimum removal of $0.2\log_{10}$ was observed for 50 C/l, while a maximum surpassing $2.6\log_{10}$ was estimated for 400 C/l. It is noteworthy that experiments with CD 200 and 400 C/l involving ESBL-*E. coli* achieved removal rates high enough to hinder detection through culture based methods, meaning that the filtered samples were below the limit of detection (LOD). For these experiments, the minimum removal efficiency for ESBL-*E. coli* was calculated on a mathematical basis, considering the method appreciation of 1cfu and the filtered volume in each case. From a comparison perspective, obtained ESBL-*E. coli* removal under 7.2 C/l/min was lower than that of sensitive *E. coli* (ANOVA p-values<0.05) while for 36 C/l/min removal was equal for 50 C/l (ANOVA p-value≈0.2) but lower for 100 C/l (ANOVA p-value<0.05). This suggests that sensitive *E. coli* is a conservative indicator for ESBL-*E. coli*, since sensitive *E. coli* is removed equally or less than ESBL-*E. coli*. The fact that ESBL-*E. coli* was removed below LOD for CD 200 and 400 C/l while sensitive *E. coli* was not, should not be misread as ESBL-*E. coli* being better removed, since concentrations of ESBL-*E. coli* were 3 orders of magnitude lower than sensitive *E. coli* in the real secondary effluent samples as depicted in Figure 3-2.

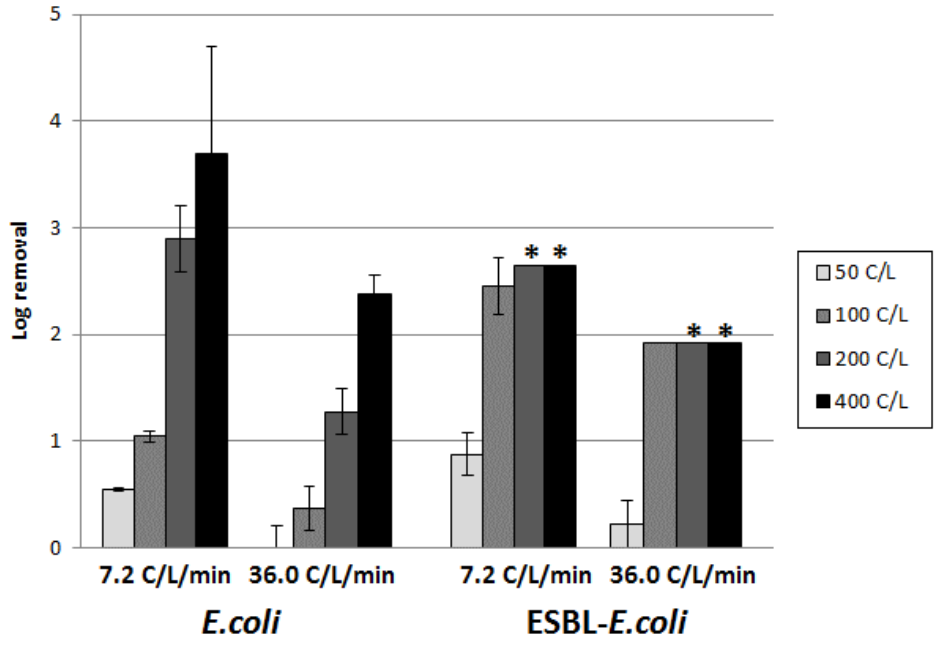


Figure 3-5 *E. coli* and ESBL-*E. coli* removal during real secondary effluent Fe-EC experiments. Note: Bars marked with an asterisk (*) indicate a minimum estimated removal, due to effluent concentration below LOD. Each bar represents the average of two values (duplicate microbial screening, single assay). Error bars indicate standard deviation.

For enterococci and VRE (Figure 3-6) removal also increased with CD for both CDRs. Enterococci removal spanned from 0.4log$_{10}$ for CD of 50 C/l, to a maximum of 3.6log$_{10}$ at 400 C/l, with attenuation of enterococci being very similar to that of *E. coli* (Figure 3-5). CDR also showed a major effect on enterococci attenuation, with removal rates at 7.2 C/l/min being up to 0.9log$_{10}$ higher than those at 36.0 C/l/min (ANOVA p-values between same CDs and either CDR<0.05 for all CDs). Regarding VRE, attenuation levels were in the same range as those of ESBL-*E. coli*, with a minimum of 0.3log$_{10}$ achieved at 50 C/l (36.0 C/l/min) and a maximum exceeding 2.5log$_{10}$ at 200-400 C/l (7.2 C/l/min), respectively. VRE removal for 200 and 400 C/l at 7.2 C/l/min, and 400 C/l at 36 C/l/min were also estimated minimum values since the resulting concentration for these experiments was below LOD. Enterococci and VRE behave in a very similar way, with VRE being removed in almost identical ratios to that of sensitive enterococci, at least for the samples with concentrations above LOD (ANOVA p-values >0.05 for both CDRs). This suggests that removal of enterococci can be used as a proxy for removal of VRE, due to the observed similarities in their attenuation patterns.

C. perfringens spores showed the most complex behavior under Fe-EC (Figure 3-7). From a CD perspective, removal increased with CD yet appeared to stagnate under 200 and 400 C/l reaching over 2log$_{10}$. ANOVA test between the 7.2 and 36 C/l/min series revealed no considerable statistical difference between them (indicating that CDR plays no major role in *C. perfringens* spore removal), while Tukey test indicated two statistically different groups, namely the 50 C/l samples, and the 100, 200 and 400 C/l samples (confirming the existence of the removal stagnation). This behavior was not observed for any other indicator organism in this study.

For somatic coliphages, removal below 1log$_{10}$ was observed for the three lower CDs assayed (50, 100 and 200 C/l), yet increasing to over 2log$_{10}$ for 400 C/l under both CDRs. CDR seems to play a less significant role for this indicator compared to bacteria, with no major difference between 7.2 and 36 C/l/min in terms of achieved removal (ANOVA p-values>0.05).

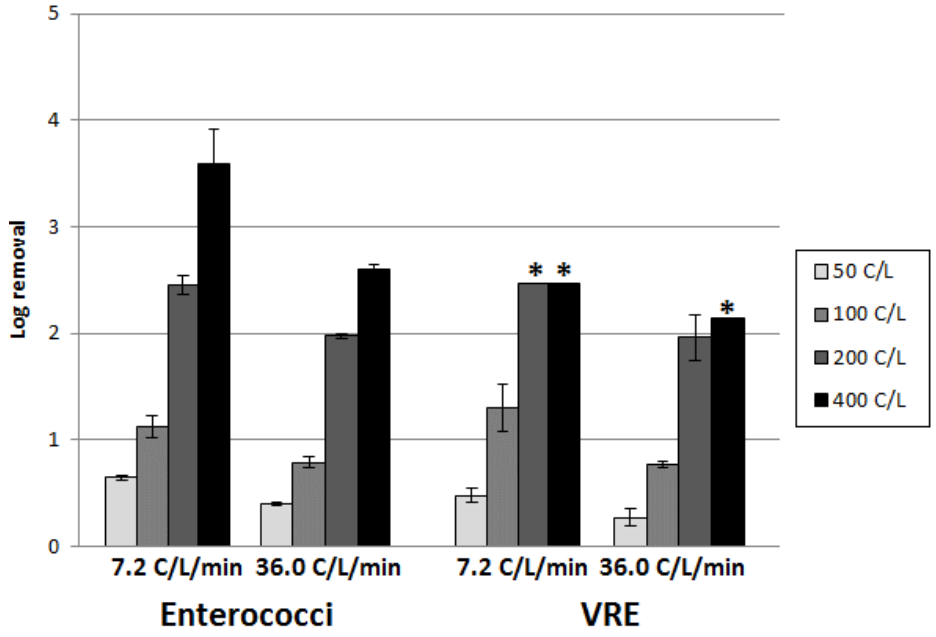


Figure 3-6 Enterococci and VRE removal during real secondary effluent Fe-EC experiments. Note: Bars marked with an asterisk (*) indicate a minimum estimated removal due to effluent concentration below LOD. Each bar represents the average of two values (duplicate microbial screening, single assay). Error bars indicate standard deviation.

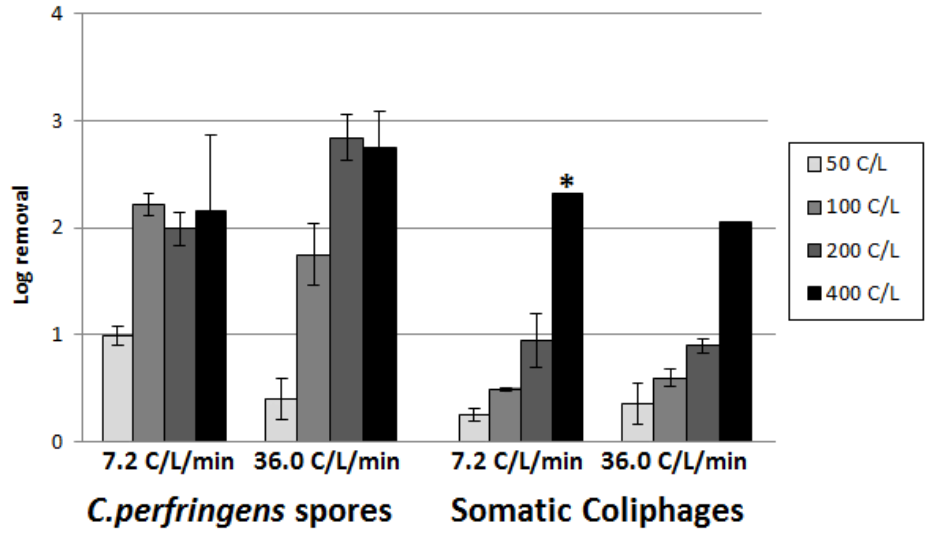


Figure 3-7 *C. perfringens* spores and somatic coliphage removal during real secondary effluent Fe-EC experiments. Note: Bars marked with an asterisk (*) indicate a minimum estimated removal due to effluent concentration below LOD. Each bar represents the average of two values (duplicate microbial screening, single assay). Error bars indicate standard deviation.

3.3.4 Nutrient removal in synthetic and real effluents.

Together with the removal of microbial indicators, PO_4^{3-}, COD, and TN removal was also measured for each of the samples under each configuration of CD and CDR in either synthetic or real effluent experiments. For synthetic HNM and LNM experiments, PO_4^{3-} removal increased rapidly with increasing CD to values below LOD (>98% removal), while TN removal displayed a decreasing trend with increasing CD for all CDRs (Figure 3-9 and 3-10). In real secondary effluents (Figure 3-8), removal of PO_4^{3-} was high, dropping from 1.50 mg/l to values below detection with associated removal rates above 99%. COD removal reached 30-40% for the higher CDs, in particular for CDR of 36 C/l/min, while TN achieved a maximum removal of 15.4% for CD 200 C/l and CDR 36 C/l/min. The influence of CDR on phosphate removal was not conclusive, since very high removal values were quickly reached, irrespective of CDR. Interestingly, COD and TN exhibit higher removal for higher CDR, opposite of what is observed for most microbial indicators.

3.3.5 Cost estimation

The operational cost of the Fe-EC process for the real secondary effluent experiments, and their achieved microbial removal for each indicator are shown in Table 3-3. The calculations indicate lower costs at lower CDRs, due to a reduced power consumption for all CDs. Operational costs from 0.01 to 0.08 €/m^3 were obtained for the experiments using real secondary effluent under a CDR of 7.2 C/l/min, while for CDR of 36 C/l/min, estimations range from 0.03 to 0.24 €/m^3. It is worth noting that lower CDRs produce better outcomes in terms of microbial removal, and also result in lower operational costs due to lower required operational voltage. From these estimates, although the iron electrode cost is the same for both scenarios, the power cost is the most important factor in the total operating cost, with an impact of about 70% for CDR of 7.2 C/l/min and 90% for CDR of 36 C/l/min. For the experimental conditions explored in this research, the combination that produced the best outcome from a microbial perspective was CD 400 C/l and CDR 7.2 C/l/min, involving an associated cost of 0.082 €/m^3. Faradaic efficiencies during real secondary effluent assays were calculated using least square method, with reporting values of 113% for CDR of 7.2 C/l/min and 114% for 36.0 C/l/min (Figure 3-11).

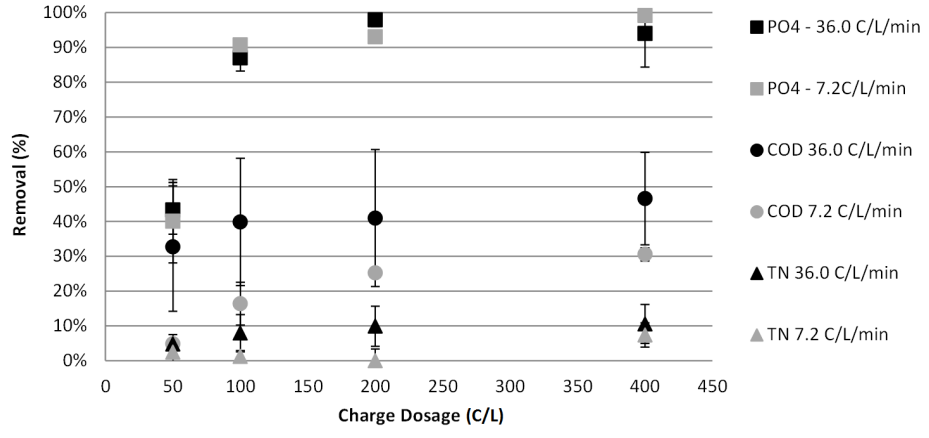


Figure 3-8 Removal of phosphate, COD and TN for real secondary effluent experiments. Each data point represents the average of four values (duplicate chemical screening in duplicate assays). Error bars indicate standard deviation.

Table 3-3 Simplified operation cost calculation for experiments conducted using real secondary effluent and associated microbial removal.

Settings				Costs			Microbial log removal					
CD (C/l)	CDR (C/l/min)	Intensity (A)	Voltage. (v)	Electric (€/m^3)	Metal Iron (€/m^3)	Operation (€/m^3)	*E. coli*	ESBL-*E. coli*	Enterococci	VRE	*C. perfringens* Spores	Somatic Coliphages
50	7.2	0.12	7.5	0.007	0.003	**0.010**	**0.6**	**0.8**	**0.6**	**0.5**	**1.0**	**0.2**
100			7.5	0.014	0.006	**0.020**	**1.0**	**2.5**	1.1	1.3	2.2	0.5
200			7.6	0.029	0.012	**0.041**	**2.9**	**>2.6**	**2.5**	**>2.5**	**2.0**	**1.0**
400			7.7	0.058	0.024	**0.082**	**3.7**	**>2.6**	**3.6**	**>2.5**	**2.2**	**>2.3**
50	36.0	0.60	27	0.025	0.003	**0.028**	**0.0**	**0.2**	**0.4**	**0.3**	**0.4**	0.4
100			27	0.051	0.006	**0.057**	**0.4**	**1.9**	**0.8**	**0.8**	**1.8**	**0.6**
200			27	0.102	0.012	**0.114**	**1.3**	**>1.9**	**2.0**	2.0	2.8	0.9
400			28	0.211	0.024	**0.236**	**2.4**	**>1.9**	**2.6**	**>2.1**	**2.8**	**2.1**

3.4 Discussion

3.4.1 Effect of water matrix on removal efficiencies

The present study confirms the influence of water matrix, i.e., synthetic versus real secondary effluent, for bacteria and virus indicator removal by EC, as well as nutrient removal. In real secondary effluents, *E. coli* removal was 1-2$\log_{10}$ lower than that observed for *E. coli* in synthetic effluents, even when the dosage of iron was doubled. Similar observations were made regarding phage ØX174, with removal also dropping by 1-2$\log_{10}$ in real secondary effluents. Although the response obtained with synthetic and real secondary effluent was similar in qualitative terms, removal obtained for *E. coli* and phage ØX174 still differs by orders of magnitude.

It was hypothesised that the complexity of the water matrix from real secondary effluents, and its higher concentration of organic matter, iron-scavenging anions and complexation agents (such as phosphates, citrates, carbonates and sulphates) are responsible for substantially reduced coagulant generation or microbial removals, coinciding with the observations of Heffron et al., (2019a), Van Genuchten et al., (2017), and Ghernaout et al., (2019). These compounds are recognized as responsible for hindering Fe^{2+} oxidation into insoluble Fe^{3+}, hence reducing coagulant precipitation and subsequent sweep flocculation. *C. perfringens* spore removal was only assessed in real secondary effluents, showing similar characteristics both in quantitative and qualitative terms with previous Fe-EC research conducted in real sewage and secondary effluents by Anfruns-Estrada et al., (2017). In the mentioned study they determined the maximum removal of *C. perfringens* spores to 2.79$\log_{10}$, with removal stagnating as the dosage of current progressed. This behaviour was also observed in the present research, particularly at lower CDRs underlining that increasing CD will not enhance spores removal beyond a certain plateau, whatever the CDR. In terms of PO_4^{3-} COD and TN removal, the obtained results are well within range of previous research, where high PO_4^{3-} removal ranging from 50% to 98%, 26% to 85% for organics and less than 30% for TN was observed (Zaleschi et al., 2013, Ikematsu, et al., 2006, Inan & Alaydin, 2014, Lacasa et al., 2011, Malinovic et al., 2016).

3.4.2 Microbial attenuation mechanisms

Removal of all bacterial indicators was in general terms very similar, irrespective of their resistance to antibiotics or their gram classification, with removal being strongly dependent on the amount and speed of iron dosage. Similar conclusions are also valid for somatic coliphages (although appearing less sensitive to the speed of dosage), but do not fully apply for *C. perfringens* spores. This evidences a differing response to the Fe-EC process for each type of microorganism. The aim of this research was not to look into the mechanisms of removal for each one, yet the body of literature recognizes three pathways for microbial attenuation, namely: a) entrapment or adsorption in the metallic hydroxide flocs, and removal by sedimentation; b) inactivation due to formation of Reactive Oxygen Species (ROS) or disinfectants; and c) inactivation due to electrical current.

According to most researchers, entrapment seems to be the dominant removal mechanism for bacteria (Ghernaout et al., 2019, Delaire et al., 2016, Delaire et al., 2015), mainly due to the affinity of their surface functional groups such as teichoic acids and phospholipids, with the EC precipitates. These functional groups are found in similar amounts in gram positive and gram negative bacterial cell walls (Delaire et al., 2016, Borrok, et al., 2005). Virus removal has been attributed to both iron hydroxide floc entrapment (Heffron et al., 2019a, Tanneru & Chellam, 2012), and inactivation by either ROS or chlorine-based oxidants formed by reduction in the anode (Heffron et al., 2019a). Formation of Cl_2 was ruled out as a mechanism of disinfection in our experiments, since Cl^- concentration was measured by ICP-MS before and after the application of current, and no variations were detected in any of the samples, also in line with the findings of Delaire et al., (2015) and Diao et al., (2004). Inactivation due to the effect of electric current has been the least reported biocidal pathway, with the research from Jeong et al., 2006 giving it a larger relevance at high current densities, from 33 to 100 mA/cm^2. It is noteworthy that throughout the experiments conducted in this chapter, current density never exceeded 20mA/cm^2, for which this pathway of removal is not regarded as dominant.

In terms of CDR in promoting either of the aforementioned mitigation mechanism, Heffron et al., (2019b) correlated ferrous iron oxidation rate and bacteriophage removal, concluding that fast oxidation of Fe^{2+} leads to a shorter exposure time and hence poorer contact between the phages and the reactive iron species, yielding a less important disinfection. In other words, implying that ROS generated during ferrous iron oxidation are a major contributor in the disinfection during Fe-EC, with the effect of these being stronger for slowly occurring oxidations. However, the selected overnight settling for all experiments likely influences oxygen diffusion into the effluent promoting the slow oxidation of ferrous iron, hence potentially impacting in the removal (besides from it having a reduced practicality on a municipal scale). The present research observed for both synthetic and real effluents an increasing removal efficiency for bacterial and viral indicators under decreasing CDRs (less prominent for viruses), even when the amount of dosed iron (and coagulation products) was identical. Although this study did not look into the mechanistic aspects of the microbial removal via Fe-EC, the observed dependency of microbial removal on CDR suggests that the production of ROS could in fact be a contributing factor during Fe-EC. The aerobic

oxidation of Fe^{2+} released during the anode oxidation produces a cascade of reactive species which includes superoxide ion ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH^-$) (Van Genuchten & Peña, 2017, Hug & Leupin, 2003, Rush, et al., 1990), all of which are known to have disinfectant properties (Tanneru & Chellam, 2012, Jeong et al., 2006, Elena Pulido 2005). This implies that microbial removal by Fe-EC could be in fact a combination of physical separation and chemical inactivation, and not just an adsorption-sedimentation phenomenon. It could also explain why spores (dormant bacterial structures, highly resistant to chemical attack) are significantly less affected than bacterial indicators by varying CD or CDR.

3.4.3 Fe-EC as a disinfection technology

When compared against typical wastewater disinfection technologies in terms of pathogen removal, Fe-EC performs in a similar way to that of conventional alternatives such as chlorination, ozonation or UV light. Typical removal values for chlorination range from 2-6$\log_{10}$ for bacteria, 0-4$\log_{10}$ for viruses and 0-3$\log_{10}$ for protozoa. Disinfection values for ozonation include 2-6$\log_{10}$ for bacteria, 3-6$\log_{10}$ for viruses and >2$\log_{10}$ for protozoa. For UV, expected performance is 2-4$\log_{10}$ for bacteria, 1-3$\log_{10}$ for viruses and 2-3$\log_{10}$ for protozoa (Collivignarelli et al., 2018, USEPA, 2012, Bitton, 2005, USEPA, 2003, Rose et al., 1996, WHO, 1989). This means that Fe-EC can in fact be regarded as an unconventional chemical-addition free disinfection technology, based on comparable performance to other classically accepted disinfection methods. Abou-Elela et al., 2012 provides reference O&M cost values for municipal secondary effluent disinfection using chlorine (32 mg/l, 15 minutes contact time), ozone (15 mg/l, 15 minutes contact time) and UV irradiation (164 mWs/cm^2, 15 minutes contact time). Cost estimates are 0.024 €/m^3 for chlorine, 0.030 €/m^3 for ozone and 0.044 €/m^3 for UV irradiation. For the lowest studied CDR of this research (7.2 C/l/min), the obtained operation costs (0.01 to 0.082 €/m^3) fall within comparable range to those obtained by Abou-Elela et al., 2012, although a proper comparison should be made on a basis of equal microbial inactivation.

3.5 Conclusions

Low voltage Fe-EC is a promising technology for microbial removal in secondary municipal effluents, with $\log_{10}$ removal comparable to those achieved by conventional disinfection methods such as chlorination, UV or ozonation.

For real secondary effluents, achieved bacterial removal exceeded 3.5$\log_{10}$, ARB removal reached or exceeded 2.5$\log_{10}$, spores were removed between 2-3$\log_{10}$ and virus elimination reached or exceeded 2.3$\log_{10}$. In synthetic secondary effluent, bacterial and virus $\log_{10}$ removal was consistently higher with 1-2 orders of magnitude.

Microbial removal was found to increase with CD, while decreasing CDRs showed a higher mitigation of bacteria, yet no significant effect on viruses or spores. The latter showed a different removal trend, with elimination reaching a plateau for medium-high CDs, this being slightly higher for higher CDRs.

Sensitive *E. coli* and Enterococci appear as conservative indicators for ESBL-*E. coli* and VRE respectively, although it must be noted that ARB determination was limited by relatively low concentrations in the secondary effluent.

Iron plates and electric consumption were the main components contributing to the costs, with the latter having the largest impact (70-90%) for the assayed conditions. For the most favourable microbial removal set of conditions (CD 400 C/l, CDR 7.2 C/l/min) the estimated unit cost of the process is 0.08 €/m^3, within comparable range to other conventional disinfection technologies as chlorine, UV or ozone.

Besides from microbe removal, Fe-EC offers additional benefits over traditional disinfection methods, such as nutrient and COD removal.

3.6 Supporting information (SI)

3.6.1 Faraday's law for electrolysis

Faraday's law provides the basic understanding of the electrochemical process during electrocoagulation, and allows to calculate the mass of released metal into the bulk solution as a function of the intensity of the current passed through it.

$$m = \frac{i \times t \times M}{n \times F} \qquad (3\text{-}3)$$

Where:

m=mass of metal released into the solution (g)
i= Current intensity (A)
t= time of current application (s)
M=Molar mass of the metal in question (for Fe=55.84 g/mol)
n= valence of the released metal ion (for Fe n=2)
F= Faraday's constant (96485.3 C mol^{-1})

Note: a current of 1A applied during 1 second is defined as 1 Coulomb.

3.6.2 Nutrient Removal during Fe-EC experiments

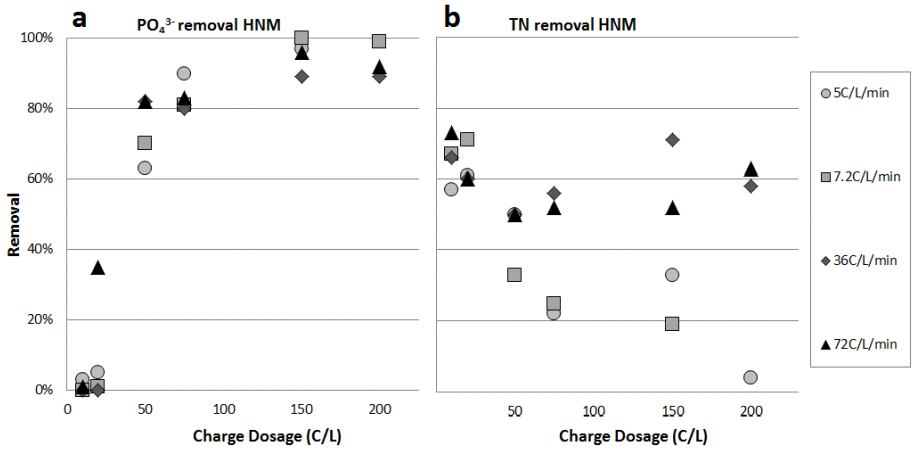


Figure 3-9 (a) PO_4^{3-} and (b) TN removal in High Nutrient Medium (HNM) Fe-EC experiments

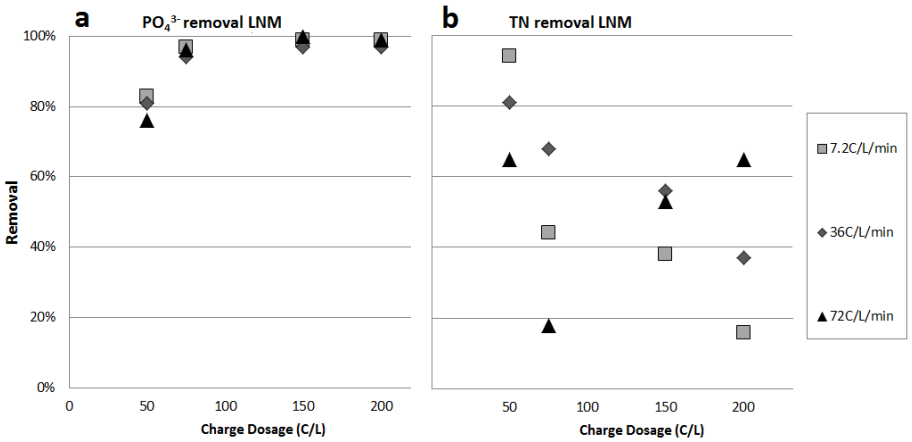


Figure 3-10 (a) PO_4^{3-} and (b) TN removal in Low Nutrient Medium (LNM) Fe-EC experiments

3.6.3 Faradaic efficiency

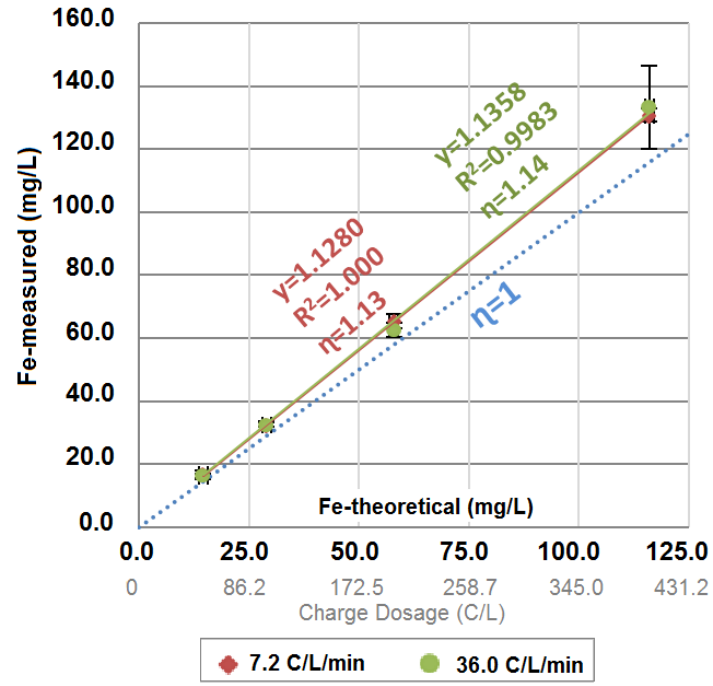


Figure 3-11 Faradaic efficiency calculation for real secondary effluent experiment (adjusted by least squares) of experiments under 7.2 C/l/min and 36.0 C/l/min. Error bars represent standard deviation (n=4). Dotted line represents 100% Faradaic efficiency.

3.6.4 Selection of Microbial Indicators

Table 3-4 Description of selected microbial indicators

Organism	Type	Characteristics	Culture	References
E. coli	Bacterial; Gram negative	Easy to handle and to culture, present in almost every human being and warm blooded animal. Classic fecal contamination indicator.	Chromocult® agar medium (ISO 9308-1), Merck Millipore.	Petri et al., 2008, Noble et al 2004, Anfruns-Estrada et al., 2017
Extended Spectrum βLactamase (ESBL) producing -*E. coli*	ARB Gram Negative	Worldwide recognition as nosocomial pathogen of concern. Listed under "serious threat" by the US Center for Disease Control	ChromID® ESBL agar medium. Biomerieux-Diagnostics (Marcy l'Etoile, France).	US CDC 2019, Réglier-Poupet et al., 2008
Enterococci	Bacterial; Gram positive	Ubiquity in human feces. Water fecal contamination indicator.	Slanetz-Bartley agar medium (ISO 7899), Merck Millipore	Al-Jassim, et al.,2015 Rosenberg Goldstein et al., 2014 Harwood et al., 2005 Anfruns-Estrada et al., 2017
Vancomycin Resistant Enterococci (VRE)	ARB Gram Positive	Worldwide recognition as nosocomial pathogen of concern. Listed under "serious threat" by the US Center for Disease Control	ChromID® VRE agar medium. Biomerieux-Diagnostics (Marcy l'Etoile, France).	US CDC, 2019 AR Threats Report Furukawa, et al., 2015 Lamba et al., 2018, Harwood et al., 2005
Somatic coliphages	Virus, bacterial phage	Abundant in wastewater. Safe to handle (non-pathogenic) and easy to culture. Size and shape resemblance to several human enteroviruses (poliovirus, norovirus,etc).	According to ISO 10705-2	Payment & Franco, 1993 Sobsey et al., 2014 Harwood et al., 2005 Anfruns-Estrada et al., 2017
C. perfringens spores	Protozoan cyst surrogate;	Water fecal contamination indicator. Pathogenic sporeforming bacterium. Extensively used as intestinal protozoan cyst surrogate (Giardia lamblia, and Cryptosporidium parvum). Easy to culture.	After pasteurization, in CHROMagar™ *C. perfringens* agar medium. Anaerobic culture in Anaerobic-Box (Fischer Scientific)	Payment & Franco, 1993 Sobsey et al., 2014 Harwood et al., 2005 Anfruns-Estrada et al., 2017

3.6.5 Real secondary effluent characteristics and assay scheduling

Table 3-5: Charaterization of real secondary effluent quality during the four sampling events

	Day 1	Day 2	Day 3	Day 4
pH	7.25	7.3	7.27	7.28
Cond. (µS/cm)	940	945	964	970
COD (mg O_2/L)	44.4	40.1	53.4	51.4
NH_4^+ (mg/L)	1.08	1.37	1.18	1.42
NO_2^- (mg/L)	1.23	0.96	0.9	0.99
NO_3^- (mg/L)	6.75	8.11	5.68	5.19
TN (mgN/L)	-	4.1	3.5	3.9
PO_4^{3-} (mgP/L)	0.4	0.23	0.49	0.32
Cl^- (mg/L)	-	109.62	115.73	117.58
Turbidity (NTU)	1.70	1.06	1.15	1.21
Aparent Color (UPt/Co)	85.44	89.38	85.44	85.44
True Color (UPt/Co)	-	53.88	73.60	85.44
TSS (mg/L)	4	1	3	6
E.coli (cfu/ml)	1.3E+02	-	1.70E+01	-
ESBL-E.Coli (cfu/ml)	6.0E-01	-	1.40E-01	-
C.perfring. Spores (cfu/ml)	1.4E+01	-	9.80E+00	-
Enterococci (cfu/ml)	-	6.70E+01	-	3.80E+01
VRE (cfu/ml)	-	3.80E-01	-	1.80E-01
Somatic Phages (pfu/ml)	-	1.12E+02	-	5.60E+01

Table 3-6 Scheduling of the real effluent assays by CD, CDR and microbial group

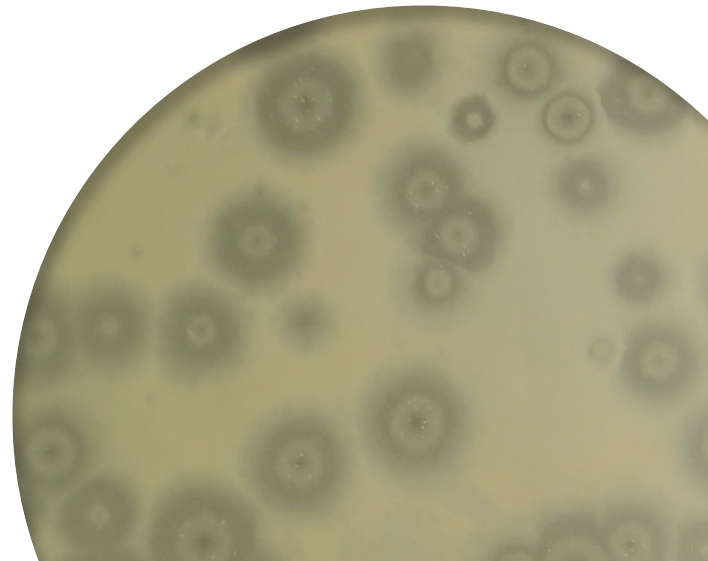
Day	CDR (C/L/min)	CD (C/L)	Assayed Indicators
1	7.2	50-100-200-400	E.coli, ESBL-E.coli, C.perfringens
2	7.2	50-100-200-400	Enterococci, VRE, Somatic Phages
3	36	50-100-200-400	E.coli, ESBL-E.coli, C.perfringens
4	36	50-100-200-400	Enterococci, VRE, Somatic Phages

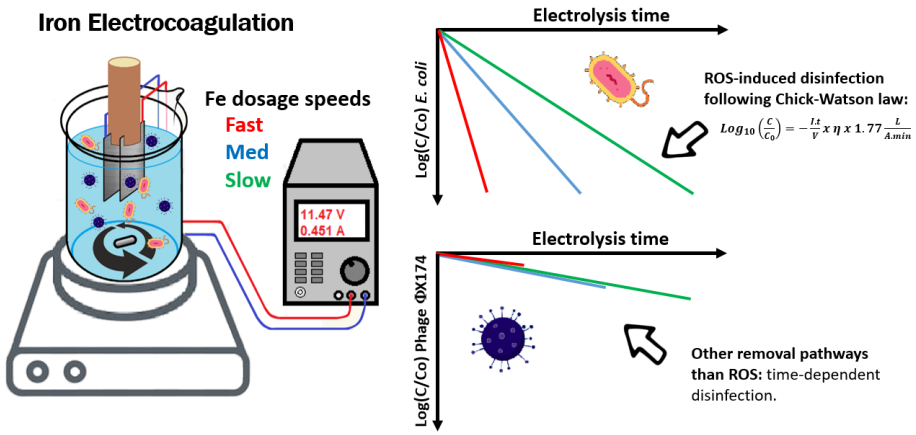
4

CHAPTER 4

Inactivation of *Escherichia coli* and somatic coliphage ØX174 by oxidation of electrochemically produced Fe^{2+}

Published as: Bicudo, B., Medema, G., & van Halem, D. (2022). Inactivation of Escherichia coli and somatic coliphage Φ X174 by oxidation of electrochemically produced Fe2+. *Journal of Water Process Engineering*, *47*(December 2021), 102683. https://doi.org/10.1016/j.jwpe.2022.102683





Abstract

Electrochemical ferrous iron (Fe^{2+}) wastewater treatment is gaining momentum for treating municipal wastewater due to its decreasing costs, environmental friendliness and capacity for removal of a wide range of contaminants. Disinfection by iron electrocoagulation (Fe-EC) has been occasionally reported in full scale industrial applications, yet controversy remains regarding its underlying elimination mechanisms and kinetics. In this study, it was demonstrated that substantial inactivation can be achieved for *Escherichia coli* WR1 (5 log_{10}) and somatic coliphage ØX174 (2-3 log_{10}). Electrochemically produced Fe^{2+} yielded similar inactivation as chemical Fe^{2+}. Reactive oxygen species (ROS)-quenching experiments with TEMPOL confirmed that *E. coli* inactivation was related to the production of Fenton-like intermediates during Fe^{2+} oxidation. The observed *E. coli* disinfection kinetics could be mathematically related to Fe-EC current intensity using a Chick-Watson-like expression, in which the amperage is surrogate for the disinfectant's concentration. We hereby show that it is possible to mathematically predict disinfection based on applied Fe dosage and dosage speed. Phage ØX174 inactivation could not be described in a similar way because at higher Fe dosages (>20 mg/l), little additional inactivation was observed. Also, ROS-quencher TEMPOL did not completely inhibit phage ØX174 removal, suggesting that additional pathways are relevant for its elimination.

4.1 Introduction

Globally, agriculture is the largest water consumer worldwide. However, 3.2 billion people currently inhabit agricultural areas prone to shortages or severe scarcity (UN-Water, 2021). The use of treated municipal wastewater is a common practice, particularly throughout Asia where over 200 million farmers make use of raw or treated wastewater for the irrigation of over 2000 km^2 of cropland (Qadir et al., 2007, 2010; Raschid-sally & Jayakody, 2008). In these cases, wastewater reuse can reduce the pressure on freshwater resources, particularly in water-stressed regions in which there can be extreme seasonal fluctuations in agricultural water availability. However, no matter how attractive municipal wastewater reclamation appears, it is still a potential source of a wide range of enteric pathogens. These include bacteria, viruses, protozoa and helminths, as well as the emerging concern of antimicrobial resistant bacteria, listed as a global health threat by WHO (*GLASS,* 2021). Hence, the reuse of municipal effluents demands careful handling of the health risks associated with it. These risks will depend on the concentration of pathogens in the treated effluent, how the treated effluent will be used and the associated exposure routes and susceptibility of the users after exposure. Therefore, the level of treatment (pathogen inactivation) for reclamation purposes depends on the particular reuse application and the likelihood and frequency of user exposure.

In this study, we evaluated the disinfection[1] capacity of the electrochemical process called iron electrocoagulation (Fe-EC), targeting its use as a municipal water reclamation technology and with a special focus on its kinetics. Fe-EC is a process which releases Fe^{2+} ions into a water stream in order to induce coagulation, as opposed to conventional Fe chemical coagulation (CC), which is usually performed by dosing $FeCl_3$ or other Fe^{3+} salts. Fe-EC produces the metallic coagulant on site by the electrochemical dissolution of Fe (or steel) plates. Fe-EC has been applied as a treatment for effluents from a wide variety of industries, such as paper and pulp, petrochemical, textile, dairy, slaughterhouses, manure, metal-plating, and others (Bergmann, 2021; Garcia-Segura et al., 2017; Hakizimana et al., 2017; Moussa et al., 2017). However, its use for municipal wastewater applications has been marginal and mainly confined to academic research (Anfruns-Estrada et al., 2017; Ikematsu et al., 2006; Llanos et al., 2014; Malinovic et al., 2016; Zaleschi et al., 2013).

[1] Disinfection is the process of water treatment to eliminate (pathogenic) microorganisms. Elimination mechanisms are inactivation of the microorganism or physical removal of the microorganism from the water matrix.

Disinfection by Fe-EC has been occasionally reported in full scale industrial applications, yet its underlying inactivation mechanisms are still not clearly understood. In general terms, three major microbe removal/inactivation mechanisms have been proposed for Fe-EC, namely, (a) sorption or entrapment of microbes to the flocculation products with subsequent removal by sedimentation (Heffron et al., 2019a; Delaire et al., 2016), (b) inactivation due to the induced electric field between the plates (Ndjomgoue-Yossa et al., 2015; Ghernaout & Ghernaout, 2010) and (c) chemical inactivation due to reactive oxygen species (ROS) (Heffron et al., 2019a; Llanos et al., 2014; Dixon & Stockwell, 2014; Tanneru & Chellam, 2012; Ghernaout & Ghernaout, 2010) or due to the in-situ formation of disinfectants and disinfectant by-products (Ndjomgoue-Yossa et al., 2015; Llanos et al., 2014).

Dixon & Stockwell (2014) define ROS as a general term that includes several partially reduced molecules containing oxygen, such as superoxide ($^{\bullet}O_2^-$), peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$). They are an integral part in the (semi)Fenton chemistry that takes place when Fe-EC is conducted under aerobic conditions, which comprises the aerobic oxidation pathway of Fe^{2+} into Fe^{3+} and the cascade of intermediate species formed in the process (Figure 4-1). Most of these species, in particular the radicals, have an extremely short half-life in the range of 10^{-6} - 10^{-9} seconds, for which their production and degradation can be considered instantaneous (Rubio & Cerón, 2021). In general terms, disinfection by ROS occurs due to oxidative damage to DNA/RNA, enzymes, proteins, cell membrane constituents (and subsequent rupture), viral capsids, phospholipid envelopes, or via the interruption of the respiratory pathway (Dimapilis et al., 2018; Kim, et al., 2021b; Villaseñor & Ríos, 2018). ROS disinfection capacity varies greatly among the different ROS species; while superoxide and hydrogen peroxide are considered the weakest ROS, compounds as hydroxyl radicals are considered the most potent.-

Hence, during Fe-EC and CC there may be different pathways for inactivation or removal of (pathogenic) micro-organisms at play. However, to utilize Fe coagulation as a disinfection method, it is critical to differentiate between these pathways, as they require different operational conditions. The objective of this study was therefore to isolate the contribution of inactivation by ROS formed during Fe^{2+} oxidation from other removal pathways, such as floc sorption and entrapment, as this differentiation is generally lacking in electrocoagulation literature. The contribution of ROS-mediated inactivation was therefore assessed for both a bacterial and a viral enteric indicator organism, namely *Escherichia coli* WR1 and somatic coliphage ØX174, respectively. For this purpose, a series of experiments with both chemical and electro-chemical dosing of Fe^{2+} were conducted, either in presence or absence of the Fenton inhibitor

TEMPOL (Hu et al., 2019). In this study, a differentiation was made between continuous and single spike dosage, as it is hypothesized that continuous generation of ROS might be beneficial for the disinfection capacity of the Fe-EC system. In addition, the dosage rate was assessed to develop a ROS-inactivation kinetics model for Fe-EC.

4.2 Materials and Methods

4.2.1 Laboratory setup and experimental design

Experiments were conducted using Fe-EC and/or CC as sources of Fe (Figure 4-2). All experiments were performed in 2 L cylindrical glass beakers mounted on identical LABNICO L23 magnetic stirrers and fitted with PTFE coated bars for stirring purposes. During all experiments, air was supplied continuously using an OASE OxyMax200 air pump, and dissolved oxygen (DO) levels were monitored continuously in order to maintain saturation in the test water.

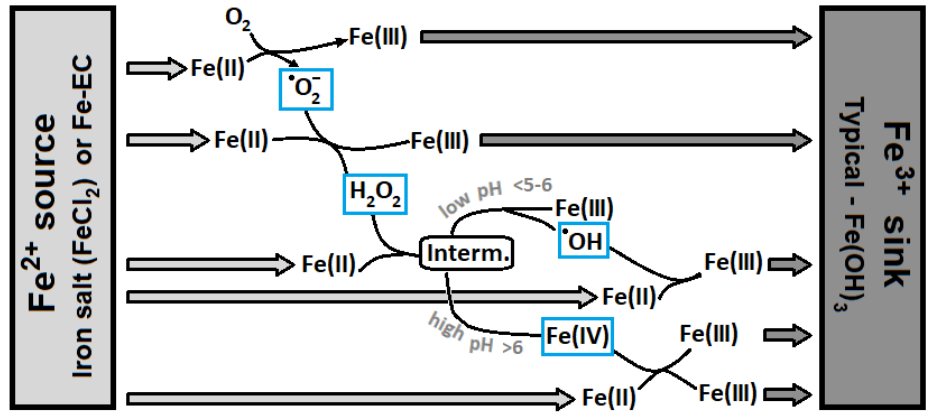


Figure 4-1 Proposed pathways of aerobic Fe^{2+} oxidation, indicating the production of ROS: superoxide radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) and high valent oxoiron (FeIV). Adapted from Hug & Leupin, 2003, Kim et al., 2011 and Van Genuchten & Peña, 2017.

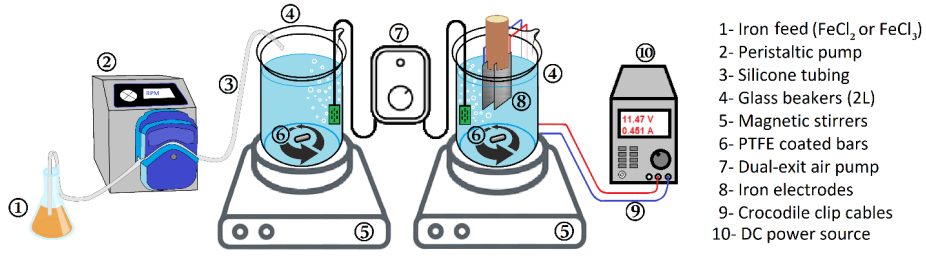


Figure 4-2 Fe dosage bench scale setup, with chemical dosage (left) and electrochemical dosage (right).

Depending on the experiment, chemical dosing was performed either by spiking the dosage (weighing and directly dosing Fe salts into the reaction beaker), or by continuously adding dosing solutions using a Watson Marlow 120U peristaltic pump. $FeCl_2$ and $FeCl_3$ reagent grade salts were supplied by Sigma-Aldrich (Germany). The Fe-EC setup included a 30 V - 3 A TENMA 72-10500 bench DC power supply, connected with crocodile clip cables to two S235 steel plates (maximum percentages: 0.14% carbon, 0.10% silicium, 0.80% manganese, 0.025% phosphorous, 0.015% sulphur, 0.010% nitrogen, 0.20% copper and 0.080% aluminium). Dimensions of the steel plates were 6 cm x 4 cm, of which 4 cm x 4 cm were submerged (2 cm remained above the test water to connect the clip cables). The plates were polished with coarse and fine sand paper before each use and mounted to the end of a plastic tube with carved parallel slots ensuring the plates remained parallel and spaced approximately 1 cm as described elsewhere (Bicudo et al., 2021; Heffron et al., 2019a; Anfruns-Estrada et al., 2017; Ndjomgoue-Yossa et al., 2015; Merzouk et al., 2009).

During each individual experiment, only two process-control parameters were adjusted, namely electrical current (amperage) and electrolysis time. These parameter combinations were selected beforehand for each experiment in order to produce a dose of 50 mgFe/l under different speeds, namely 5.0, 2.5, and 1.0 mgFe/l/min, as oxidation kinetics are relevant for this research. The maximum Fe dosage rate (5.0 mgFe/l/min) was selected based on the capacity of the power supply not to reach/exceed its maximum voltage (30 V). Since experiments were conducted at room temperature, the need to minimize 'spontaneous' inactivation of *E. coli* limited the duration of the experiments, which in turn defined the lower Fe dosage rate (1.0 mgFe/l/min). An intermediate Fe dosage speed (2.5 mgFe/l/min) was added as a third dataset.

Four groups of experiments were performed in order to determine the role of oxidizing Fe^{2+} in ROS-mediated disinfection (Figure 4-3) and the kinetics involved. For all experiments, synthetic water containing a buffer solution and indicator microbes was prepared (described in section 2.2). The contribution of ROS-mediated disinfection was assessed in two ways, namely, 1) comparing inactivation by Fe^{2+} or Fe^{3+} and 2) ROS quenching while dosing Fe^{2+} ($FeCl_2$) (see 2.4). To determine whether the source of Fe had any impact on ROS-mediated disinfection, a third group of experiments compared microbial inactivation using CC or Fe-EC as the Fe^{2+} source (including ROS quenching for each case). Lastly, Fe-EC disinfection kinetics were studied by operating the system at different dosage rates. Additional information can be found in Table 4-2.

For all experiments, the stirring speed was set to 200 rpm, which induced intense mixing and turbulence, and prevented the formation of macroscopic flocs. Samples

were collected immediately after the Fe dosing was stopped (while the stirring was still on), in order to avoid any sedimentation. Collected samples were used immediately for microbiological and physical/chemical characterization.

4.2.2 Synthetic water matrix

For all experiments, the synthetic water matrix was either 0.02M or 0.04M tris-HCl buffer ($(HOCH_2)_3CNH_2$) (Table 4-2), selected by its capacity to buffer at a pH of approximately 7.5-7.7 (similar to that of municipal secondary effluents), its absence of Fe or ROS scavengers (such as PO_4^{3-}, CO_3^{2-}, Ca^{2+}), and its moderate conductivity (avoiding the addition of electrolytes for Fe-EC).

4.2.3 Microbial spike preparation

Two non-pathogenic organisms were used to spike the synthetic effluents, namely *E. coli* WR1 (NCTC 13167) and somatic coliphage ØX174 (ATCC 13706-B1), a bacterial and viral indicator respectively, as previously used in Bicudo et al., 2021. *E. coli* WR1 frozen stock was thawed and grown in TYGB broth (Tryptone Yeast Extract Glucose Broth) for 3 hours at 37 °C to concentrations of $\approx 1\times10^8$ cfu/ml, then centrifuged at 10.000 rpm during 10 minutes. The obtained pellet was re-suspended in PBS pH 7.2 to a concentration of $\approx 1x10^9$ cfu/ml, stored at 4 °C and used within 24 hours. Phage ØX174 was propagated following the ISO 10705-2:2000 method (Water quality — Detection and enumeration of bacteriophages — Part 2: Enumeration of somatic coliphages), to concentrations of approximately $\approx 1x10^9$ pfu/ml. *E. coli* WR1 and phage ØX174 were dosed into the test liquid to initial concentrations of $\approx 1\times10^5$ cfu/ml and $\approx 1\times10^4$ pfu/ml, respectively.

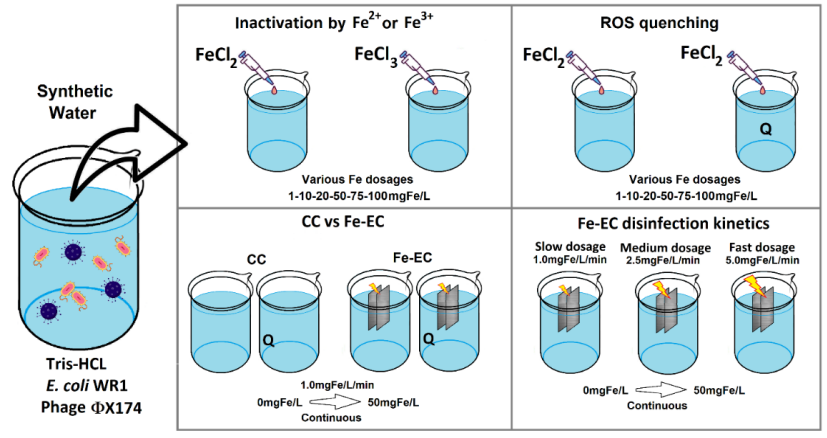


Figure 4-3. Schematic description of the four Fe disinfection experiments. Letter "**Q**" indicates the beaker was dosed with an ROS-Quencher. Pipette represents single-event chemical dosage, while the arrow represents continuous dosage either by CC ($FeCl_2$ solution dosed with peristaltic pump) or Fe-EC (Fe dosed electrochemically under a constant current).

4.2.4 TEMPOL as quencher for Fe2+-mediated ROS

In order to inhibit the disinfection by ROS produced following the aerobic oxidation of Fe^{2+}, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (also known as 4-Hydroxy-TEMPO, or TEMPOL) was selected as ROS quencher. TEMPOL is the most studied of the nitroxides, mainly due to its low molecular weight and high cell permeability (Wilcox & Pearlman 2008). Its most remarkable properties include the superoxide catalysis, the catalytic destruction of H_2O_2 by catalase-like reactions and the hindering of toxic hydroxyl radical production.

The reaction between TEMPOL and Fe^{2+} cations was described by Mitchell et al., 1990:

$$\boldsymbol{RR'NO^{\bullet} + Fe^{2+} \Rightarrow RR'NOH + Fe^{3+}} \quad (4\text{-}1)$$

In this reaction, the nitroxide (indicated by the reducible functional group -$NO^{\bullet}$) reacts on a 1:1 molar ratio with Fe^{2+}, to produce the oxidized oxoammonium (-NOH) and Fe^{3+}. In this way, nitroxides accept the electrons from reduced Fe complexes, therefore outcompeting oxygen, and thus preventing the production of oxygenated radicals. This equimolar reaction between Fe^{2+} and TEMPOL was instrumental during ROS-quenching experiments later described in this chapter. During these experiments, reagent grade TEMPOL (Sigma-Aldrich –Germany), was weighed, added directly and diluted into the synthetic water before any addition of Fe.

4.2.5 Analytical methods

E. coli screening and quantification was performed by membrane filtration according to APHA-Standard Methods for the Examination of Water and Wastewater, 23rd Edition. Samples were filtered in 0.45 µm cellulose acetate membrane filters, and placed on Chromocult® Coliform Agar (Merck) selective media (in which *E. coli* WR1 yields distinct purple colonies), and incubated at 37 °C for 24 hours. Screening of somatic coliphages was performed by pour plate technique following ISO 10705-2:2000. Total Fe was measured with Spectroquant®Iron Cell Test (1-50 mgFe/l) using a Spectroquant®NOVA60 (Merck, Germany) photometer, while Fe^{2+} was measured with Hach LCK 320 Fe test kits (0.2-6 mgFe/l). Hydrogen peroxide measurements were conducted following the Ghormley triiodide method (Frew et al., 1983) with filtered samples (0.45 µm) using a Genesys 10S UV-Vis (Thermo Scientific) at 350 nm wavelength. All samples analyzed for H_2O_2 were processed within 60 seconds of extraction. For Fe-EC experiments, free chlorine tests were conducted in filtered samples using the DPD method (Spectroquant- Supelco) in order to rule out the production of Cl_2 compounds that could account for unwanted disinfection.

4.2.6 Data analysis

A reduction in the concentration of the microorganisms is expressed as log removal values since this term is widely used, but here referred to as either *removal* or *inactivation* based on the involved elimination mechanism. Comparison between data series of somatic coliphage ØX174 or *E. coli* concentrations in synthetic effluents under different conditions was performed using Spearman's Rank-Order Correlation, which calculates rank correlation coefficient (R_s) and p-value. R_s determines the strength of the correlation between two datasets (R_s>0.9 indicates very strong correlation). The p-value determines the likelihood of two data series to be co-relatable by mere chance (p-value <0.05 shows a strong correlation beyond chance between data sets). Spearman was preferred over Pearson's correlation due to the monotonic behavior of most plots to be correlated (section 3).

4.3 Results

4.3.1 Inactivation by Fe^{2+} or Fe^{3+}

Experiments comparing *E. coli* WR1 and somatic coliphage ØX174 inactivation by varying dosages of either Fe^{2+} or Fe^{3+} are depicted in Figure 4-4. No significant difference in terms of floc characteristics were observed after 1 hour of continuous stirring, for samples dosed with either $FeCl_2$ or $FeCl_3$ (Figure 4-13). For both selected microbial indicators, inactivation using $FeCl_3$ (Fe^{3+}) was negligible across all assayed dosages, yet the response towards $FeCl_2$ (Fe^{2+}) was considerably different. *E. coli* inactivation increased with increasing Fe^{2+} dosage, with an approximate 0.05 $\log_{10}$ per mg Fe/l dosed. At the maximum dosage of 100 mg Fe/l, *E. coli* inactivation approached 5 $\log_{10}$. The inactivation of ØX174 showed a biphasic pattern; up to 10-20 mg Fe^{2+}/l inactivation was more rapid (close to 1.5 $\log_{10}$), which was similar to *E. coli*, while at higher Fe^{2+} concentrations, inactivation levelled off, reaching approximately 2.5 $\log_{10}$ at 100 mg Fe/l. All retrieved samples dosed with $FeCl_2$ showed full Fe^{2+} oxidation after 1 h of stirring (Fe^{2+} below Limit of Detection - LOD), though given the relatively high pH and DO, it is likely that this condition had been achieved within minutes. It may be concluded that under intense mixing (to prevent sedimentation) and aerobic conditions, dosage of Fe^{2+} demonstrated to have considerable inactivation properties that were not observed for Fe^{3+} under identical conditions. This indicates that the oxidation of Fe^{2+} itself, and potentially the production of intermediate ROS, plays a critical role in Fe-based disinfection.

4.3.2 ROS quenching

To investigate the contribution of ROS to *E. coli* and ØX174 inactivation during Fe^{2+} oxidation, Fe^{2+} was dosed chemically with and without the presence of an equimolar amount of the ROS-quencher TEMPOL. TEMPOL counteracted the bactericidal properties of oxidizing Fe^{2+} for *E. coli*, with under 1 $\log_{10}$ inactivation at all dosages (Figure 4-10). According to Equation 4-1, the interaction between TEMPOL and Fe^{2+} should lead to oxidation of the latter into Fe^{3+} without O_2 acting an as electron acceptor, avoiding the formation of the superoxide radical, and thus halting the ROS cascade. All samples (with and without TEMPOL) showed full Fe oxidation after 1 hour of stirring (Fe^{2+} below LOD). Inactivation of *E. coli* and phage ØX174 under a dose of 50 mgFe/l is displayed in Figure 4-5. The complete dosage series is presented in Figure 4-10. For both indicators, beakers in which only TEMPOL was dosed showed almost identical microbial concentrations as the blank (negligible inactivation), confirming that the quencher itself did not exhibit inactivation properties. *E. coli* inactivation reached approximately 3 $\log_{10}$ when the reaction was not quenched, dropping to <0.5 $\log_{10}$ with TEMPOL added. These results indicate that the observed inactivation during Fe^{2+} oxidation was related to the formation of reactive intermediates, and that this inactivation pathway could be almost completely suppressed by the addition of the Fenton inhibitor TEMPOL. This also indicates that the primary elimination process was inactivation rather than entrapment in flocs. Phage ØX174 demonstrated a similar inactivation as *E. coli* under the presence of Fe^{2+} (≈2.8 $\log_{10}$) without TEMPOL, while inactivation was still significant after the addition of TEMPOL (1.5 $\log_{10}$). The observation that ØX174 disinfection in the presence of TEMPOL is not fully suppressed suggests the contribution of an elimination mechanism other than ROS, such as entrapment in flocs. No production of Cl_2-based compounds (<0.01 mg/l total Cl_2) was detected during these or any of the experiments described in this chapter, hence this inactivation mechanism was ruled out.

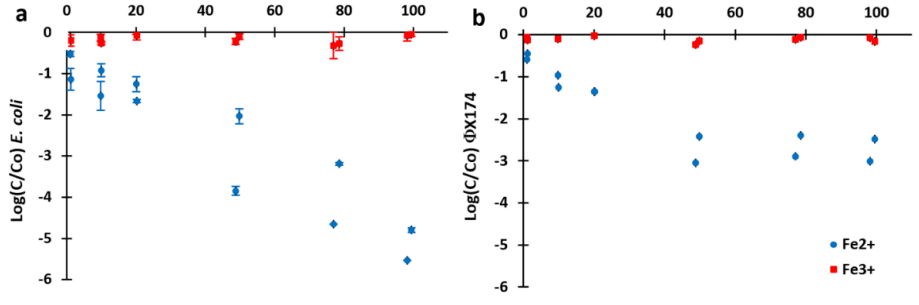


Figure 4-4 Inactivation of (a) *E. coli* WR1 and (b) somatic coliphage ØX174 during dosing 0-100 mg/l of either Fe^{2+} or Fe^{3+}. Error bars represent standard deviation. All experiments were performed in duplicate, and all microbial determinations were executed in triplicate.

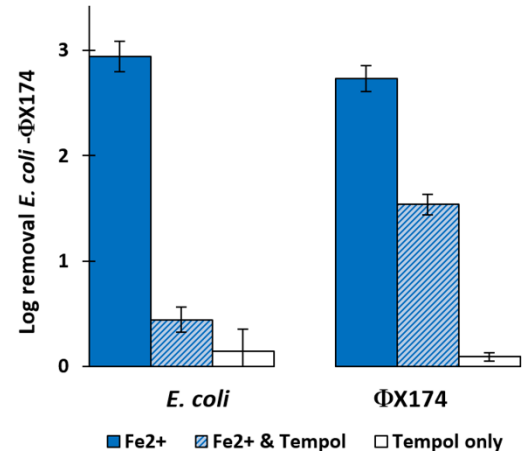


Figure 4-5 Log Removal of *E. coli* WR1 (left) and somatic coliphage ØX174 (right) under Fe^{2+}, Fe^{2+}+ TEMPOL, and only TEMPOL (equimolar concentrations of 0.9 mM ≈ 50 mgFe/l and/or 154.5 mgTEMPOL/l). Error bars represent standard deviation. All experiments were performed in duplicate and all microbial determinations were executed in triplicate.

4.3.3 CC versus Fe-EC

To determine the impact of the source of Fe^{2+} in inactivation, experiments using an identical and constant Fe supply rate (1.0 mgFe/l/min) were conducted, either by CC or Fe-EC. For *E. coli* WR1, almost identical inactivation was obtained whether the Fe source was either chemical ($FeCl_2$) or electrochemical (Figure 4-6a). This indicated that the source of Fe^{2+} was irrelevant for inactivation as long as the produced Fe^{2+} dosage was similar (Spearman's Rank Correlation test between averaged data sets determined R_s≈1 with a p-value of 0.001). For both CC and Fe-EC, the Fe was dosed in a constant manner instead of in a single spike, as instant dosing is not possible with Fe-EC. When comparing Fe^{2+} continuous dosing (Figure 4-6a,c) against instant dosing (Figures 4-4 and 4-5) for inactivation of *E. coli*, it is apparent that for constant dosing the same maximum inactivation is achieved after dosing only half the Fe dose (50 mgFe/l) than for the single spike experiments (100 mgFe/l). For phage ØX174, experiments with non-quenched Fe^{2+} also produced similar inactivation results whether the Fe source was chemical ($FeCl_2$) or electrochemical, as depicted in Figure 4-6c. This indicated that the source of Fe^{2+} is also irrelevant for phage ØX174 inactivation as long as the produced Fe dosage is similar (Spearman R_s=0.97; p-value=0.001). Regarding the performance of continuous Fe dosing compared to a single spike (Figures 4-4 and 4-5), 50 mgFe/l continuous dosage yielded similar inactivation of ØX174 as a single spike dose (≈2 $\log_{10}$ inactivation). For experiments involving quenched Fe^{2+} dosage (with added TEMPOL), inactivation datasets coming from the *E. coli* experiments with chemical and electrochemical sources still show a statistically strong correlation (Spearman R_s=0.86, p-value=0.05), but inactivation is in either case almost completely

mitigated under the presence of TEMPOL (Figure 4-6b). Inactivation was ≈1 $\log_{10}$ at the maximum Fe dosage (50 mgFe/l). Data for ØX174 experiments involving quenched Fe^{2+} dosage (Figure 4-6d), on chemical versus electrochemical sources again showed a statistically strong correlation (Spearman R_s=0.93, p-value=0.02). However, as opposed to the strong reduction of the inactivation rate observed for *E. coli* (Figure 4-6b versus 4-6a), addition of TEMPOL yielded only a small reduction of the inactivation rate for either of the Fe sources (Figure 4-6d versus 4-6c).

Altogether, these results conclusively show that irrespective of the type of assay (quenched/non-quenched) or the type of microbial indicator, there is no statistical difference between inactivation induced by Fe^{2+} coming from either CC or Fe-EC. This implies that during Fe-EC, the contribution of electrolysis-specific inactivation pathways (due to electric fields or to Cl_2 formation) was negligible.

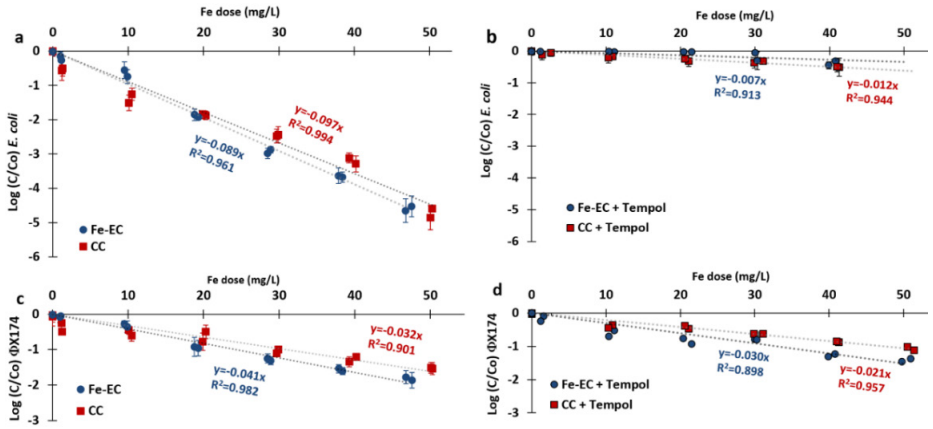


Figure 4-6 Inactivation of *E. coli* under CC and Fe-EC without TEMPOL (a) and with TEMPOL (b), and inactivation of phage ØX174 under CC and Fe-EC without TEMPOL (c) and with TEMPOL (d). Error bars represent standard deviation. All experiments were performed in duplicate, and all microbial determinations were executed in triplicate.

4.3.4 Fe-EC inactivation kinetics

In order to investigate Fe-EC inactivation kinetics, experiments were conducted with three different dosing rates, namely 5.0, 2.5 and 1.0 mgFe/l/min (fast, medium and slow dosage, respectively). For each of these settings, the Fe supply was stopped once the cumulative dosage reached 50 mgFe/l in order to produce the same end concentration.

For *E. coli* WR1, the faster the Fe dosage, the higher the inactivation rate. Interestingly, inactivation stopped abruptly once the electric charge was interrupted, which means that the inactivation produced during the Fe-EC stage offered no residual effect once

the current ceased (Figure 4-11). This implies that disinfectants produced during the supply of Fe^{2+} were extremely short lived. The plot Ln(C/Co) versus electrolysis time (Figure 4-7) shows a good correlation between the averaged datasets and linear trendlines, with the slope being the inactivation rate, in which the ratios between the inactivation rates (5.2/3.0/1.0) are similar to the ratios of the current intensity and of Fe production speed (5.0/2.5/1.0). When plotted against the Fe dosage (Figure 4-7b), there is an overlap between the Ln(C/Co) plots, indicating that inactivation is directly proportional to the dosed Fe for all dosage speeds.

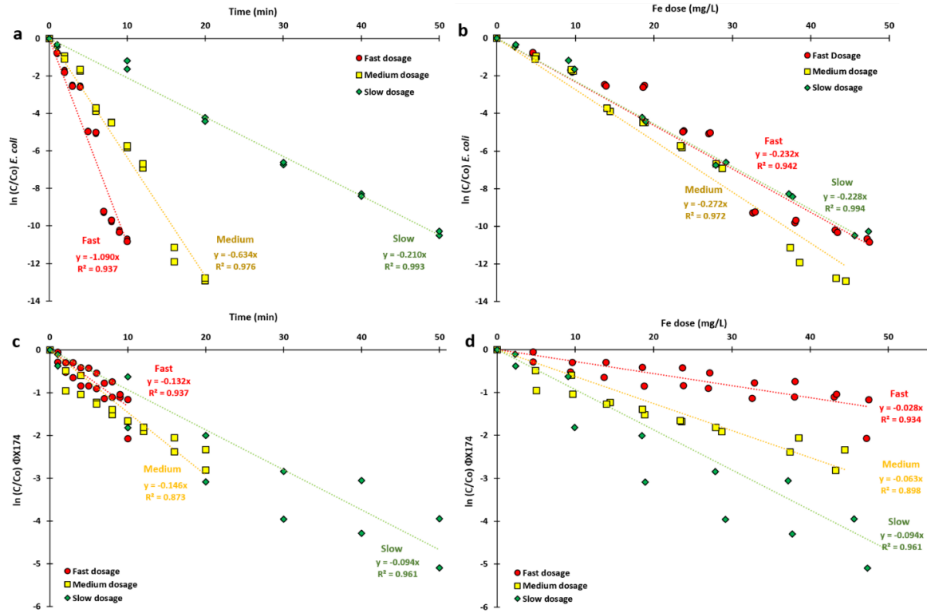


Figure 4-7 Ln(C/Co) plot for *E. coli* WR1 concentration during the application of fast, medium and slow electrochemical Fe dosage as a function of (a) time and (b) dosed Fe, and Ln(C/Co) plot for phage ØX174 concentration during the application of fast, medium and slow electrochemical Fe dosage as a function of (c) time and (d) dosed Fe. All experiments were performed in duplicate, and all microbial determinations were executed in triplicate.

For phage ØX174, results showed different inactivation kinetics than *E. coli*: the inactivation rate (the slope in Figure 4-7c) appeared numerically comparable for all experiments. This means that the inactivation rate progressed similarly irrespective of dosing speed as long as the electric current was being applied, yet was independent of the magnitude of such current (instead of proportional as for *E. coli*). Since each configuration delivered different amounts of Fe per unit time, shorter Fe dosage times (faster dosage speeds) yielded less total inactivation than slower dosages, as depicted in Figure 4-7d. For 50 mgFe/l, the slow dosage speed (1.0 mgFe/l/min) produced ≈4.7

Ln inactivation (1.8 $\log_{10}$), whereas the fast dosage speed (5.0 mgFe/l/min) reached only 1.2 Ln (0.7 $\log_{10}$). The averaged Ln(C/Co) diagrams for each different charge speed condition show sufficient linearity as to assume a linear regression to provide an adequate fit, even though the R^2 values were somewhat lower than those obtained for *E. coli*. For all cases, the decrease in the concentration of phage ØX174 ceased abruptly once the electric current supply was interrupted (as was the case with *E. coli*), once again suggesting that Fe-EC produced no residual disinfection, and that the disinfectants produced were extremely short lived (Figure 4-12).

4.3.5 Fe speciation and H_2O_2 measurements

During the Fe-EC inactivation kinetics experiments, screening of Fe^{2+} and Fe^{3+} was performed to verify that the conversion of Fe^{2+} into Fe^{3+} was a steady-state process. H_2O_2 screening was performed simultaneously, to confirm the onset of the ROS cascade. Fe screening (Figure 4-8) showed, for all cases, a very stable and relatively low Fe^{2+} concentration being established and measured during electrolysis, simultaneous with a stable Fe^{3+}production. Since Fe is electrochemically dosed as Fe^{2+} (Lakshmanan et al., 2009), and Fe^{3+} is produced at a steady rate, this means that Fe^{2+} is readily converted to Fe^{3+}. This was expected due to relatively high pH (7.4-7.7) and dissolved oxygen close to saturation (>7.5 mgO_2/l). Once the current was interrupted, an abrupt drop in the Fe^{2+} concentration was observed, readily stabilizing at ≈1 mgFe/l for the remainder of the screening. It is important to note that in all cases inactivation only occurred during the time in which Fe^{2+} was being steadily supplied (and steadily oxidized), and halted abruptly once Fe^{2+} stopped being supplied (hence stopped being oxidized). For all experiments, Faradaic efficiency of the Fe-EC was determined ≈95-96%.

Based on the measured Fe^{2+}plateau, pH and dissolved oxygen concentrations during Fe-EC, theoretical Fe^{2+} oxidation rates (or Fe^{3+} production) were calculated following the methodology proposed by Stumm & Lee (1961) expressed in Equation 4-2. Calculations are detailed in Table 4-4.

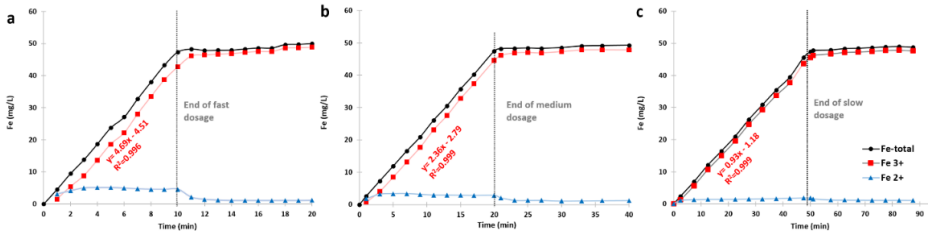


Figure 4-8 Fe profiles (Total Fe, $Fe^{2+}$and Fe^{3+}) during (a) fast dosage (b) medium dosage and (c) slow dosage. The best fitting linear equation and R^2 for the Fe^{3+} series (during Fe supply) is indicated in each case, together with the timestamp in which Fe supply is stopped. All experiments were performed in duplicate.

$$-\frac{d[Fe^{2+}]}{dt} = k.[Fe^{2+}].p_{O_2}.[OH^-]^2 \quad (4\text{-}2)$$

Where:

k is the kinetic constant 1.5 ± 0.5 $x10^{13}$ $L^2mol^{-2}atm^{-1}min^{-1}$

$[Fe^{2+}]$ is the molar concentration of the Fe present in the Fe^{2+} form

p_{O2} is the partial pressure of oxygen (atm)

$[OH^-]$ is the molarity of the OH^- ion

Theoretical versus observed oxidation rates are displayed in Table 4-1:

Table 4-1 Theoretical versus observed average Fe^{2+} oxidation rate, as a function of pH and D.O for each dosage condition.

	DOSAGE		
	Fast	**Medium**	**Slow**
pH	7.75	7.70	7.70
D. O. (mg/l)	7.6	7.8	9.1
Fe^{2+} plateau (mg/l)	4.8	2.9	1.6
d[Fe^{2+}]/dt (mg/l/min) - Stum & Lee	**4.78**	**2.29**	**1.29**
d[Fe^{2+}]/dt (mg/l/min) - measured	**4.69**	**2.36**	**0.93**

These theoretical Fe^{2+} oxidation values are numerically very similar to the measured production rate of Fe^{3+} in each case (4.69, 2.36 and 0.93 mgFe/l/min, respectively), confirming that the obtained Fe^{2+} concentrations and oxidation rates during the application of electric current corresponded to that of a (pseudo) steady-state.

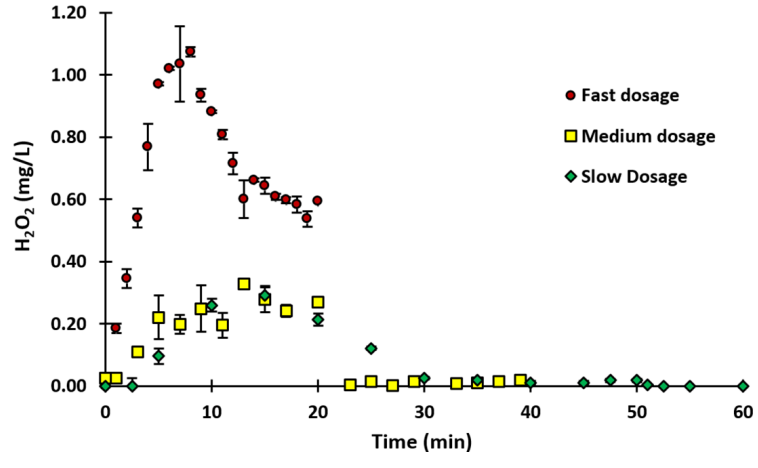


Figure 4-9 Average H_2O_2 production profiles during fast, medium and slow Fe dosage. All experiments were performed twice. Error bars represent standard deviation.

Samples for H_2O_2 measurement were collected every 1, 2 or 5 minutes for fast, medium and slow dosage respectively, and processed according to the Ghormley triiodide method after 0.45 µm filtration, as turbid samples yield very high false readings. The maximum observed value for H_2O_2 occurred during fast dosage, reaching ≈1.07 mg/l (53.7 µM) at t=8 min, while for the medium and slow dosage experiments the maximum was approximately at 0.25 ± 0.05 mg/l (13.9 µM), as depicted in Figure 4-9. During slow dosage, a sudden drop of H_2O_2 concentration from 0.27 mg/l to below detection was observed once the current was disconnected, suggesting that the H_2O_2 conversion rate into intermediates (Figure 4-1) is much faster than the experimental measurement time. This could imply that the actual H_2O_2 value inside of the beaker at the moment of sample extraction could be larger than the value actually measured 60s later, this being an important limitation of the H_2O_2 measurement method. It is unlikely however, that H_2O_2 was responsible for disinfection since the required concentrations and exposure time for inactivation are orders of magnitude larger than the ones observed (Labas et al., 2008). However, the production of H_2O_2 is a necessary intermediate step in the ROS-cascade, mediating in the production of hydroxyl radicals and/or ferryl ions (the main disinfecting species related to Fe-ROS), as depicted in Figure 4-1.

4.4 Discussion

4.4.1 Inactivation of E. coli and phage ØX174 by Fe2+ oxidation: role of ROS

Electrochemically produced Fe^{2+} yielded significant inactivation in synthetic secondary effluent at pH 7.5-7.7 at room temperature (20 °C) under intensive mixing and aeration, reaching 5 $\log_{10}$ for *E. coli* at 100 mg/l Fe^{2+}. It was suggested that the oxidation of Fe^{2+} promotes the formation of bactericidal ROS species (Anfruns-Estrada et al., 2017), while Fe^{3+} is unable to do so given its already oxidized state (Jeong et al., 2006; Vatansever et al., 2013). This hypothesis was tested by the introduction of the Fenton inhibitor TEMPOL in order to verify if the anoxic oxidation of Fe^{2+} into Fe^{3+} would lose its disinfection capacities, as the quencher would theoretically accept the produced electron (instead of the O_2) preventing the ROS formation (Nyui et al., 2018; Lewandowski & Gwozdzinski, 2017; Soule et al., 2007). Indeed, the addition of TEMPOL resulted in the almost complete inhibition of the inactivation of *E. coli*, underlining the value of dosing Fe in its reduced state.

For phage ØX174, electrochemically produced Fe^{2+} also resulted in inactivation, but the net result was lower than for *E. coli* (2 versus 5 $\log_{10}$ at 100 mg/l Fe^{2+}). A biphasic inactivation pattern was observed for ØX174, with faster inactivation up to 10-20 mg/l Fe^{2+} and slower inactivation between 20-100 mg/l Fe^{2+}. For the phage, the quencher did reduce inactivation, but to a considerably smaller extent than for *E. coli*, pointing towards an additional removal pathway other than ROS. Such an alternative pathway

was proposed by Kim et al., 2011, who determined that un-oxidized Fe^{2+} achieved measurable MS2 phage inactivation, although it proved to be weak under aerobic conditions and relatively low Fe^{2+} concentrations, as in our experiments. Zhu et al., 2005 found that MS2 bacteriophages adsorb to the Fe oxyhydroxide flocs due to their negative surface charge, and Kim et al., 2021a demonstrated that virus entrapment and inactivation take place during Fe-EC. Heffron et al., 2019a, and Heffron et al., 2019c proposed that phage inactivation was promoted by longer exposure time of the viruses to the ROS, which aligns with our findings for different Fe-EC dosing rates. However, in our experiments, TEMPOL did not fully shield phage ØX174 from inactivation (Figure 4-6c and 4-6d) since a decrease in plaque counts was observed even when ROS were believed to be suppressed. This points to either a separate simultaneous inactivation process or to marginal ROS production due to O_2 competition for Fe^{2+} that could not be sufficiently quenched by TEMPOL.

4.4.2 Fe-EC inactivation kinetics of phage ØX174 and E. coli

Inactivation of phage ØX174 increased with apparent linearity with the Fe-EC dosage time. The variation of the value k (0.094 to 0.140 min^{-1}) is relatively small considering the 500% variation of the current intensity, meaning that the inactivation rate is largely irresponsive to it. With this, we conclude that exposure time to oxidizing Fe^{2+} is the main driver in phage inactivation:

$$Ln\left(\frac{N}{N_0}\right) = -k_n.t \quad (4\text{-}3)$$

Where:
-k_n is a first order rate constant (ranges from 0.094 to 0.140 min^{-1}, derived from Fig 7c).
-t is the electrolysis time (min)

Phage ØX174 inactivation was found to be dependent of time, but not of the dosed Fe. The Ln(C/Co) plot for *E. coli* WR1, however, clearly showed linearity towards the Fe dose. Therefore, the expression linking *E. coli* WR1 inactivation to the dosed Fe can be written as follows:

$$\boldsymbol{Ln\left(\frac{N}{N_0}\right) = -K.Fe_{dosed}} \quad (4\text{-}4)$$

Where:
K= first order rate constant
N= concentration of *E. coli*
N_0= starting concentration of *E. coli*
Fe_{dosed}= cumulative dosage of Fe

The data series obtained for fast, medium and slow dosage had very similar slopes, varying from 0.23 to 0.27 $mgFe^{-1}$ with an average slope of 0.24 $L.mgFe^{-1}$, being therefore selected as the value K. The Fe dose can in turn be expressed as the product of Fe dosing rate (d[Fe]/dt) and electrolysis time (Equation 4-5), hence:

$$Ln\left(\frac{N}{N_0}\right) = -0.24.\frac{d[Fe]}{dt}.t \quad (4\text{-}5)$$

Based on Faraday's law (equation 3-3), the term *d*[Fe]/*d*t can be obtained as follows:

$$m_{Fe} = \frac{\mathrm{I.t.}M}{n.F}\eta \xrightarrow{/Vol} [Fe] = \frac{\mathrm{I.t.\ 55.85g.mol^{-1}}}{V.2.96485.3\ \mathrm{C\ mol^{-1}}}\eta \xrightarrow{/dt} \frac{d[Fe]}{dt} = \frac{\mathrm{I.\ 2.90x10^{-4}g.C^{-1}}}{V}\eta \quad (4\text{-}6)$$

Where:
m_{Fe} = mass of released Fe (g)
I = Intensity of the electric current (A)
t = time of electrolysis (s)
M=Molar mass of the sacrificial anode metal (Fe=55.85 g/mol)
n= valence of the released metal ion (for Fe n=2)
F= Faraday´s constant (96485.3 C/mol)
[Fe] = Concentration of Fe (g/l)
V = Sample volume (L)
η = Faradaic efficiency (0.95 – 0.96 during these experiments)

Converting concentrations and time into mgFe and min respectively in Equation 4-6, and substituting in Equation 4-5, a simple expression can be obtained for the Ln(N/No), resulting in Equation 4-7:

$$Ln\left(\frac{N}{N_0}\right) = -\frac{I.t}{V}.\eta\ .4.08\frac{L}{A.min} \quad (4\text{-}7)$$

Since both the assay volume and Faradaic efficiency are assumed to be constant, the obtained expression has the same structure as the well-known Chick-Watson equation (Watson, 1908), namely:

$$Ln\left(\frac{N}{N_0}\right) = -K_{cw}.C.t \quad (4\text{-}8)$$

In this expression K_{cw} is a specific inactivation constant, t is the exposure time to the disinfectant, and C is the concentration of such disinfectant. In our particular case, the current intensity behaves as a pseudo-disinfectant, not because the electric current is producing inactivation, but because the release of the disinfectant's precursor (Fe^{2+}) is directly proportional to the current intensity (I).

Applying a logarithmic base change:

$$Log_{10}\left(\frac{N}{N_0}\right) = -\frac{I.t}{V}x\ \eta\ x\ 1.77\frac{L}{A.min} \quad (4\text{-}9)$$

This expression shows that for a given time [min] and sample volume [L], the logarithmic removal value of *E. coli* WR1 is directly proportional to the current intensity [A]. The adjustment coefficient (1.77 $L.A^{-1}.min^{-1}$) is then a function of the environmental conditions (pH, DO, temperature, etc.). To the best of our knowledge, this expression is the first to link disinfection with electric current during Fe-EC.

4.2.3 Chemical or electrochemical Fe^{2+}

When comparing inactivation results of CC with Fe-EC, it may be concluded that the origin of the Fe^{2+} source did not play a significant role in disinfection. It could be argued, however, that the final hydrolysis products and the floc formation process are different, even though both experiments were conducted under identical test conditions (pH, temperature, mixing intensity, DO). The differences between chemical and electrochemical floc forming processes were extensively studied by Harif et al., (2012), who concluded that the coagulation/flocculation mechanisms using either coagulation method are similar yet not identical. It was found that electrocoagulation produced more fragile and porous flocs, which are easily compacted and restructured. The high shear conditions in our experiments prevented the formation of macroscopic floc-like structures (7-11 µm range; Table 4-3), producing what could best be described as fine dust-sized particles. The flocs, however, did not play a significant role in the elimination of *E. coli,* as the addition of TEMPOL almost completely stopped inactivation. For ØX174, inactivation was less prominent and also less affected by TEMPOL, suggesting that another elimination process (e.g., entrapment in flocs) was also important. This could differ between CC and Fe-EC, however, the potential contribution of differences in $Fe(OH)_3$ floc structure on microbial entrapment was beyond the scope of this study. What is apparent is that experiments using Fe^{3+} did not produce observable differences with the no-treatment blank for either microbial indicator, pointing out that Fe^{3+} or its particulates offered no measurable disinfection properties (inactivation nor floc entrapment) under these high shear conditions.

4.5 Conclusions

In this study it was demonstrated that *E. coli* WR1 and somatic coliphage ØX174 are inactivated during Fe^{2+} oxidation, irrespective of dosing Fe chemically or electro-chemically. In control experiments with Fe^{3+}, no inactivation was observed for either microbe. ROS-quenching experiments with TEMPOL confirmed that *E. coli* inactivation (5 log_{10}) is related to the production of Fenton-like intermediates

(ROS) during Fe^{2+} oxidation. The observed *E. coli* inactivation kinetics could be mathematically related to the Fe^{2+} oxidation rate, which under aerobic conditions is also directly proportional to the Fe-EC current's intensity. The inactivation process then follows a Chick-Watson-like expression, in which the amperage behaves as a surrogate for the disinfectant's concentration. Phage ØX174 removal/inactivation (2-3 log_{10}) could not be described in the same way, as at higher Fe dosages (>10 mg/l) removal efficiency dropped rapidly. Also, ROS-quencher TEMPOL did not completely inhibit phage ØX174 inactivation, suggesting that additional pathways are relevant for virus removal.

4.6 Supplementary information

4.6.1 Experimental phases and their characteristics

Table 4-2 The four phases comprising this study, and their characteristics.

Phase	Study	Water matrix	Fe-source		Fe dosage type	Fe dosage range	Assay time
1	Fe^{2+}/Fe^{3+} Inactivation	2L of 0.04M **Tris-HCl**	$FeCl_2$ [0.09M]		Dosed at once t=0	1-10-20-50-75-100 mgFe/l	60min
			$FeCl_3$ [0.12M]				
2	ROS quenching	2L of 0.04M **Tris-HCL**	$FeCl_2$ [0.09M]		Dosed at once t=0	1-10-20-50-75-100 mgFe/l	60min
		2L **Tris-HCl** [0.04M] **TEMPOL:** Matching Fe molarity					
3	CC vs Fe-EC	2L **Tris-HCl** [0.02M]	$FeCl_2$ [0.09M]		Continuous 24 ml/h (peristaltic pump)	From 0 to 50 mgFe/l [from 0 to 0.9mM] **(1.0 mgFe^{2+}/l/min)**	50min
		2L **Tris-HCl** [0.02M] **TEMPOL:** 154.5 mg/l [0.90mM]					
		2L **Tris-HCl** [0.02M]	Fe-EC		Continuous DC current 0.115A		
		2L **Tris-HCl** [0.02M] **TEMPOL:** 154.5 mg/l [0.90mM]					
4	Fe-EC inactivation kinetics	2L **Tris-HCL** [0.02M]	Fe-EC		Continous DC current 0.575A first 10:00min	From 0 to 50mgFe/l **(5.0 mgFe^{2+}/l/min)**	20min
					Continous DC current 0.288A first 20:00min	From 0 to 50mgFe/l **(2.5 mgFe^{2+}/l/min)**	40min
					Continous DC current 0.115A first 50:00min	From 0 to 50mgFe/l **(1.0 mgFe^{2+}/l/min)**	1:40h

4.6.2 Inactivation profiles during ROS quenching experiments

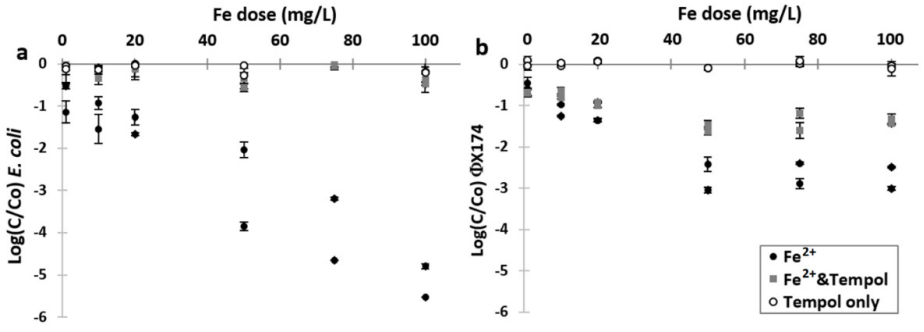


Figure 4-10 Microbial inactivation of *E. coli* WR1 (left) and somatic coliphage ØX174 (right) under equimolar dosages of Fe^{2+}, Fe^{2+}+ TEMPOL, and only TEMPOL. Error bars represent standard deviation. All experiments were performed in duplicates, and all microbial determinations were executed in triplicate.

4.6.3 Microbial decay during Fast, Medium and Slow Fe-EC

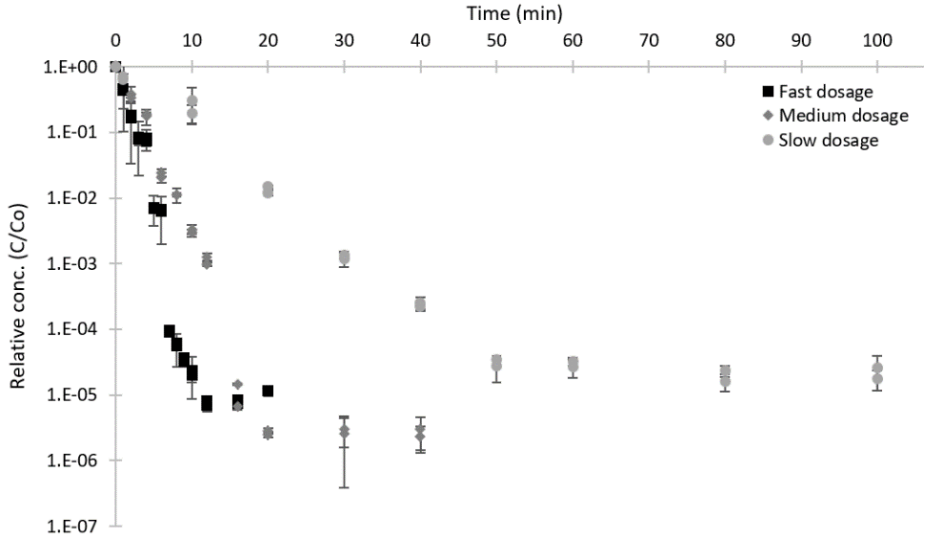


Figure 4-11 Relative concentration plot of *E. coli* under Fast, Medium and Slow Fe dosage during Fe-EC (10, 20 and 50 minutes respectively), and during supplementary mixing (10, 20 and 50 minutes respectively) without addition of current. Error bars represent standard deviation. All experiments were performed in duplicate, and all microbial determinations were executed in triplicate.

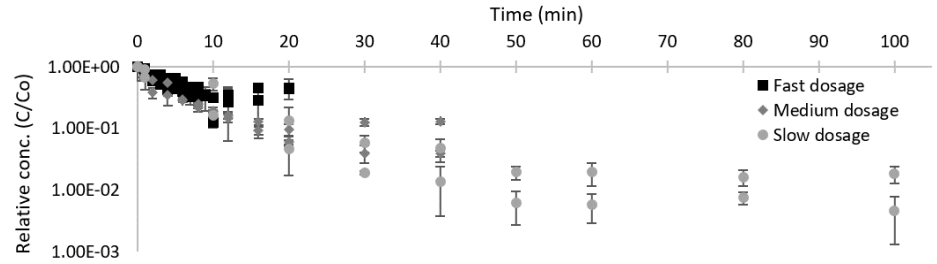


Figure 4-12 Relative concentration plot of somatic coliphage ØX174 under Fast, Medium and Slow Fe dosage during Fe-EC (10, 20 and 50 minutes respectively), and during supplementary mixing (10, 20 and 50 minutes respectively) without addition of current. Error bars represent standard deviation. All experiments were performed in duplicate, and all microbial determinations were executed in triplicate.

4.6.4 Floc microscopy

Together with the microbial experiments, samples of the mixed bulk liquid (intense mixing and no sedimentation) were observed under a Keyence VHX digital microscope, in order to characterize the size and size distribution of the flocs formed in each case (Figure 4-13). A large number of pictures (>20) was taken for each experiment, and finally five random pictures were selected to derive average floc area, maximum and minimum diameter of the obtained flocs (Table 4-3).

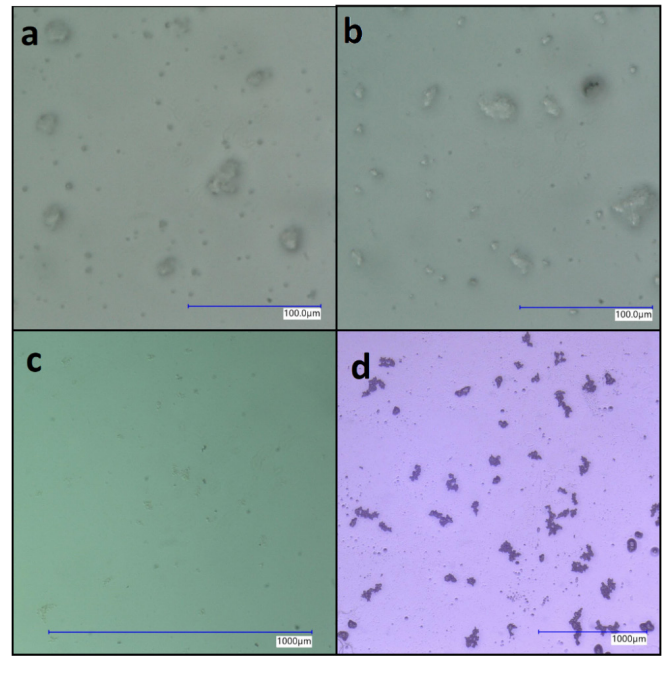


Figure 4-13 Digital microscopy of obtained flocs during the different Fe-related experiments, namely (a) $FeCl_2$, (b) Fe-EC, (c) Fe-EC+TEMPOL and (d) $FeCl_3$. All pictures correspond to a 10 mgFe/l dosage.

Table 4-3 Floc characteristics (average cross section area, maximum and minimum diameter) obtained by digital microscopy of at least 5 pictures for each case (randomly selected), using the Keyence VHX digital microscope particle analysis software.

	Average floc characteristics		
Coagulant	**Area (µm²)**	**Max diam. (µm)**	**Min diam. (µm)**
$FeCl_2$	65.5	9.6	6.9
Fe-EC	82.2	10.9	7.8
Fe-EC (+Tempol)	486	35	20
$FeCl_3$	1152.8	47.7	25.8
$FeCl_3$ + Jar Test	285341	1286	631

4.6.5 Fe^{2+} oxidation rate calculation by Stum & Lee equation

Table 4-4 Fe^{2+} oxidation rate calculation according to Stum & Lee (1961) for the three assayed Fe dosage speeds. Measured values highlighted in grey.

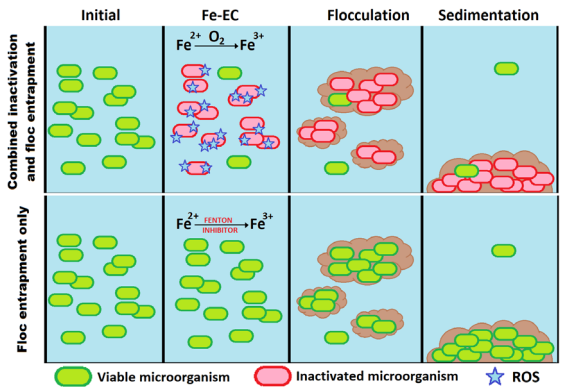
	Iron dosage speed			
Magnitude	**Fast**	**Med**	**Slow**	**Units**
pH	7.75	7.7	7.7	-
pOH	6.25	6.3	6.3	-
[H+]	1.78E-08	2.00E-08	2.00E-08	M
[OH-]	5.62E-07	5.01E-07	5.01E-07	M
D.O	7.6	7.8	9.13	mgO2/l
$[O_2]$	2.4E-04	2.4E-04	2.9E-04	M
K	1.5E+13	1.5E+13	1.5E+13	$L^2M^{-2}atm^{-1}min^{-1}$
Fe(II)	4.8	2.9	1.63	mg/l
Fe(II)	8.59E-05	5.19E-05	2.92E-05	M
dFe(II)/dt	8.56E-05	4.11E-05	2.31E-05	M/l/min
dFe(II)/dt	**4.78**	**2.29**	**1.29**	mg/l/min

5

CHAPTER 5

Disinfection during iron electrocoagulation: Discerning inactivation and floc entrapment

Published as: Bicudo, B., Werff, B. Van Der, Medema, G., & Halem, D. Van. (2022). *Disinfection during Iron Electrocoagulation : Differentiating between Inactivation and Floc Entrapment for Escherichia coli and Somatic Coliphage ØX174*. https://doi.org/10.1021/acsestwater.2c00230



Abstract

Electrochemical water treatment is gaining increasing popularity due to its wide range of potential applications, its decreasing costs and its suitability as a decentralized treatment alternative, but mainly due to it being considered a "green technology". In the field of municipal wastewater treatment, the use of Iron Electrocoagulation (Fe-EC) has been marginal, and although disinfection has been reported, its underlying mechanisms are not fully understood, for which significant controversy remains. In this study microbial inactivation during Fe-EC was evaluated as a two-component process, namely: physical removal by microbial floc sorption/entrapment, and inactivation by Reactive Oxygen Species (ROS) produced by (semi)Fenton reactions. Using the faecal indicators *Escherichia coli* WR1 and somatic coliphage ØX174 suspended in a synthetic water matrix, the role of ROS and the role of flocculation were quantitatively evaluated. Fenton inhibitor TEMPOL was used to quench ROS production during Fe-EC. At circumneutral pH, ROS were found to be highly detrimental to *E. coli*, yet only mildly damaging for phage ØX174 (≈3.9 $\log_{10}$ and ≈0.8 $\log_{10}$ inactivation respectively). Inactivation for both indicators increased under acidic conditions (pH 5.5), likely due to the formation of hydroxyl radicals (•OH), exceeding 5.1 $\log_{10}$ for *E. coli* and 1.5 $\log_{10}$ for phage ØX174. The ROS inactivation pathway is linked to the oxidation of ferrous iron (Fe^{2+}), being independent of flocculation conditions. Flocculation experiments demonstrated that there is strong positive correlation between orthokinetic-like flocculation conditions, floc sedimentation, and microbial removal, meaning that floc entrapment is a major removal pathway following Fe-EC. When compared to control experiments in which no flocculation was induced, orthokinetic flocculation produced an additional 3.1 $\log_{10}$ and 4.4 $\log_{10}$ removal for *E. coli* and phage ØX174 respectively, also in presence of Fenton inhibitor TEMPOL. We conclude that ROS production is not a prerequisite for removal of *E. coli* and phage ØX174, however, it does offer an additional disinfection barrier, which increases the robustness of Fe-EC for water treatment.

5.1 Introduction

Water reclamation is a generic name for processes designed to turn municipal sewage or industrial effluents into water that can be used for a wide range of purposes, from drinking, irrigation, aquaculture, dust control and cooling, to cleaning or construction (Exall & Vassos, 2012; Saidan et al., 2020) According to FAO (Winpenny et al., 2010) the largest consumer of reclaimed water globally is agriculture, accounting for over 20 million hectares irrigated in over 50 countries, 10% of the world's croplands. Despite the obvious advantages of wastewater reclamation, such as reducing pressure on freshwater sources and avoiding pollution in receiving waterbodies, it still carries important health risks to its users, as reclaimed water may harbor a wide range of pathogenic microorganisms. These include helminth eggs, bacteria, viruses, protozoa that are associated with a high disease burden now, and (emerging) antibiotic resistant bacteria, regarded as one of the most significant threats to global health by the WHO in 2021. As a result, municipal effluent reclamation demands special attention for its associated health risks, which will vary according to the type of water reuse, susceptibility and exposure routes of its users, and concentration of pathogens in the reclaimed effluents. Therefore, the extent to which sewage requires to be treated for adequate reuse is both a function of the specific reuse intended, and the probability of its users of being exposed to it.

In this research, we explore the microbial disinfection mechanisms produced during Iron Electrocoagulation (Fe-EC) for water reclamation, as previous studies have highlighted its benefits for OMP degradation as well as nutrient, heavy metal and arsenic removal (Malinovic et al., 2016; Hamdan & El-Naas, 2014; Amrose et al., 2013; Lacasa et al., 2011; Moreno-Casillas et al., 2007; Bazrafshan et al., 2006). During Fe-EC, an electric current is conducted between two (or more) electrodes immersed in a liquid. As a consequence, the metallic Fe^0 constituting the anode will dissolve as Fe^{2+} into solution (Jiménez et al., 2012; Lakshmanan et al., 2009; Sasson et al., 2009), while on the surface of the cathode water molecules are reduced to OH^- and H_2, as indicated in equations 5-1 and 5-2.

$$Fe^0 \rightarrow Fe^{2+} + 2e^- \quad (5\text{-}1)$$

$$2H_2O + 2e^- \rightarrow 2OH^- + H_2 \quad (5\text{-}2)$$

Fe-EC is especially advantageous in off-grid applications in which coagulant supply chains and storage are difficult. It also offers notable advantages over traditional Fe-salt coagulation: the H_2 production in the cathode aids in floc removal by flotation, it does not react with alkalinity and it does not increase conductivity, since the Fe is directly dissolved into solution without the need of a counter ion in the salt. However, dosage of chemical coagulants can be performed regardless of the water conductivity and instantaneously in a single injection, while electrocoagulation requires current and time (as described by Faraday's law) and a sufficiently conductive liquid. If the process is conducted under aerobic conditions, then Fe^{2+} will further oxidize to Fe^{3+}. It is during the ferrous Fe oxidation process that a series of so-called Reactive Oxygen Species (ROS) are produced, some of which have been identified as powerful oxidizing agents with attractive disinfectant properties. According to Hug & Leupin (2003), this ROS pathway starts with the production of the superoxide radical ($^{\bullet}O_2^-$) which progresses into H_2O_2 to trigger a Fenton process, finishing with either hydroxyl radical ($^{\bullet}OH$) or high valent Fe species such as ferryl radical (Fe(IV)) depending on the pH. Under acidic conditions (pH<6), the predominant species will be the hydroxyl radical, while under more basic conditions ferryl radical will predominate. Inactivation by ROS is a consequence of severe oxidative damage to proteins, enzymes, RNA/DNA, viral capsids and phospholipid envelopes, damage and rupture of cellular membranes, or the interruption of the cellular respiratory pathways (Galeano et al., 2019; Dimapilis et al., 2018; Kim et al., 2021b; Villaseñor & Ríos, 2018). The hydroxyl and ferryl radicals are the most detrimental to microbial cells, as these lack enzymatic deactivation pathways against them (Giannakis, 2018; Vatansever et al., 2013). ROS-mediated inactivation by oxidizing Fe^{2+} has received increasing attention, particularly for virus and bacterial inactivation (Bicudo et al., 2022; Delaire et al., 2016; Heffron et al., 2019c; Jeong et al., 2006; Kim et al., 2021b; Tanneru & Chellam, 2012), although the inactivation kinetics associated to it are still not fully understood. To make the issue more complex, ROS inactivation is not the only process associated to microbial disinfection during Fe-EC, as microorganisms are to a very large extent trapped and/or adsorbed into the hydrolysis products, typically insoluble Fe(III)-hydroxides. These two processes are not just simultaneous, but also coincide spatially and target the same microorganisms, rendering the characterization of each disinfection process conceptually challenging. The objective of this research was therefore to differentiate between Fe-EC disinfection pathways, specifically ROS-induced inactivation versus floc entrapment.

To do so, Fe-EC experiments were conducted at various pH, flocculation conditions and under presence/absence of an ROS quencher using water containing the bacterium *E. coli*, and somatic coliphage virus ØX174, given their extensive use as faecal indicators in the water research field (Blount, 2015; Payment & Franco, 1993).

5.2 Materials and methods

5.2.1 Water matrix and microbial indicators

Experiments were conducted using a water matrix comprised by demineralized water, and 175 mg/l NaCl added for conductivity purposes as well as to prevent osmotic shock to the spiked microbial indicators. Non-pathogenic microbial indicators were selected to be spiked into the test water, namely *E. coli* WR1 (NCTC 13167) and somatic coliphage ØX174 (ATCC 13706-B1). Dilution buffer for all experiments was Phosphate Buffer Saline (PBS), with a buffer strength of 0.01M phosphate buffer and a pH of 7.3 ± 0.1. Propagation of *E. coli* WR1 was performed in TYGB broth (Tryptone Yeast Extract Glucose Broth) incubated at 37 ^{o}C to concentrations of ≈1×10^8 cfu/ml, centrifuged and re-suspended in PBS to concentrations of ≈$1x10^9$ cfu/ml, stored at 4 ^{o}C and used within 24 h of production. Propagation of phage ØX174 was performed as per ISO 10705-2_2000, obtaining concentrations ≈$1x10^9$ pfu/ml. For all experiments, the indicators were dosed at concentrations of ≈$1x10^6$ cfu/ml and $1x10^5$ pfu/ml respectively. Quantification of *E. coli* was performed by culture methods, either by membrane filtration or spread plating technique (depending on the expected concentration range) as described in APHA-Standard Methods for the Examination of Water and Wastewater, 23rd Edition. Chromocult® Coliform Agar (Merck) was the selected medium for *E. coli* growth and quantification of colonies. Quantification of phage ØX174 was performed by pour-plate, as per ISO 10705-2_2000.

5.2.2 Laboratory setup

Experiments were conducted in cylindrical 1 l glass beakers as depicted in Figure 5-1. The power source was a dual 30 V - 3 A TENMA 72-10500 bench DC supply, connected using crocodile clip cables to two parallel ARMCO steel plates (maximum percent- ages: 0.14% carbon, 0.10% silicium, 0.80% manganese, 0.025% phosphorous, 0.015% sulphur, 0.010% nitrogen, 0.20% copper and 0.080% aluminium). The plates were square-shaped (40 mm X 40 mm) with an thin elongation protruding parallel to one of the sides (40 mm X 5 mm) in order for it to serve as a dry contact for the clip cables outside of the water. Before each use, plates were polished with coarse and fine sand paper and rinsed with demineralized water. Plates were then fitted in parallel slots carved at the end of a PVC tube, guaranteeing they remained parallel and spaced to exactly 10 mm from each other, as described elsewhere (Anfruns-Estrada et al., 2017; Heffron, et al., 2019a; Merzouk et al., 2009; Ndjomgoue-Yossa et al., 2015). Beakers were fitted with magnetic PTFE coated bars, placed on LABNICO L23 magnetic stirrers, and stirred during the course of the electric dosage. During all experiments, air was supplied continuously using an OASE OxyMax200 air pump to maintain oxygen saturation (>8 mgO_2/l). During the experiments in which the effect of the flocculation/sedimentation was assessed, the beakers were moved into a Velp Scientifica JLT6 Jar

Test immediately after the application of the current had concluded. Settings for the jar test experiments are further described in Table 5-1.

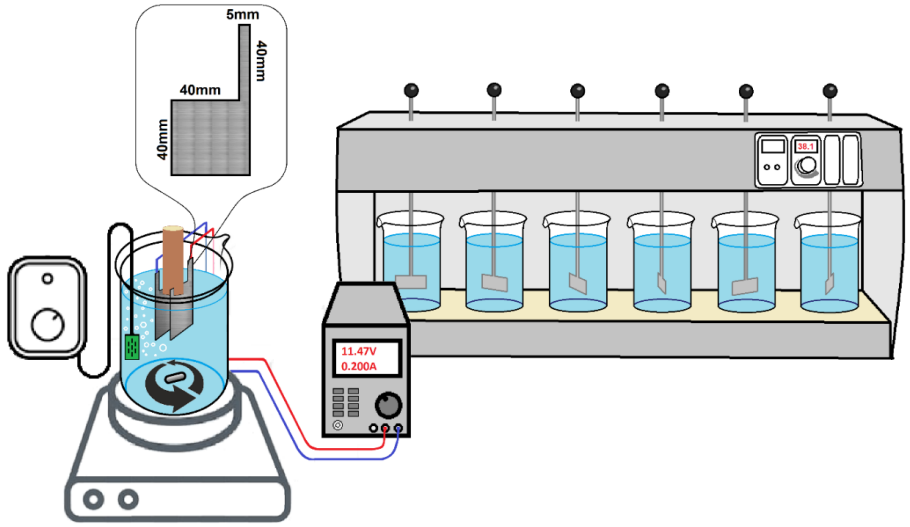


Figure 5-1 Experimental setup; Fe-EC unit with DC magnetic mixing, power supply, air supply, details of size and shape of Fe electrodes and jar test used for flocculation/sedimentation.

5.2.3 Experimental overview

For all experiments, Fe was dosed electrochemically by applying a constant 200 mA current during 15 minutes in 1 l beakers containing microbial suspensions, producing a Faradaic (theoretical) Fe dosage of approximately 52.1 mgFe/l, according to Faraday's law of electrolysis.

Experiments in which the effect of ROS in inactivation was assessed, were conducted under the presence or absence of the nitroxide 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (also known as 4-Hydroxy-TEMPO or TEMPOL), as performed by Hu et al., (2019), which was also performed under varying pH values. TEMPOL is a known Fenton inhibitor that promotes the catalysis of the superoxide radical, together with catalase-like destruction of H_2O_2, and the hindering of toxic hydroxyl radical production. It is the most studied nitroxide, due to its more affordable cost, high cell permeability and low molecular weight (Wilcox & Pearlman 2008). Beakers selected for Fenton quenching were dosed with 200 mg of reagent grade TEMPOL (Sigma-Aldrich –Germany) in crystal form, after weighing and dosing directly into the test beakers before the application of electric current. For the pH experiments, three levels of pH were selected, 5.5, 7.5 and 8.5. The stabilization of pH was conducted

before the application of current by manually dosing HCL [1 M] or NaOH [1 M], and continued during the application of current. Three samples were produced during each experiment, namely: before the application of Fe-EC, immediately after the application of Fe-EC, and after 4 h of settling.

To examine the effect of flocculation on disinfection, five different flocculation settings were tested, ranging from no flocculation nor sedimentation, to proper orthokinetic flocculation with adequate settling time, immediately after the application of the electric charge. The characteristics of each mixing/settling condition (time, intensity, mixing device) are described in Table 5-1.

The selected settings for the different mixing and sedimentation conditions were chosen to represent improving flocculation/sedimentation conditions, ranging from; i) no sedimentation (Fe-EC only), ii) Fe-EC and settling without flocculation (No Flocculation), iii) inadequate flocculation (Poor Flocculation), iv) suboptimal flocculation (Semi-orthokinetic), and v) optimal flocculation (Full orthokinetic). The latter represents standard orthokinetic flocculation conditions as recommended for municipal drinking water or chemical wastewater treatment throughout design literature (Crittenden et al., 2017; Eslamian, 2016; Shammas, 2010; Wiesner et al., 1987). For experiments without flocculation or sedimentation, (Fe-EC only) bulk liquid samples were collected immediately after the current was removed, while for the rest of the assays supernatant samples were collected after the 4 h sedimentation stage.

Samples of the mixed bulk liquid during and following Fe-EC were observed under a Keyence VHX digital microscope, in order to characterize the size and size distribution of the formed flocs. A large number of pictures (>25) was taken for each set of experiments, from which average floc area, and maximum/minimum floc diameter were obtained.

Table 5-1 Flocculation and sedimentation settings.

	Flocculation	Settling
Fe-EC only	No flocculation	**No**
No Flocculation	No flocculation	**4h**
Poor Flocculation	10 minutes @ 1000-1100 rpm with magnetic Stirrer	
Semi-orthokinetic	10 minutes @ 100rpm (G=62s^{-1}) with Jar Test	
Full orthokinetic	10 minutes @ 100rpm (G=62s^{-1}) + 10 minutes @ 50rpm (G=22s^{-1}) with Jar Test	

5.2.4 Statistical analysis

Data series of somatic coliphage and *E. coli* inactivation were analyzed with the statistical test ANOVA (analysis of variance) in order to determine if the different pH conditions (section 3.1) or flocculation conditions (section 3.2) introduced statistically significant differences. In all cases, the obtained data was comprised by triplicate microbial sampling in duplicate assays (n=6). For microbial indicators presenting varying inactivation patterns under different settings, Tukey's (honest significance) range test was used to verify significance between all possible combinations of them.

5.3 Results

5.3.1 ROS-induced inactivation

To distinguish the contribution of ROS-inactivation produced during Fe-EC from physical separation produced during flocculation/sedimentation, the ROS quencher TEMPOL was added before the current was applied. For *E. coli*, results demonstrated that Fe-EC yielded 3.9 $\log_{10}$ removal in the bulk liquid, which dropped to 0.6 $\log_{10}$ in the presence of the ROS quencher. ANOVA test confirmed that inactivation under quenched and non-quenched conditions is statistically different (p-value<0.01). Hence, quenching of ROS prevented *E. coli* removal by 3.3 $\log_{10}$. Phage ØX174 showed a very different behaviour, as removal seemed to be unaffected by the presence of the ROS quencher (ANOVA p-value >>0.05). During the Fe-EC process ≈0.8 $\log_{10}$ removal was achieved for phage ØX174, which was significantly lower than for *E. coli* (Figure 5-2).

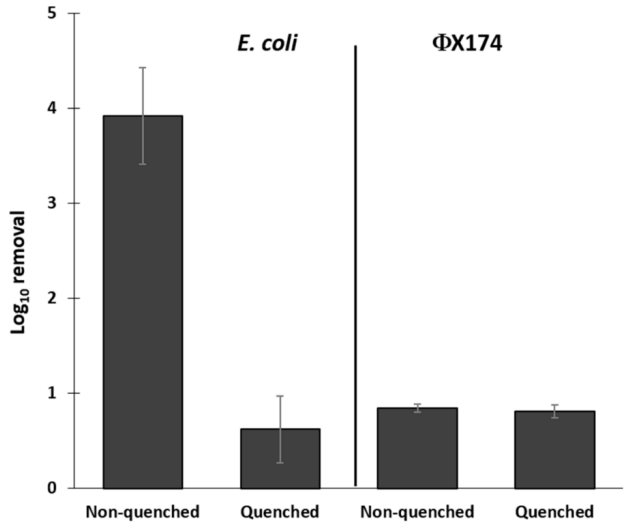


Figure 5-2 Log_{10} removal plot of *E. coli* WR1 and phage ØX174 immediately after Fe-EC (pH 7.5), in absence (non-quenched) and presence (quenched) of the Fenton-inhibitor TEMPOL. Experiments were performed in duplicate, and microbial screening was performed in triplicate. Error bars indicate standard deviation.

The solution's pH has been reported to affect the ROS speciation, specifically for the Fenton reaction products $^{\bullet}OH$ (hydroxyl radical) and Ferryl Fe (FeIV), as the former is produced under acidic conditions (pH<6) while the latter is predominant at more basic conditions (pH>6) (Hug & Leupin, 2003; Kim et al., 2011; Van Genuchten & Peña, 2017). The hydroxyl radical is regarded as a more potent inactivation agent than the high-valent Fe radical, implying that a fixed amount of oxidizing Fe^{2+} should be more detrimental to microorganisms if the oxidation is produced under more acidic conditions. In order to demonstrate this, similar quenching experiments were conducted using 3 different pH values, namely; pH 5.5 to achieve production of hydroxyl radical, pH 7.5 as in the previous experiments (similar to pH of secondary effluents), and a pH of 8.5 to promote production of high-valent Fe (FeIV). For all cases the pH was kept constant during the Fe-EC by the addition of acid or base.

For *E. coli*, results indicate a better overall removal efficiency under acidic conditions (pH 5.5), exceeding 5.1 $\log_{10}$ (Figure 5-3). For pH 7.5 and 8.5, inactivation reduced to ≈3.9 $\log_{10}$ and ≈1.2 $\log_{10}$, respectively. ANOVA and Tukey range tests point to a clear statistical difference between the three ROS inactivation plots (p-value<0.01), indicating that ROS inactivation increases strongly with decreasing pH. This pH-dependant effect on *E. coli* inactivation is in-line with the reactivity of the produced Fenton intermediates, illustrating that the main driver in these experiments for *E. coli* attenuation was ROS-mediated inactivation. It is worth noting that for pH 5.5, even though 5.1 $\log_{10}$ inactivation was observed, Fe oxidation was incomplete as 44%-48% of Fe^{2+} still remained in solution. This means that in a scenario in which Fe^{2+} is fully oxidized into Fe^{3+}, *E. coli* ROS-inactivation is expected to be even higher, as larger amounts of ROS would be produced. This however was not achieved during the timespan of the experiments due to the low Fe^{2+} oxidation rate under acidic conditions.

For phage ØX174, results showed that the disinfection fraction attributable to ROS-mediated inactivation was significantly larger (≈1.5 $\log_{10}$) for pH 5.5, than for pH 7.5 or 8.5. This could indicate a slightly larger sensitivity of phage ØX174 to the more aggressive hydroxyl radical, whose presence is expected at pH 5.5, but negligible at pH>6. ANOVA test identified a statistical difference between the ROS inactivation plots (p-value<0.05), while the Tukey range test isolated such difference between the pH 5.5 data set and the remaining two datasets (pH 7.5 and 8.5), indicating that ROS inactivation is statistically similar for pH 7.5 and 8.5, but increases significantly under acidic conditions.

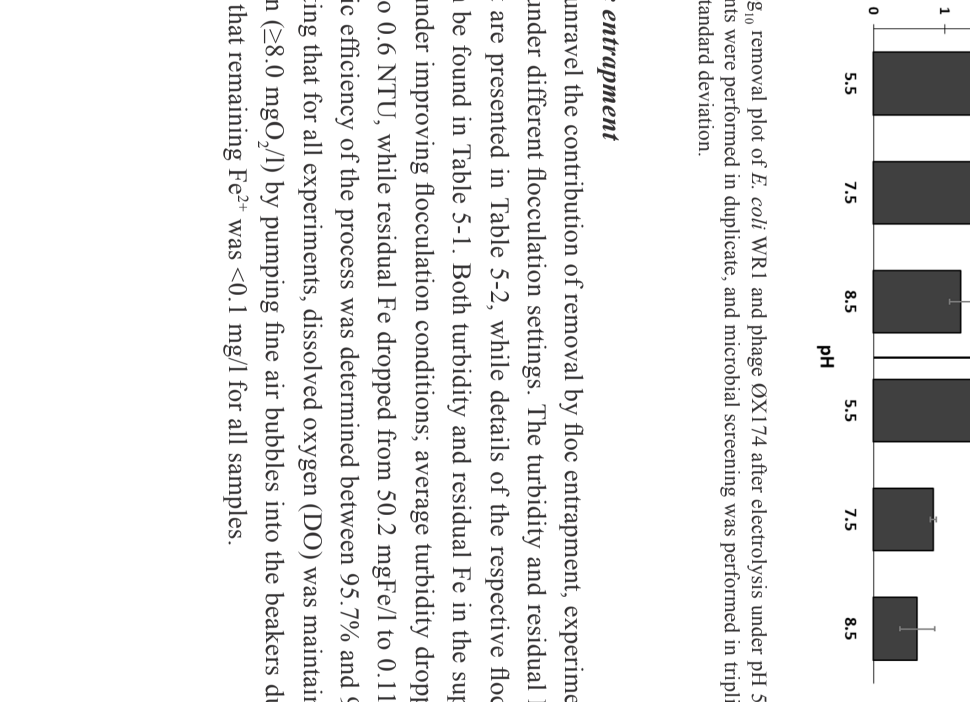


Figure 5-3 Log_{10} removal plot of *E. coli* WR1 and phage ØX174 after electrolysis under pH 5.5, 7.5 and 8.5. Experiments were performed in duplicate, and microbial screening was performed in triplicate. Error bars indicate standard deviation.

5.3.2 Floc entrapment

In order to unravel the contribution of removal by floc entrapment, experiments were conducted under different flocculation settings. The turbidity and residual Fe in the supernatant are presented in Table 5-2, while details of the respective flocculation settings can be found in Table 5-1. Both turbidity and residual Fe in the supernatant decreased under improving flocculation conditions; average turbidity dropped from 68.9 NTU to 0.6 NTU, while residual Fe dropped from 50.2 mgFe/l to 0.11 mgFe/l. The Faradaic efficiency of the process was determined between 95.7% and 96.5%. It is worth noting that for all experiments, dissolved oxygen (DO) was maintained close to saturation (≥8.0 mgO_2/l) by pumping fine air bubbles into the beakers during the Fe-EC, and that remaining Fe^{2+} was <0.1 mg/l for all samples.

Table 5-2 Turbidity and residual Fe concentrations for the five assayed flocculation conditions.

	Fe-EC only	No Flocculation	Poor Flocculation	Semi-orthokinetic	Full orthokinetic
					
Turbidity after Fe-EC (NTU)	69.7	66.3	67.9	70.4	70.1
Avg. turbidity after 4 h settling (NTU)	N/A	6.6	2.8	0.9	0.6
Avg. dosed total Fe (actual) (mg/l)	50.3	50.3	50.1	49.9	50.2
Supernatant total Fe - 4 h setling (mg/l)	N/A	4.80	1.80	0.25	0.11
Fe removal by settling (%)	N/A	90.4%	96.4%	99.5%	99.8%
E. coli removal (log_{10})	3.9	4.2	4.6	5.6	7.0
ØX174 removal (log_{10})	0.8	0.8	0.9	3.5	5.2

The control experiment with Fe-EC only, showed a concentration decrease of 3.9 log_{10} for *E. coli* and 0.8 log_{10} for phage ØX174, with all the dosed Fe still being in suspension at the moment of sampling (Table 5-2). Subsequent experiments conducted under identical Fe-EC were subjected to progressively improving flocculation conditions (all including 4 hour sedimentation), in which decreasing turbidity and residual iron were observed, simultaneously with increasing microbial removal in the supernatant. Remaining Fe particles in the supernatant were typically amorphous $Fe(OH)_3$ microscopic flocs of 7-12 µm average diameter (digital microscope observations) which could be fully removed by 0.45 µm membrane filtration. The best results in terms of Fe, turbidity and microbial removal from the supernatant were obtained for Full orthokinetic flocculation conditions. It must be noted however, that when compared to Semi-orthokinetic conditions, Full orthokinetic conditions offered just a marginal improvement in terms of Fe and turbidity removal, yet a considerable improvement in microbial removal. This demonstrates that removing approx. 0.3% additional Fe (from 0.25 mg/l to 0.11 mg/l), though apparently insignificant, adds a valuable 1.4 log_{10} removal for *E. coli* and 1.5log_{10} removal for phage ØX174.

For all assayed configurations, *E. coli* appeared to be inactivated during the Fe-EC stage (3.9 log_{10}), after which additional inactivation was a consequence of improving floc removal, thus suggesting that the surviving bacterial cells were attached to the flocs. ROS-inactivation of phage ØX174 was poor for the first three configurations (0.8-0.9 log_{10}), rapidly increasing as flocculation acquired orthokinetic characteristics, also suggesting that incremental disinfection was a consequence of improving floc removal, and that the surviving viable viruses were also attached to the flocs.

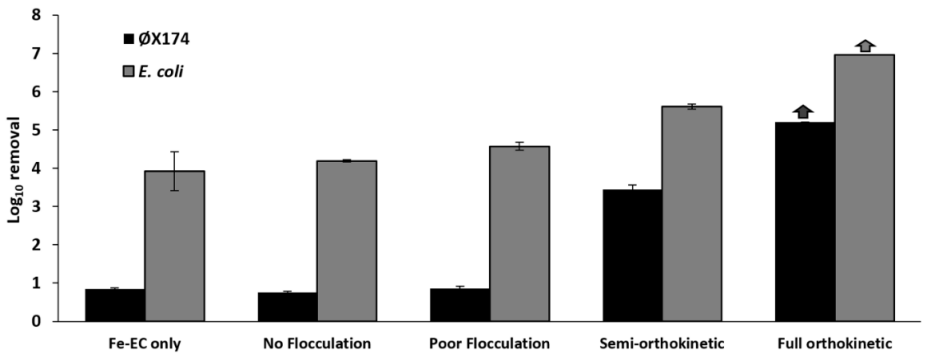


Figure 5-4 Log_{10} removal plot of phage ØX174 and *E. coli* for each flocculation configuration/removed Fe (%), following Fe-EC. Note: Bars marked with an arrow (↑) indicate a minimum estimated removal due to microbial concentration below detection limit. Experiments were performed in duplicate, and microbial screening was performed in triplicate. Error bars indicate standard deviation.

5.3.3 Coupling of mechanisms

The cumulative log_{10} removal after Fe-EC electrolysis and after sedimentation, either in absence or presence of the ROS quencher TEMPOL is displayed in Figure 5-5. For these experiments, the full orthokinetic flocculation conditions were applied, with 4 h of settling time. For *E. coli*, cumulative log_{10} removal under quenched conditions is on average lower than that obtained for non-quenched conditions, although such discrepancy was statistically insignificant (ANOVA p-value >>0.05). For phage ØX174, quenched and non-quenched data was virtually identical, with no statistical differences (ANOVA p-value >>0.05). Figure 5-5 suggests that *E. coli* are likely to be partially inactivated prior or during entrapment (by Fenton intermediates) leading to a mix of viable and non-viable *E. coli* in the Fe flocs, whereas ØX174 coliphages are predominantly removed by sedimentation with a significant portion of them being still viable.

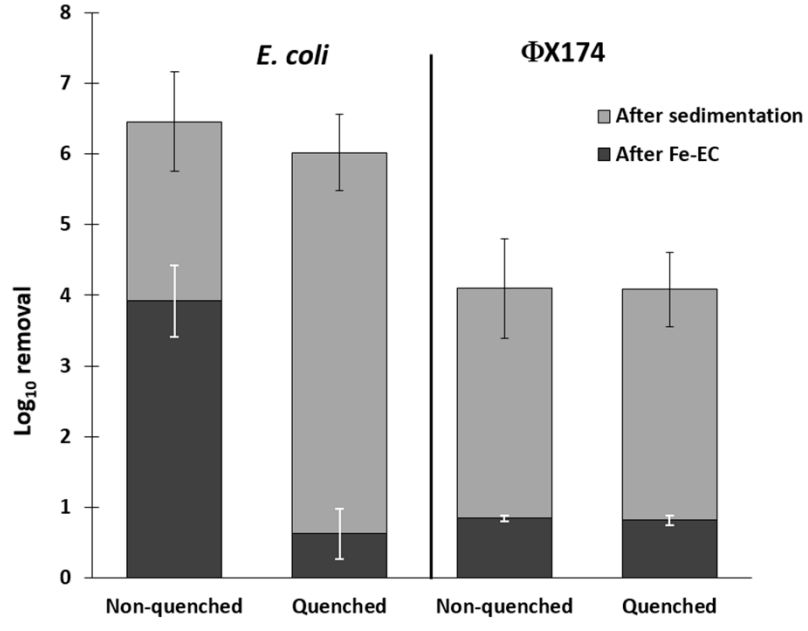


Figure 5-5 Log_{10} removal plot of *E. coli* WR1 and phage ØX174 after Fe-EC, with and without the presence of the Fenton-inhibitor TEMPOL after Fe-EC and after 4h settling, indicating the independent contributions of ROS inactivation and floc sedimentation. Experiments were performed in duplicate, and microbial screening was performed in triplicate. Error bars indicate standard deviation.

5.4 Discussion

The presented results have shown that Fe floc removal was positively correlated to decreasing microbial concentrations. This indicates that both microbial indicators show affinity to be better incorporated into the floc structures and/or adsorbed onto their surface as flocculation/sedimentation conditions improve (Delaire et al., 2016; Heffron et al., 2019a). *E. coli* removal increased gradually from 3.9 $\log_{10}$ to >7.0 $\log_{10}$ (below detection). For phage ØX174, a sharp improvement in removal reaching 3.5 $\log_{10}$ was observed after introducing semi-orthokinetic conditions, further increasing to >5.2 $\log_{10}$ (below detection) under fully orthokinetic conditions. This type of flocculation is known to produce larger and more compact flocs with better settling ability (Crittenden et al., 2017; Shammas, 2010; Wiesner et al., 1987), meaning that floc sedimentation and microbial removal are faster. Under these orthokinetic conditions, it was found that *E. coli* inactivation was a result of ROS produced during Fe^{2+} oxidation, given an effective Fe-dose of approximately 50 mgFe/l. ROS inactivation appears primarily linked to the production of the hydroxyl radical (•OH) and to a lower extent to the ferryl radical (FeIV). This is concluded from the observation that under acidic conditions disinfection was orders of magnitude more efficient, which is theoretically in agreement with •OH formation (Nieto-Juarez & Kohn, 2013; Hug & Leupin, 2003).

ROS production is a direct consequence of ferrous Fe oxidation (Hug & Leupin, 2003; Kim et al., 2011; Van Genuchten & Peña, 2017), which co-exists spatially and temporarily with the floc entrapment process. As presented in Figure 5-3, at a pH of 7.5 these ROS are highly detrimental to the bacterial indicator *E. coli* (3.9 $\log_{10}$), but dropped to 0.6 $\log_{10}$ when ROS were suppressed by the Fenton inhibitor. Conversely, this same ROS disinfection mechanism does not appear to affect the virus indicator ØX174 at circumneutral pH, as there is no difference in removal under quenched or non-quenched conditions, due to a reduced sensitivity of ØX174 towards the Fe(IV) radical, or to a removal process which is either ROS- independent (e.g., inactivation by Fe^{2+} or by the induced electric current) or mediated by other ROS species unable to be quenched by TEMPOL (Bicudo et al., 2022). Under acidic conditions (pH 5.5) a marginal, yet statistically significant improvement in phage ØX174 inactivation was observed, evidencing a mild sensitivity to •OH radicals. However, other researchers have suggested that this could be a simple consequence of better virus sorption onto the Fe-hydroxides at lower pH, promoting aggregation and subsequent decrease in plaque counts (Nieto-Juarez & Kohn, 2013), and not necessarily the effect of more toxic radicals. Regardless, reducing pH appears to be a poor means of enhancing virus disinfection during Fe-EC, while improvement of flocculation conditions is the major determinant in virus removal, mainly by enhancing irreversible floc-adsorption and separation, as has also been reported elsewhere (Kim et al., 2021b; Heffron et al., 2019; Nieto-Juarez & Kohn, 2013)

Although inactivation by ROS is clearly an interesting pathway for disinfection during Fe-EC, the overall disinfection efficiency of either indicator was statistically the same for quenched and un-quenched conditions. This could cast doubt on the real advantage of using reduced Fe forms to conduct coagulation, as opposed to using more conventional Fe-based coagulants such as $FeCl_3$ for which disinfection by sorption/sedimentation has been reported (Payment & Armon, 1989). However, it is imperative to consider that ROS production offers an additional disinfection barrier, particularly for *E. coli*, which could be attractive for water reclamation or drinking water treatment, as was also suggested by Durán Moreno et al., 2003. Municipal effluent water treatment using ferrous-based oxidation has been shown to produce sludge with lower microbial loads (Ramírez Zamora et al., 2002), which is advantageous for its handling and prospective agriculture reuse applications.

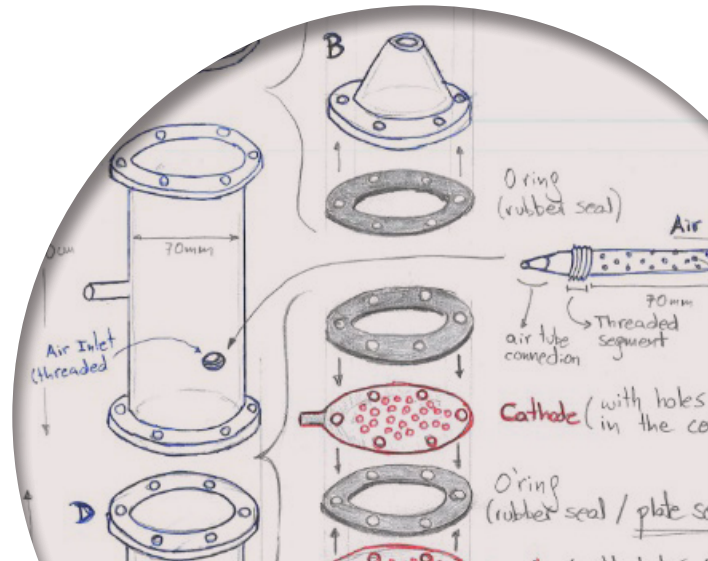
5.5 Conclusions

It may be concluded that during Fe-EC, floc entrapment demonstrated to be an effective microbial removal process, strongly depending on flocculation conditions, being notably enhanced by proper orthokinetic conditions. Particularly for phage ØX174, which was found to be hardly sensitive to ROS, removal patterns closely followed that of Fe removal. For *E. coli*, disinfection by ROS was found to reach 3.9 $\log_{10}$, whereas subsequent floc entrapment during sedimentation added another 3.1 $\log_{10}$. With 7.0 $\log_{10}$ *E. coli* removal, Fe-EC may be considered an effective disinfection technology. pH was found to largely influence *E. coli* inactivation by ROS, with 5.1 $\log_{10}$, 3.9 $\log_{10}$ and 1.2 $\log_{10}$ for pH 5.5, 7.5 and 8.5, respectively. This indicates that hydroxyl radicals are more effective in inactivating *E. coli* than ferryl radicals. However, the overall necessity of ROS to achieve this log removal may however be debated, as experiments where ROS was quenched by TEMPOL showed no significant difference. As such, inactivation of *E. coli* by ROS during Fe^{2+} oxidation may be considered of value for microbiologically safer sludge, as well as an additional double barrier for disinfection.

6

CHAPTER 6

Outlook



6.1 Culture based tracking of ARB and ASB in wastewater treatment

Antibiotic resistance usually makes the headlines in relation to deadly hospital infections or zoonotic diseases that threaten our livestock, but ARB are literally all around us, and that includes our own digestive tract. Hence, many concerning ARB strains can be found in our faeces, our sewage, and in our surface waters: researchers like Blaak et al., 2015, Huijbers et al., 2020 and Varela et al., 2013 directly correlated specific *ESBL-E. coli* and VRE strains from WWTPs and hospitals to those found in surface waters, while Schmiege et al., 2021 and Paulshus et al., 2019 further suggested that the general community is the main *ESBL-E. coli* reservoir. In this thesis it was found that the studied ARB (ESBL-*E. coli* and VRE) are present in sewage all year round and in very stable concentrations. Furthermore, the ratio between them and their sensitive analogues (*E. coli* and enterococci) also remained relatively stable throughout the full extent of both sampling campaigns (June 2018 to December 2018 and November 2020 to March 2021). Interestingly, in the non-disinfected effluent of the studied AS-WWTP, the ratio of *ESBL-E. coli* to *E. coli* and VRE to enterococci was similar to the ratio in the influent, indicating no selective advantages or disadvantages of the resistant strains over the sensitive strains. Moreover, this similar behaviour of resistant and sensitive strains was also observed in disinfection processes such as chlorination, Fe-EC and spontaneous decay. A recent review by Zaatout et al., 2021, determined that this ratio between ESBL-*E. coli* and *E. coli* is actually increasing over the years, and that the highest ESBL-*E. coli* loads originate from hospitals. These conclusions might not be applicable to all ARB strains, as a recent study by Zhi et al., 2020 on urinary-tract resistant ESBL-*E. coli* strains showed high adaptability of the resistant strain to survive wastewater treatment operations, particularly disinfection, increasing the ratio of resistant to sensitive *E. coli*.

The implications of these results derived from this dissertation are various, the most evident being that sensitive and resistant strains of bacterial species appear to behave in an identical manner in wastewater and during wastewater treatment in terms of removal by activated sludge treatment, in line with the observations from Sorgen et al., 2021. Oxidation-based disinfection processes such as chlorination and electrocoagulation also showed no differences in inactivation between sensitive and resistant populations, conclusion also reached by Giannakis et al., 2018 when asserting that "*antibiotic resistance does not imply resistance to oxidative treatment*". This statement also appears true for spontaneous decay, as the die-off patterns between sensitive and resistant *E. coli* and Enterococci were virtually indistinguishable from one another. This implies that sensitive *E. coli* and Enterococci, are good proxies for estimating the presence and concentration of ESBL- *E. coli* and VRE in sewage and effluents (disinfected and non-disinfected alike) once the usual ratios between

them has been established. These results were obtained from the largest WWTP in the Netherlands, and its findings agree with a previous and broader countrywide study in the Netherlands using similar ARB indicators (Schmitt et al., 2017). It is worth noting that most drinking water, wastewater, surface water and reuse water quality standards and regulatory bodies around the world already rely on either or both of these FIB for assessing the microbial safety of water, and our findings indicate that safeguarding water systems on the basis of the traditional FIB also offers protection against the ESBL-*E. coli* and VRE. No drinking, wastewater, reuse or bathing water quality standard in existence is currently including numerical standards for ARB or ARG, partially because of the difficulties and lack of consensus on what a suitable indicator could be. Current state of the art AR screening in research is based almost exclusively on molecular methods (qPCR, metagenomics, etc.), which are in many cases out of reach for water utilities and regulatory bodies, particularly in the global south, due to lack of resources, equipment and especially of trained personnel. The advantage of the culture methods that were applied in this study is that it allows for viable resistant bacteria to be enumerated, while qPCR methods would be less suitable to evaluate the efficacy of disinfection and decay processes, as well as human and animal exposure to ARB via the environment. On the other hand, genomic methods are better suited to evaluate the fate of ARG in water treatment and the environment. This dichotomy between methods has not passed unnoticed by the research community, and has been addressed by Berendonk et al., 2015, Manaia, 2017, Pruden et al., 2021, Leonard et al., 2022, and by Liguori et al., 2022, who provides a detailed framework with recommendations for standardized AR monitoring in municipal and surface waters, combining culture and molecular methods based on specific objectives of the monitoring programme and on the type of sampling locations.

This thesis proposes that the current evaluation approach for water safety and treatment efficacy measurement of water systems through the use of FIB is also protective against ESBL-*E. coli* and VRE, and possibly against other ARB present in our wastewater streams. It also shows that under conditions in which the ratio between ARB and ASB is known and stable, FIB can be used to infer presence and concentration of certain indicator ARB in any water stream impacted by municipal effluents, without the need of resorting to complex methodologies demanding the use of molecular methods. It must be noted that literature on culture methods for ARB prediction from FIB is indeed scarce, yet the approach has already yielded promising results, such as the study of Van Heijnsbergen et al., 2022 who developed and validated a model predicting ESBL-*E. coli* concentrations in bathing waters based on *E. coli* measurements. It is then recommended that future research on ARB and wastewater treatment (globally) looks into incorporating this ASB/ARB-ratio dimension in

sewage and surface water AR-research, in order to contribute to the normalization and standardization of ARB/ARG monitoring. This environmental data should be centralized and accessible, in similar manner to that of clinical-ARB surveillance initiatives such as the European Antimicrobial Resistance Surveillance Network (EARS-Net) or the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) in USA. This should be a valuable input for policymakers to determine the optimal AR monitoring strategies and guidelines, and eventually design tailor-made risk assessment frameworks.

6.2 Disinfection by Fe-EC: the double barrier

Disinfection by Fe-EC is the antagonistic result of two independent processes, namely floc-sorption/sedimentation, and inactivation by Fenton-type reactive species, also known as ROS. While the former is essentially a function of flocculation conditions (mixing velocity gradient and sedimentation time), the latter is a function of aerobic oxidation of dosed Fe^{2+}. The aerobic oxidation of the Fe^{2+} released via Fe-EC produces a cascade of ROS that commences with the Superoxide radical, and is subsequently followed by H_2O_2 and finally Ferryl iron (Fe (IV)) or Hydroxyl radical based on the bulk's pH. The reaction between the formed H_2O_2 and released Fe^{2+}, typically known as the Fenton reaction, is receiving increasing attention through processes like Photo-Fenton, Electro-Fenton and Sono-Fenton due to its remarkable capacity for organic matter degradation and disinfection (O'Dowd & Pillai, 2020; Qi et al., 2020; Robles et al., 2020; Xu et al., 2020). Because these reactions also take places during Fe-EC, the process can simultaneously be regarded as a physical/chemical treatment and an Advanced Oxidation Process (AOP).

During Fe-EC, microorganisms can be separated from the water by physical sorption/entrapment in the coagulation products (flocs), and can also be inactivated by the Fenton-type ROS, processes normally occurring simultaneously and in the same physical volume. Disinfection results from the overlapping action of these two processes, and discerning their independent effect can be complex, as inactivated microbes can be entrapped in the flocs while entrapped viable microbes can simultaneously be inactivated by the ROS. Quenching of ROS proved a suitable strategy for quantifying the contribution of inactivation, achieved by exposing microbial suspensions to Fe-EC in either the presence or absence of these ROS-quenchers. The differences in microbial concentrations between quenched and unquenched experiments after Fe-EC was attributed to the inactivation by ROS (Hu et al., 2019), though it must be noted that a careful selection of the quencher is critical: it must not exhibit disinfectant properties, it must be cell-permeable, and it must not complex with or hamper the oxidation of the released Fe^{2+}. Although TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl)

meets these criteria in full, several other compounds of the nitroxide family could also be regarded as suitable quenchers for Fe-EC and other Fenton-type applications.

ROS-inactivation of bacterial indicator *E. coli* could be described following Chick-Watson kinetics, in an expression in which the applied current intensity is used as the disinfectant concentration, providing highly accurate estimations for disinfection. *E. coli* inactivation under the presence of the Fenton quencher was in all experiments virtually negligible, demonstrating its high sensitivity to ROS radicals. Conversely, inactivation of phage ØX174 was only partially responsive to ROS, as disinfection under the presence of the Fenton quencher was reduced, but still significant. Inactivation kinetics could be correlated with a Chick model with time as the only variable, thus indicating that neither the iron dosage nor Fe^{2+} oxidation rate were fully behind ØX174 inactivation: exposure to either electric field or directly to Fe^{2+} have also been proposed as contributors for ØX174 and MS2 phage inactivation by Heffron et al., 2019; Jeong et al., 2006 and Kim et al., 2011. The dependency of ROS inactivation with pH was included in the research as it was suggested by Hug & Leupin, 2003; Kim et al., 2011 and Van Genuchten & Peña, 2017b that more aggressive species such as the hydroxyl radical ($^{\bullet}OH$) were formed at acidic conditions (pH<6) and less aggressive forms such as Ferryl iron (Fe(IV)) were formed at basic conditions (pH>6). Experimental results clearly demonstrate that inactivation of *E. coli* and phage ØX174 are higher under acidic conditions (pH 5.5) than under basic conditions (pH 7.5 and 8.5). However, the intentional decrease of pH for disinfection purposes is seldom used in real municipal wastewater treatment applications, and it is also not recommended for Fe-EC in pursuit of more aggressive radical formation: over-acidification to pH levels below 5 can greatly reduce the Fe^{2+} oxidation rate, effectively hampering ROS production and halting inactivation. The results obtained in this dissertation should not be interpreted as that effluent acidification is advisable for Fe-EC disinfection, but rather to indicate that Fe-EC is an advantageous disinfection technology when effluents are of a slightly acidic nature (pH 6-7). Instead of trying to control pH, Fe-EC should best used for disinfection purposes in combination with H_2O_2, arrangement that increases the ratio between produced ROS and dosed Fe, yet without influencing the type of ROS produced.

Flocculation, the second Fe-EC disinfection component discussed in this dissertation, bears a great influence of microbial sorption/entrapment and later sedimentation, mainly by improving the settling ability of the microbe-laden flocs formed during the Fe-EC process. Results indicate that performing Fe-EC without the incorporation of a proper flocculation stage yields microscopic flocs (10-20 µm diameter) which require impractically long times to settle (>12h), which prevents the removal of the leftover

viable microorganisms by sedimentation. The addition of a proper flocculation stage using conventional orthokinetic settings (e.g. by the use of a Jar Test), resulted in greatly reduced sedimentation times (<4h), reduced residual Fe and turbidity in the supernatant, and reduced microbial concentrations, hence significantly improving the water quality for prospective reuse applications. This unique combination of microbial entrapment in flocs, simultaneous with ROS release by ferrous iron aerobic oxidation provides a double protection barrier with major implications from a water system safety perspective, as disinfection will still happen even if flocculation is performed sub optimally, or fails to be performed at all. It is highly advisable for full scale Fe-EC municipal applications to include a flocculation process either during or immediately following the application of electric current, as this greatly enhances sedimentation with a subsequent decrease of microbial and solid content in the treated effluent, and increase in quality. **Fe-EC offers a high potential for adoption in the municipal sector, not only for wastewater treatment but also for drinking water production. Although nutrient removal with Fe-EC has been extensively reported, further research is due on the study of the disinfection kinetics in real surface water and wastewaters together with the removal/degradation of OMP, both of which are essential for prospective Fe-EC water reuse schemes. Future research on these lines should also look at ways to reduce the Fe-dosage and/or enhance the ROS production, for example by dosing H_2O_2 as a Fenton-catalyst.**

The Fe-EC experiments described in this dissertation make use of real municipal effluents for general performance and disinfection evaluation (Chapters 2-3) and synthetic effluents for the mechanistic description of the ROS inactivation processes and flocculation optimization (Chapters 4-5). It is worth noting that the composition of the water matrix has a large impact on the disinfection capacity of Fe-EC, as results clearly show orders of magnitude in the differences in disinfection between real and synthetic water types for equal Fe dosages. Real municipal effluents are laden with a large number of compounds that interfere with Fe^{2+} oxidation by scavenging, such as phosphates, carbonates and NOM. Synthetic water formulations usually overlook the complexities of real municipal effluents and offer lower scavenger strength, permitting for the unobstructed formation of flocs and ROS by Fe^{2+} oxidation, mainly to simplify the process and allow for an easier mechanistic description of it. The study of the Fe-EC disinfection processes and of the interactions between Fe and its scavengers is too complex to be performed in real wastewater, as its composition changes daily, and due to large number of confounding variables present in it (dissolved and suspended compounds of very diverse nature). The fact that in this dissertation several core results were derived from synthetic water matrixes does not disqualify them from being applied in real effluents: they provide a conceptual framework and qualitative

mechanistic description, that requires calibration for each particular effluent quality. Still, synthetic water matrixes should be avoided when estimating disinfection capacity of a given Fe-EC setting, as these tend to overestimate disinfection and underestimate costs. **Hence, it is critical to consider the advantages and limitations of real and synthetic water matrixes during the study of Fe-EC, as extrapolations can lead to oversimplification of results and overestimation of its qualities.**

6.3 More than a disinfection process: Fe-EC

Disinfection with Fe-EC results from a combination of physical removal and inactivation by radicals (double barrier), being consistently effective against a broad range of microorganisms including bacteria, viruses, protozoan indicators and ARB, and can achieve disinfection comparable to conventional methods such as chlorine, ozone, or UV-radiation, but with the advantage of the double barrier as was demonstrated in this thesis. It is also comparable from a cost perspective and has no associated DBPs, making it a green and attractive disinfection technology. Additionally, it features several advantages that conventional disinfection methods cannot deliver, including an almost complete removal of phosphorous (Inan & Alaydin, 2014; Lacasa et al., 2011; Zaleschi et al., 2013), turbidity (Cotillas et al., 2014; Espinoza-Quiñones et al., 2009), effective adsorption of arsenic and toxic metals (Amrose et al., 2013; Delaire et al., 2015; Espinoza-Quiñones et al., 2009; Van Genuchten & Peña, 2017a), and ROS-mediated degradation of organic matter (Moreno-Casillas et al., 2007; Zaroual et al., 2006), OMPs and other recalcitrant compounds (Maher et al., 2019; X. Xu & Zhu, 2004; Yoosefian et al., 2017; Zodi et al., 2011). Hence, more than a disinfection process, Fe-EC should be regarded as a robust all-round water reclamation technology, with numerous potential applications, particularly in the municipal sector. It is worth noting however, that many of these Fe-EC advantages achieved by the production of ROS do not derive from the use of electric current to produce Fe^{2+}, but rather from the use of Fe^{2+} itself, meaning that these observations can also be extrapolated to chemical coagulation provided that ferrous salts are used (i.e. $FeCl_2$, $FeSO_4$) instead of the more conventional ferric salts (i.e. $FeCl_3$ or $Fe_2(SO_4)_3$). ROS-mediated *E. coli* and phage ØX174 inactivation for example, was shown to be identical following the oxidation of Fe^{2+} either electrochemically dosed with Fe-EC or chemically dosed with $FeCl_2$ (Chapter 4), both in the presence and absence of the ROS-quencher TEMPOL, further supporting this statement.

Although the application of Fe-EC can appear impractical from an operational perspective, it offers specific advantages unmatched by chemical coagulation: It can be operated off-grid with the use of solar panels and without relying on a continuous supply of metallic salts, which can be an advantage in remote and isolated locations.

Iron plates do not expire nor degrade when wet, and require little storage space. No operator nor dosing pumps are required for the preparation and injection of the coagulants as these are produced on site, dosage can be reliably correlated to the current intensity, and the process can be easily automated. Fe-EC also produces less sludge than chemical coagulation methods, demanding lower sludge dewatering and disposal costs. Additionally, Fe-EC does not increase conductivity as metallic salts do, this being a critical element for reuse applications in agriculture and for drinking water production. **Overlooked for decades due to higher electricity costs, Fe-EC is a very promising, all-round technology for municipal effluent reclamation, acting not only on microbial contaminants but also on other parameters relevant for reuse, such as solid content, nutrients, and organic load, including that of OMPs and other recalcitrant organic compounds.** Upgrading the technology from a batch-type laboratory setup into continuous-flow units remains a significant research gap that requires further attention before upscaling to municipal level can be attempted.

6.4 Upscaling Fe-EC

The data included in this dissertation regarding Fe-EC was obtained exclusively from batch experiments, with understandably reduced capacity for producing large volumes of treated effluents. Upscaling the process, meaning operation in continuous flow mode, is a necessary step towards upgrading the technology readiness level of Fe-EC for municipal reuse applications. The findings described in this dissertation have been the basis for the design and construction of a continuous flow unit (10 l/h), currently being tested in TU Delft laboratories, for OMP, heavy metal, and arsenic removal as well as for disinfection in real municipal secondary effluents (Figure 6-1).

Promoting the removal of flocs by flotation with the cathode-released H_2, enhancing flocculation conditions within the unit, and optimization of the electrode design to facilitates the air passage remain the most challenging aspects of the upgrade to continuous flow operation, and recommended focus points for future research on the process.

By the time of this dissertation's drafting, preliminary Fe-EC experiments in the continuous flow unit using real 2ry effluents complemented with heavy metals (Arsenic (V), Copper, Zinc, Chromium and Vanadium), showed very promising results, ranging from ≈55% for Copper, to >99% for Arsenic and Chromium (Figure 6-2). Similar experiments on OMP removal are currently underway.

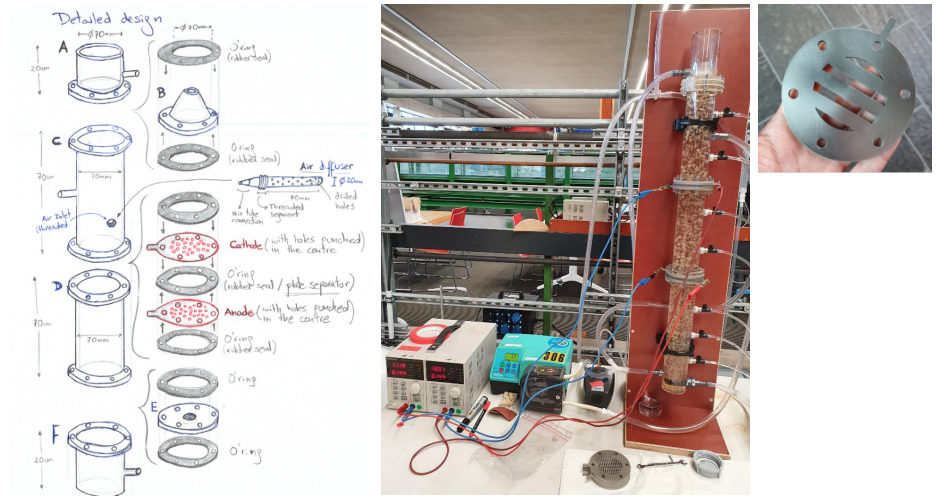


Figure 6-1: Initial sketch of the continuous flow Fe-EC unit-October 2021 (left), and assembled Fe-EC continuous flow unit –March 2022 with detail of the flange-type electrodes (right).

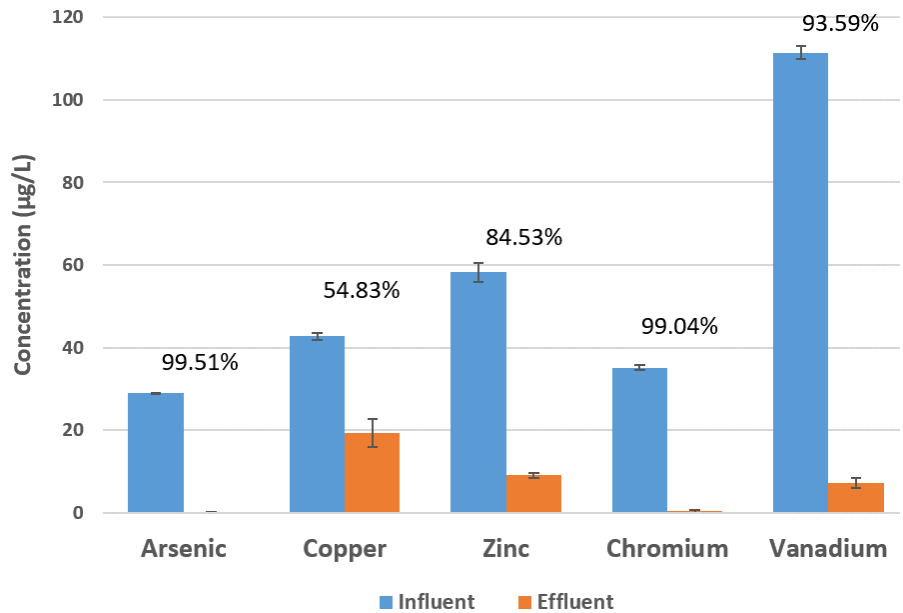


Figure 6-2 Preliminary data on heavy metal removal percentages using continuous flow Fe-EC on 2ry effluents complemented with Arsenic (V), Copper, Zinc, Chromium and Vanadium. Experiments were performed in duplicate, and samples were analysed in triplicates. Error bars represent standard deviation.

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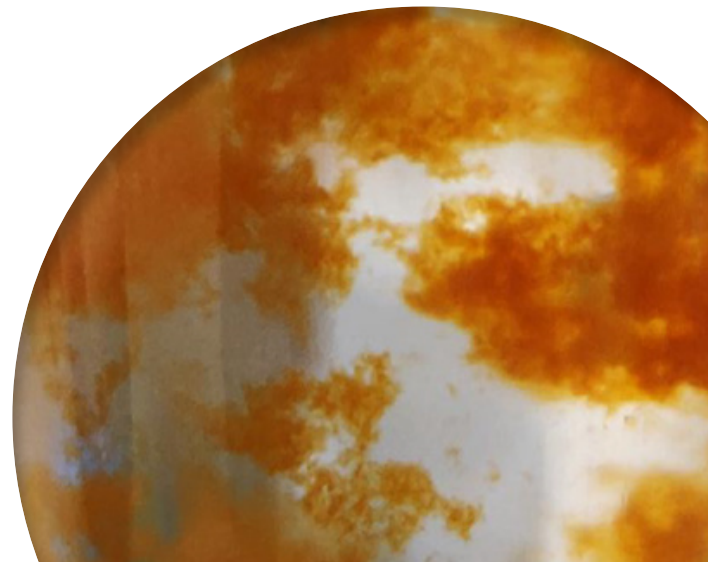

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Curriculum Vitae

Bruno Bicudo Pérez was born on November 13th 1985 in Montevideo, Uruguay. In 2004, he began with the bachelor in Civil Engineering in the Engineering Faculty of the Uruguayan's Republic University. There he developed an interest for water treatment, majoring in Environmental Hydraulics in 2010. From his graduation onwards, Bruno worked in the private sector in Uruguay and later in drinking water treatment projects in Angola. In 2013, he received a full scholarship in UNESCO-IHE (Delft, The Netherlands) to pursue a MSc. in Sanitary Engineering. His thesis on growing Anammox in a MBR for main line wastewater denitrification earned him a Cum Laude.

Back in Uruguay, he continued working in the private sector on design of municipal drinking water treatment plants, before moving to Peru in 2016 to work in rural water and sanitation projects throughout the country. In 2018, he started his PhD in the Delft University of Technology, Sanitary Engineering Section, under the supervision of Prof. dr. Gertjan Medema and Prof. dr. Doris van Halem. As a part of the larger LOTUS^{HR} programme, his PhD focused on municipal effluent reclamation with non-conventional technologies, specifically aiming at microbiological quality and removal of antibiotic resistant organisms. Iron Electrocoagulation (Fe-EC) was his technology of choice, which he used for 4 years to elucidate microbial removal and disinfection, inactivation kinetics, and behaviour of antibiotic resistant organisms.

Bruno currently works as a WASH officer for the International Organization for Migration (IOM) in Ukraine, assisting water utilities whose infrastructure was damaged during the ongoing Russo-Ukrainian war.

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