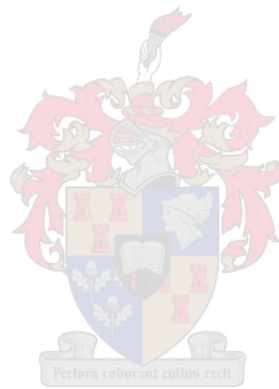# Comparative study for the transformation of Emerging Contaminants and Endocrine Disrupting Compounds: Electrochemical Oxidation and Biological Metabolism

by

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Master of Science
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Declaration:

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March 2020

Abstract:

As little as 1% of all water sources is fresh water accessible for use and it is increasingly be polluted by anthropogenic materials such as solid and chemical waste. Previous studies have shown that various organic micropollutants are not effectively removed by conventional water treatment processes and persist in natural water sources. The primary aim of this study was not to detect and monitor micropollutant distribution but rather to investigate two degradation processes, electrochemical oxidation and microbial degradation, as well as the resulting transformation products. It was hypothesized that microbial degradation will produce less toxic transformation products than the harsh process of chemical oxidation. Two micropollutants, sulfamethoxazole and carbamazepine, were chosen based on their widespread detected and persistence in the environment. The CabECO technology harnesses the process of electrochemical oxidation to produce ozone in the aqueous phase for water treatment and produced >2 mg/L of ozone at the suggested operating parameters. Ozonation of environmental water sources showed some success in reducing the microbial load, however, several orders of magnitude of microbes remained after treatment, especially in samples with high COD. It also proved effective in the abatement of SMX and CBZ, reducing the micropollutant concentration to below detection limits within 1 min. However, the endocrine disrupting effect of the compounds required up to 4 hours of exposure time to ozone to eliminate the estrogenic/anti-estrogenic activity. Although effective for SMX and CBZ, the CabECO technology is less effective against a broad suite of micropollutants and environmental samples, where non-target pollutants scavenge the ozone. Microbial degradation of SMX and CBZ was more effective by nutrient limited biofilms than by planktonic counterparts. Even though biodegradation was less effective than ozonation, the transformation products proved to be less toxic. Nutrient limited biofilms are scarce in natural system, as most natural and waste water is high in nutrients, therefore the application thereof for micropollutant removal would be a post-secondary treatment step or 'polishing step' for water treatment systems. The possibility of combinations of treatment processes should be further investigated to optimize a system that can effectively reduce micropollutants as well as the eco-toxicological footprint.

Opsomming:

Slegs 1% van alle water bronne is vars water wat toeganklik is vir gebruik en word al meer deur mens gemaakte materiale soos soliede en chemiese afval, besoedel. Vorige studies het gewys dat verskeie organiese mikro-besoedelstowwe nie deur konventionele water behandeling verwyder word nie en dat dit voortduur in natuurlike water bronne. Die primêre doel van hierdie studie was nie om mikro-besoedelstowwe te meet en te monitor nie, maar eerder om twee degradasie prosesse, elektrochemiese oksidasie en mikrobiese degradasie, asook die gevolgelike transformasie produkte te ondersoek. Die hipotese was die mikrobiese afbraak minder toksiese transformasie produkte gaan produseer as die sterk proses van chemise oksidasie. Twee mikro-besoedelstowwe, sulfamethoxazole en carbamazepine, was geselekteer op grond van hul wyd verspreide opsporing en voortdurendheid in die omgewing. Die CabECO tegnologie gebruik die proses van elektrochemiese oksidasie om osoon in die waterige fase met die doel vir water behandeling. Die CabECO tegnologie produseer >2 mg/L osoon en toon sommige effektiwiteit om die mikrobiese lading van omgewings water monsters meningsvol te verlaag, alhoewel veelvuldige ordegroottes van mikrobes steeds teenwoordig is, veral in monsters met hoë CSB. Dit was ook effektief in die afbraak van SMX en CBZ, deur dit te verwyder tot onder deteksie limiete binne 1 min se behandeling. Selfs met effektiewe afbraak binne 1 min, het die endokrien versteurende effek tot 4 ure se blootstelling geverg om die estrogeniese/anti-estrogeniese aktiwiteit te verwyder. Alhoewel effektief vir SMX en CBZ, is die CabECO tegnologie minder effektief teen 'n wye reeks van mikro-besoedelstowwe en omgewings monster, waar nie-teiken stowwe die ozoon aas. Mikrobiese afbraak van SMX en CBZ was meer effektief deur nutriënt beperkte biofilms as deur planktoniese ewewigte. Selfs al was bio-afbraak minder effektief as osoon behandeling, was die transformasie produkte minder toksies. Nutriënt beperkte biofilms is skaars in natuurlike sisteme, aangesien meeste natuurlike en afval water hoog in nutriënte is. Daarom sal die toepassing daarvan vir mikro-besoedelaar verwydering toegepas word as 'n na-sekondêre behandelings stap of 'n 'polishing' stap in water behandelings sisteme. Die moontlikheid van gekombineerder behandelings prosesse moet verder ondersoek word om 'n sisteem te ontwikke wat beide mikro-besoedelstowwe en die eko-toksilogiese voetspoor effektief verwyder.

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Glossary:

Abbreviations:

ACM	Acetaminophen
AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
AOP	Advanced Oxidation Process
ATZ	Atrazine
BPA	Bisphenol A
BSA	Bovine Serum Albumin
BZT	Benzotriazole
CabECO	Carbon Based ElectroChemical Oxidation
CAF	Caffeine
CBZ	Carbamazepine
CEC's	Contaminants of Emerging Concern
CFU	Colony Forming Unit
COD	Chemical Oxygen Demand
CPRG	Chlorophenol Red Galactopyranoside
DCF	Diclofenac
DiOH-CBZ	Dihydroxy Carbamazepine
DO	Dissolved Oxygen
DOC	Dissolved Organic Compounds

E$_2$	17β-Estradiol
EC	Electrical Conductivity
EC	Electrical Current
EC's	Emerging Contaminants
EDC's	Endocrine Disrupting Compounds
EP-CBZ	Carbamazepine Epoxide
EPS	Extracellular Polymeric Substances
HDPE	High Density Polyethylene
HSD	Honest Significance Difference
LCMS	Liquid Chromatography Mass Spectroscopy
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MRM	Multiple Reaction Monitoring
NOAE	No Observed Adverse Effect
NOM	Natural Organic Matter
O$_3$	Ozone
OD	Optical Density
ORP	Oxidation Reduction Potential
PPCP's	Pharmaceutical and Personal Care Products
RAS	Return Activated Sludge
RO	Reverse Osmosis
SEM	Scanning Electron Microscopy

SHBG	Sex Hormone-Binding Globulin
SMX	Sulfamethoxazole
SOP	Standard Operating Procedure
SPE	Solid Phase Extraction
SWA	SafeWaterAfrica
TMP	Trimethoprim
TOC	Total Organic Carbon
TSA	Tryptic Soy Agar
UV	Ultraviolet
WWTP	Wastewater Treatment Plant
WWTW	Wastewater Treatment Works
YEAS	Yeast Anti-Estrogen Screen
YES	Yeast Estrogen Screen
YM	Yeast Mould

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Chapter 1

Introduction

1.1 General introduction:

Fresh water is an essential part of all life on Earth and we humans are no exception. Water management dates back thousands of years, with the aim to control floods and droughts, enhance agricultural practices and to meet the demands of sanitation of an ever growing world population (Angelakis and Zheng, 2015). Fresh water is defined as water that contains less than 1000 mg/L of dissolved solids (Zaman and Sizemore, 2017). Based on this definition less than 1% of the world's water reserves is fresh water that is accessible to us.

One of the greatest challenges our society faces today is the alarming rate at which the human population is growing. With the world population at 7.53 billion people in 2017, fresh water is becoming an increasingly scarce resource (World Bank, 2019). The major sources of freshwater pollution derives from industrial waste, agricultural runoff and domestic waste (Zaman and Sizemore, 2017). With increase in population, anthropogenic activity also increases, thus the pollution of the finite fresh water sources is increased. The fast rate of urbanization contributes to the problem, as domestic wastewater becomes loaded with increasing concentrations of pollutants. According to The World Bank, the percentage of the world population that lives in urban areas has increased from 33% in 1960 to 55% in 2017 and is estimated to increase to 68% by 2045. This problem also hits home, as South Africa's urban population has increased from 46% to 64% in the last 55 years (World Bank, 2019).

Advances in the scientific and medical fields have resulted in the consumption and use of thousands of pharmaceuticals and personal care products (PPCP's) that get excreted, either in their original form or as metabolites, into wastewater (Clara et al., 2005). Most of these PPCP's and a class of pollutants called endocrine disrupting compounds (EDC's) are detected in surface waters across the world and wastewater treatment works (WWTW) effluent is a major point of discharge for these contaminants in rivers, streams and surface waters (Archer et al., 2017; Clara et al., 2005). EDC's are classified as chemicals and hormones which are suspected to have an impact on human and wildlife endocrine systems (Clara et al., 2005). In a study by Heberer (2002) it was reported that certain micropollutants detected in drinking water have been traced back to municipal sewage. Recently these micropollutants have been found all over the world, from remote lakes in the Himalayas to pristine mountain streams in the Swiss-Italian Alps (Guzzella et al., 2011; Guzzella et al., 2018).

Conventional WWTW's are not equipped to reduce the load of these chemical pollutants as they are designed to remove organic waste, heavy metals and only a selected few chemicals. These micropollutants typically range from the ng/L to μg/L range in wastewater effluents (Benner et al., 2013) and various recent studies have confirmed that many PPCP's and their transformation products pass through most WWTW's untreated and that WWTW's serve as a site of accumulation of some persistent compounds, rather than removal thereof (Archer et al., 2017; Luo et al., 2014; Loos et al., 2013). Various studies report adverse negative health effects on aquatic organisms, such as reduced reproductive success and disruption of population gender dynamics, which could be attributed to endocrine disruptors (Sonnenschein and Soto, 1998; Sumpter, 1998).

Many advances have been made in the field of micropollutant removal using various treatment technologies. In a review by Klavarioti et al. (2009), they summarized and evaluated various advanced oxidation processes (AOP's) for the abatement of PPCP's. The degradation of compounds in wastewater treatment by advanced oxidation relies on the principle of oxidation by highly reactive hydroxyl ions. Removal efficiencies, however, vary greatly depending on the AOP's used, or combinations thereof, and the chemical properties of the contaminants in question (Klavarioti et al., 2009).

The SafeWaterAfrica project, funded by the European Union (EU) Horizon 2020 initiative (https://safewaterafrica.eu/en/home), aims to provide an alternative technology to address the problem of access to clean drinking water in Southern African rural areas. The project relies on the utilization of the CabECO technology (carbon based electrochemical oxidation) to produce potable drinking water from environmental water sources. With the electrolysis of water by the CabECO technology, various free radicals are generated in the aqueous phase that have the potential to reduce chemical and microbial contaminants. The technology has the benefit of generating ozone directly in the aqueous phase, without gas-liquid transfer. The final design proposes a completely off the grid and decentralized system and it poses a range of potential benefits over other drinking water treatment technologies. CabECO requires low voltage and direct current, meaning it can be run off solar panels, is safe and easy to use and maintain, requires no specialized personnel to handle dangerous chemicals and has a long expected lifespan. However, during the run of the project, it was found that the system was not easy to use and maintain, and constant attention by skilled personnel was required.

Biological degradation of micropollutants is also widely investigated, as this could be a cost effective and environmentally friendly alternative. Some minor success has been achieved for the removal of selected micropollutants with the use of microbial communities (Falås et al., 2013). However the process is not universal, the removal efficiencies vary greatly with compound structures, aerobic and anaerobic processes, suspended and attached growth, as well as climate and retention time of the treatment system (Wang and Wang, 2018; Benner et al., 2013; Falås et al., 2016).

The primary problem of micropollutant removal, in comparison to the standard wastewater treatment processes removing nitrogen, carbon and phosphorous, is the vast diversity of compounds. It is estimated that as many as 300 million tons of anthropogenic chemicals makes its way to natural water sources every year (Schwarzenbach, 2006) and with more than 100 000 unique substances registered, the list of detected micropollutants in the natural environment is growing daily (ECHA, European Chemicals Agency, 2019). Keeping in mind the irregularities in the removal efficiency of different compounds using individual treatment processes, the idea of hybrid treatment is gaining popularity as the most feasible way to deal with this challenge. This involves the combination of two or more removal methods to cater for a wider range of micropollutants in terms of degradation (Grandclément et al., 2017). This is especially relevant to biological degradation of micropollutants; wastewater treatment plants are not designed to completely remove all organic molecules, since that will be prohibitively expensive. Micropollutants occur at lower concentrations than the acceptable levels of the organic loading in wastewater discharge.

Based on the current knowledge of the extent of chemical pollutants in our water sources, the potential health implications and the need for efficient and cost effective methods of removal thereof, we compared two processes for the primary aim of micropollutant degradation, one reliant on microbial metabolism, the other a physico-chemical process that aims to eliminate both micropollutant and biological contaminants.

1.2 Hypothesis:

It was hypothesized that electrochemical oxidation by the suite of free radicals generated with the CabECO technology is an effective method for micropollutant removal from water as well as an effective sterilization method for various types of environmental waters. It was hypothesized that combinations of pollutants might influence the effect of ozonation on

pollutant transformation, test in controlled and environmental conditions. It was also hypothesized that a microbial community would switch to micropollutant metabolic transformation when in a state of starvation, leading to micropollutant degradation. Additionally to both hypotheses, it was also hypothesized that microbial degradation will produce less toxic transformation products than ozonation.

It is widely known that ozonation is effective in reducing micropollutant concentrations in water samples, however, little is known about the transformation products formed during this process. Due to the nature of highly reactive oxygen species, it is hypothesized that even with effective degradation of a single parent compounds, various potentially toxic and active transformation products may form as a result of the oxidation reaction. In comparison to biological degradation of micropollutants, it is less likely that toxic or active metabolites will form as a living organism will favor inactive or less harmful metabolites.

1.3 Aims:

1) To determine the impact of electrochemical oxidation on

   a) The absolute concentrations of two representative micropollutants, individually and in combination

   b) The toxicity of two representative micropollutants, individually and in combination

   c) A suite of environmental micropollutants in river water

   d) The microbiome survival rate and morphology of river and household greywater at different levels of contamination

2) To determine the impact of metabolically starved biofilms on

   a) The absolute concentrations of two representative micropollutants, individually and in combination

   b) The toxicity of two representative micropollutants, individually and in combination

1.4 Objectives:

1) To design, install and optimize a laboratory prototype of the CabECO technology for maximum ozone production at safe operation concentrations

2) To use cell counts and scanning electron microscopy to determine cell concentrations and investigate cell morphology of environmental microbiomes before and after treatment with the CabECO technology

3) To use LCMS to quantify the absolute concentrations of two representative micropollutants, as well as a complex suite of micropollutants, and comparing complex water matrices, including river and greywater

    a) Before and after treatment with the CabECO technology

    b) Before and after exposure to metabolically starved biofilms

4) To use recombinant yeast strains, with the human estrogen receptor, to determine potentially endocrine disrupting effects of two representative micropollutants, as well as a complex suite of micropollutants, and comparing complex water matrices, including river and greywater

    a) Before and after treatment with the CabECO technology

    b) Before and after exposure to metabolically starved biofilms

Chapter 2

Review of Literature

2.1 Introduction:

All life on Earth is dependent on water and even more so on fresh water. Water sources across the globe are constantly being polluted by various man-made chemicals. These chemicals persist in surface waters and accumulate over years due to neglect to effectively remove them from waste water (McDowell et al., 2005).

Municipal wastewater treatment works (WWTWs) are generally not equipped to deal with complex pharmaceuticals, as they were built and upgraded with the principal aim of removing easily or moderately biodegradable carbon, nitrogen and phosphorus compounds present in WWTW influent in concentrations to the order of mg/L, and high numbers of heterogeneous microbial communities. Micropollutants in raw wastewaters are generally in the range of 10^{-3}–10^{-6} mg/L, in addition, their chemical and physical properties, namely solubility, volatility, adsorbability, absorbability, biodegradability, polarity and stability, vary greatly, with obvious implications on their behaviour during the treatments and consequently their removal efficiencies (Verlicchi et al., 2012).

The major sources of these contaminants are industrial, domestic and agricultural, where these sources introduce harmful and persistent chemicals into the aquatic environment (Archer et al., 2017). Pharmaceuticals and personal care products (PPCP's) are increasingly investigated as emerging contaminants in aquatic ecosystems (Fent et al., 2006). Many pharmaceuticals are not completely metabolized in the human body, so both unmodified parent compounds and metabolites are excreted and can enter the water cycle via wastewater. Due to incomplete removal of many pharmaceuticals in wastewater treatment works (WWTWs), these micropollutants are emitted into the aquatic environment. An additional source of pharmaceuticals in the aquatic environment is their application in livestock followed by fertilization with manure (Boxall et al., 2004).

The presence and especially the persistence of PPCP's and their metabolites in the aquatic environment has raised concern due to the potential ecological and health risks associated with the chronic low level exposure to these compounds (Cunningham et al., 2010; Kostich et al., 2014).

2.2 Environmental impact of frequently detected micropollutants:

The frequent detection of micropollutants in environmental water sources, be they domestic, industrial or agricultural in origin, has raised various concerns about the chronic low level of exposure of these chemicals to organisms in the environment. Exposure to many of these chemicals have the potential to interfere with human and wildlife endocrine systems, resulting in increased human endocrine-related diseases and adverse health and population effects in aquatic ecosystems (WHO/ICPS, 2002). Endocrine disrupting compounds (EDCs) are defined as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" (WHO/ICPS, 2012).

Additionally, the frequent discharge of sub-acute concentrations of micropollutants, including antimicrobial compounds, into the environment, has led to the distribution and development of antimicrobial resistance mechanisms and genes. The occurrence of antimicrobial resistance results in untreatable infections and increases in morbidity and mortality across the world (Keen and Fugère, 2017).

2.2.1 Endocrine Disruption:

EDCs bring forth the above proposed effects on exposed organisms and their populations by interacting with the endocrine system in various ways. The most profound mechanisms of interaction of EDCs include mimicking the structure (and hence, function) of naturally occurring hormones, inhibiting the production thereof or blocking/enhancing hormone-receptor binding in humans and various wildlife species (Soares et al., 2008). EDCs can influence the endocrine system by various mechanisms, including sensitizing hormones, interfering with nuclear receptor binding, irregular oxidative stress responses and steroid hormone metabolism (Maqbool et al., 2016).

Possible EDCs include industrial chemicals, pharmaceuticals and personal care products, agricultural chemicals (pesticides, herbicides, fertilizers and agricultural pharmaceuticals) and plastics and their by-products (Diamanti-Kandarakis et al., 2009; De Jager et al., 2013). EDCs are a diverse group of chemicals with different chemical classes and vary greatly in potency, resulting in great inconsistency with regards to predicting compound effect on the natural environment, especially when mixtures of chemicals are taken into consideration (De Jager et al., 2013). EDCs are usually present in the environment at concentrations below the necessary levels needed for acute toxic effects or the no observed adverse effect

(NOAE) levels for most individual endocrine disruptors (Pinto and Reali, 2009). However, the exposure to a mixture of a diverse range of chemicals and chronic low level exposure, increases concern regarding endocrine disruption potential and the dose addition effect (Kortenkamp et al., 2007; Muncke, 2009). Long term chronic EDC exposure have been reported to be associated with obesity, diabetes, developmental issues, cardiovascular diseases and reproductive abnormalities (Erler and Novak, 2010; Diamanti-Kandarakis et al., 2009; Murray et al., 2007; Muncke, 2009). EDCs impacting estrogen function and production is highly linked to the development of several cancers (Metzler et al., 1998; De Jager et al., 2013).

Various individual micropollutants have been associated with disrupting estrogen function, leading to several adverse health effects in humans. One such chemical is the plastic-derived bisphenol A (BPA), which is known to cause estrogenic effects in humans and wildlife (Muncke, 2009). BPA, in the low µg/L range, has been linked to obesity, diabetes, male and female reproductive tract abnormalities and increased incidence of cancer, specifically breast and prostate cancer (Hugo et al., 2008).

Diabetes occurrence has been linked to environmental micropollutants that could possibly interact as estrogenic hormones (Porta, 2006). Estrogen receptors is involved in maintaining glucose metabolism, therefore, any estrogen analogues can bring forth a response by binding to these receptors, disrupting glucose homeostasis and insulin release regulation (Maqbool et al., 2016). Low doses of estrogen analogues (µg/L), such as BPA, have been known to disrupt the glucagon pathway, an important hormone in the regulation of blood sugar levels (Alonso-Magdalena et al., 2005). With irregularities in blood sugar regulation, obesity and consequently cardiovascular diseases can be expected (Collins, 2005; Poirier and Després, 2003).

Endocrine disruptors can target various hormonal systems that form part of the endocrine system, such as the thyroid system (Maqbool et al., 2016). Estrogenic compounds, ones that interact with the production and/or binding of estradiol to nuclear estrogen receptors, are known to cause disruptions in the human thyroid system, leading to growth and metabolic disorders, and in severe cases can lead to mental health problems and ultimately brain damage (Bergman et al., 2012). It has also been reported that thyroid hormone is targeted by the metabolites of benzene containing organic pollutants (Maqbool et al., 2016).

2.2.2 Anti-microbial resistance:

The widespread occurrence, as well as the increase, of antibiotic resistance is a global epidemic that threatens the human populations' health and food security (Keen and Fugère, 2017). The widespread and incomplete use of antibiotics, coupled with incomplete removal during wastewater treatment processes, have resulted in exposing the natural environment to a dilute solution of antibiotics over the last few decades. Agricultural use of antibiotics heavily contributes to the problem as well, where in the United States of America (USA) it is estimated that livestock farming uses about eight times more antibiotics than human consumption (Mellon et al., 2001).

Rizzo et al. (2013) has identified WWTWs and aquatic ecosystems receiving WWTWs discharge as hotspots for antimicrobial resistance (AMR) and AMR genes. These nutrient-rich environments with frequent antibiotic compound discharge from WWTWs, agricultural runoff and industrial effluent, together with naturally occurring organisms, provide the perfect environment for the propagation and horizontal gene transfer of AMR genes and mechanisms (Davies and Davies et al., 2010; Miran et al., 2018; Anderson et al., 2015; Rizzo et al., 2013).

2.3 Frequently detected micropollutants:

Due to the widespread detection of micropollutants across the globe and the many possible impacts the chronic low-dose exposure thereof may have on the natural environment, this topic has gained vast popularity amongst researchers and conservationists alike. With over 100,000 unique registered chemical products that can end up in waste water and environmental waters, the list of frequently detected micropollutants are ever growing (ECHA, European Chemicals Agency, 2019). Table 2-1 summarises a short list of frequently detected chemicals of concern, or emerging contaminants (ECs), to the immediate environment they are detected in.

Table 2-1: Selection of frequently detected micropollutants across the world

Compound type	Compound name	Located	Detection level	Reference
Anti-epileptic	Carbamazepine	Germany	250 ng/L	Ternes et al., 1998
		USA	185 ng/L	Metcalfe et al., 2013
		Canada	120 ng/L	Metcalfe et al., 2013
		South Africa	3.2 µg/L	Archer et al., 2017
Antibiotics	Sulfamethoxazole	Germany	1 µg/L	Radke et al., 2009
		USA	0.03 - 1 µg/L	Heberer et al., 2002
		South Africa	34.5 µg/L	Archer et al., 2017
	Clarithromycin	Germany	360 ng/L	Baumann et al., 2015
		Spain	141 ng/L	López-Serna et al., 2012
		France	>1 µg/L	Feitosa-Felizzola and Chiron, 2009
β-blocker	Atenolol	Germany	400 ng/L	Küster et al., 2007
		Canada	1100 ng/L	Lapen et al., 2008
		Switzerland	404 – 678 ng/L	Maurer et al., 2007

Anti-Inflammatory	Ibuprofen	Germany	0.6 ng/L	Weigel et al., 2002
		UK	3 µg/L	Bound and Voulvoulis, 2006
		Poland	6 – 74 µg/L	Stafiej et al., 2007
	Diclofenac	Germany	15 µg/L	Jux et al., 2002
		Italy	5.45 µg/L	Andreozzi et al., 2003
		France	0.89 µg/L	Andreozzi et al., 2003

Table 2-1 summarizes a condensed list from amongst thousands of micropollutants; these selected based on their frequent detection and investigation. Due to their persistence in the environment and the fact that these micropollutants are man-made in origin, many of them are used as human bio-markers, indicating the impact of human activity on the environment (Baumann et al., 2015). Among a diverse range of pharmaceuticals found in water sources, Carbamazepine and Sulfamethoxazole are found at high frequencies and were selected as the two main compounds for investigation in this study (Zhang et al., 2008; Gomez-Ramos et al., 2011; Archer et al., 2017). In comparison to some developed countries, many micropollutants are detected at higher concentrations in South African waste water effluents, this could be a possible indication of WWTW's not functioning optimally in South Africa.

2.4 Carbamazepine:

Carbamazepine (CBZ), an benzodiazepine derivative with a tricyclic structure, is one of the most widely prescribed drugs for the treatment of epilepsy, bipolar disorder and other psychotherapy applications; however, CBZ has a distinct chemical structure and properties that can affect its environmental behavior (Fertig and Mattson, 2008). CBZ's method of

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action is on a neurological level, inhibiting the release of glutamate at the glutamatergic synapse, regulating the voltage-gated Na$^+$ receptors (Katzung and Trevor, 2015). CBZ and the benzodiazepine class of drugs it belongs to, are surrounded by a fair amount of controversy regarding the adverse health effects associated with long-term, chronic administration. Some of these adverse effects have been known to be associated with hyperthyroidism, hypertension and serum sex-hormone levels in males and females (Löfgren et al., 2006; Vainionpää et al., 2004). Carbamazepine has been reported to interact with a variety of other therapeutic drugs, such as oral contraceptives, antibiotics and cardiovascular disease related medication (Drugs.com, 2019).

2.4.1 Detection of CBZ in environmental water:

The widespread prescription and use of CBZ has resulted in the occurrence thereof in the natural environment across the globe. In 2002 the annual consumption in Spain was approximately 25 tons, which increased up to 32 tons in 2006 (De la Fuente et al., 2007), and in the United Kingdom (UK) 40 tons is prescribed each year (Jones et al., 2002). The global consumption of CBZ is estimated to be approximately 1000 tons per year (Zhang and Geiben, 2010). Carbamazepine is among the most frequently detected micropollutants in wastewater effluents at relatively high concentrations of about 1 µg/L (Clara et al., 2004; Verlicchi et al., 2012). CBZ can be found in many streams and rivers across the globe at concentrations averaging 250 ng/L in Germany, at 185 ng/L in the Detroit River, USA and at 120 ng/L in Lake Ontario, Canada (Ternes et al., 1998; Metcalfe et al., 2003). However, in South Africa, Carbamazepine has been found at concentrations up to 3.2 µg/L in surface waters and waste water effluents (Archer et al., 2017). CBZ has been identified as one of the most concerning compounds frequently found in South African wastewater (Archer et al., 2017; Odendaal et al., 2015).

CBZ passes through most conventional WWTWs with moderate to low removal efficiencies (Jankunaite et al., 2017; Chen et al., 2014; Kunkel and Radke, 2012), in fact, WWTWs seems to serve as a site for accumulation of CBZ rather than degradation (Archer et al., 2017). The persistence of CBZ becomes significant, when keeping in mind the side effects and adverse outcomes CBZ can cause in non-target human and wildlife health upon exposure.

2.4.2 Endocrine disruption of CBZ:

As mentioned above, the chronic long-term use of CBZ is associated with adverse health effects. Additionally, CBZ has the potential to cause an endocrine disruptive effect with chronic exposure, especially regarding thyroid hormone function and levels. In a previous study, CBZ exposure resulted in decreased levels of serum thyroid hormone in prepubescent girls (Vainionpää et al., 2004). In a study done by Reis et al., (2013), CBZ was shown to decrease semen quality and quantity in men exposed to the drug, suggesting some form of interacting with testosterone activity.

Löfgren et al., (2006) also found that women taking CBZ had decreased levels of testosterone and increased serum levels of sex hormone-binding globulin (SHBG). The resulting decrease in SHBG lowers serum testosterone and estradiol, causing menstrual disorders in women taking CBZ. These adverse health effects in female reproductive hormone function is suspected to be caused by the induction of the hepatic P450 enzyme system, by the administration of CBZ (Perucca et al., 2004). These findings are supported by Mikkonen et al., (2004), where men and young boys had reduced serum sex-hormone levels and increased SHBG, due to induction of hepatic enzymes.

CBZ and/or the biotransformation products as a result from biological degradation, resulted in moderate toxic effect on *Vibrio Fischeri*, when in aqueous solution, however, other organisms showed no sensitivity towards CBZ or its metabolites. Even with a 95% removal efficiency of CBZ, the toxicity of the sample remained high due to the formation of active metabolites which potentially increases toxicity (Bessa et al., 2019). Various other studies also reported that even though CBZ is removed, toxicity remains problematic due to active metabolites formed (Bessa et al., 2019; Russell et al., 2015)

2.5 Sulfamethoxazole:

Sulfamethoxazole (SMX) is a bacteriostatic antibiotic part of the sulphonamide class. SMX's mechanism of action is attributed to the inhibition of synthesis of dihydrofolic acid by bacteria, by competitively binding to certain enzymes. By inhibiting dihydrofolic acid synthesis, the administration of SMX results in reduced synthesis of DNA and nucleotides in bacteria (DrugBank, 2019). SMX is usually administered in combination with Trimethoprim

(TMP), resulting in slower development of resistance to both SMX and TMP (Wright et al., 1999).

Sulfamethoxazole (SMX) is one of the top-selling antibiotics. After oral application, it is only partly metabolized in the human body and approximately 45-70% of a SMX dose is excreted in the urine within 24 hours. Göbel et al. (2005) determined a removal efficiency of SMX of 62% in a Swiss WWTP. In laboratory reactors an elimination rate for SMX of 84% has been measured; the cause of this elimination was reported to be primarily microbial degradation. Only <0.1% of SMX was removed by adsorption to sewage sludge and hydrolysis of SMX was also not a relevant removal process (Letzel et al., 2009).

2.5.1 Detection of SMX in environmental water:

In 2001, 53.6 tons of SMX were sold in Germany, and measured in effluents of German WWTWs at concentrations in the range of several hundred ng/L up to 1000 ng/L (Radke et al., 2009). In surface waters, SMX was determined at concentrations between 0.03 and 1 µg/L in the United States and it is a common contaminant of groundwater with maximum concentrations measuring more than 1 µg/L (Heberer et al., 2002; Barnes et al., 2008). In a review by Archer et al. (2017) it was found that Sulfamethoxazole concentrations reached up to 34.5 µg/L in WWTW's influent and 3.68 µg/L in surface waters in South Africa. However, in WWTWs, only 15-25% is present as the unchanged drug while 43% is present as N4-acetyl-sulfamethoxazole, and 9-15% is present as sulfamethoxazole-N1-glucuronide, the main human metabolites of SMX (Gomez-Ramos et al., 2011; Radke et al., 2009). Three additional metabolites made up to 4-10% of the total concentration.

2.5.2 Antimicrobial resistance and endocrine disruption of SMX:

Due to the constant low-level exposure of SMX to the environment from waste water discharge and direct human pollution (lack of sanitation, incorrect disposal of drugs, agricultural runoff, etc.) of environmental water sources, the environmental impact and eco-toxicological effects need to be investigated. One of the major concerns regarding the persistence of SMX in aquatic environments, is the development of antimicrobial resistance of the chronically exposed organisms in the immediate environment (Wright et al., 1999; Desforges et al., 1993)

Wright et al. (1999) found that a gram-negative, aerobic group of bacilli, the *Enterobacteriaceae*, can cause various infections in humans, which includes *Escherichia coli*. SMX resistance has been increasing in this gram-negative group in recent years, especially in the waste water sector and environmental waters that receives waste water discharge (Desforges et al., 1993).

In a 10 year-long study, with more than 40000 *Staphylococcus aureus* isolates, it was found that SMX resistance was significantly increased from less than 1% to 4% of the isolates. Interestingly, the strains that became resistant all came from outpatient sources, such as environmental waters and waste water effluent (Vicetti Miguel et al., 2019). The conclusion was drawn that constant low level exposure to the environment is increasing the occurrence of antimicrobial resistant organisms, as the inpatient isolates showed no increase in resistance, possibly due to the high doses of SMX administered to patients, eradicating the organisms causing the infections (Vicetti Miguel et al., 2019).

SMX, in combination with TMP, is a commonly prescribed antibiotic in newborn babies for the treatment of pneumonia and sepsis, especially in Asian countries. However, there is concern for the cause of neurotoxicity with the prolonged use of SMX in neonates (Thyagarajan and Deshpande, 2013). Concerning reports have stated that SMX administration can cause kernicterus in newborn babies, a brain disorder caused by increased levels of the pigment bilirubin. It is thought that SMX displaces the bilirubin from binding sites, leading to increased serum bilirubin levels, eventually crossing the blood-brain barrier, and accumulating in the brain tissue (Silverman, 1959; Silverman, 1960). It is reported that the acetylation of SMX by metabolism could result in metabolites that increase the risk of kernicterus in neonates (Thyagarajan and Deshpande, 2013).

2.6 Waste water treatment and micropollutant abatement:

Conventional waste water treatment processes such as sedimentation, flocculation, activated sludge and filtration is ineffective at micropollutant removal, as primary design did not include the abatement of micropollutants (Petrie et al., 2015; von Sonntag and von Gunten, 2012). New technologies and processes are needed for efficiently, and economically, transforming and reducing micropollutant concentrations to biologically inactive products and levels in waste water (Wang et al., 2018; Schwarzenbach et al., 2006). Various technologies exist that have potential to degrade several micropollutants, such as

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advanced oxidation processes (AOP's) and adsorption, but economically these options are not viable as of yet (Wang et al., 2018). The adsorbents and oxidation processes applied are quickly depleted by the high loads of organic matter and inorganic salts that typically occur in waste water, thus requiring high economic input to deliver the desired level of micropollutant removal (Petrie et al., 2015). Furthermore, the vast number of micropollutants in waste water have a diverse range physicochemical properties and the effect of complex mixtures of micropollutants cannot be addressed by a single, economic process (Wang et al., 2018; Petrie et al., 2015; von Sonntag and von Gunten, 2012).

It is clear that no perfect treatment exists for the abatement of micropollutants from waste water as of yet, however, many treatment options do exist that could potentially reduce the load of micropollutants (Klavarioti et al., 2009). AOP's harness the high reactivity of free radicals to oxidize persistent, bio-toxic and compounds that are recalcitrant to biotransformation to various transformation products and eventually biologically inactive products. Biological degradation with activated sludge has also shown potential in micropollutant degradation, but due the high loads of non-target organic matter present in waste water, full scale application results in less than ideal removal efficiencies (Wang et al., 2018). Some physical treatment technologies, such as ultra-filtration and UV exposure have reported success in selected micropollutant removal, however, the high cost associated with these technologies makes them non-feasible options for large scale water treatment (Zheng et al., 2014).

The potential removal of several classes of micropollutants by ozonation, as well as the benefits of ozone over other treatment technologies, as well as the application thereof in water treatment will be discussed below. The SafeWaterAfrica project is a multinational endeavour researching the application of electrochemical generated ozone in a decentralized water treatment system. Additionally, biological degradation of micropollutants is gaining popularity and will be discussed in this chapter, due to the low cost and environmental footprint of implementation, as well as the added benefit that the transformation products from other treatment technologies are known to be more susceptible to biotransformation. Biological degradation might be a possible polishing step in water treatment (Klavarioti et al., 2009; Hoigné et al., 1998).

2.7 SafeWaterAfrica:

The SafeWaterAfrica project is a multi-national endeavor for potable water provision for Southern African rural areas. The scope of the project is to harness the CabECO technology to generate ozone in the aqueous phase with the aim to reduce microbial load as well breakdown harmful chemical contaminants in various water sources. Carbon based ElectroChemical Oxidation (CabECO) harnesses the principle of the electrolysis of water for the generation of ozone and other free radicals such as hydroxyl radicals to be used in water treatment.

The CabECO cell was initially designed for industrial uses such as reducing chemical oxygen demand of industrial effluent. In this project, its potential application as an autonomous and decentralized water treatment system for application in rural and peri-urban area was investigated, which can potentially degrade harmful pollutants and at the same time efficiently eliminate microbial contaminants.

The implementation of CabECO follows a "Made in Africa, for Africa" approach, where the local population will be trained to operate and maintain the technology. CabECO requires low voltage and direct current, meaning it can be run off solar panels and has a long expected lifespan. However, lifespan is relative to the hardness of the water being passed through the cell. Hardness components react with end products of the process and deposits on the electrodes, reducing efficiency over time as these scaly deposits reduce contact area on the electrodes. The final design of the SafeWaterAfrica system have several pre-treatment steps, such as electro-coagulation and micro- and nano-filtration, which would deal with most of the water components which might cause problems with the integrity of the electrodes, due to scale formation. In the case of compromised electrodes, maintenance would include disassembling the cell and soaking the electrodes in a solution of diluted hydrochloric acid (<10% v/v) when efficiency is influenced (Nishiki et al., 2011).

The original conceptional design of the CabECO-based treatment system was designed to have a holding tank for storing water for later use. This holding tank is meant to serve as a vessel for allowing reaction time for the produced free radicals with the target pollutants.

Figure 2-1: A) Proposed design of SafeWaterAfrica decentralized water treatment system.

Due to the formation of several by-products from the reaction of ozone with specific compounds such as chlorine and bromide, which can be present in many water sources, some pre-treatment may be necessary for the implementation of CabECO. The reaction of ozone with bromide generate bromate, a potent human carcinogen, and can be produced by the reaction at relatively low bromide concentrations (50 mg/L) (Wang et al., 2018). Similarly, chlorine-ozone reactions can generate chloride, a carcinogenic and bio-toxic by-product (Siddiqui, 1996; Sijimol et al., 2015). This is a major drawback of the technology as a decentralized water treatment system, requiring extra pre-treatment steps. However, the CabECO cell has specific carbon electrode modifications, which may reduce the formation of these bio-toxic by-products while operating at low current densities (SafeWaterAfrica, 2015).

2.7.1 Electrochemical generation of ozone for water treatment:

Triatomic oxygen, ozone (O_3), results from the rearrangement of atoms when oxygen molecules are subjected to high-voltage electric discharge and when atmospheric oxygen is irradiated with UV light. Ozone is a bluish gas with a characteristic odour and strong oxidizing properties (Hoigné et al., 1985). Ozone was officially discovered in 1840 by

German scientist Christian Friedrich Schönbein when he noticed the characteristic smell of ozone during the electrolysis of water. In 1886 the potential of ozone to disinfect water was discovered and by 1893 the first full scale drinking water plant using ozone was operational in the Netherlands. Throughout the 20th century the use of ozone in water treatment and food preservation gained popularity and pose several advantages over other oxidants such as chlorination. However, chlorination is still preferred over ozonation for water disinfection due to the single fact that chlorine can be produced in much higher quantities (Oxidationtech.com, 2019). Currently, there are more than 3000 ozone-based water treatment installations all over the world and more than 300 potable water treatment plants in the USA (Rice et al., 2000). This widespread application is a clear indication of the efficacy and usefulness of ozone.

The technology relies on the principle of specifically designed membranes: metal electrodes which are coated with conductive boron-doped polycrystalline sp^3-bonded carbon. When water is passed through the cell connected to a power source, it will promote the formation of strong oxidants. When there is current flow in the cell, an anode reaction favors the generation of oxygen [1] and ozone [2] in that order (Nishiki et al., 2011).

$$2 H_2O = O_2 + 4 H^+ + 4 e^- \qquad [1]$$

$$3 H_2O = O_3 + 6 H^+ + 6 e^- \qquad [2]$$
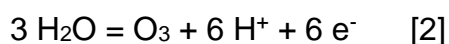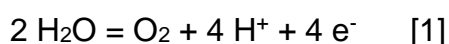
Simultaneously, a cathode reaction generates hydrogen gas [3].
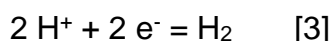
$$2 H^+ + 2 e^- = H_2 \qquad [3]$$

Other oxidants may also be formed during this process, such as hydroxyl and hydrogen peroxide, but are not thermodynamically favored. Tap water is typically chlorinated and an additional anode reaction can occur where chlorine is transformed to hypochlorite, chlorate and perchlorate (Nishiki et al., 2011). These compounds may exhibit some biological toxicity at high levels, such as in the case in humans, where they interfere with cellular iodine uptake leading to carcinogenic effects (Sijimol et al., 2015).

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2.7.2 Physical factors influencing ozone production and stability:

Ozone is relatively unstable in aqueous solutions, it decomposes continuously into oxygen according to a pseudo first-order reaction (Tomiyasu et al., 1985). The half-life of ozone in distilled water at 20 °C is generally considered to be 20 to 30 min, but varies considerably in literature (Wynn et al., 1973; Wickramanayake et al., 1984). Physicochemical properties of ozone and the solution properties are closely related to ozone's efficacy and solubility in the aqueous phase, and thus these properties will be discussed (Khadre et al., 2001).

The pH greatly affects the stability of ozone in aqueous solutions. In a study done by Kim et al. (1998) it was found that the stability of ozone in aqueous solution was the highest at a pH of 5 and that the stability of ozone decreased as pH rises, and no ozone was detected at a pH of 9. The rate at which ozone degrades in aqueous solution is more rapid at higher pH due to the catalytic activity of the hydroxyl radical. It has been reported that ozone has a stronger bactericidal effect at lower pH (6) when compared to higher pH (8) (Kim et al., 1998). This is supported by various studies where ozonation at lower pH values had a greater disinfection capability than higher pH values of the medium (Khadre et al., 2001; Farooq et al., 1977; Foegeding et al., 1985). When treating SMX with ozone, there seems to be better removal at slightly higher pH than at lower solution pH values, with 100% removal of SMX at 8.2 pH compared to 93.5% SMX removal at 4.7 pH (Gao et el., 2014). It was found that SMX removal is driven by a direct attack from molecular ozone to the molecule, which might explain why higher pH is favoured for SMX ozonation, due to the deprotonation at higher pH that makes SMX more reactive to molecular ozone (Lui et al., 2012). There is limited literature on how pH influences removal efficiency of other micropollutants by ozonation.

Temperature strongly influences the solubility as well as the decomposition rate of ozone in the aqueous phase. Lower temperatures increase solubility of ozone in solution whereas higher temperatures cause ozone to be less stable in an aqueous solution (Khadre et al., 2001). However, various studies have reported that there is little to no differences in the disinfection of microbes with ozone at different temperatures. Even though lower temperatures result in higher residual ozone concentrations, the increased reactivity of ozone with higher temperatures compensates for the loss in residual ozone concentration (Anchen et al., 2001; Kinman et al., 1975). Conflicting results have been shown in literature where increased temperature resulted in a greater disinfection rate than lower temperatures,

as well as where lower temperatures yielded better disinfection than higher temperatures (Khadre et al., 2001).

When ozone is generated in the aqueous phase, as in the case of the CabECO technology, the composition of the aqueous phase can greatly influence the ozone concentration. Ozone-consuming compounds is highly undesirable, as such compounds readily react with ozone and limiting the capability of ozone to react with the intended targets, such as microbes or certain chemical traces (Khadre et al., 2001). Easily degraded organic matter present in water is problematic as ozone concentration will be reduced significantly. It's reported that when added as little at 20 mg/L bovine serum albumin (BSA) it can significantly reduce the residual ozone concentration in the aqueous phase (Kim et al., 1998). Organic compounds may also lead to the formation of unknown and potentially harmful by-products when exposed to ozone, which is undesirable in drinking water treatment and the food processing industry (Khadre et al., 2001).

Humic acid comprises a large part of natural organic matter (NOM) in most environmental waters and ranges in concentration from 0.1 – 20 mg/L, varying in composition depending on the source thereof (Rodrigues et al., 2008). For ozonation, humic acid greatly influences the ability of ozone to degrade certain compounds. For instance, Gao et al. (2014) found that ozonation was highly effective in degrading SMX at a wide concentration range with >95% removal efficiencies. However, with increasing humic acid concentrations they found that at 5 mg/L the removal efficiency of SMX by ozone was reduced to less than 4%. When considering that WWTW's effluent and environmental waters possibly have a significant amount of organic matter present (the possible areas of implementation), this is an important observation as this might result in insufficient removal efficiencies of some micropollutants, due to quick depletion of ozone by non-target matter (Gao et al., 2014).

System pressure correlates directly with the solubility of ozone in the aqueous phase, as described by Henry's Law (Sotelo et al., 1989). Higher pressure results in less ozone diffusing into the atmosphere as the increased force promotes the solubility of ozone.

2.7.3 Ozonation for water disinfection:

For application in water disinfection, ozone treatment is advantageous to various other popular options such as chlorination and UV treatment. Ozone has a short half-life compared

to other disinfection chemicals, resulting in no residual chemical in treated water after a short period. Also, ozone is one of the strongest oxidizing agents, meaning that it will target almost any organic material it comes in contact with, including most microorganisms (Khadre et al., 2001). A major drawback of ozone for water treatment is that ozone must be generated onsite, due to its unstable nature.

Ozone is a powerful antimicrobial agent that is suitable in both the gaseous and aqueous states for application in the food and drink industry. Molecular ozone or its decomposition products inactivate microorganisms rapidly by reacting with intracellular enzymes, nucleic material and components of their cell envelope, spore coats, or viral capsids (Khadre et al., 2001).

Molecular ozone reactions are selective and limited to unsaturated aromatic and aliphatic compounds and ozone oxidizes these compounds through cycle-addition to double bonds (Bablon et al., 1991). One possible explanation for the rapid inactivation of microorganisms and bacterial spores by ozone, may include the oxidation of sulfhydryl groups, which are abundant in microbial enzymes. The reaction of ozone with polysaccharides has a relatively low rate constant and leads to the breakage of glycosidic bonds and the formation of aliphatic acids and aldehydes (Bablon et al., 1991). Reaction of ozone with primary and secondary aliphatic alcohols can lead to formation of hydroxy-hydroperoxides, precursors to hydroxyl radicals, which in turn react strongly with cellular hydrocarbons (Anbar et al., 1967). Perez et al. (1995) showed that N-acetyl glucosamine, a compound present in the peptidoglycan of bacterial cell walls and in viral capsids, is resistant to the action of ozone in aqueous solution at pH 3 to 7. Glucosamine reacts fast with ozone, whereas glucose is relatively resistant to degradation. This observation may explain, at least in part, the higher resistance of gram-positive bacteria compared to gram negative ones; where gram-positive bacteria contain more peptidoglycan in their cell walls. The action of ozone on amino acids and peptides is significant, especially at neutral and basic pH (Khadre et al., 2001). Ozone attacks either the nitrogen atom or the R group or both on the macro-structure on the amino acid molecule. Ozone reacts slowly with saturated fatty acids. Unsaturated fatty acids are readily oxidized with ozone and cyclic products are formed. Ozone reacts quickly with nucleobases, especially thymine, guanine, and uracil. Reaction of ozone with the nucleotides releases the carbohydrate and phosphate ions (Ishizaki et al., 1981).

2.7.4 Ozonation for micropollutant removal:

Due to the strong oxidizing capabilities of ozone, it is extensively used in water treatment, specifically drinking water preparation and less often in waste water treatment. Ozone is used for controlling several characteristics of water, such as odor, color and taste control, disinfection and the degradation of micropollutants (McDowell et al., 2005). The oxidation of several PPCP's by ozone and hydroxyl radicals can lead to the degradation of various anthropogenic drugs during water treatment, however, efficiency of ozone treatment for micropollutant transformation is directly related to the concentration of the ozone applied, the physical and chemical characteristics of the target compounds and the composition of the water source (Gómez-Ramos et al., 2011). According to Wang et al. (2018), a drawback of ozone for micropollutant abatement is that it is a selective oxidant and will only react with organic compounds that have electron-rich moieties like aromatic rings, olefins, deprotonated amines and reduced sulphur-containing functional groups, whereas micropollutants lacking electron-rich moieties will prove recalcitrant toward ozone transformation (McDowell et al., 2005).

The CBZ molecule contains a double bond with a tricyclic structure, which is recalcitrant to various types of removal technologies. However, CBZ was identified as having a high rate constant with ozone (McDowell et al., 2005). The two major metabolites of CBZ has been identified as 10,11-dihydro-10,11-dihydroxy-CBZ (DiOH-CBZ) and 10,11-epoxy-10,11-dihydro-CBZ (EP-CBZ) (Bahlmann et al., 2014). In contrast, ozonation of CBZ results in the formation of a vast suite of transformation products that can potentially form as part of several multi-step breakdown pathways, and which is driven by various factors such as pH, temperature and concentration of both CBZ and ozone (McDowell et al., 2005; Lee et al., 2017). CBZ has more than 13 known transformation products as a result of a reaction to molecular ozone (Lee et al., 2017). It was suggested that functional organic groups are cleaved at the nitrogen containing ring of CBZ to form some of the most prominent ozonation transformation products, as well as the distribution of protons across the molecule (McDowell et al., 2005).

McDowell et al. (2005) found that CBZ is degraded relatively fast by ozone, where >95% of the initial concentration of CBZ was removed from samples within 20s, however, at the same time four major transformation products were formed that persisted longer exposure times of ozone and proved recalcitrant to complete degradation at the end of ozone exposure (15 min).

In a study by Gao et al. (2014), a comparative study was performed on chlorine, ozone and permanganate as different oxidizers for SMX degradation. Results obtained from that study clearly showed that SMX is most efficiently degraded by ozone, compared to the other two oxidizers. However, it was found that pH influenced the degradation of efficiency of SMX, where a higher pH favored the removal of SMX. At a pH of 4.6, 7.0 and 8.2, the removal efficiency of SMX by ozonation (2 min) was 93.5%, 96.6% and 100% respectively. The transformation of SMX by ozonation was found to be an effect of a direct attack to the molecule from ozone rather than some of the other free radicals that may be generated by the electrochemical process (Lui et al., 2012). The favored degradation of SMX at higher pH is due to the deprotonation of SMX, resulting in a molecule that is more reactive to molecular ozone (Lui et al., 2012).

SMX is known to have 5 oxidation products as a result of ozone exposure that can be detected (Gau et al., 2014). The proposed pathways for the transformation of SMX by ozonation is reported to be dependent on the various functional groups or structures that interact with the ozone. SMX transformation by SMX is known to be a result of the oxidation of the methyl group on the isoxazole ring, forming a carboxylic acid group (Gómez-Ramos et al., 2011). Furthermore, the dihydroxylated transformation product of SMX, suggested to be due to ozonation, is believed to be caused by the oxidation of the carbon double bond of the isoxazole ring of SMX. The ozonation of SMX could also result in oxidation of the amine group on the benzene ring, cleavage of the S-N bond and the hydroxylation reaction on the benzene ring (Gau et al., 2014).

Similar to many other organic compounds, the ozone derived oxidation products formed from parent pharmaceuticals, including CBZ and SMX, may have a higher susceptibility to biological degradation than the parent compounds (Hoigné et al., 1998; Klavarioti et al., 2009).

2.8 Microbial degradation of micropollutants:

One of the major classes of origin for micropollutants is pharmaceuticals used by humans for self-medication and agricultural use (Celiz et al., 2009). The very nature of pharmaceuticals is to interact with living organisms and be metabolized. Biological degradation of micropollutants is heavily investigated, as this could be a cost effective and environmentally friendly water treatment option. Metabolism of pharmaceuticals typically

involves oxidation, reduction and/or hydrolysis, resulting in more polar and soluble and therefore, more mobile transformation products (Celiz et al., 2009). The cytochrome P450 group of enzymes (found in all lifeforms) metabolize most organic compounds by adding functional groups such as –OH, -SH, -NH2 or –COOH during metabolism to form more hydrophilic products. These biotransformation products can maintain therapeutic activity, binding to proteins and other cellular components, causing disruption of cellular components (Celiz et al., 2009).

2.8.1 Microbial biotransformation of micropollutants:

Approximately 25–30% of the orally administered dose of carbamazepine is excreted unchanged from the human body while absorbed carbamazepine goes through excessive metabolism (Zhang et al., 2008). 10,11-epoxycarbamazepine (EP-CBZ) is a therapeutically active metabolite, which is excreted from the body at approximately 2% of the orally administered dose, and was consequently detected in reclaimed wastewater in a concentration range of 50–120 ng/L (Bahlmann et al., 2014). 10,11-dihydro-10,11-*trans*-dihydroxycarbamazepine (DiOH-CBZ), which is not therapeutically active, is the most common carbamazepine metabolite found in urine (30% of the oral dosage). DiOH-CBZ is the predominant metabolite detected in reclaimed wastewater in concentrations equal to or higher than the parent compound (Leclercq et al., 2009).

Once organic micropollutants are introduced into various water sources, they may undergo structural changes due to the environmental conditions, which alters their behavior in the environment. Upon entering water systems, micropollutants may undergo hydrolysis, adsorption to biomass and sediment as well as biodegradation (Li et al., 2010). Letzel et al. (2008) showed that SMX is resistant to adsorption to sewage sludge and hydrolysis in water. Photodegradation has shown some promise in the removal of SMX in surface waters with direct sunlight, however, this is not a feasible option for treating large volumes of water as only the top layer of water is exposed to direct sunlight (Lam et al., 2005).

The removal of SMX in biological waste water treatment works during anaerobic fermentation has been studied and the degradation of SMX has been linked to the high biomass density of microbial activity in WWTWs. Although some minor removal successes have been achieved with biological degradation in WWTWs, the high microbial load and the conditions present in a WWTWs is not typical of natural water bodies (Drillia et al., 2005). It was also found that the degradation rate constant of SMX was higher with the addition of an

organic carbon source in river systems (Radke et al., 2009). Humic acid, a component present in natural water bodies, has yet to be investigated whether the presence thereof can influence SMX biodegradation.

The physicochemical parameters of the environment may play an important role in the predominant SMX resistant and degrading microbes that is isolated from environmental water samples containing moderate to high levels of SMX. Xu et a. (2011) found that *Bacillus* strains were the predominant SMX degrading bacteria found in a waste water treatment works' return activated sludge, followed by aerobic *Acinetobacter strains.* In another study, *Bacillus* and *Vibrio* strains were isolated as the predominant SMX resistant organisms from shrimp farming ponds (Le et al., 2005). However, *Pseudomonas, Flavobacterium* and *Aeromonas* have also been found to be resistant to SMX. (Akinbowale et al., 2006).

Bacillus was found to be the predominant species with resistance to SMX in environmental water samples and thus the potential to biotransform SMX with a high removal efficiency in environmental water sources (Xu et al., 2011). However, further studies are needed to determine the effect of environmental factors that influence the physiology of possible organisms with the ability to degrade SMX, as well as how these factors can be adjusted and controlled to enhance SMX biotransformation even further. Xu et al. (2011) states that microbial activity plays an important role in the biotransformation of SMX. They found that the degradation of SMX was significantly slower in the sterile control compared to a batch culture of 2 *Bacillus* strains, which implies that the microbes biotransformed SMX. However, the batch cultures required up to 37 days to reach a removal efficiency of 74.7% for SMX, which is an impractical time scale for treatment of large volumes of water, as conventional WWTWs have a retention time of 1-2 days.

The degradation kinetics of SMX vary greatly depending on temperature, as the half-life of SMX in water is five times greater at 4 °C than at 25 °C (Xu et al., 2011). Also, the degradation rate constant of SMX is also higher at increased temperatures. Microbial activity is also higher at 25 °C and the degradation of SMX could also be dependent on catalytic enzyme processes, which is sensitive to temperature (Wolff et al., 1999).

Guo et al . (2014) studied the degradation of SMX by *Phanerochaete chrysosporium* and showed a 53% removal efficiency after 24 h and reached a maximum removal efficiency of 74% at the end of the experiment. It was found that laccase, a lignin-degrading enzyme produced by *P. chrysosporium* was partly involved in the biotransformation process. The

authors suggested that a degradation process using ligninolytic fungi for antimicrobial micropollutants could be considered as an alternative to conventional water treatment processes or as a potential polishing step in these processes.

2.8.2 Micropollutant biotransformation by suspended cell cultures versus mixed community biofilms:

Biofilms pose several advantages over planktonic cells, such as increased resistance to antimicrobial compounds, a wider metabolic range due to aerobic and anaerobic zones in the biofilm structure, inter-species cooperation and horizontal genetic transfer, as well as increased protection from physical forces (Stewart and William Costerton, 2001; Wolcott et al., 2013; Madsen et al., 2012). The physical structure of biofilms may also benefit the degradation of micropollutants, as the three-dimensional structure and the presence of an EPS can increase the retention time of molecules in the biofilm structure (Rice et al., 2005). The EPS matrix may also facilitate improved extracellular enzyme activity and concentrate the micropollutants to levels at which the cells derive more energy from such metabolism than the energy spent to produce the required enzymes for biodegradation and overall maintenance. However, biofilms also pose a challenge in equilibrium dynamics due to the attachment and persistence of the biomass, rather than constant regeneration of biomass in planktonic state, likely acting as a sink until surface liquid exchange reaches saturation/equilibrium.

These characteristics of biofilms can prove beneficial and necessary for the abatement of micropollutants in water. Horvath et al. (1972) found that co-metabolism plays an important role in the biodegradation of organic micropollutants as well as better removal efficiencies by naturally occurring microbial communities than by pure cultures of microbes.

2.8.3 Carbon-starved biofilms for the degradation of micropollutants:

Nutrient limited biofilms or 'hungry biofilms' act differently to biofilms in nutrient rich environments (Rice et al., 2005; Kaplan et al., 2010). This is an important consideration in the use of microbial degradation for the abatement of micropollutants, as most natural water systems and final phases of water treatment systems are usually very nutrient poor. Nguyen et al. (2011) found that in a state of active starvation, biofilms showed an increase in antibiotic resistance. Several antibiotics prove to be persistent to some water treatment processes and are detected in high levels in water systems. The increased resistance of nutrient limited biofilms to antibiotics may be beneficial in the degradation process of

persistent antibiotics. Nutrient starved biofilms prove to be overall more robust than biofilms grown up on high nutrient levels, an additional benefit that these biofilms will be able to withstand harsh environmental changes and fluctuations in water quality and constituents (Rice et al., 2005).

Although nutrient limitation may aid the microbial degradation of certain micropollutants, Kaplan et al. (2010) states that biofilm dispersal is regulated by several factors, including nutrient ques. This might prove problematic, as nutrient limitation triggers frequent dispersal or sloughing events in biofilms, thus altering the degrading capabilities of biofilms. Adequate biomass in biofilms as well as biofilm structure are important factors in the increased degradation capabilities of organic micropollutants by biofilms (Stewart and William Costerton, 2001; Wolcott et al., 2013; Madsen et al., 2012).

2.9 Conclusions:

The primary problem of micropollutant removal, in comparison to the standard wastewater treatment processes removing nitrogen, carbon and phosphorous, is the vast diversity of compounds, the low affinity of bacteria for compounds in Michaelis-Menten kinetics due to the low concentrations at which micropollutants occur. It is estimated that as many as 300 million tons of anthropogenic chemicals makes its way to natural water sources every year (Schwarzenbach, 2006) and with more than 100 000 unique substances registered, the list of detected micropollutants in the natural environment is growing daily (ECHA, European Chemicals Agency, 2019). Keeping in mind the irregularities in the removal efficiency of different compounds using individual treatment processes, the idea of hybrid treatment is gaining popularity. This involves the combination of two or more removal methods to cater for a wider range of micropollutants in terms of degradation (Grandclément et al., 2017).

However, before the combination of treatment processes can be considered, individual treatment processes must be fully investigated and validated to better understand each process's benefits and drawbacks. Different treatment processes vary in removal efficiencies for different micropollutants and thus the combination of treatment modules should be carefully considered to ensure the optimal performance for micropollutant removal.

Chapter 3

Microbial disinfection and micropollutant transformation by Carbon-based ElectroChemical Oxidation (CabECO) technology

3.1 Introduction:

The investigated technology is a Carbon-based ElectroChemical Oxidation (CabECO) process for water treatment applications. Advanced Oxidation Processes (AOP's) for drinking water treatment has been described as early as 2001 (Fryda et al., 2001), and relies on the principle of specifically designed membranes: metal electrodes which are coated with conductive boron-doped polycrystalline sp^3-bonded carbon. When water is passed through the cell connected to a power source, it will promote the formation of strong oxidants. When there is current flow in the cell, an anode reaction favors the generation of hydroxyl radicals, which in turn react with each other to produce ozone.

Other oxidants may also be formed during this process, such as hydroxyl and hydrogen peroxide, but are not thermodynamically favored. Tap water is typically chlorinated and an additional anode reaction can occur where chlorine is transformed to hypochlorite, chlorate and perchlorate (Nishiki et al., 2011). These compounds may exhibit some biological toxicity at high levels, such as in the case in humans, where they interfere with cellular iodine uptake, which may result in carcinogenic effects (Sijimol et al., 2015).

The CabECO cell was initially designed for industrial uses, such as reducing microbial and chemical (COD) loads of industrial effluent, but here the prospective application thereof was investigated for an autonomous and decentralized water treatment system for rural and peri-urban areas, which can hypothetically degrade harmful pollutants and at the same time efficiently eliminate microbial contaminants. However, knowledge is limited on the possible toxic byproduct formation and efficiency of the technology when applied in natural water systems, thus there is a need for more research and in-field testing of the CabECO technology, and more broadly, advanced oxidation.

It should also have a long lifespan, which is relative to the hardness of the water being passed through the cell. Hardness components react with end products of the process and deposits on the electrodes, reducing efficiency over time as these scaly deposits reduce contact area on the electrodes. Regular current inversion minimizes the incidence of scaling, and maintenance would include disassembling the cell and soaking the membrane in a solution of diluted hydrochloric acid (<10% v/v) when efficiency is influenced (Nishiki et al., 2011). This maintenance is still considered simple compared to other water treatment systems. However, the focus of this study is based on the efficiency of the system and not the development of the system.

Two micropollutants, Carbamazepine (CBZ) and Sulfamethoxazole (SMX), were selected for this study on the basis of their widespread use and persistence in the environment (Jankunaite et al., 2017; Chen et al., 2014; Kunkel and Radke, 2012). In a recent review by Archer et al. (2017) on pharmaceutical and personal care products (PPCP's) and endocrine disrupting compounds (EDC's) in South African surface waters, CBZ and SMX were among the most concerning compounds in terms of concentrations at which they were detected. The methods for chemical analyzes of these two compounds are also well validated in our research group. Carbamazepine is a commonly prescribed anti-epileptic drug, with over 40 tons being prescribed in the UK every year (Jones et al., 2002) and Sulfamethoxazole is a top selling broad spectrum antibiotic used primarily in urinary tract infections with over six million prescriptions in South Africa alone (Osunmakinde et al., 2013).

Within this chapter the capacity of CabECO is investigated for two main functions. Firstly, to effectively reduce microbial load in water samples and to investigate the effect of the ozone on cell morphology. Secondly, to break down representative micropollutants that typically persist in environmental waters and waste water treatment works (WWTW) effluent, as well as to reduce the endocrine interaction of some of these chemical pollutants.

Two potential benefits of the ozone produced by CabECO technology were investigated: (1) a reduction in microbial load and persistence, and (2) a reduction in micropollutants. However, with the reduction in micropollutant parent compound, there comes a potential increase in a suite of toxic breakdown products. Thus, this work also investigated the endocrine disruption and toxicity potential of the whole water chemical footprint.

3.2 Materials and Methods:

3.2.1 CabECO performance optimization:

The CabECO cells were connected in a stainless steel piping system (Figure 3-1), where the flow paths could be controlled to let water pass through either one of the cells individually or over both in series. By means of various valves throughout the piping system and a Watson-Marlow 630S peristaltic pump the pressure and flowrate could be adjusted. With the use of an electronic control box (Figure 3-2), the current connected to the cells could be adjusted, as well as the inversion rate. The electronic supply control box had the function to invert the direction of current flow through the CabECO cell, thus preventing buildup of

deposits on the electrodes as the anode and cathode were inverted every 5 min, reversing the action of deposit buildup.

In order to optimize the technology; flowrate, pressure and current were tested at the possible combinations of the gradations summarized in Table 3-1. These combinations were also tested for ozone generation with a single CabECO unit as well as 2 units in series. These parameters were chosen upon recommendations and instructions from the lead application partner in the SafeWaterAfrica project, namely Virtual Consulting Engineers. These parameters were chosen upon calculations to achieve the highest charge density (Ah/L), thus resulting in more oxidants available for reaction. Different sampling points (A, B and C) along the various flow paths allowed for sampling water either directly after passing over either membrane (A, B), or further downstream of both membranes (C).

Table 3-1: Gradations and combinations of the adjustable parameters tested for the optimization of the CabECO system.

40 L/h Flowrate								
Current (Amp)	2		3		4		5	
Pressure (bar)	1	2	1	2	1	2	1	2
80 L/h Flowrate								
Current (Amp)	2		3		4		5	
Pressure (Bar)	1	2	1	2	1	2	1	2
300 L/h Flowrate								
Current (Amp)	2		3		4		5	
Pressure (Bar)	1	2	1	2	1	2	1	2
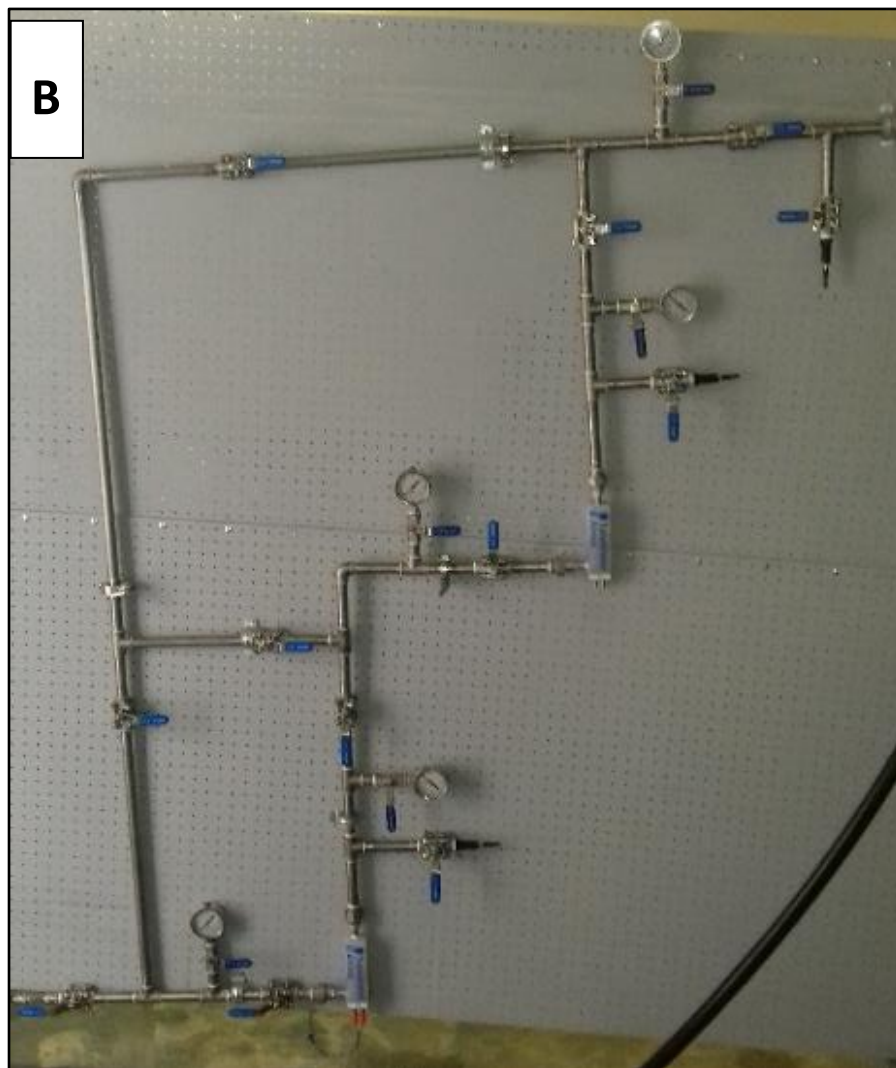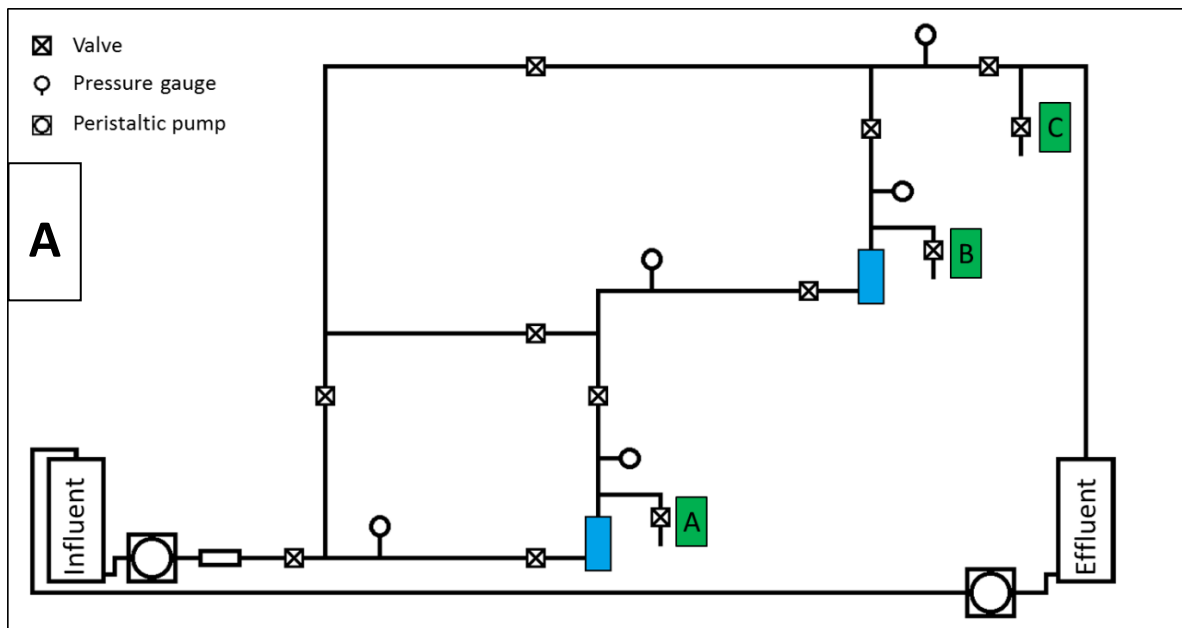
Figure 3-1: A) CabECO laboratory prototype schematic. The position of the CabECO cells is indicated in blue and the various sampling points in green. B) CabECO laboratory prototype.
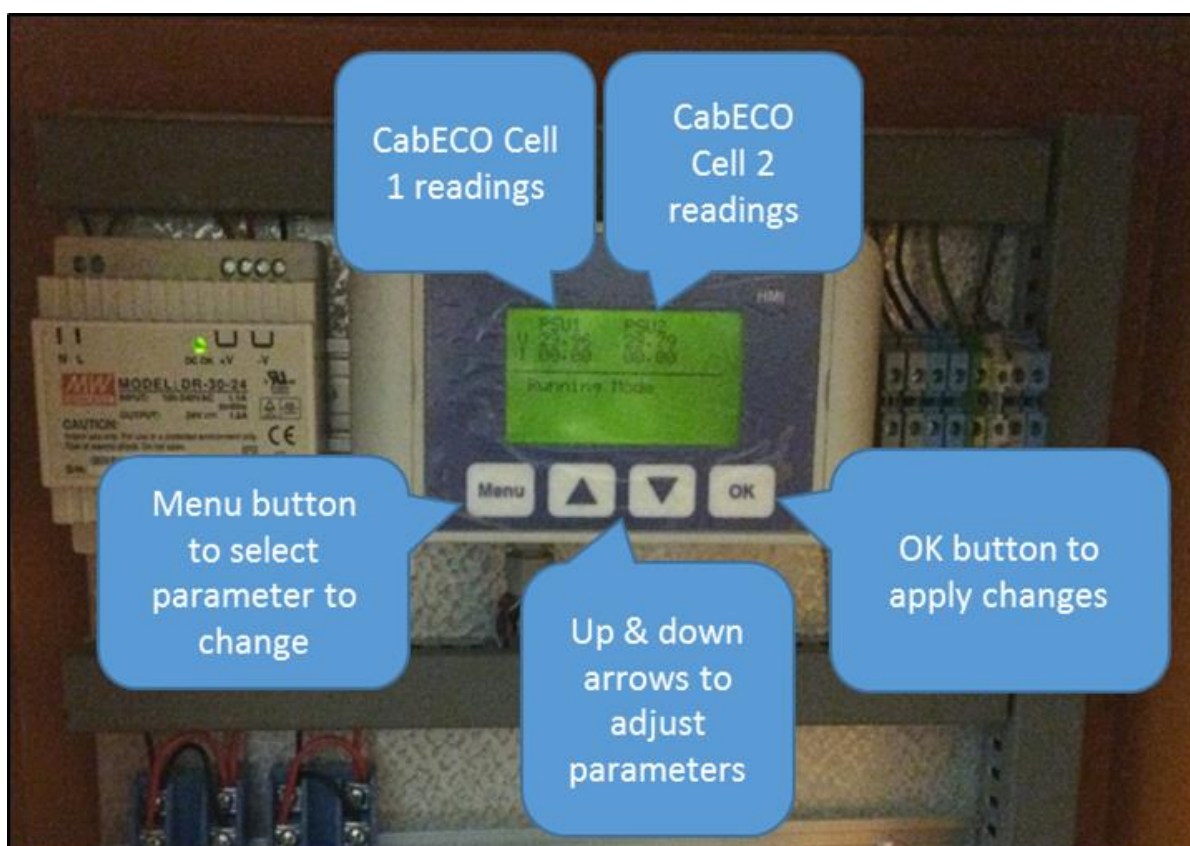
Figure 3-2: Power supply unit of CabECO, designed to adjust and monitor control parameters.

Due to severe droughts in the Western Cape, South Africa at the time of this study, a system of collection tanks and pumps were designed and installed (Figure 3-1) to recycle the water used in these experiments as far as possible. Water collected in the effluent tank was pumped back to the influent tank when uncontaminated water was being used, for instance when water was being cycled through the system to allow for the membranes to reach optimal ozone production, which could take up to 30 min.

Once the system was installed and operational, various experiments were conducted as indicated in the above mentioned summary (Table 3-1) of the adjustable parameters (pressure, number of CabECO cells, flowrate and current) to optimize the formation of ozone in the aqueous phase. Ozone concentration was determined with the Spectroquant Ozone Test (Merck, Germany) by measuring the color change photometrically when ozone reacts with DPD (N,N Diethyl-1,4 Phenylenediamine Sulfate). Additionally, the pH, Electrical Conductivity (EC) and Oxidation-Reduction Potential (ORP) were measured at all the different combinations of parameters.

After optimizing flowrate, pressure, current strength and number of CabECO cells for efficient ozone generation, whilst considering the fact that ozone is highly corrosive and a

health hazard to users at high levels of exposure, all subsequent experiments were run at 60 L/h, 1 bar and 3 Amp, unless otherwise specified. Before any experiments were started, the system was run for at least 30 min with normal tap water to allow for the system to stabilize, as well as monitoring ozone concentration. This resulted in an aqueous ozone concentration ranging from 2-3 mg/L, which is high enough to obtain significant results regarding microbial sterilization and micropollutant degradation, while the ozone concentration is still low enough to not corrode the piping network and damage the actual CabECO cells.

In order to optimize treatment and sampling time windows, green colored (food-grade coloring, Robertsons®, 1 mL/L) tap water was passed through the system to determine how long it would take a sample to reach each sampling point.

3.2.2 Microbial Disinfection by CabECO:

To determine the disinfection capacity of CabECO with regards to real world applications as opposed to distilled water or tap water experiments, water samples were collected according to the sampling procedure of ISO 5667-6 (2010) from two different rivers in the Stellenbosch region, South Africa (Figure 3-3), one unpolluted and the other severely polluted. At each sampling site, dissolved oxygen (DO), pH and electrical conductivity (EC) were measured on site and a collected sample was analyzed for chemical oxygen demand (COD). These conditions were collected at 3 different time points: in the dry season (January), at the start of the rainy season (March) and during the rainy season (May) to confirm expected pollution levels of the two rivers. The less polluted sample was from the upper part of the Eerste River from the Jonkershoek Nature Reserve, before entering the residential area of Stellenbosch. The visibly polluted river sample was from the Plankenbrug River after running past an informal settlement and an industrial business park. Also, domestic shower greywater samples were collected and left stagnant for 2 days, which represented a sample with even higher total organic content (TOC) (visibly turbid and foul smelling).

Figure 3-3: Map of the Stellenbosch region and the 2 river sampling sites indicated by the red stars (-33.940619;18.889808 for Eerste River and -33.931050;18.889808 for Plankenbrug River).

Water was collected at the 2 river sites, Eerste River, Plankenbrug River, as well as shower greywater (10 L of each), each sample was separately passed through the system at 60 L/h and collected at sampling point B (Figure 3-1). The system was run on tap water for 30 min to allow ozone generation to reach optimal levels and then the influent was switched to the respective sample. At 60 L/h the sample would pass through the entire system in less than 40 s and samples presented as treated by CabECO would only have been collected after 2 min to ensure there is no dilution effect from the tap water. This protocol was decided upon since CabECO could not run indefinitely on environmental samples, due to the scale of sample containers and waste disposal volumes that would be required, as well as protecting the CabECO cells against harsh samples. When the influent was switched to the respective sample, 2 min were allowed and ozone concentration was measured immediately at sampling point B after each sample was passed through, as well as the control ozone concentration of tap water to determine the effect of sample pollution level on the measurable dissolved ozone produced by the CabECO cells. Additionally, a 500 mL Schott bottle was filled to the rim (to reduce headspace for ozone to evaporate into) at sampling point B and sealed immediately and left undisturbed for 1 hour to represent an exposure time to the ozone generated over the membrane. After 1 hour the samples were opened, diluted in triplicate (0.9% m/v NaCl) and counted on Tryptic Soy Agar (3 g/L) and Yeast Mold

Agar plates to determine cell concentration (colony forming units (CFU/mL). Optical density (OD) was measured at 640 nm with a Spectroquant (Merck, Germany) before and after treatment for the 3 source waters. Untreated controls were also assessed for cell concentration and OD for river water and greywater.

3.2.3 Scanning Electron Microscopy:

To determine changes in cell morphology of microbes treated with CabECO, treated samples were fixed on filters and subjected to Scanning Electron Microscopy (SEM). In addition to microbial disinfection in terms of cell concentration, the effect of the CabECO system on cellular morphology was studied before and after treatment with the aqueous oxidation products generated by the CabECO membranes.

Samples were collected from the Eerste River, Plankenbrug River and shower greywater as described in the previous section. In addition, a control with two pure strains was prepared, to visualize the effect of ozone exposure on the cell structure of both a Gram positive and Gram-negative organism. Laboratory strains of *E. coli* and *S. aureus* were grown up in 10 % Tryptic Soy Broth (TSB) at 37 °C until the absorbance readings exceeded 0.8 A, correlating to 1×10^7 CFU/mL, as pre-determined with growth curves. Sterile RO water (10 L) was inoculated with 10 mL of the overnight cultures of both *E. coli* and *S. aureus*, resulting in a concentration of 1×10^4 CFU/mL for each of the two strains.

Samples were separately passed over the CabECO membranes, collected in 500 mL Schott bottles and sealed immediately. A residence time of 1 hour was allowed for the ozone to react with the microbes in the samples. Samples (100 mL) were then passed through a 0.7 µm filter and a 10 mm by 10 mm square was cut out and placed in a sterile petri dish. The samples were fixed to the filters using the method as described by Joubert et al. (2017). Briefly, filter blocks were fixed in 2 % glutaraldehyde at 4 °C, then in 0.1 M osmium tetroxide, 3 washes with ddH_2O water, followed by dehydration with increasing concentrations of ethanol (50%, 70%, 90% and 100%). Untreated samples were also prepared and fixed in the same manner, to serve as the controls.

The samples were placed on aluminum stubs by using carbon conductive double sided tape and sputter-coated with a carbon. After the coating the samples were loaded onto a

Merlin Field Emission SEM (Zeiss, Germany) operated at 2–4 kV, using InLens SE (secondary electron) and SE2 detection and a line averaging noise reduction algorithm.

3.2.4 Glassware deactivation:

For the use of glassware in chemical analysis for the micropollutants in question, an experiment was set up to determine the effect of chemical interaction of the micropollutants with the hydroxyl groups on the glass surfaces. A basic protocol in the laboratory for cleaning glassware used in chemical analysis is to rinse glassware thoroughly with MeOH followed by RO water and repeated twice. Glassware treated in this way was taken as the control in this experiment, compared to silanization, which is reported to decrease micropollutant interaction with the chemically charged glassware surfaces.

For de-activation and silanization, glassware was cleaned and completely dried, rinsed thoroughly with 5% DMDCS for 15 minutes, followed by 2 Toluene washes for 1 minute each, 2 methanol washes for 1 minute each and left to dry. Lastly, glassware was once more rinsed with RO water before use (Archer et al., 2017). Different volumes of the chemicals were used for different container sizes, but typically enough liquid was added to the containers to fully coat the internal surfaces when swirled around. For a 1 L Schott bottle, 50 mL of each chemical was added and swirled to contact the entire internal surface.

To compare the effect of glassware deactivation, two 1 L Schott bottles were prepared with MeOH and RO water (control method) and two with the salinization method described above. All the bottles were filled with 1 L dh20, spiked with CBZ and SMX to a final concentration of 500 µg/L and 3000 µg/L, respectively, and left undisturbed for 24 hours to interact with the glass surface. After 24 hours duplicate samples from each bottle were extracted and subjected to LCMS analysis (see section 3.2.8).

3.2.5 Ozone exposure time optimization for micropollutant degradation (CBZ and SMX):

RO water was passed through 2 CabECO cells and collected at sampling valve B (Figure 3-1) in 2 sets of 3 glass 500 mL Schott bottles and spiked with CBZ and SMX, respectively. Passing microbial and chemical contaminants through the system was avoided initially to

preserve the integrity of the membranes while optimizing the protocol. The first set of Schott bottles were filled to the brim with the CabECO treated water, which was analyzed for ozone concentrations (Merck, HC850565) and immediately spiked with CBZ, sealed with caps and left undisturbed for different time intervals (1 min, 15 min, 30 min and 240 min) to determine the efficiency of the technology over different exposure times. The second set of bottles were treated in the same manner with the only exception that they were spiked with SMX. The micropollutants were spiked to a final concentration of 500 µg/L for CBZ and 3000 µg/L for SMX, which is approximately 100 times the environmental concentrations recorded for these compounds in South African waste waters (Archer et al., 2017). This study aims at describing proof of concept, and environmental data was used as a basis to determine these higher concentrations to ensure effect and demonstrate the hypothesis. Future studies can look at lowest thresholds of each phenomenon. An additional 2 sets of 3 bottles (500 mL) were filled to 500 mL with untreated RO water and each spiked with CBZ and SMX to the same concentrations as above. This served as the control in this experiment and is represented as 0 min treatment time in Figure 14. The samples were processed for LCMS analysis as described Section 3.2.8.

3.2.6 Maximum and minimum capacity of CabECO for micropollutant degradation:

After determining the effective length of exposure time, the technology was tested for micropollutant degradation at the maximum and minimum ozone production capacity of the system. The minimum ozone concentration was at 0.99 ± 0.01 mg/L and the maximum ozone concertation was at 4.24 ± 0.27 mg/L. The experiment was repeated as described above with CBZ and SMX but incubated for 1 hour and 4 hours only. In addition, an extra sample was prepared with both SMX (3000 µg/L) and CBZ (500 µg/L) in combination, to investigate how the combined treatment of these compounds result as well the effect of higher concentrations of pollutants.

3.2.7 Micropollutant degradation of environmental waters by CabECO:

After initial success with the CabECO cells for the breakdown of CBZ and SMX, the technology was tested against a broader suite of micropollutants in environmental samples. Water was collected at the 2 river sites, Eerste River, Plankenbrug River as well as shower

grey water (10 L of each), and was passed through the system at 60 L/h and collected at sampling point B. In each case, the system was run on tap water for 30 min to allow the ozone generation to reach optimal levels and then the influent was switched to the respective sample. As described above, colored tap water was passed through the system during optimization to determine how long it would take a sample to reach sampling point B. At 60 L/h the sample would pass through the entire system in less than 40 s and thus samples considered 'treated' by CabECO were collected after 2min, to eliminate any dilution effects from the tap water. This protocol was decided upon since CabECO could not run indefinitely on environmental samples, due to the flow rate, the scale of samples containers and waste disposal volumes that would be required, as well as minimizing the exposure of CabECO cells to contaminants. When the influent was switched to the respective sample, 2 min were allowed and a 1 L Schott bottle as well as a 500 mL Schott bottle were filled to the rim, sealed immediately and left undisturbed for 1 hour (incubation time selected based upon the results of previous experiments). After 1 hour the 500 mL bottle was split into triplicate samples of 100 mL each and then subjected to processing for LCMS analysis of the following compounds: Carbamazepine (CBZ), Sulfamethoxazole (SMX), Acetaminophen (ACM), Benzotriazole (BZT), Diclofenac (DCF), Caffeine (CAF) and Atrazine (ATZ). The 1 L bottle was split into triplicate samples of 300 mL each for the yeast estrogen screen.

To verify detection levels and accuracy of micropollutants in the environmental samples at low concentrations, the experiment was repeated with the modification that all 3 source water samples (river water and greywater) were spiked with a cocktail of the analyzed compounds at 200 µg/L final concentration each and then passed through the CabECO system in the same manner. The addition of the cocktail of micropollutants were done on the basis of anticipating that several compounds will be below the limit of detection, especially in the less polluted Eerste River and the shower greywater that will contain a limited variety of micropollutants.

3.2.8 Liquid Chromatography Mass-Spectroscopy (LCMS):

Solid Phase Extraction (SPE) for LCMS was performed as described by Petrie et al. (2016). Briefly, Oasis HLB 3 cc Extraction cartridges were conditioned with 2 mL of MeOH followed by 2 mL of MilliQ water at a rate of less than 1 mL/min. Samples were collected (100 mL) and the corresponding internal standards were added to a final concentration of 50 µg/L (in

the final eluted sample), before filtering through a 0.7 um filter. The filtered sample was then passed through the cartridge at a rate of no more than 5 mL/min. After allowing for complete drying of the cartridge, the sample was eluted from the cartridge with 4 mL of MeOH under gravity and collected in 5 mL glass test tubes. All glassware used during SPE and subsequent LCMS processing procedures were rinsed with MeOH and distilled water and repeated two times. The eluted samples were dried under nitrogen until completely dry and reconstituted in 500/1000 μL (depending on required concentration of final sample) of MeOH. After resuspension by vortexing, 175 μL of the sample was transferred to polypropylene LCMS vials with glass inserts and subjected to LCMS analysis at the Stellenbosch University Central Analytical Facility (CAF). When samples immobilized on the column were kept for later analysis, after the drying step of the cartridge, it was wrapped in parafilm and aluminum foil and stored in airtight plastic bags at -30 °C.

Samples were analyzed using an in-house UPLC-MS/MS method developed for a Water Research Commission (WRC) project (K5/2733//3; Archer et al., unpublished). An ultra-performance liquid chromatograph (UPLC; Waters AQUITY) was used to acquire chromatography of target analytes. Separation of the target analytes was achieved using de-ionised water (MilliQ) containing 0.1% formic acid (Mobile phase-A) and 100% HPLC-grade methanol (Mobile phase-B). Starting conditions were 100% mobile phase-A which were maintained for 0.2 min and then reduced to 10% mobile phase-A over 6.8 min and to 0% mobile phase-A over 0.1 min. This was returned to 100% mobile phase-A over a period of 0.4 min and maintained for 2.5 min to allow for re-equilibration. The total run time was 10 min. This method used a reversed-phase BEH C18 column (Waters AQUITY, 1.7μm pore size, 2.1 x 100mm) equipped with a 0.2μm in-line column filter. The column temperature was maintained at 50°C. The flow rate of the mobile phases was set at 0.4 ml/min and a sample injection volume of 2μL. The UPLC was coupled with a triple quadrupole mass spectrometer (Xevo TQ-MS, Waters AQUITY) equipped with an electron spray ionisation source. All the analytes were determined using a positive ionisation mode (ESI+). Nitrogen was used as both nebulising and desolvation gas, and argon as collision gas (Archer et al., unpublished).

Acquisition of the LC-MS data was achieved using a multiple reaction monitoring mode (MRM) using two fragment ions for each compound where possible. The optimised MS/MS parameters for the target analytes are shown in Table 3-2. Linearity of a reference standard calibration curve for each target analyte was achieved using a 10-point concentration range

ranging from 1 µg/L to 750 µg/L, in the same solvent as the re-constituted water samples (MeOH). The integration of the analyte standard curves and surface water sample concentrations were determined using the TargetLynx software (Version 4.1, Waters) (Archer et al., unpublished).

Table 3-2: Details of the chromatographic retention times and mass spectrometry parameters used in the LC-MS method to estimate the concentrations of micropollutants in environmental water and laboratory studies samples (Adapted from Archer et al. Unpublished).

Contaminant of Emerging Concern (CEC)	RT (min)	Parent m/z	Daughter 1			Daughter 2			Corresponding Internal Standard
			m/z	CV (V)	CE (eV)	m/z	CV (V)	CE (eV)	
Acetaminophen	2.14	152.0	110.0	20	25	93.0	20	25	Methamphetamine-d5
Caffeine	3.01	195.0	138.0	38	15	110.0	38	23	Methamphetamine-d5
Benzotriazole	3.24	120.0	65.0	30	20	92.0	30	15	Naproxen-d3
Sulfamethoxazole	3.33	254.0	156.0	20	25	147.0	20	25	Sulfamethoxazole-13C6
Carbamazepine	5.21	237.0	194.0	20	25	179.0	40	38	Carbamazepine-d10
Atrazine	5.47	217.0	174.0	25	20	96.0	25	20	Atrazine-d5
Naproxen	5.94	231.0	185.0	20	10	-			Naproxen-d3
Diclofenac	6.66	296.0	250.0	15	15	215.0	15	15	Naproxen-d3
MDMA-d5	2.61	199.1	165.1	20	15	-			-
Methamphetamine-d5	2.71	155.0	92.0	25	15	121.0	25	15	-
Cocaine-d3	3.44	307.0	185.0	10	15	85.0	10	25	-
Sulfamethoxazole-13C6	3.35	260.0	162.0	20	15	114.0	20	20	-
Carbamazepine-d10	5.16	247.0	204.0	30	25	-			-
Atrazine-d5	5.45	222.0	179.0	25	20	101.0	25	25	-
Naproxen-d3	5.92	234.0	188.0	20	10	170.0	20	20	-

3.2.9 Sample preparation for Yeast estrogen screen/Yeast anti-estrogen screen:

The yeast-based screen for estrogenic activity of the samples followed the protocol described by Sohoni and Sumpter (1998). Briefly, a recombinant yeast strain of *Saccharomyces cerevisiae* (obtained from Prof JH van Wyk, University of Stellenbosch), transfected with the human estrogen receptor (hER) gene and a plasmid containing an estrogen response element-linked lac-Z gene, was used. Successful binding of ligands in the water samples (steroids and other EDCs) to the receptors in the yeast cells initiates the expression of the *lac-Z* reporter gene which encode for the enzyme β–galactosidase in the assay. The β–galactosidase then metabolises chlorophenol red galactopyranoside (CPRG), which results in a colour change of the assay medium, indicating a dose-dependent activity of the ligands to bind to the estrogen receptor. Once the chemical components have been extracted (as described below) from a water sample it can be exposed to the recombinant

yeast strain to determine the estrogenic equivalence (representing the potential to disrupt endocrine activity) by measuring the CPRG colour change photometrically.

Solid Phase Extraction (SPE) for the Yeast screens was performed as described by Petrie et al. (2016) for LCMS sample preparation, with minor modifications. Briefly, Oasis HLB 6 cc Extraction cartridges were conditioned with 4 mL of MeOH followed by 4 mL of MiliQ water at a rate of less than 1 mL/min. Samples were collected (300 mL) and filtered through a 0.7 µm filter. The filtered sample was then passed through the cartridge at a rate of no more than 5 mL/min. After allowing for complete drying of the cartridge, the sample was eluted from the cartridge with 6 mL of MeOH under gravity and collected in 10 mL glass test tubes. All glassware used during SPE and subsequent LCMS processing procedures were rinsed with MeOH and distilled water and repeated two times. The eluted samples were dried under nitrogen until completely dry and reconstituted in 600 µL of MeOH. The samples were transferred to polypropylene glass vials and stored for no more than a day at 4 °C before subjected to the yeast screen.

3.2.10 Yeast Estrogenic Screen (YES):

The recombinant yeast strains were inoculated in assay medium which consisted of 45 mL of Minimal medium (Table 3-3), 5 mL of 20 % Glucose solution, 1.25 mL of L-Aspartic acid (4 mg/mL), 0.5 mL of Vitamin solution, 0.4 mL of L-Threonine (24 mg/mL) and 0.125 mL of Copper(II)Sulphate solution (0.319 mg/mL) and incubated for 48 hours on an orbital shaker at 26°C. After incubation, the yeast was inoculated into fresh assay medium at a concentration of approximately 4×10^7 cells/mL and containing 0.5 mL of CPRG (10 mg/mL).

Table 3-3: Medium components for 1 L of Minimal medium for yeast estrogen screen.

13.6 g KH_2PO_4	1.98 g $(NH_4)_2SO_4$	4.2 g KOH
0.2 g $MgSO_4$	50 mg L-leucine	50 mg L-histidine
50 mg Adenine	20 mg L-arginine-HCL	20 mg L-methionine
30 mg L-tyrosine	30 mg L-isoleucine	30 mg L-lysine-HCL

25 mg L-phenylalanine	100 mg L-glutamic acid	375 mg L-serine
150 mg L-serine	1 mL $Fe_2(SO_4)_3$ (0.8 g/L)	1 L dd H_2O
Adjusted pH to 7.1		

Serial dilutions of the samples were prepared and 10 μL of each dilution was transferred to a sterile 96 well flat bottom plate (Costar, 3370, Sigma) and allowed to evaporate.

All samples and concentration factors were analysed in triplicate in the same assay plate. A standard curve for the steroid hormone 17β-estradiol (E_2; CAS 50-28-2; Sigma) was included for each assay plate in 12 serial dilutions as a positive control, ranging from 1.3 to 2724.0 ng/L. Blank wells were also included in each assay plate containing only assay medium without any hormone spike or sample extracts. After evaporation, all the wells on the plate were inoculated with 200 μL of the assay medium containing the CPRG and the yeast inoculum. The final volume of 200 μL in the wells amounts to a diluted final concentration of 1/20 for the samples. The assay plates were then allowed to incubate on a shaker for 72 hours at 30°C under dark conditions.

After 72 hours of incubation, or when an adequate colour change was observed in the dilution range of the E_2 standard curve, the absorbance of the assay plates were read with a plate reader at 570 nm for a colour change of the CPRG and at 620 nm to quantify turbidity to assess cell toxicity.

3.2.11 Yeast Anti-Estrogenic Screen (YAES):

For the YAES assay the same protocol was followed as described in the YES section with the following modifications: All wells in the assay plate (with the exception of blank wells) were coated with an EC_{50} of E_2 (calculated from an E_2 standard curve from the conventional YES assay) with a concentration of 45 ng/L. Two sets of blank wells were prepared, one set with only the assay medium and no samples or steroid hormone spikes and one set with the EC_{50} concentration of the E_2 spike and the assay medium. Instead of an E_2 standard curve,

serial dilution of a Tamoxifen stock solution (7.4303 mg/L) was prepared, which served as the reference standard curve for the YAES (7.4303 mg/L – 3.6281 μg/L). The assay plates were then allowed to incubate on a shaker for 72 hours at 30°C under dark conditions.

After 72 hours of incubation, or when the a balance range of colour change was observed in the dilution range of the Tamoxifen standard, the absorbance of the assay plates were read with a plate reader at 570 nm for a colour change of the CPRG and at 620 nm for turbidity.

3.2.12 Statistical analysis:

Statistical analyses were performed on all data collected during this study. To assess if variation between individual samples were significant, unpaired t-tests were done. For determining significance of variance between various sample means, a one-way analysis of variance (ANOVA) and the Tukey's Honest Significance Difference (HSD) test was performed as *post hoc* analysis. Significant variance was accepted with a probability value of less than 0.05 ($P < 0.05$), indicating that there is sufficient evidence at a 95% confidence interval that differences in sample means was significant.

3.3 Results and Discussion:

3.3.1 CabECO performance optimization:

With the design, build and installation of a new CabECO system (Figure 3-1), came various challenges regarding troubleshooting, optimization and method validation for a standardized method before any subsequent experimentation could begin. The efficiency of the CabECO technology relies on the strong oxidants generated by the electrochemical process and thus optimal ozone generation was the main aim of the optimization studies. Ozone is a highly corrosive and hazardous gas, therefore the desired ozone production had to be performed in a way that would not compromise personnel safety and the integrity of the piping network. Proper ventilation and limited operation times reduced health risks to a minimum and non-corrosive materials (stainless steel and plastic) were used to avoid corrosion of the system.

Optimal operating conditions were determined, and subsequently run with 2 CabECO units in series, at 60 L/h flowrate, 2 bar pressure and 5 Amp, resulting in maximum charge density for the prototype. These settings allowed for a generated ozone concentration of about ±4.5 mg/L on average, sufficient for disinfection and micropollutant degradation. However, the theoretical amount of ozone due to electron transfer efficiency is expected to be higher and the less ozone measured could be attributed to reaction with the materials of the system and some off-gassing of ozone during the sampling process. The flowrate of 60 L/h is towards the lower limits of the system, but 60 L of contaminated waste per hour that had to be dealt with appropriately, already proved challenging, and thus higher flowrates were avoided. The highest settings for pressure and current were selected to ensure high ozone concentrations.

Optimization also included determining the effect of the technology on pH, EC and ORP, to verify that significant observations made was in fact due to reaction with ozone and not influenced by changes in these physicochemical parameters.

Figure 3 shows that as flowrate increases, the ozone concentration in the aqueous phase decreases (One-way ANOVA, $P < 0.05$). The difference in ozone concentration between 80 L/h (1.375 mg/L) and 300 L/h (1,084 mg/L) is however not significant ($P > 0.05$). On the basis of these results, it was decided that all subsequent optimization experiments would be conducted at 40 L/h, facilitating highest ozone generation. At 40 L/h a single CabECO cell

can produce up to 2 mg/L ozone, whereas when the flow is increased to 300 L/h the cell will only be able to produce slightly more than 1 mg/L of ozone. This was expected, as the water that is passed over the membrane has less contact time with the carbon-coated electrodes, thus allowing less time for the generation of energetic compounds such as free radicals from the water molecules (Christensen et al., 2013). The data in Figure 3-4 (A) represents the averages of 5 different CabECO cells, each tested individually. Figure 3-4 (B) shows how the different cells performed individually and the same trend was observed, where ozone concentration decreases with increased flowrate. There was noticeable variation between the different cells, and the two cells that performed best overall were selected for further experimentation.
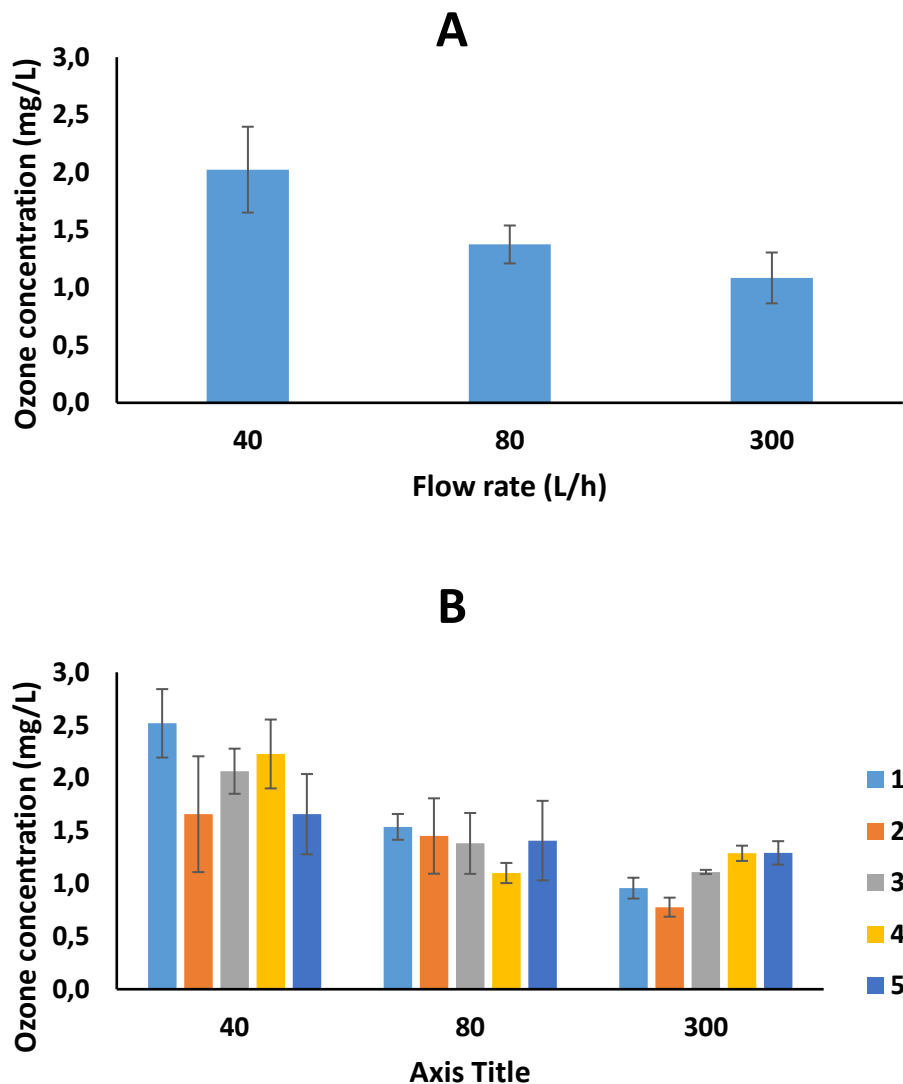
Figure 3-4: Ozone concentration produced by various CabECO cells at different flowrates. A) Is the average of 5 CabECO cells combined and B) is how the cells performed individually.

When 2 cells were connected in series, the ozone produced by the system reached levels of up to 5 mg/L (Figure 3-5) when run at optimal conditions, whereas a single cell only produced about 2.5 mg/L ozone under the same conditions. Two CabECO units generate twice as much ozone as a single unit, but more tests with extra units are needed to confirm linearity between number of CabECO units and ozone generated. As indicated before, this can be explained by the longer exposure time to the carbon-coated electrodes as the water passes over two membranes consecutively, rather than one (Christensen et al., 2013). Thus, more of the water molecules are energized with ozone and free radicals. Along with flowrate and number of CabECO cells, current (2 -5 Amp) and system pressure (1-2 bar) also influences the CabECO cells' capacity to produce ozone when water is passed over the membranes. With each increase in current applied to the cells there was a significant

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increase in the aqueous ozone concentration and even more so when two cells were connected to the system in series (One-way ANOVA, $P < 0.05$). When comparing 2 bar to 1 bar system pressure, more ozone was generated at the higher pressure, even more so at higher current strength and in series (One-way ANOVA, $P < 0.05$). However, single CabECO cells showed no significant differences in ozone concentration between 4 and 5 Amp ($P > 0.05$). At 2 bar there is a significant increase in the aqueous ozone concentration (20 – 25%) at 4 and 5 Amp when using 2 cells. This can be explained by Henry's law (Sotelo et al., 1989) which dictates that the solubility of a gas is directly correlated to pressure, temperature and concentration of the gas. An increase in pressure causes the solubility of ozone to increase and results in higher aqueous ozone concentrations. The higher physical pressure in the system also reduces any diffusion from the aqueous phase into any available headspace. The effect is more profound with increased current and 2 cells in series, as there are more oxidants produced at these conditions.
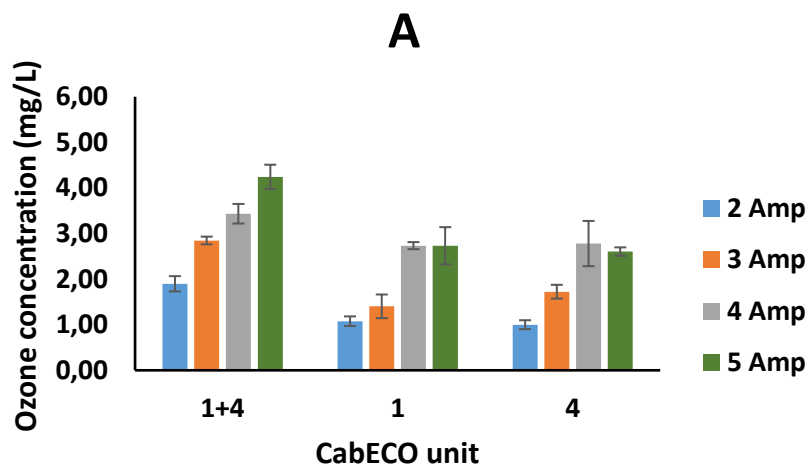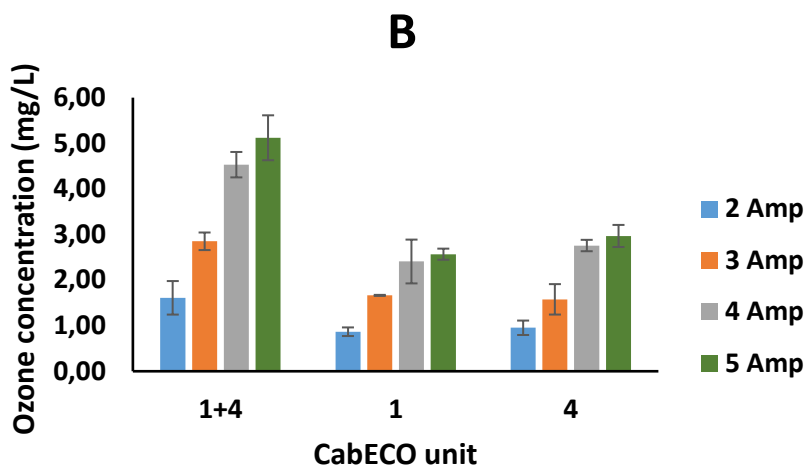
Figure 3-5: The effect of current on the performance of CabECO cells to produce ozone in the aqueous phase at (A) 1 bar and (B) 2 bar.

To further investigate the CabECO technology as an option for water treatment, some of the physical characteristics of the treated water were monitored alongside ozone production, such as pH (Figure 3-6) and EC (Figure 3-7), since these parameters all have an influence on (1) microbial persistence and tolerance to environmental stimuli, and (2) the solubility and partitioning of micropollutants. The control sample of the water that was passed through the CabECO system had an average pH of 6.18. After exposure to the cells, the pH of the samples was slightly reduced to a pH of about 6 at 1 bar and a pH varying between 5.8 and 6 at 2 bar. The changes in the pH with the different gradations, though some are significant (One-way ANOVA, $P < 0.05$), showed no observable trend and further testing is required to determine effect. However, the decrease in pH when pressure was increased from from 1 bar to 2 bar is significant ($P < 0.05$), possibly due to the increased solubility of CO_2 at higher pressure (Haghi et al., 2017). Although these pH changes are significant, a pH change of 0.2 – 0.3 is negligible to report in the scope of this project. Small pH changes such as these will have little to no effect on most microbial life in environmental samples as the small reduction in pH is still well within limits of acidophilic and neutrophilic microbes (Krulwich, et al., 2011), as well as solubilized chemical stability. The slight decrease in pH is also in line with fluctuations in environmental and service provided water. All pH values of treated water samples still fall within acceptable standards for potable water (SANS 241, 1999; Department of Water Affairs and Forestry, 1996).
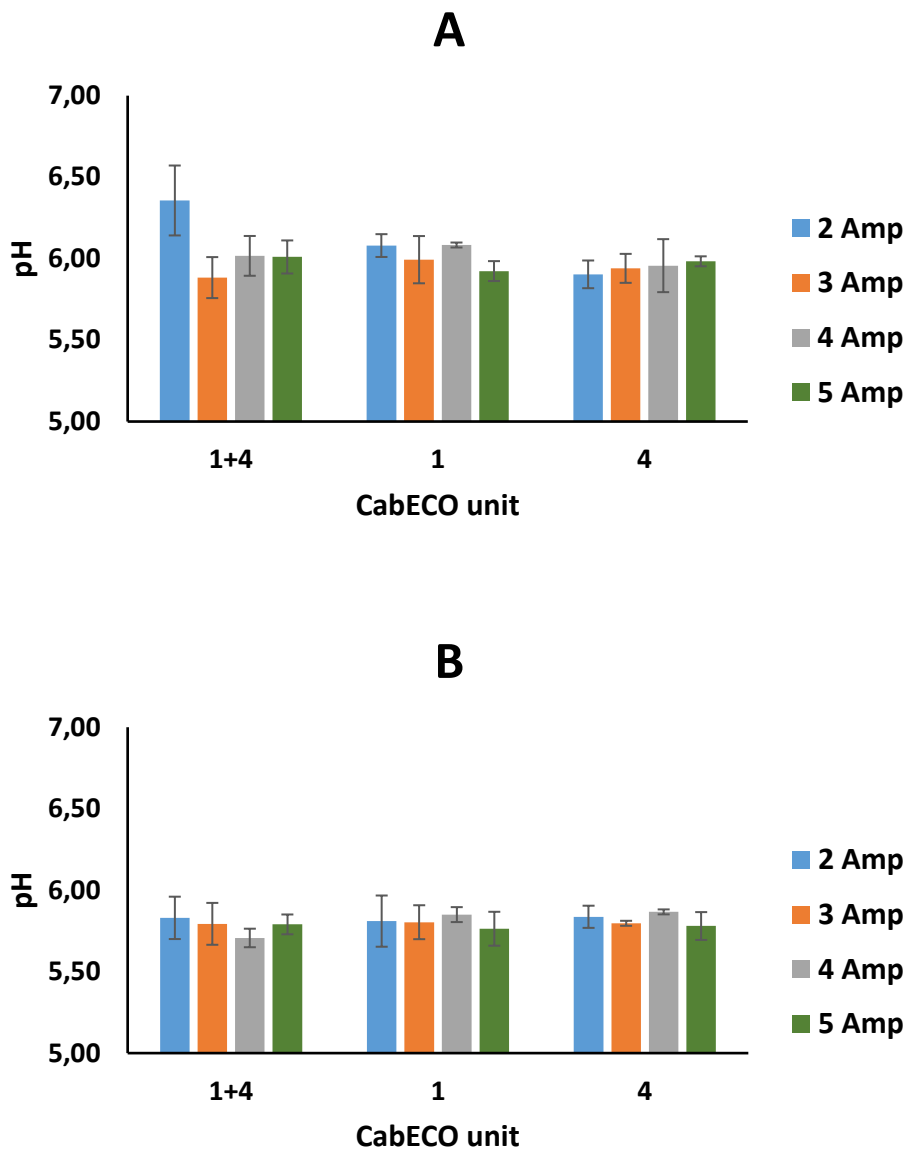
Figure 3-6: pH changes of water treated with the CabECO technology at A) 1 bar and B) 2 bar pressure.

The average EC of the control sample was 2.5 (±0.5) mV/m. There were varied results in EC of CabECO treated water, while variation between the current gradations were insignificant (One-way ANOVA, $P < 0.05$). However, when two CabECO units were combined, there was a slight increase in EC. There was however significance in the variation between 1 bar and 2 bar pressure for CabECO unit #1 ($p < 0.05$). CabECO treatment had little to no effect on the electrical conductivity of the water treated.

A

B
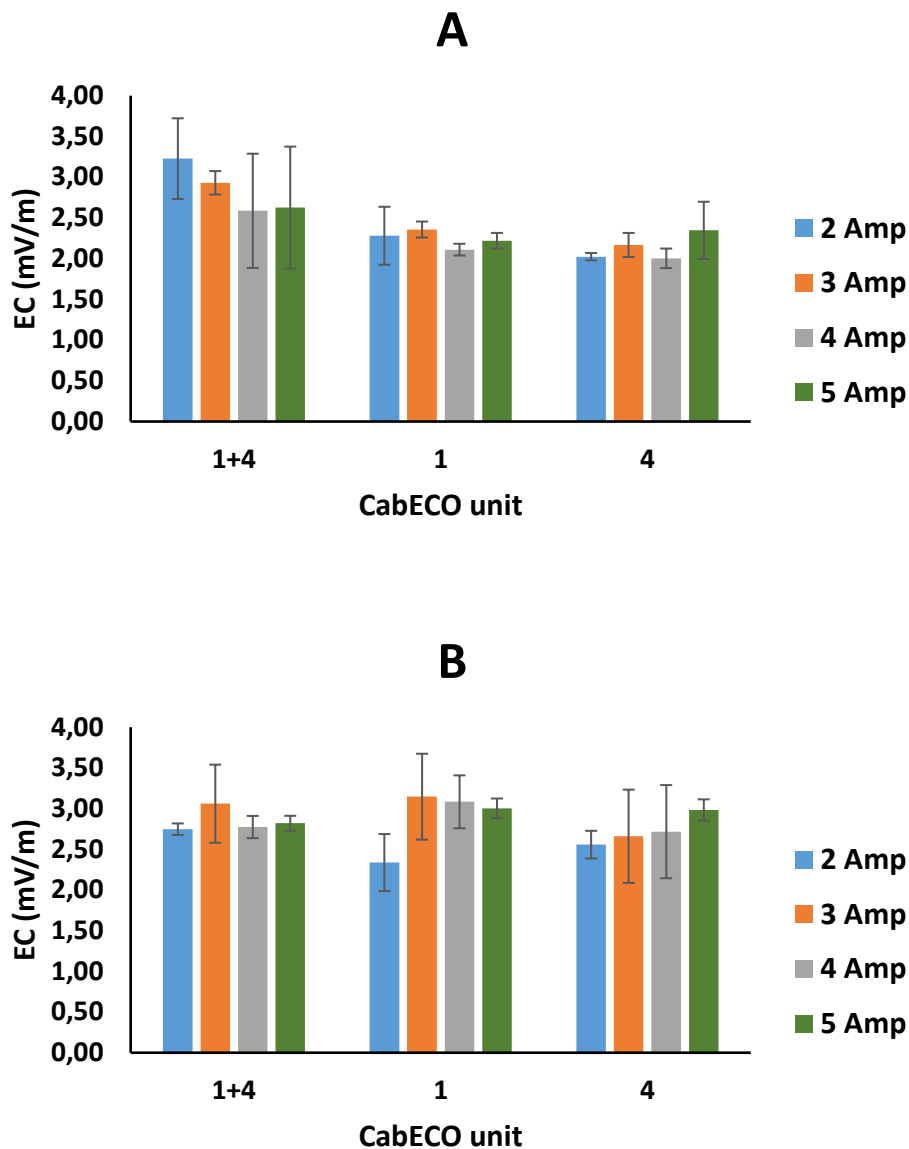
Figure 3-7: Changes in electrical conductivity (EC) of water treated with the CabECO technology at A) 1 bar and B) 2 bar pressure.

Ozone is a strong oxidant, thus the generation thereof in the aqueous phase will lead to a sharp increase in the oxidation reduction potential (ORP). Newman (2004) suggests that an ORP of 650 mV is the minimum threshold for disinfection of contaminated water. Chlorine is generally used as the final disinfection step of water treatment and ozone has an oxidation potential of 1.5 relative to chlorine (Newman, 2004). When measuring ORP during the optimization period of the CabECO system, inconclusive results were obtained. With increased ozone generation, an increase in ORP is expected, although the ORP measurements did not follow such trend (Figure 3-8 A1 and B1). ORP values that were well below the 650 mV threshold were measured even though the ozone concentration measured (Figure 3-5) was higher than what is known to be needed for disinfection. The

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less than expected ORP can be explained by the rapid off-gassing of ozone as soon as the sample was taken to insert the ORP probe. Initially ORP would be in excess of 1000 mV but would decrease rapidly to below 400 mV before a stable measurement could be taken. This is supported by the fact that when sampling from the system, a strong odour of ozone could be smelled as soon as the sampling tap was opened and furthermore supported by the rapid decrease in ORP after 10 min (Figure 3-8 A2 and B2). Due to the inconsistent ORP measurements, statistical analysis at this point would have proven inconclusive. An inline ORP probe would improve the accurate quantification of ORP of the CabECO system, but the high levels of ozone measured was nevertheless sufficient for disinfection and micropollutant abatement.
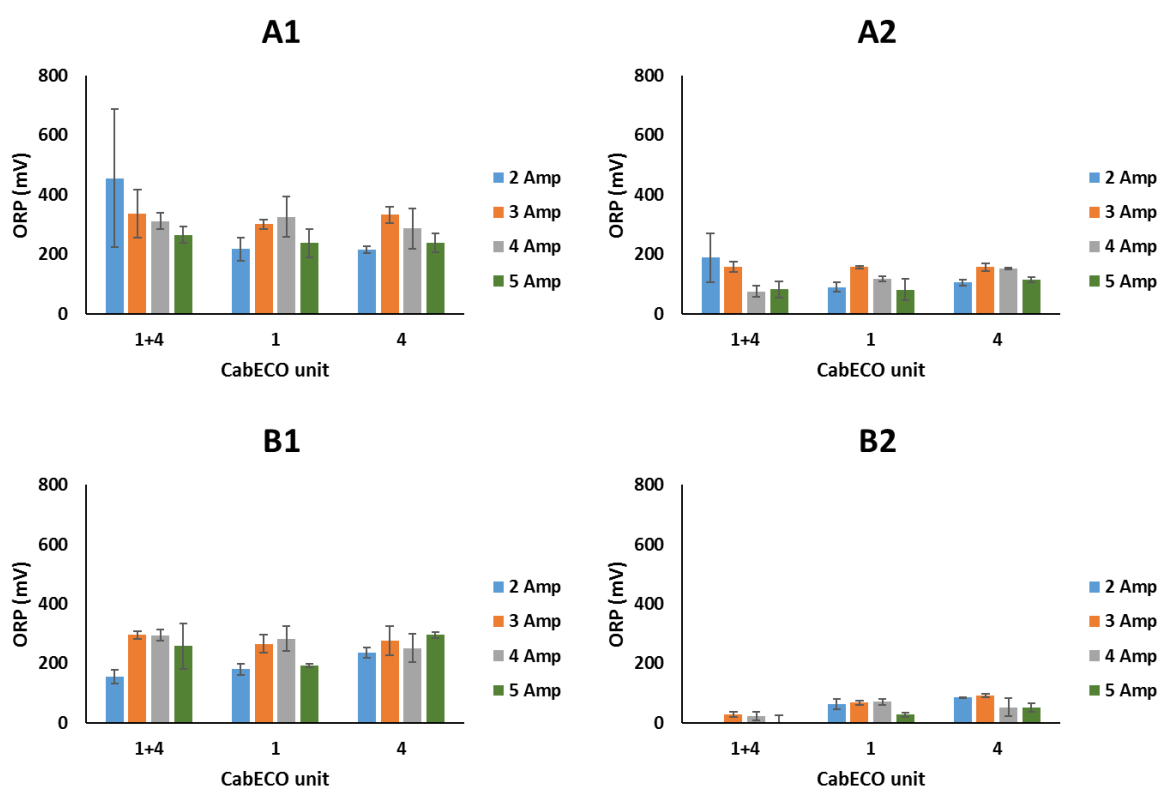
Figure 3-8: Changes in the oxidation-reduction potential of water treated with the CabECO technology at A1) 1 bar immediately after exposure, A2) 1 bar 10 min after exposure, B1) 2 bar immediately after exposure and B2) 2 bar 10 min after exposure.

3.3.2 Disinfection by CabECO:

Ozone concentrations measured in the CabECO system (Figure 3-9) were reduced significantly (One-way ANOVA, $P < 0.05$) as the organic load levels of the samples increased (Table 3-4). The measured ozone concentration was reduced from 1.53 ± 0.13

mg/L in the tap water control to 0.57 ± 0.02 mg/L for the shower greywater (highest TOC). The same effect was seen between the two environmental river samples, where higher ozone concentrations were achieved in the Eerste River, compared to the Plankenbrug River samples. The higher COD, EC and lower DO of the Plankenbrug River in comparison to the Eerste River could be the reason for the decreased ozone concentration measured in the Plankenbrug River sample. This observation can be ascribed to the higher concentration of pollutants that bind to the highly reactive free radicals generated and act as a sink, decreasing the ozone available for measurement. This is important to note as the efficiency of the system to reduce microbial load and break down chemical pollutants will be reduced as the pollution level of the sample increases. As the ozone binds to the high concentrations of complex molecules, the ozone available to target microbes and micropollutants is lower, thus increased ozone concentrations will be necessary to achieve the same level of sterilization in water systems with high COD and complex pollutant sources.

Table 3-4: COD and physicochemical parameters of the two river sampling sites over 3 time points.

		January	March	May
Plankenbrug River	**COD (mg/L)**	331.2 ± 201.1	423.7 ± 118.3	194.0 ± 103.8
	DO (mg/L)	1.06	1.38	2.50
	EC (µS)	686.07	737.90	587.07
	pH	6.60	6.45	6.99
Eerste River	**COD (mg/L)**	40.8 ± 3.7	45.7 ± 8.5	75.3 ± 55.9
	DO (mg/L)	9.65	7.85	10.45
	EC (µS)	71.58	76.15	85.60

	pH	7.53	7.67	7.33
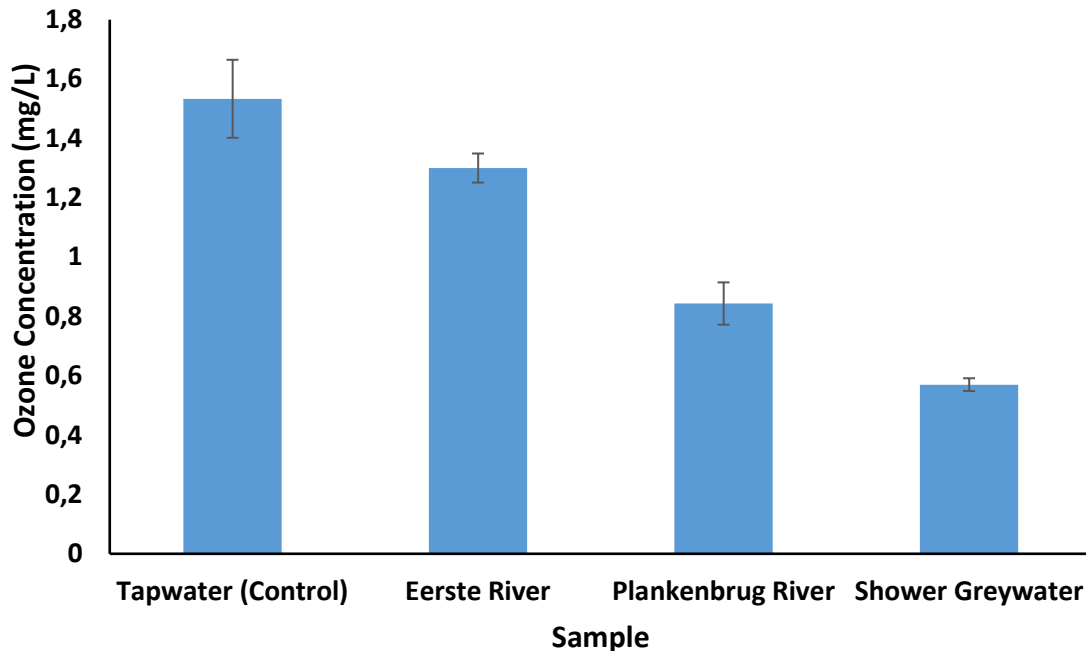
Figure 3-9: Aqueous ozone concentration produced by CabECO when treating 3 different environmental water samples: Tap (Control), Eerste River (<polluted), Plankenbrug River (>polluted) and Greywater (Shower).

CabECO was effective in substantially reducing the microbial load in all 3 types of samples; the mildly polluted Eerste River, the severely polluted Plankenbrug River and the shower grey water. Shower greywater was added as a sample as greywater storage and re-use was a common occurrence in the Western Cape with a severe drought in the area at the time of this study. Household greywater was collected and stored for flushing toilets, watering plants, etc. In contrast to potable water, complete sterilization is not necessary, but odour control and contaminant reduction are desirable. The CabECO cells effectively reduce the microbial load in all 3 sample types by more than 97% (Figure 3-10) compared to the initial untreated samples, which resulted in a log 2, log 3 and log 3 reduction in the Eerste River, Plankenbrug River and shower greywater samples, respectively. The treatment of the Eerste River water resulted in a reduction of 1.48×10^3 to 4.3×10^1 CFU/mL and in the Plankenbrug River of 1.48×10^5 to 2.2×10^2 CFU/mL. It is important to note that even with a log 3 reduction in CFU/mL for the shower greywater, that the initial cell concentration for this sample was very high at 1.0×10^7 CFU/mL and still had 2.0×10^4 CFU/mL left after treatment

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with CabECO. Even though the more polluted samples would deplete more of the aqueous ozone, there was still ozone left to reduce the microbial load detectable by these methods. It is expected that the high COD that is associated with polluted water samples would scavenge all the ozone, effectively reducing the ozone concentration to 0 mg/L before it has interacted with any of the target pollutants, in this case microbes. The ozone concentrations reported here was measured within 1 min of taking the sample, as per instruction of the ozone concentration kit. If the sample was left out for a reasonable time, the ozone concentration would be much less, due to the reaction with the COD. It is important to consider that COD is determined after a 2 hour digestion step at 148 °C to free any COD that is bound to organic matrices and thus, many of the ozone molecules won't be scavenged by the COD immediately and some ozone molecules would be free to interact with the free floating microbes in the water. Although there is a significant reduction in the microbial loads of the water samples, there are still several orders of magnitude of microbes present in the water after ozone treatment and the system is not entirely effective in disinfecting environmental water samples.
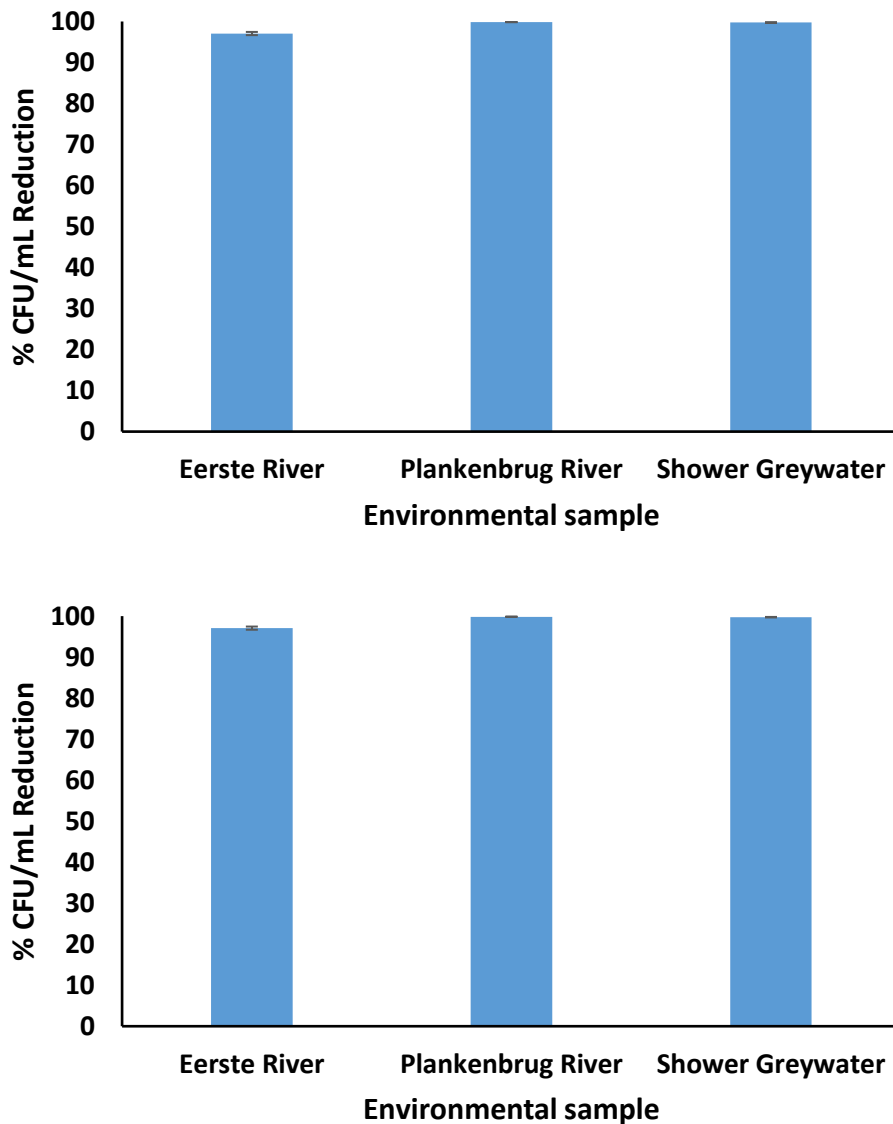
Figure 3-10: The efficiency of CabECO for microbial disinfection for 3 different environmental samples; The Eerste River, Plankenbrug River and Shower Greywater determined on A) TSA and B) YM plates.

Scanning Electron Microscopy (SEM) was used to visually investigate the effect of CabECO-produced ozone on microbial cell morphology. Figure 3-11 A show *S. aureus* cells prior to ozone treatment. The cells are intact, with a round shape characteristic of *S. aureus* and appear to be viable (Henriques et al., 2009; Thanomsub et al., 2002). However, the sample treated with ozone (Figure 3-11 B) shows cells with irregular cell shapes, the round structure is lost and the circled section in Figure 3-11 B shows a *S. aureus* cell that has been ruptured by the treatment of ozone. Notably, although the cell survival experiments showed a 95% reduction in colony forming units, microscopy shows that some cells appear undamaged and intact post-treatment, correlating with survival studies and demonstrating that the treatment parameters are not 100% effective. Even with the high percentage reduction in microbial contaminants, there is several orders of magnitude left after treatment with ozone

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from the CabECO system. Strong oxidants such as ozone oxidize amino acids in cell membranes and denature membrane proteins, causing disruption to the ultra-cellular structure and eventually causing the cell to lyse and be destroyed (Thanomsub et al., 2002) (César et al., 2012). Glucosamine, a compound present in bacterial peptidoglycan, reacts with ozone and may lead to disruption of the cell wall (Khadre et al., 2001).
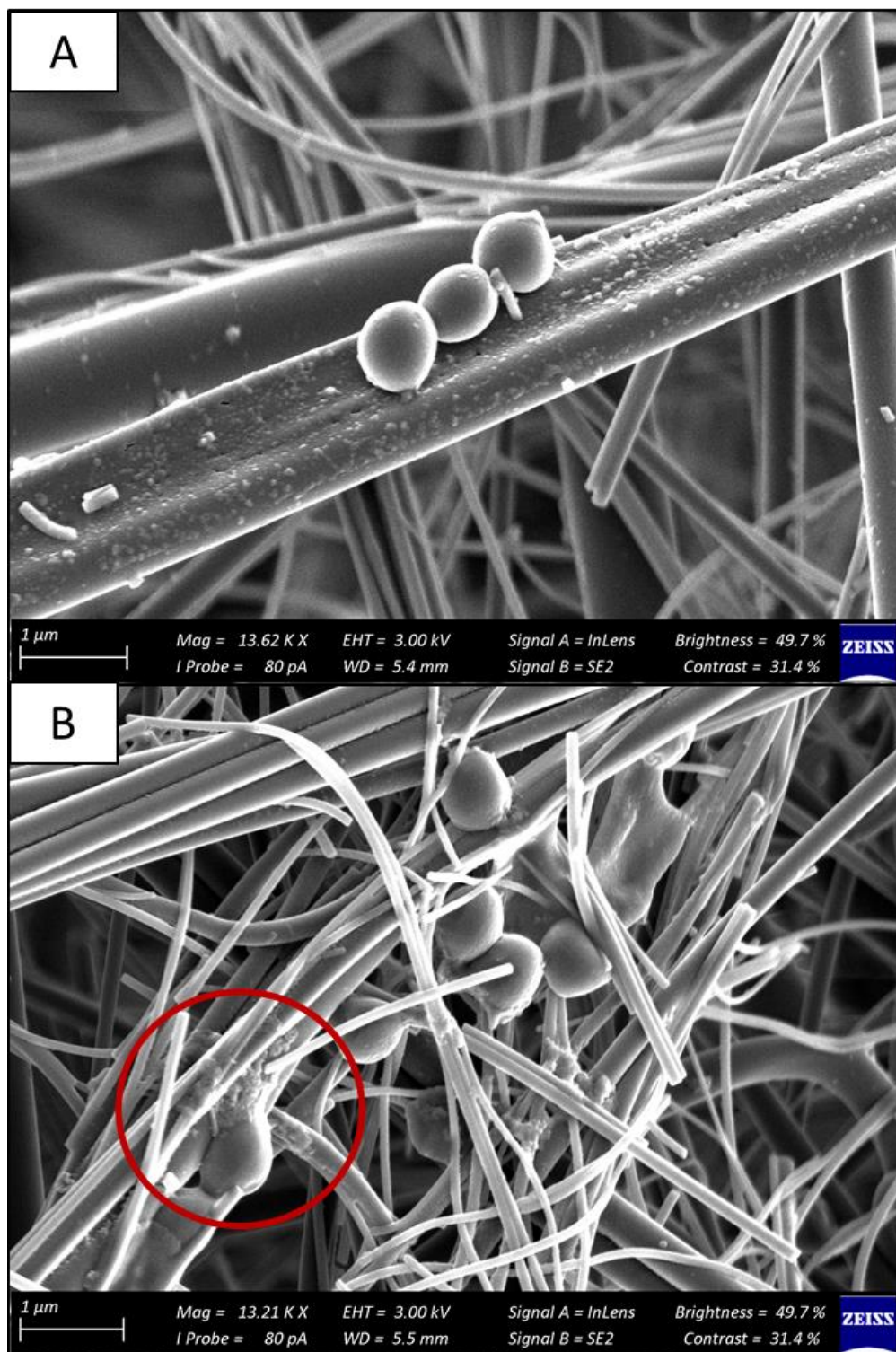
Figure 3-11: SEM images of S. aureus before (A) and after (B) ozonation for 1 hour (damaged cell circled).

SEM of environmental samples from Plankenbrug River and Eerste River water did not show individual cells, but rather large clusters of microbial cells (Figures 3-12 A and 3-13 A) imbedded in organic mass, correlating with the pollution level data in Table 3-4. This can be expected, as flocs or surface associated biofilms will tend to form in natural waters rather than suspended, individual cells. Figure 3-12 A shows how the clusters of cells in the untreated Plankenbrug River sample are thicker and more frequent than the treated sample (Figure 3-12 B) which are dispersed, smaller and less regular. The cells in the untreated sample appear in a thick matrix of numerous cells, whereas the treated sample shows a lack of the thick clusters, with only a few cells that survived the treatment. The same observation can be made from the SEM images from the Eerste river sample (Figure 3-13). The large clusters of cells as in the untreated samples were qualitatively less prevalent after ozonation. Although some cells still remain and damaged or disrupted cells are difficult to be distinguished from the heterogeneous matrices, the reduction in occurrence, along with the significant reduction in colony forming units (Figure 3-10) confirm that CabECO effectively reduced the microbial load of the environmental samples, by disrupting cell structure and heterogeneous cellular-EPS masses. Ozone is also a widely used method for disrupting biofilms, as demonstrated by Dosti, et al. (2005).
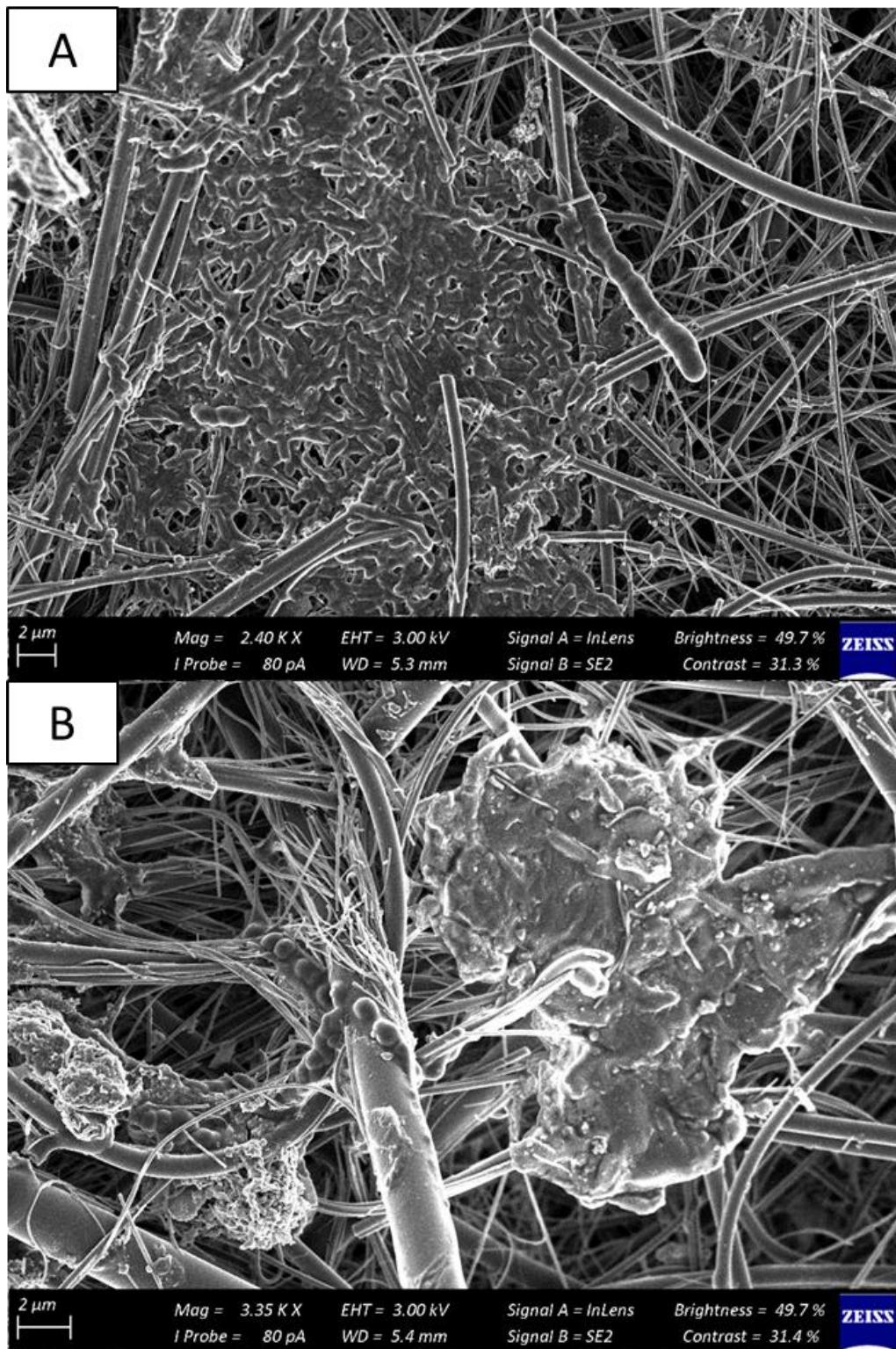
Figure 3-12: SEM images of untreated A) untreated Plankenbrug River water and B) treated Plankenbrug River water. Note the difference in magnification as indicated by the scale bar.

A

2 μm	Mag = 8.62 K X	EHT = 3.00 kV	Signal A = InLens	Brightness = 49.5 %
	I Probe = 80 pA	WD = 5.5 mm	Signal B = SE2	Contrast = 31.4 %

ZEISS

B

2 μm	Mag = 2.55 K X	EHT = 3.00 kV	Signal A = InLens	Brightness = 49.9 %
	I Probe = 80 pA	WD = 5.5 mm	Signal B = SE2	Contrast = 31.7 %

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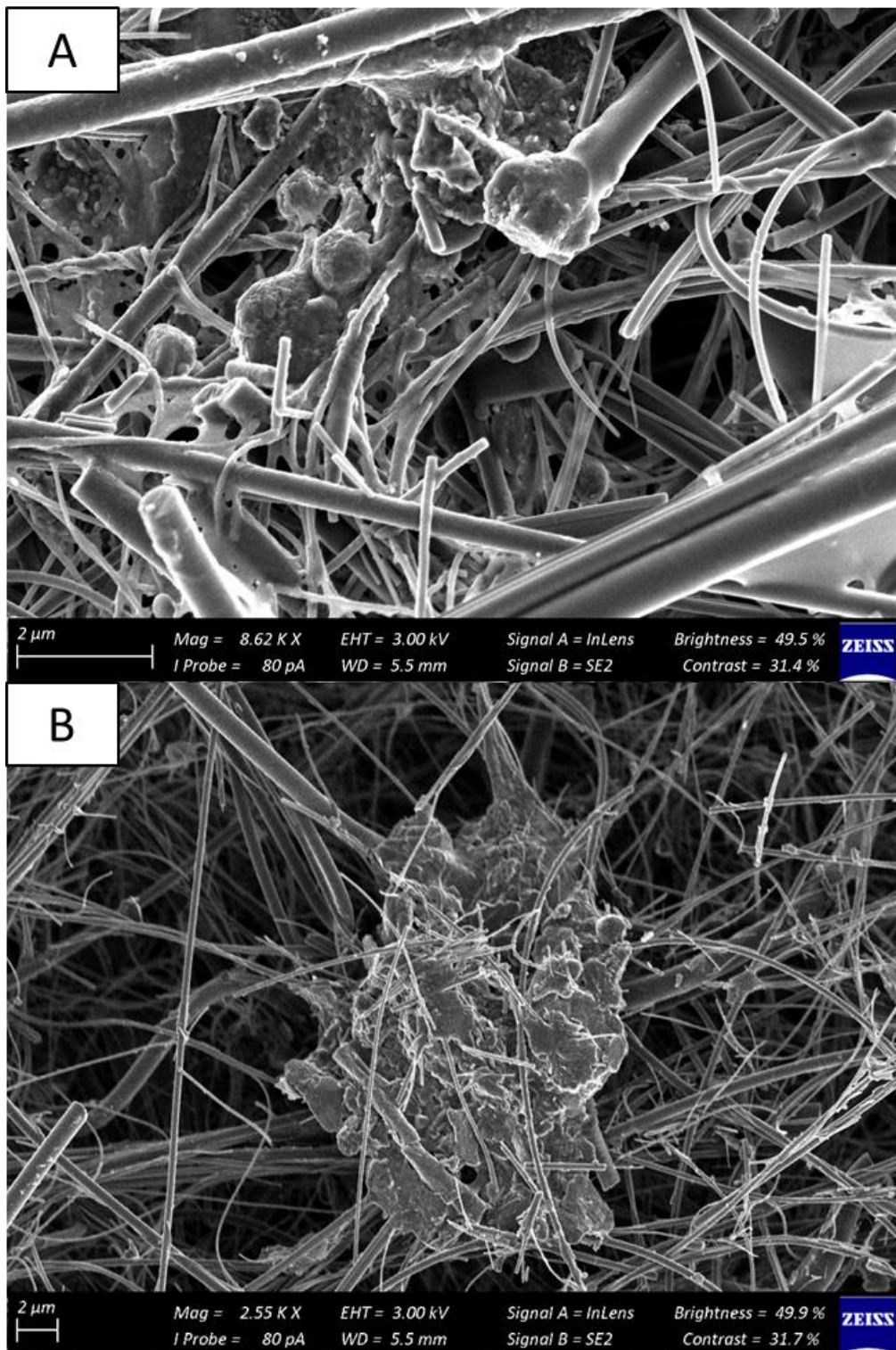
Figure 3-13: SEM images of CabECO treated A) untreated Eerste River water and B) treated Eerste River water. Note the difference in magnification as indicated by the scale bar.

3.3.3 Glassware deactivation:

The impact of ozonation on micropollutants is much less than its impact on microbial cells. In order to assess micropollutant stability during CabECO treatment, the binding of micropollutants to glassware first had to be determined. In addition, the effect of silanization (the process of covering the mineral components of glassware with organofunctional alkoxysilane molecules to prevent the binding of the pollutants of interest) was assessed. It is an expensive and time-consuming process, with a large chemical waste impact and thus the necessity of glassware silanization was assessed to decide whether to include it in the Standard Operating Procedure (SOP). The control and silanized samples had no significant differences in the concentration measured after the 24 hour incubation period for both CBZ and SMX (Figure 3-14) (One-way ANOVA, $P < 0.05$). Similar experiments in our research group were conducted in previous studies, where the adsorption of micropollutants onto glassware surfaces was investigated. Comparable results were found, that a negligible amount of the relevant compounds, in comparison to the measurement range of interest, binds to the Si-OH groups on the glass surfaces (Stone, 2017, unpublished).

Measured concentrations of both CBZ and SMX were less than what was spiked with, possibly due to losses during sample processing for LCMS analysis. The percentage recovery of the corresponding internal standards accounts for these losses. Although this loss was not ideal, these results were only integrated qualitatively and not quantitatively and conclusions could still be made with a good degree of certainty, as both CBZ and SMX showed a 0% loss from the control glassware and the desalinized glassware. All experiments in this study were done at relatively high concentrations and no conclusions were made from small changes in the concentrations of the micropollutants, except when stated otherwise.

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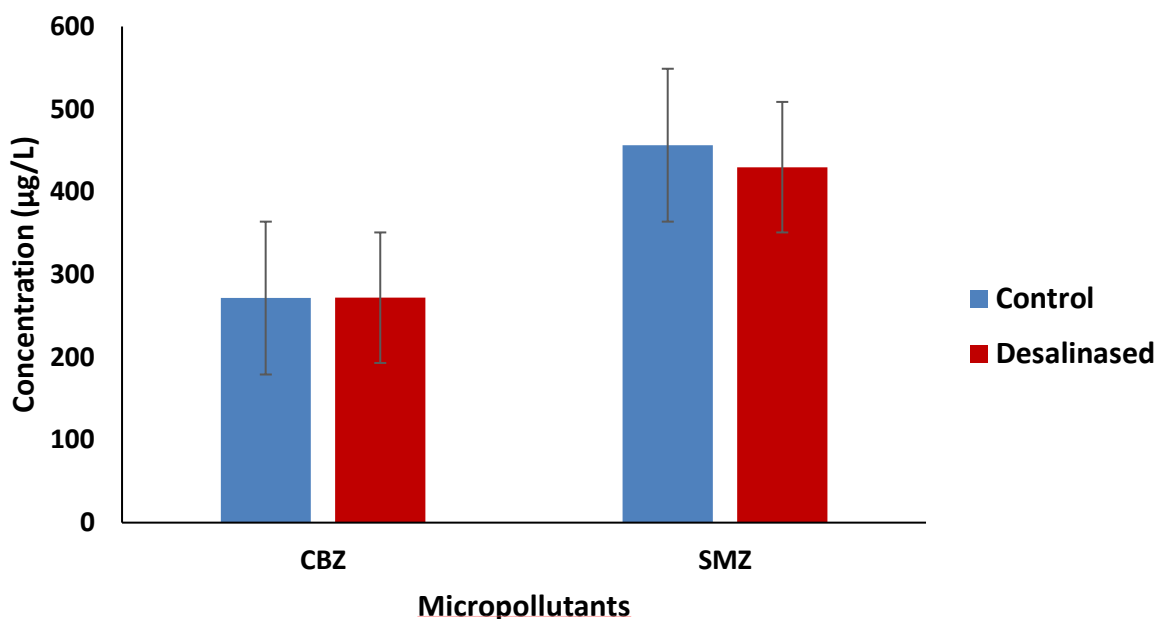
Figure 3-14: The effect of glassware deactivation on the concentration of two micropollutants, CBZ and SMX, in glass containers versus control glassware.

Taking this into consideration, any adsorption onto the glassware could be considered negligible for CBZ and SMX at the concentrations used in this study. These results, especially when duplicated, served as motivation to save time and reagents, and importantly, reduce the amount of toxic chemical waste. All glassware for subsequent experiments for chemical analysis therefore used the methanol method.

In a review by Petrovic in 2014, it is stated that the physical and molecular properties of compounds differ greatly and glassware adsorption may have a profound effect, depending on which pharmaceuticals are being investigated. Various studies suggest different sample containers, ranging from silanized glassware, High Density Polyethylene (HDPE) containers and amber glass bottles, depending on the compounds of greatest interest (Suri et al., 2011; Baker and Kasprzyk-Hordern, 2011; Vanderford et al., 2011). In the study by Baker and Kasprzyk-Hordern (2011), the percentage recovery from silanized and non-silanized glassware were tested from 27 compounds, where some of the compounds, especially those containing amines, had significant binding to the glass surfaces and others not. The current project primarily studied CBZ and SMX, and thus silanization was not relevant, however, for the larger suites, previous studies show it might have been relevant for caffeine, Benzotriazole and Acetaminophen (Baker and Kasprzyk-Hordern, 2011).

3.3.4 Ozone exposure time optimization for degradation of CBZ and SMX:

The first experiment to test CBZ and SMX degradation showed promising results, in terms of a reduction in the concentration of parent compounds. The ozone generated by CabECO was highly effective in breaking down the parent compounds of CBZ and SMX (Figure 3-15) with an effective reduction of both compounds from the shortest exposure time (1 min). This can be expected as ozone is an oxidant and the reaction thereof will be immediate with any susceptable compound (Guo et al., 2018). Different treatment times of up to 30 min had no changes in the ability of CabECO to almost completely remove CBZ and SMX from the samples. A one-way ANOVA inferred a 95% confidence interval, that all the treated time points differed significantly from the 0 min exposure time.

For micropollutant abatement, the action of ozonation is driven by the oxidation of the compounds with the ozone and/or hydroxyl radicals that can be formed by the decomposition of ozone with water constituents (von Sonntag and von Gunten, 2012). Ozone is one of the strongest oxidizers, however it is a selective oxidant and favours the reaction with electron-rich compounds such as phenols, olefins, reduced sulfur groups and deprotonated amines. In contrast, hydroxyl radicals are less selective and react freely with almost all types of organic materials. Very little hydroxyl radicals are generated in the ozonation process harnessed by the CabECO technology (Guo et al., 2018) and therefore, the majority of the micropollutant abatement in this study can be ascribed to the oxidation by ozone, although hydroxyl radicals can also contribute to some small extent (Nishiki et al., 2011).

It is well known that the ozonation of CBZ and SMX effectively reduces their concentration in various types of waters. For CBZ, various studies show 75 – 100% removal efficiency, depending on the method of ozone exposure and treatment time (20 min maximum) (Doll and Frimmel, 2005; Hua et al., 2006; Pereira et al., 2007). Good removal effeiciency of SMX was reported in literature with removal ranging from 88 – 100%, but SMX required longer exposure times overall in comparison with CBZ, with treatment times reaching up to 60 min (Abellan et al., 2007; Gonzalez et al., 2007; Dantas et al., 2008). In contrast to these studies, where SMX may require longer exposure times for degradation, we report an immediate (1 min) almost complete degradation of SMX. This might be due to the high concentrations of SMX these studies used in their reseach, whereas we worked with lower concentrations of SMX, closer to environmental levels. The CabECO cells seem to have the advantage of very high removal efficieny within a very short period of time in comparison to other technologies

(Table 3-5). The CabECO cells showed >96% removal efficiency for both CBZ and SMX after 1 min of exposure time. Possible reasons for CabECO being such a good competitor for micropollutant abatement is due to the direct contact with the ozone generating membrane, as the CabECO cell is designed to increase turbulence over the membrane as the water is passed through the cell. Additionally, CabECO has the advantage of harnessing the ozone in the aquoues phase and therefore not losing any reactive capacity by having to transfer ozone gas from the gas to aquoues phase.

Table 3-5: Comparisons between different technologies for the abatement of CBZ and SMX.

CBZ			
Type of Treatment	**Percentage Removal**	**Treatment Time**	**Reference**
CabECO	97.5%	1 min	
TiO$_2$ Photo-catalysis	99%	90 min	Carabin et al., 2015
Solar photo-Fenton	96%	45 min	Miralles-Cueuvas et al., 2014
E-Beam Radiation	85.4%	5 min	Zheng et al., 2014
SMX			
CabECO	96.7%	1 min	
E-Beam Radiation	88.7%	5 min	Zheng et al., 2014

TiO₂ Photo-catalysis	98%	30 min	Nashuhoglu et al., 2011
UV-C Photolysis	99%	10 min	Nashuhoglu et al., 2011
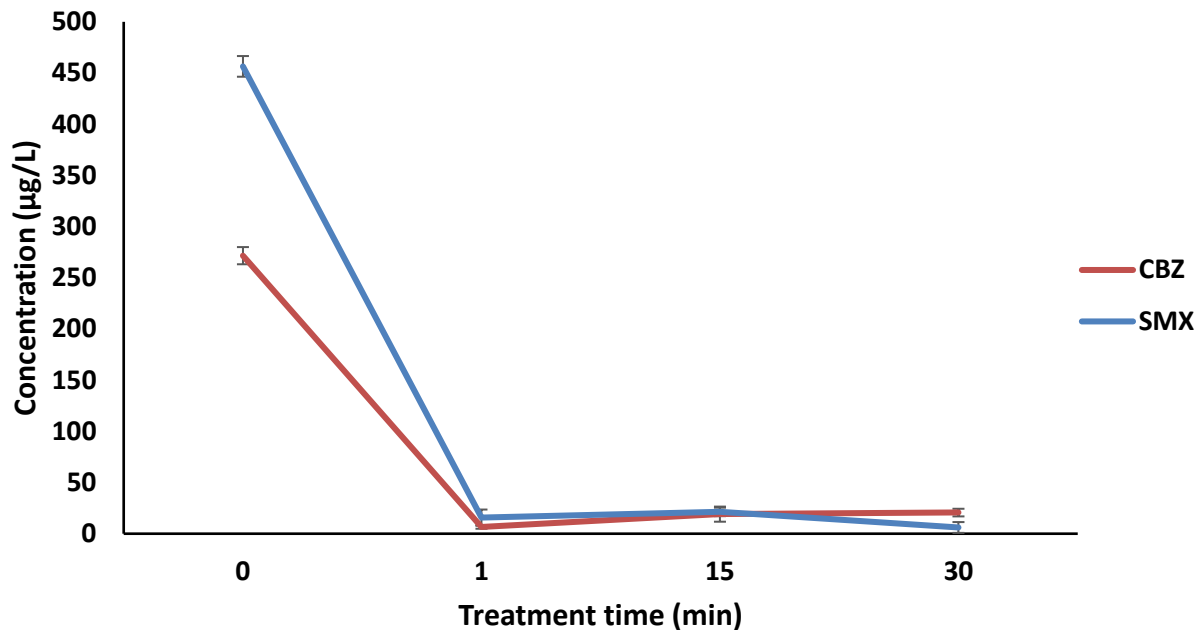
Figure 3-15: The degradation of CBX and SMX by the CabECO system at various treatment times.

3.3.5 Maximum and minimum capacity of CabECO for micropollutant degradation:

With CabECO being a system with adjustable parameters (flowrate, pressure and current strength) to control the generated ozone concentration, it was assessed whether the minimum ozone generation could remain effective in micropollutant abatement. Thus, the lower and upper limits of the technology were compared, in order to evaluate how to run subsequent analyses. Ozone is a hazardous and highly corrosive gas and the health and safety of personnel as well as the integrity of the CabECO system should be considered (Khadre et al., 2001). Defining the minimum effective ozone concentration would therefore be advantageous. Figure 3-16 A and B show that at lowest settings of CabECO (resulting in 0.99 ± 0.01 mg/L ozone), removal efficiencies of both CBZ and SMX are significantly less in comparison to the high dose of ozone from the highest settings (4.24 ± 0.27 mg/L ozone concentration) (One-way ANOVA, $P < 0.05$). As in the previous section (section 3.3.4), there

is little difference between 1 and 4 hours of exposure time for CBZ and SMX ($P > 0.05$). Minimum ozone production resulted in only about 20 % removal efficiency for SMX and almost a 0 % reduction in CBZ concentration, whereas at maximum ozone production 80 – 90% reduction is showed for both CBZ and SMX. The decrease in removal efficiency is due to decreased ozone production, and micropollutant molecules in excess of the ozone molecules. The ozone is depleted in oxidation reactions and the majority of the compounds remains in the sample. Similar studies also found that higher ozone concentration is linked to better removal efficiencies (Klavarioti et al., 2009). It is important to note that when the two pollutants were combined, resulting in a higher concentration of compounds overall, the efficiency of the CabECO system was significantly compromised, even at the maximum ozone generation settings, when compared to the removal of single compounds at maximum ozone generating settings ($P < 0.05$). The same effect was observed in environmental samples with increasing pollution levels (Figure 3-9 and Table 3-4) which reduced the freely available ozone, by the increasing levels of organic load. More pollutants deplete the ozone molecules, despite the assumption that ozone is far in excess of micropollutant concentrations in the samples, and results in a percentage of the pollutants remaining unchanged. This could also be due to the fact that the molecules combine in a form that protects from oxidation, although unsupported and limited literature exist on how CBZ and SMX associate chemically.
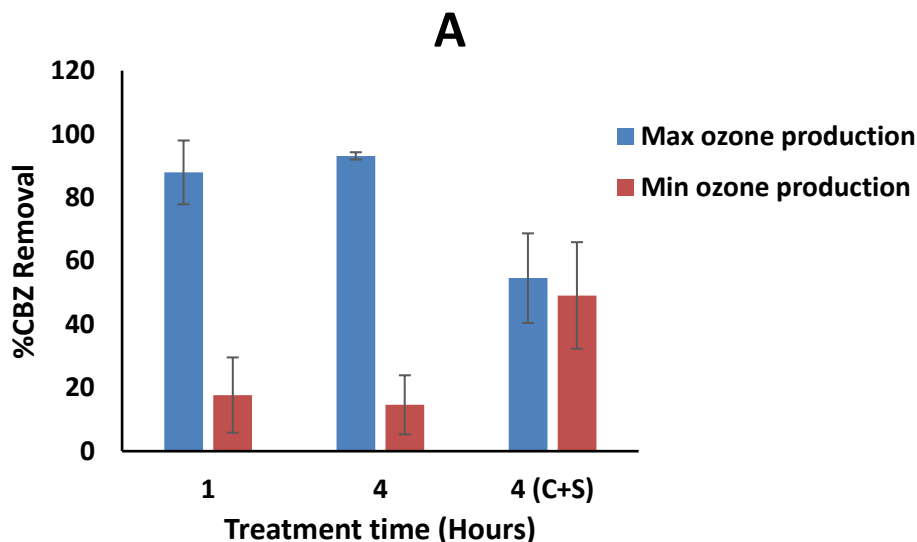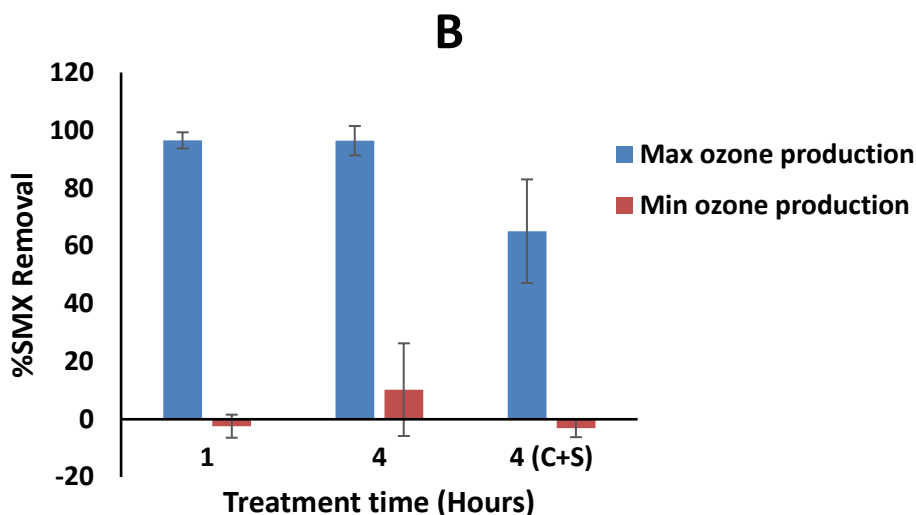
Figure 3-16: The percentage removal of A) CBZ and B) SMX by CabECO at maximum and minimum ozone production over different treatment times as well as the percentage removal of each when in combination with each other.

With this information on the degradation of parent compounds, the question arise: what are the transformation products? However, with the increasingly large and diverse suite of pollutants and degradation products this question becomes redundant, complex and expensive. CBZ alone has more than 30 known human metabolites and up to 13 predicted transformation products from ozonation (Bahlmann et al., 2014; Lee et al., 2017). Bahlmann et al. (2014) reports that some of the metabolites of CBZ remain therapeutically active and some have a higher eco-toxicological concern than CBZ itself. SMX can be metabolized into 5 major metabolites and a predicted 6 ozonation transformation products with potential for increased toxicity (Radke et al., 2009; Gómez-Ramos et al., 2011).

In light of the complexity and expenses associated with tracking individual parent compounds' and transformation products' fate post treatment, it was decided to rather investigate the toxicity of the mixtures of daughter and transformation products. In the next section, this is assessed by concentrating the total micropollutant footprint of the water sample via an Oasis HLB 6 cc column, pre- and post-CabECO treatment, and exposing it to the Yeast Estrogen and Anti-Estrogen Screen. The potential of the micropollutant footprint to disrupt the endocrine pathway transformed into the yeast is assessed as an indicator of the toxicity of the sample, as these endocrine effects have been associated with a number of health indices. These endocrine disruptors have been linked to decreased fertility, increased incidence of cancers, birth defects, increases in spontaneous abortions and

several physiological disorders in various organisms (mostly aquatic) (Soto and Sonnenschein, 2010; Sharma, 2011; Archer et al., 2017).

In this work, the yeast anti-estrogen screen produced the clearest, statistically significant results indicating the effect of the technology on the micropollutant footprint of water samples, and was thus selected as the technique of choice. Figure 3-17 shows the results of the yeast anti-estrogen screen for both CBZ and SMX when treated by CabECO for different exposure times to the generated ozone, trapped in a retention vessel. Before treatment with CabECO, CBZ shows an anti-estrogenic effect relative to the EC_{50} of the E_2 spike (a pre-determined amount known to disrupt endocrine activity, measurable in the yeast assay), inhibiting the expected estrogenic response of the E_2 spike. When CBZ is exposed to ozone for 1, 5 and 60 min, the anti-estrogenicity thereof is slowly decreased as the CBZ is being degraded, until CBZ exhibits no estrogenic/anti-estrogenic effect at all after 240 min of ozone treatment. The differences in anti-estrogenicity of CBZ between consecutive exposure times shows little significance ($P > 0.05$), however, when 0 min exposure is compared to 60 min and 240 min exposure time, the decrease in anti-estrogenicity is significant ($P < 0.05$). This is in contrast to figure 14, which demonstrates an almost complete removal (98% removal efficiency) of CBZ after only 1 min. It is evident that even though the parent compound is removed, the transformation products can still be active and have an effect on the life and health of aquatic organisms and ultimately humans (Archer et al., 2017).

Prior to CabECO treatment, SMX also showed an anti-estrogenic effect relative to the EC_{50} of the E_2 spike, where SMX is hindering the expected 100% estrogenic response of the E_2 spike. However, after only 1 min of ozone exposure, there was a statistically significant change is the estrogenicity/anti-estrogenicity of the sample containing SMX, compared to the untreated sample (One-way ANOVA, $P < 0.05$). After ozone treatment, SMX no longer interferes with the E_2 response, but rather shows >100% estrogenicity relative to the E_2 spike, meaning that this sample can now elicit a hormonal response by binding to the hER on the recombinant yeast cells. Again, as with CBZ, we reported a near 100% removal efficiency of SMX after 1 min of exposure to ozone (Figure 3-15), where in reality the transformation products of this process appear to remain active and requires longer treatment times to eradicate an estrogenic response. With increased exposure to ozone, SMX becomes slowly less estrogenic until at 240 min exposure, where SMX has very little hormonal activity left. It is important to note that the differences in estrogenicity relative to the different treatment times (1 – 60 min) are insignificant ($P > 0.05$), however, relative to

the E_2 spike, there is significant changes ($P < 0.05$) in estrogenicity when comparing the different exposure times.

From these results we can conclusively report that CabECO is an effective system for the degradation of CBZ and SMX from water samples as well as the reduction of anti-estrogenic responses of micropollutants in the yeast estrogenic screens. However, it is only effective if the ozone concentrations reported here can be reproduced at industrial scale, which seems improbable. This data supports the implementation of a residence column in the final design of an operational CabECO system for adequate reaction time for the ozone with contaminants.
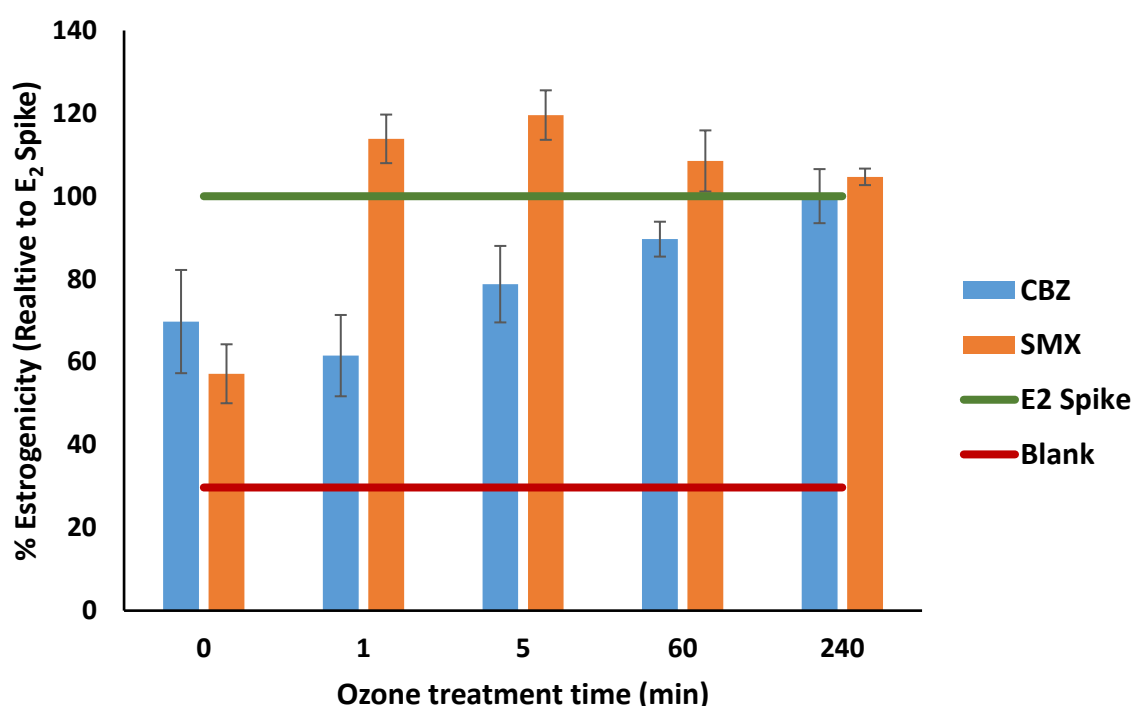
Figure 3-17: Estrogenic effect of CBZ and SMX relative to an EC_{50} E_2 spike, over different exposure times (0 – 240 min) to ozone generated by the CabECO technology.

3.3.6 Micropollutant degradation of environmental waters by CabECO:

CabECO showed promising results for the abatement of CBZ and SMX in controlled laboratory experiments. The technology was tested against environmental water samples, as envisioned for application, and was shown to be less effective as the pollutant load is increased. Environmental samples have high loads of dissolved organic compounds (DOC) as well as a broader suite of micropollutants (Archer et al., 2017), which as mentioned above

might prove persistent despite ozone exposure, due to their chemical structure and properties (Guo et al., 2018).

Figure 3-18 shows good removal of CBZ, ACM and SMX, with removal efficiencies upward of 90 % for all three of these compounds in all three types of environmental samples. However, in shower greywater, quantified as the most polluted sample in terms of microbial load and capacity to deplete the generated ozone (Figure 3-9 and Figure 3-10), even with good removal efficiencies for CBZ, ACM and SMX it became increasingly difficult to obtain good reproducibility, with higher standard deviation, whereas in the less polluted samples variation was lower.

Some of the analyzed compounds had low reproducibility and consistency between the different types of samples. Overall, BZT, DCF, CAF and ATZ were persistent during ozone exposure, with the highest removal percentages only reaching 30% and the lowest no removal. ATZ was the least sensitive to ozone as it showed very little to no removal. Hua et al. (2017) also found ATZ to be a persistent compound during ozone treatment of a river in Canada.

With environmental samples, the methodological challenges of generating accurate chemical analysis results increase significantly as various components in the samples influence the analytical procedures. Different types of matrices are in the water, other chemical pollutants and metabolites/by-products of the analyzed compounds are but a few of the factors that could cause challenges (Petrovic, 2014). Background noise and the potential for cross-measurement of compounds with similar column residence times are both potential risks introduced with environmental analyses of complex matrices. In addition, the effect of residual ozone on the internal standard concentrations remains a potential risk, as internal standard percentage recovery post LCMS analysis is influenced, thus jeopardizing the quantification of sample losses during extractions and processing for analysis. This is evident in Figure 3-18 as variation became high and conflicting results are present for some of the more persistent compounds. DCF, CAF and ATZ show negative removal for some of the samples, although inconsistent between the different sample types. Sorption of micropollutants onto solid matrices is a well-known phenomenon and is exploited to waste water treatment to aid in micropollutant removal from the aqueous phase (Torresi et al., 2017). The observed negative removal might be due to release of the compounds from organic matter in the samples which it adsorbed to, as the organic matter is being degraded

by the ozone treatment. Organic matter is removed prior to measurement by filtration, and thus these results report primarily on purely soluble compounds in the aqueous phase.
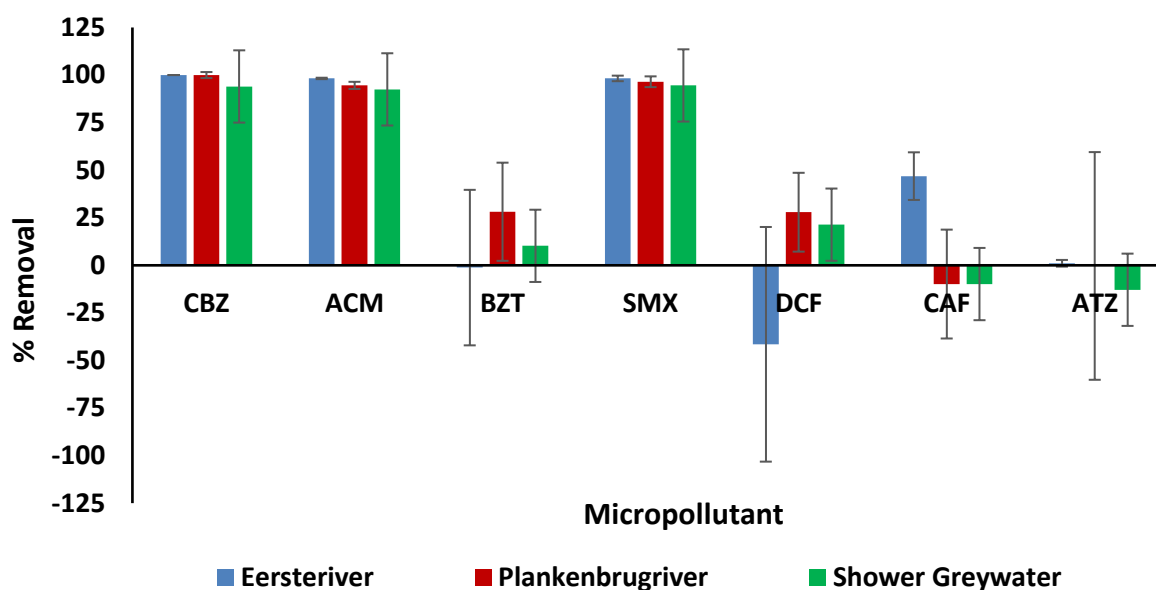
Figure 3-18: CabECO removal efficiency of a broad suite of selected micropollutants in environmental water samples; Eerste River, Plankenbrug River and Shower Greywater.

The same samples analyzed for micropollutant removal, as shown in Figure 3-18, were used in a yeast anti-estrogen screen to determine the estrogenic effect of an environmental sample before and after ozone exposure for 1 hour. Figure 3-19 shows that the Plankenbrug River is about 130% estrogenic relative to the E_2 spike, before ozone treatment. After ozone exposure generated by the CabECO technology, estrogenicity remained relatively stable with no reduction in estrogenicity (one-tailed T-test, $P > 0.05$). Even though various compounds were removed by the CabECO technology (Figure 3-18) in the Plankenbrug sample, it can be expected that some estrogenicity will remain as several compounds were resistant to ozone degradation. Furthermore, it was reported previously (Figure 3-15 and Figure 3-17) that even though CBZ and SMX were almost completely degraded after 1 min exposure time to ozone, that these compounds needed up to 240 min retention time for complete inactivation relative to the yeast estrogenic screen.

Environmental samples may contain naturally occurring estrogens as well, such as phyto-estrogens produced by plants and myco-estrogens from fungi, which may also contribute to the estrogenicity of the untreated sample (Kuiper, 1998).

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The inability of CabECO to effectively reduce estrogenicity of the Plankenbrug sample can further be explained by the release of potentially estrogenic compounds from organic matter being disturbed and degraded by the CabECO process. Toressi et al. (2017) states that river biofilms have a high affinity to sorb organic micropollutants and from the microbial disinfection section above, the conclusion can be made that biofilm structure and flocs are disturbed by the CabECO process, thus opening the possibility that some compounds could have been released during exposure and contributing to the estrogenicity of the sample. When the sample was diluted by half, estrogenicity decreased, not adding to the estrogenicity of the E_2 spike, identifying a limit of detection in this assay. Although the diluted sample did not show effect on the yeast assay in this study, there is numerous studies implying the negative health effects of chronic low level exposure to EDC's (Kuiper, 1998; Swart et al., 2011; Swart and Pool et al., 2007).

In Figure 3-19, where the Plankenbrug River sample was spiked with the cocktail of micropollutants (200 μg/L each), the initial estrogenicity almost reached 200%, effectively being twice as potent as the EC_{50} E_2 spike. This is expected as the high concentrations of the spiked micropollutants would be mostly in the aqueous phase and contribute to the overall estrogenicity of the samples. In the spiked sample, CabECO treatment significantly reduced the estrogenicity of the sample (one-tailed T-test, $P < 0.05$), although the sample still exhibited a strong estrogenic response. Longer exposure to ozone may better degrade contributing compounds until no effect relative to the yeast estrogen screen is seen, as observed with both CBZ and SMX requiring up to 4 hours of ozone exposure to completely have no effect on estrogenicity (Figure 3-17).
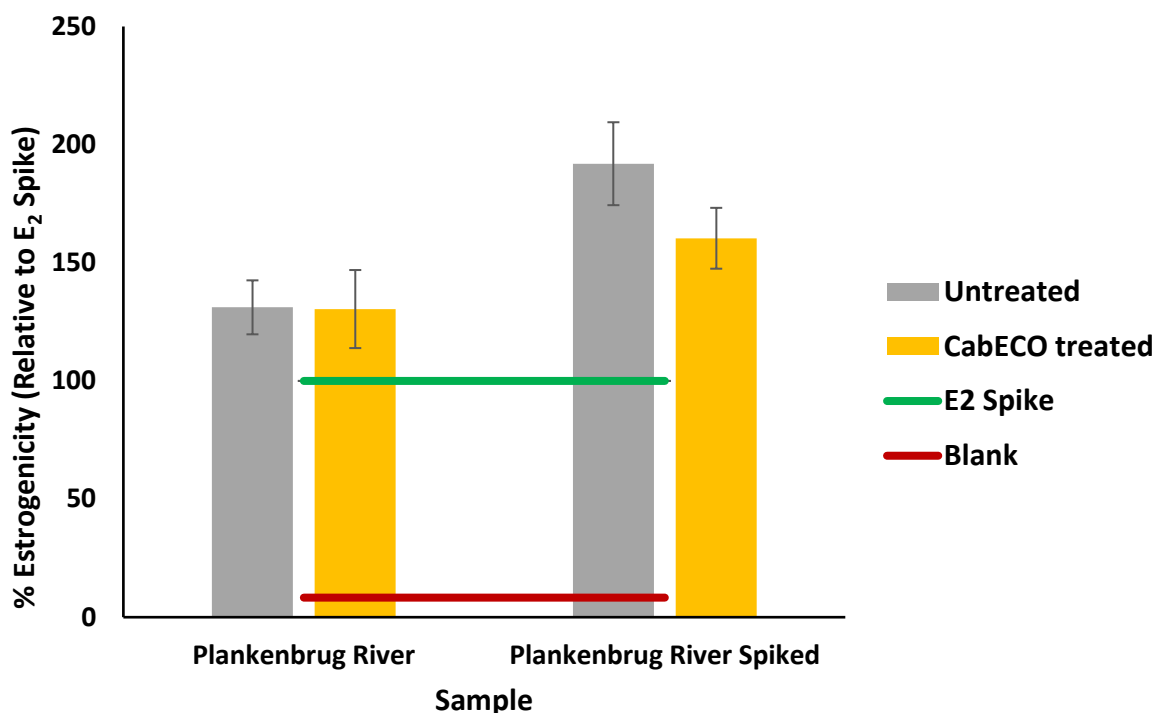
Figure 3-19: Estrogenic effect of a Plankenbrug River sample at environmental micropollutant concentrations and an environmental sample spiked with a cocktail of micropollutants, relative to an EC50 E2 spike before and after CabECO treatment.

When investigating the endocrine disrupting potential of the Eerste River before and after CabECO treatment using the yeast anti-estrogen screen (Figure 3-20), it is interesting to note that the initial environmental sample of the supposedly less polluted sample (Table 3-4) is showing a significantly more potent estrogenic effect than the Plankenbrug River at environmental concentrations (one-tailed T-test, $P < 0.05$). According to Swart el al. (2011), the Eerste River, close to the sampling site in this study, showed some level of estrogenicity possibly due to phyto and myco-estrogens from the environment. In comparison to the Plankenbrug sampling site, the Eerste River site is more surrounded by agricultural land and it is well known that several herbicides and pesticides have a strong estrogenic effect (Kuiper, 1998). These factors may be an explanation as to why the Eerste River shows more estrogenicity than the Plankenbrug River. However, CabECO treatment resulted in a statistically significant decrease in estrogenicity in both of the Eerste River samples (one-tailed T-test, $P < 0.05$), possibly meaning that the responsible compounds in this sample are more easily degraded than the ones in the Plankenbrug River sample, due to the lower organic load in the Eerste River. The same effect is seen in the spiked Eerste River samples,

high initial estrogenicity and a significant reduction when exposed to the ozone generated by the CabECO technology.
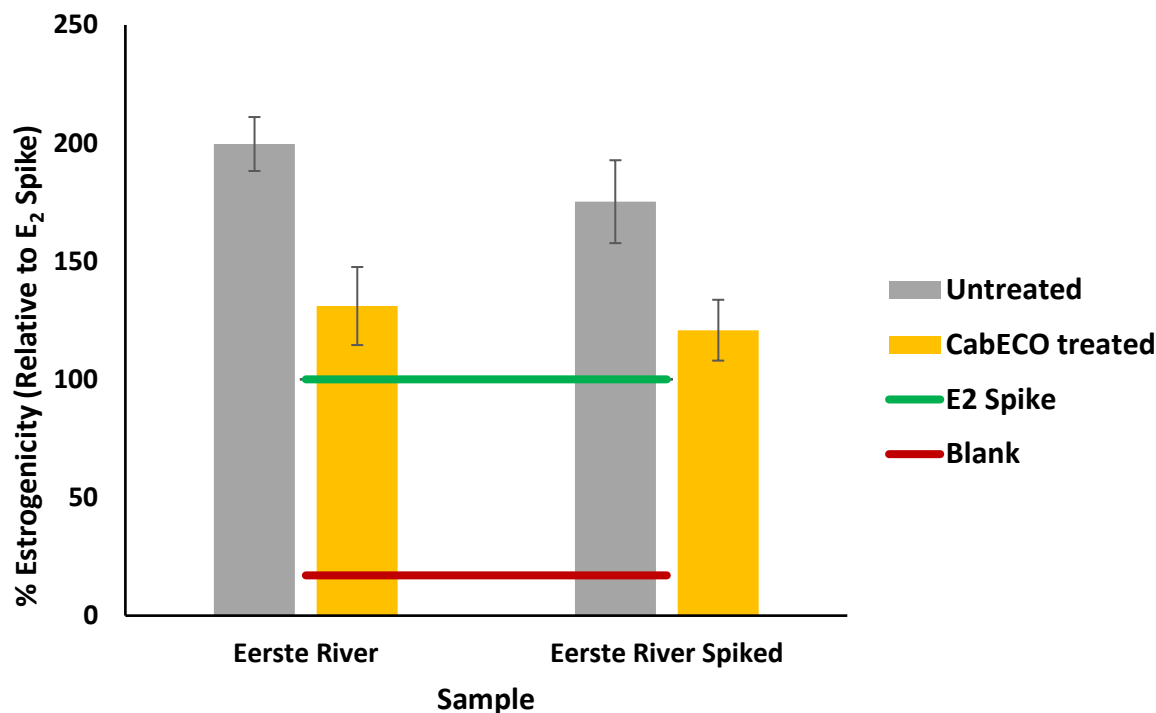
Figure 3-20: Estrogenic effect of an Eerste River sample at environmental micropollutant concentrations and an environmental sample spiked with a cocktail of micropollutants, relative to an EC50 E2 spike before and after CabECO treatment.

3.4 Conclusions:

In conclusion, the CabECO technology is a promising technology for water treatment in a controlled environment, however, there are still many issues to be addressed and the technology is still in its infancy. The ozone generated by the electrochemical process is sufficient for the disinfection of various environmental samples as well as the abatement of several micropollutants, however not to the standards of national drinking water regulations. The anti-estrogenicity of both CBZ and SMX was reduced by CabECO until no observed effect was seen in the yeast anti-estrogen screen after 4 hours, however, up until 4 hours of treatment, both micropollutants were still affecting the uptake of 17β-Estradiol by the recombinant yeast strains. The treatment time of 4 hours was achieved in a controlled laboratory setting and the implications of achieving this in large scale application should be considered.

Hydraulic retention time is extremely important to include in the final design of this system. To eliminate the estrogenic footprint of both CBZ and SMX, up to 4 hours of ozone exposure

was needed, even though the parent compounds have been almost completely degraded after 1 min of exposure. Increased exposure time will also enhance the disinfection capability of the technology. An in-line retention tank, maintained at system operation pressure, will limit any off-gassing of ozone, as was the case in this study, thus ensuring that the maximum amount of ozone gets in contact with target pollutants in the water. It should also be considered that with increased retention times, more ozone will be scavenged by the chemical oxygen demand (COD) of environmental water sources and that the generated ozone will be completely depleted within a certain time frame, relative to the COD of the water treated.

For the application of this system in a real-world scenario it is important to consider that ozone concentration is reduced considerably, with a substantial portion of the ozone being depleted by various sources of organic matter other than the target compounds and the microbes. Thus, CabECO appears better suited as a disinfectant or "polishing step" after water treatment. The standard procedures for generating potable water (flocculation, sedimentation, filtration) always demand the addition of a disinfectant, post-treatment. Currently, chlorine is the go-to disinfectant, but has the disadvantage of being hazardous to human health and having a negative environmental impact. CabECO might prove an alternative for the disinfection step in water treatment, or for polishing water with a micropollutant footprint, if more studies are conducted to optimize the technology for real-world scenario application. For disinfection, it has the disadvantage of lacking residuals, due to the instability of ozone. Thus, retreatment before use, after storage, would be recommended.

When environmental samples were treated with CabECO some compounds such as Diclofenac, Benzotriazole, Caffeine and Atrazine still proved recalcitrant to degradation with ozonation. Plankenbrug River and Eerste River water samples remained estrogenic after 1 hour of ozone exposure, although there was some decrease, further experimentation is required to determine optimal ozone exposure time.

It is recommended for future studies that ozone exposure times should be further investigated to accurately determine the optimal exposure times for the maximum efficiency in removal of a broad suite of micropollutants such as in environmental rivers. However, when exploring longer exposure times, the accompanying challenges should be carefully considered to include economic feasibility. The recycling of water through the CabECO system should also be further explored, how a broad suite of micropollutants and their eco-

toxicological effects react when a previously exposed sample is exposed to a fresh, high dose of ozone for a second and possibly a third time, thus increasing the charge density. Advanced oxidation, where hydroxyl radicals are utilized as the oxidant should also be investigated, as it is a less selective oxidant as ozone. Furthermore, a wider range of eco-toxicological assays, extending various trophic levels should be incorporated to assess the final toxicity footprint of treated samples. As the CabECO technology is investigated as an option for drinking water treatment, disinfection studies should include indicator organisms, such as coliforms, to compare to national drinking water standards (SANS 241) and not only total cell concentration before and after treatment.

Chapter 4

Micropollutant transformation by microbiological metabolism

4.1 Introduction:

Biological degradation of micropollutants currently receives notable attention, as this could be a cost effective and environmentally friendly water treatment option. Metabolism of pharmaceuticals typically involves oxidation, reduction and/or hydrolysis, rendering these molecules more polar and soluble and therefore, potentially lead to more mobile transformation products (Celiz et al., 2009). The cytochrome P450 group of enzymes (found in all lifeforms) metabolize most organic compounds by adding functional groups such as –OH, -SH, -NH$_2$ or –COOH during metabolism to form more hydrophilic products. These biotransformation products can maintain therapeutic activity and bind to proteins and other cellular components, causing disruption of cellular components (Celiz et al., 2009).

The primary problem of micropollutant removal, in comparison to the standard wastewater treatment processes removing nitrogen, carbon and phosphorous, is the vast diversity of compounds, the low concentrations of micropollutants and the low affinity for removal by microbes. It is estimated that as many as 300 million tons of anthropogenic chemicals makes its way to natural water sources every year (Schwarzenbach, 2006) and with more than 100 000 unique substances registered, the list of detected micropollutants in the natural environment is growing daily (ECHA, European Chemicals Agency, 2019). Success has been achieved for the removal of selected micropollutants with the use of microbial communities (Falås et al., 2013). However, the process is not universal, the removal efficiencies vary greatly with compound structures, aerobic and anaerobic processes, suspended and attached growth, as well as climate and retention time (Wang and Wang, 2018; Benner et al., 2013; Falås et al., 2016).

Two micropollutants, Carbamazepine (CBZ) and Sulfamethoxazole (SMX), were selected for this study on the basis of their widespread use and persistence in the environment (Jankunaite et al., 2017; Chen et al., 2014; Kunkel and Radke, 2012). In a recent review by Archer et al. (2017) on pharmaceutical and personal care products (PPCP's) and endocrine disrupting compounds (EDC's) in South African surface waters, CBZ and SMX were among the most concerning compounds in terms of detection concentrations and persistence. The method for chemical analysis of these compounds is also well validated in our research group. Carbamazepine is a commonly prescribed anti-epileptic drug, with over 40 tons being prescribed in the UK every year (Jones et al., 2002) and Sulfamethoxazole is a top selling broad spectrum antibiotic used primarily in urinary tract infections, with over six million prescriptions in South Africa per year alone (Osunmakinde et al., 2013).

The chemically stable aromatic rings in the structure of CBZ and SMX are partly responsible for these micropollutants' recalcitrance in the environment and WWTWs (Yu et al., 2013). Wu et al. (2011) found only a 1% removal of SMX by microbial degradation, however, SMX biotransformation products may be back transformed to the parent compound in the presence of microbial enzymes (Göbel et al., 2005). Kümmerer (2008) reports on microbial adaption, where micropollutant exposed microbial communities developed the ability to degrade the respective micropollutants. Similarly, it was found that when exposing a biofilm reactor to SMX for a second time, the biofilm showed increased removal of SMX, compared to the first exposure (Martínez-Hernández et al., 2016).

This chapter reports on the capacity of microbial metabolism to break down a range of micropollutants that typically persist in environmental waters and WWTW effluent. The ability of microbial metabolism to reduce endocrine disruption by some of these pollutants was also assesses. We investigated the potential advantages of carbon-starved biofilms for the removal of selected micropollutants, compared to suspended cell cultures of the same inoculum from a WWTWs return activated sludge, aiming for pre-evolved/adapted sludge, with exposure to high levels of micropollutants. However, with the reduction in micropollutant parent compound, there comes a potential increase in a suite of active biotransformation products. Thus, this work also investigated the endocrine disruption and toxicity potential of the whole water chemical footprint.

4.2 Materials and Methods:

4.2.1 Inoculum preparation:

The removal capacity of biological systems was compared to that of electrochemical degradation, by exposing biofilms to micropollutants as the sole carbon source. These biofilms were cultivated from a heterogeneous microbial community sampled from a local wastewater treatment works, with known exposure to the pollutants under study.

All subsequent laboratory based experiments were inoculated with a defined volume of inoculum grown up on M63 medium. The medium contained per 1000 ml: 2 g $(NH_4)2SO_4$, 13.6 g KH_2PO_4, 0.5 mg $FeSO_4 \cdot 7H_2 0$, 1 mL of a 1 M $MgSO_4 \cdot 7H2O$ solution, 0.1 mL of a 0.5% Vitamin B_1 (thiamine), 10 mL of a 20% glucose solution and 5 mL of a 20% CasAmino acid solution (Elbing and Brent, 2002). The medium was prepared as described by Elbing and Brent (2002) with the only exception that glucose/glycerol was substituted with a high dose of the respective micropollutants: Carbamazepine (500 µg/L) and Sulfamethoxazole (3000 µg/L). These concentrations are representative of 100× that of the highest environmental concentrations recently recorded in South African waste water treatment works effluent (Archer *et al.*, 2017). A grab sample, collected in a sterile 50 mL Falcon tube from Athlone wastewater treatment works' aeration tank, was used as the initial inoculum. After vortexing (1 min), 1 mL of the collected sample was added to 3 L of the micropollutant-enriched M63 media (M63*) and grown aerobically with agitation (7 days, 26 °C, 120 RPM). Thereafter, every 7 days, 1 mL of the culture was transferred to a sterile 3 L flask of M63* medium. This was repeated three times to select for a microbial community capable of growing on the experimental concentrations of Carbamazepine and Sulfamethoxazole, to be used in all subsequent biodegradation experiments. The culture from the third flask in the passage series was added to an 80% glycerol solution to a final concentration of 40% glycerol, which was then aliquoted to sterile cryogenic tubes and stored at -30 °C. For biofilm inoculation, the culture was thawed, 100 µL of the culture was added to 100 mL of M63* media and grown up at 26 °C to the inoculation concentration, pre-determined in growth curves of absorbance and colony forming units.

4.2.2 Biodegradation of CBZ and SMX in batch reactors:

Batch reactor experiments were designed to determine the extent of microbial degradation of CBZ and SMZ over 20 days. A total of 6 Erlenmeyer flasks (3 L) were filled with 2 L of sterile M63 medium, the glucose substituted with Carbamazepine to a final concentration of 500 µg/L and a set of 3 flasks were inoculated with 500 µL of inoculation culture, while 3 flasks were kept sterile.

Both the sterile and inoculated flasks were incubated on a shaker (120 RPM) at 30 °C for 20 days. Ideally the experiment would have been run at 26 °C, closer to environmental temperatures, but the limitation of a big enough shaker forced the shift to 30 °C. At 3 time points, (prior to inoculation, 10 days and 20 days), 100 mL and 300 mL were sampled aseptically from each batch reactor to be subjected to SPE for LCMS analysis and the yeast anti-estrogen screen (YES), respectively (sections 3.2.8 – 3.2.11).

In tandem, 6 additional flasks were prepared in the same manner with the only modification that Carbamazepine was replaced by Sulfamethoxazole to a final concentration of 3000 µg/L.

4.2.3 Biodegradation of CBZ and SMX by carbon-starved biofilms:

To investigate the ability of biofilms to metabolically transform the selected micropollutants (CBZ and SMX) biofilms were grown up in flowcells (Wolfaardt et al., 1994) inoculated from the freezer stocks, prepared as described in the section above. Modified M63 medium was pumped from a sterile container through 1.6 mm inner diameter (3 mm outer diameter) silicone tubing to the flowcell by means of a Watson Marlow peristaltic pump. The flowcell consists of a Perspex block with a machined cavity covered by a glass microscope slide kept in place by silicone sealant. In front of the flowcells a glass bubble trap was inserted to reduce the exposure of air bubbles that might have reached and disrupt the biofilm (He et al., 2011). For this experiment 8 flowcell channels were constructed, where the first two channels were supplemented with M63 medium with the carbon source replaced by CBZ to a final concentration of 200 µg/L. The third and fourth channels were fed by M63 where the carbon source was replaced by SMX to a final concentration of 200 µg/L. The fifth and sixth channels were fed by M63 medium where the carbon source was replaced by both CBZ and SMX, combined, to a final concentration of 100 µg/L each. Lower micropollutant

concentrations were chosen on the basis of reducing cost of analytical grade standards of CBZ and SMX, due to the long cultivation time required to establish a mature biofilm on a limited carbon source. Channels 7 and 8 were fed by the same medium as 5 and 6. Channels 1 to 6 were each inoculated with the inoculum culture as described above, by injecting 200 µL of the culture directly into the flowcells with a 26 gauge needle and sterile 1 mL syringe. Channels 7 and 8 were kept as duplicate sterile controls and were not inoculated. Figure 4-1 shows a schematic diagram of how the system was designed.
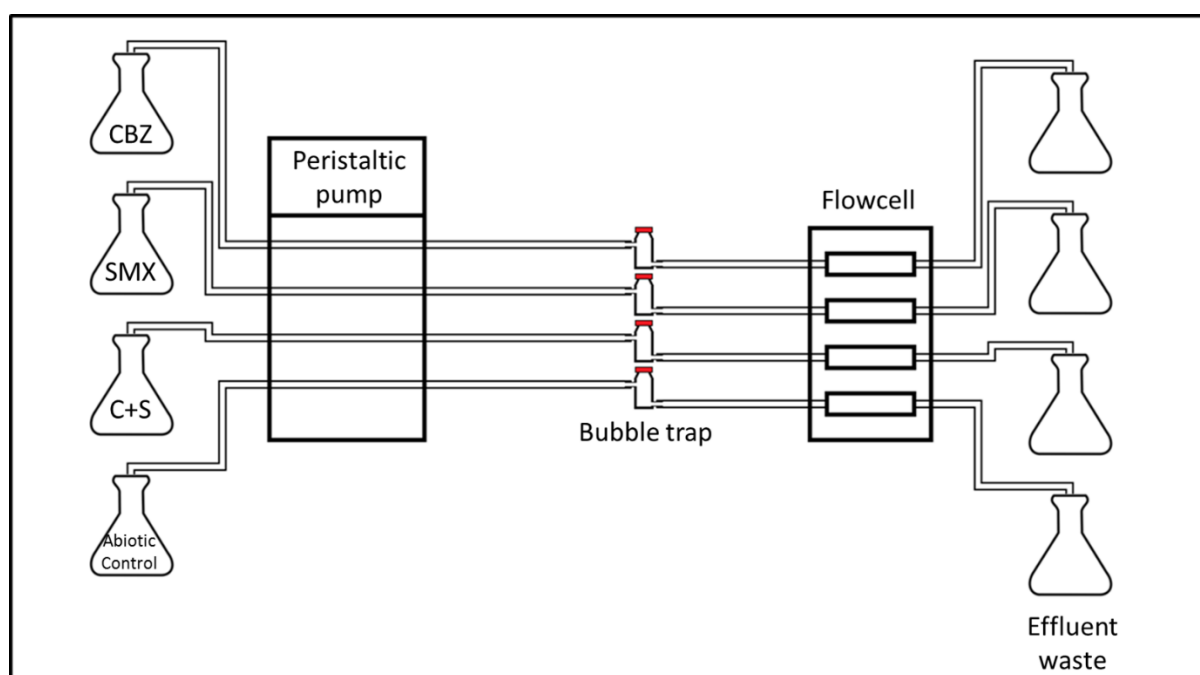
Figure 4-1: Schematic design of carbon-starved biofilm setup in the laboratory.

Prior to inoculation, the flowcell system was sterilized with 10% sodium hypochlorite solution for 2 hours, followed by sterile dH_2O for 10 hours. The respective media was then pumped through the system for 2 hours before inoculation. During inoculation and 1 hour afterwards, flow was switched off and the silicone tubing was clamped off to allow the cells from the inoculum to adhere to the flowcell chamber before flow was switched back on.

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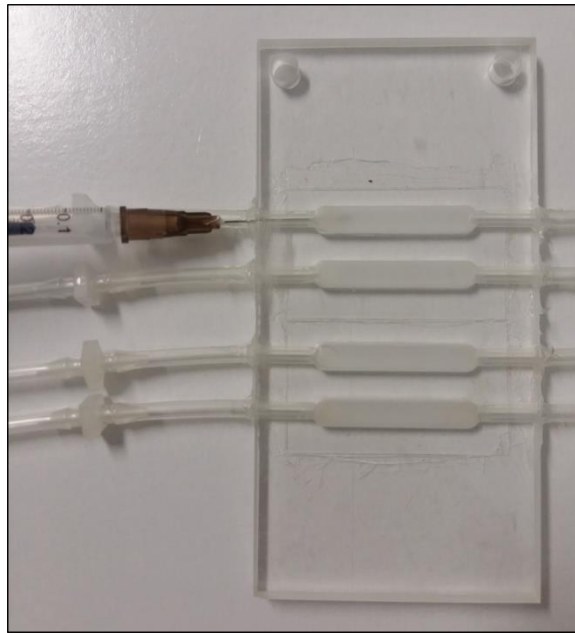
Figure 4-2: Flowcell inoculation with 200 µL of culture with a sterile syringe.

After inoculation, the flow of the medium was resumed, and the optical density of the effluent of each channel was measured. On the effluent side of the flowcell, 1.5 mL of the effluent was collected and the optical density (600 nm) was measured using a Spectroquant Pharo 300 spectrophotometer (Merck) every 6 hours. Additionally, 1 mL of the effluent was collected in a 1.5 mL Eppendorf tube and a serial dilution ($10^0 - 10^{-7}$) was made in sterile 0.8% saline solution. The dilutions were plated out on 3 g/L TSA in triplicate and incubated at 26 °C for 48 hours and the respective colony forming units were counted.

Using the optical density measured from the flowcell effluent, the growth phases of the biofilms were monitored. When steady state was reached, several days were allowed for biofilm maturation. After 15 days, 100 mL and 300 mL was sampled from each influent container as well as from the effluent containers for LCMS analysis and the yeast anti-estrogen screen, respectively, as described in sections 3.2.8 – 3.2.11.

4.3 Results and Discussion:

This section discusses and compares the microbial biodegradation of CBZ and SMX by suspended cell cultures and carbon-starved biofilms. The estrogenic profiles of biologically transformed CBZ and SMX were also monitored for both planktonic and the biofilm cultures for the duration of incubation, to assess the estrogenic effect of the metabolites produced during metabolism, as an indication of endocrine disruption. Biofilms pose several advantages over planktonic cells, such as increased resistance to antimicrobial compounds, a wider metabolic range due to aerobic and anaerobic zones in the biofilm structure, inter-species cooperation and horizontal genetic transfer, as well as increased protection from physical forces (Stewart and William Costerton, 2001; Wolcott et al., 2013; Madsen et al., 2012). The physical structure of biofilms may also benefit the degradation of micropollutants, as the three-dimensional structure and the presence of an EPS can increase the retention time of molecules in the biofilm structure (Rice et al., 2005). The EPS matrix may also facilitate improved extracellular enzyme activity and concentrate the micropollutants to levels at which the cells derive more energy from such metabolism than the energy spent to produce the required enzymes for biodegradation and overall maintenance. However, biofilms also pose a challenge in equilibrium dynamics due to the attachment and persistence of the biomass, rather than constant regeneration of biomass in planktonic state, likely acting as a sink until surface liquid exchange reaches saturation/equilibrium.

Ultimately, the biodegradation of CBZ and SMX will be compared to the transformation of these compounds by electrochemical oxidation, as presented in *Chapter 3*.

4.3.1 Biodegradation of CBZ and SMX in batch reactors:

Degradation of CBZ over a 20 day incubation period with an inoculum originally derived from a return activated sludge (RAS) sample of a WWTW could not be accounted for by microbial metabolism (Figure 4-3 A), because, although there was an 80% removal of CBZ from the bioreactor after 10 and 20 days of incubation at 30 °C on a shaker (120 RPM), the sterile control also had an 80% reduction in CBZ after 10 and 20 days incubation, and thus microbial metabolism appeared to have no influence on CBZ removal. A one-way ANOVA with a Tukey's HSD multiple comparison test was performed on all the data points in Figure 4-3 A, to determine if any variation between the samples had significance ($P < 0.05$). None of the samples varied significantly from each other, not in the sterile control compared to the

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inoculated sample, nor between 10 and 20 days of incubation. The loss of CBZ during incubation in both the control and the metabolically active samples could be due to a number of factors. Binding to the glassware surface is not probable as this possibility was tested in the previous chapter (Figure 3-14) and the effect of CBZ binding to the hydroxyl groups on the bioreactor glass surface is negligible. Natural (photo-degradation) degradation of CBZ in the sterile control seems unlikely as CBZ is such a persistent and recalcitrant chemical in the environment (Jankunaite et al., 2017). However, the high temperature (30°C) and consistent mixing on a shaker (exposure to high levels of oxygen) may have promoted free radicals in the aqueous phase, leading to degradation. The consistency in anti-estrogenicity (Figure 4-3 B) supports the theory that CBZ concentration remains the same over the 20 day period and that the lack of CBZ degradation in the incubated samples may have been due to detection errors, or due to transformation products that did not influence the estrogenic effect of the sample.

In contrast to our results on CBZ biodegradation, various studies have reported to some extent success in the biodegradation of CBZ. However, these studies carried out their degradation studies at very high concentrations of CBZ relative to the environment, whereas removal by these microbial communities were significantly lower at environmentally relevant concentrations (Ha et al., 2016; Jelic et al., 2012). Raeke et al. (2005) found that CBZ binds to dissolved organic matter (DOM), becoming undetectable and unavailable to be metabolized. This effect was found to be more profound at lower CBZ concentrations and increasing DOM/CBZ ratios, which might explain as to why less success is found with the biodegradation of CBZ when lower CBZ concentrations is used. As CBZ is bound to DOM, very little is available for biodegradation and it becomes ineffective for microbes to spend energy on metabolizing the compound for little return. With the combination of low availability in water sources with high DOM content and the low energy yield from degradation relative to other labile carbon sources, microbial metabolism of micropollutants from waste water seems unfeasible.

CBZ shows some anti-estrogenicity (Figure 4-3 B) in the yeast anti-estrogenic screen, where at 0 days CBZ possibly inhibits the binding of estradiol to the human estrogen receptor (hER), or hinders β-galactosidase expression at multiple possible sites along the pathway (Routledge and Sumpter, 2007). Samples containing CBZ only shows a 70% estrogenic response relative to the estradiol spike. CBZ is an anti-convulsion drug in the treatment of epilepsy by interacting with neurological transmitters, however, serum sex-hormones

concentrations, such as estrogen, androgen and progesterone have also been known to be decreased and supports the data above, that CBZ is exhibiting some anti-estrogenicity (Death et al., 2005). When CBZ is incubated with an inoculum from a waste water treatment works' return activated sludge (RAS) for 10 and 20 days, there seems to be no change in the anti-estrogenicity of the samples. This could be expected, as in Figure 4-3 A, where CBZ removal by microbial degradation in the bioreactor was quantified, there was no degradation of CBZ by the microbes in the bioreactor. Various studies have reported that CBZ is very recalcitrant to biodegradation as well as other treatment methods for micropollutant abatement and passes through most conventional WWTW unchanged (Jankunaite et al., 2017; Chen et al., 2014; Kunkel and Radke, 2012). However, it is possible that CBZ is not degraded by microbes due to the low energy yield they will get for their metabolism of CBZ. Over the period of 20 days, the turbidity of the reactor increased as the microbes started to grow, but if CBZ was the only carbon source, the microbes would not have grown. The other components in the M63 medium, especially the CasAmino acids, can support the growth of the microbes, even though the CBZ was not metabolized. Further investigation is needed to determine at which level of the yeast assay CBZ inhibits β-galactosidase expression, although binding to estradiol to bring forth structural changes and thus hindering binding to the hER seems most probable (Death et al., 2005).

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B
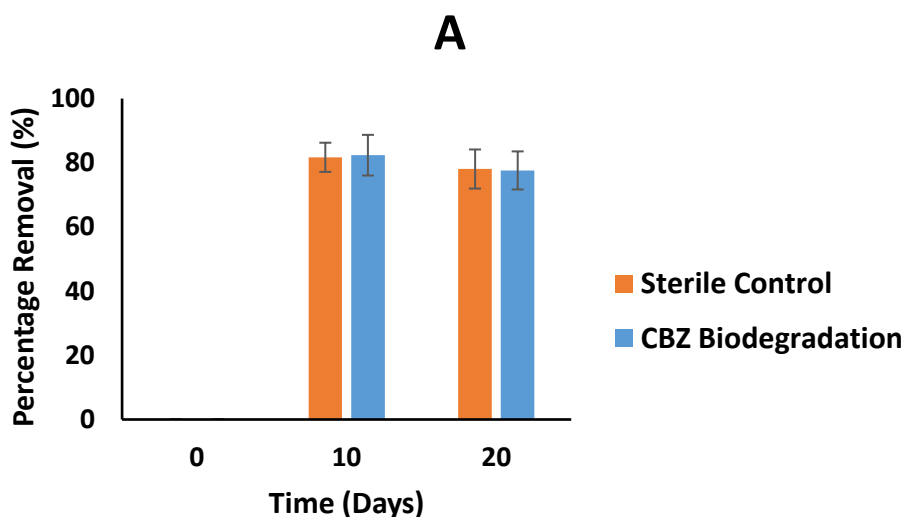
Figure 4-3: A) Percentage removal of CBZ and B) Estrogenic effect of CBZ relative to an EC50 Estradiol spike, over a 20 day incubation period with an inoculum isolated from a returned activated sludge sample of a WWTW.

The biodegradation of SMX with an inoculum isolated from a return activated sludge (RAS) sample of a WWTW was investigated over a 20 day incubation period (Figure 4-4 A). Although there is a significant percentage removal of SMX over the incubation period, it is also seen in the sterile control, making it unlikely to be due to microbial metabolism. However, at the 10 day time point, there is a difference in SMX concentration between the sterile control and the inoculated bioreactor. This suggests some microbial metabolism of SMX, which is further supported by the sharp change in estrogenicity of the SMX sample (Figure 4-4 B). The difference in the percentage removal of SMX between the sterile reactor and the inoculated reactor could also be explained by the back transformation of SMX transformation products to the parent compound when certain microbial enzymes are present (Celiz et al., 2009). Although SMX is a persistent compound in the environment and is resistant to biodegradation, there have been other studies reporting success in the breakdown of SMX by microbes, however, these successes were reached only with very high concentrations of SMX relative to environmental concentrations (Al-Ahmad et al., 1999; Mulla et al., 2018).

The yeast anti-estrogen screen revealed a significant anti-estrogenic effect exhibited by the SMX relative to the estradiol spike (Figure 4-4 B). When SMX was present in the sample, the estrogenic response was <60% relative to the estradiol spike, thus hindering the binding of E_2 to hER. After 10 days in the bioreactor, the inoculated sample had a significant

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reduction in anti-estrogenicity (one-way ANOVA, $P < 0.05$). When biodegraded, SMX shows no hindrance to the estradiol-hER binding as estrogenicity is now equivalent to 115% estrogenic activity relative to the estradiol spike. In this instance, after 10 days of microbial interaction, SMX shows an addition effect to the estradiol spike, indicating that SMX has been metabolized and the metabolites possibly act as an estrogenic compound. This change can be ascribed to the microbes in the bioreactor as the sterile control shows no significant changes in estrogenicity/anti-estrogenicity. This theory of biodegradation is supported by various studies where SMX is degraded by microbes (Al-Ahmad et al., 1999; Mulla et al., 2018). Even though Figure 4-4 A is inconclusive in terms of quantifying SMX degradation, the fact that there is significant differences ($P < 0.05$) in the estrogenicity/anti-estrogenicity of the inoculated and sterile bioreactors, further supports the theory that the RAS biofilm is interacting with SMX, causing either a transformation or acting as a sink.

It is interesting to note that after 20 days in the bioreactor, the SMX sample (Figure 4-4 B) had a return to an anti-estrogenic effect. However, this effect was less profound than at day 0 before any interaction with the inoculum and was still significantly less anti-estrogenic than the sterile control sample at day 0.

Although biodegradation of SMX is relatively well studied, there is very limited literature on the effect of SMX and its biodegradation by-products on human sex hormone systems as in the yeast estrogen screen. This return to anti-estrogenicity could be due to back-transformation of daughter compounds to parent compounds, or, more likely, if acting as a binding sink, the microbial community reaching a steady state and an accompanied equilibrium transfer of the compounds back into the aqueous environment. Notably, the micropollutants are measured in the aqueous phase, with the microbes filtered out of suspension. Further studies are needed to optimize micropollutants partitioned into the solid particulate matter.
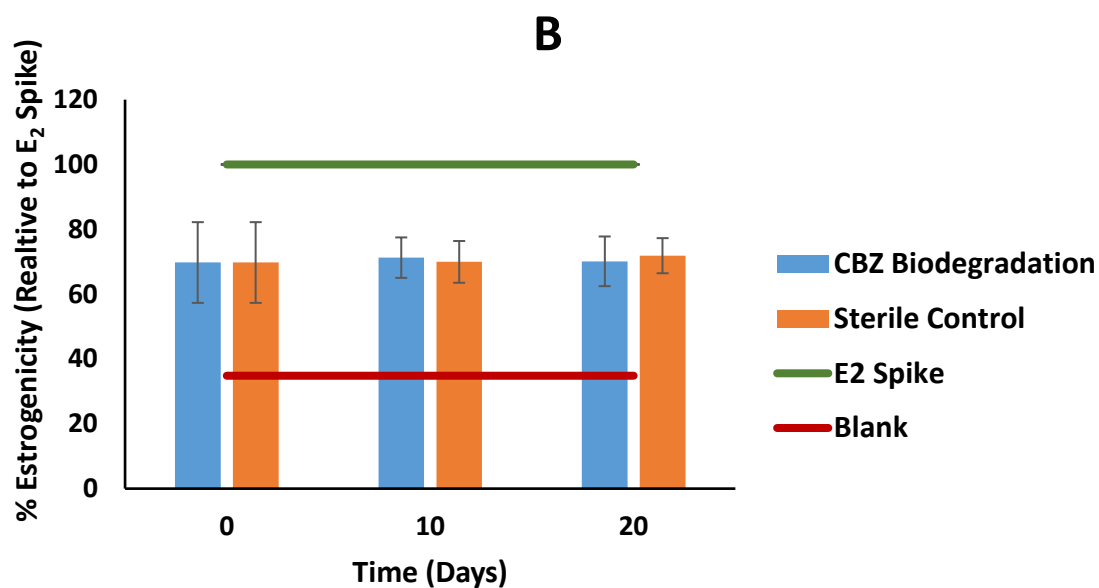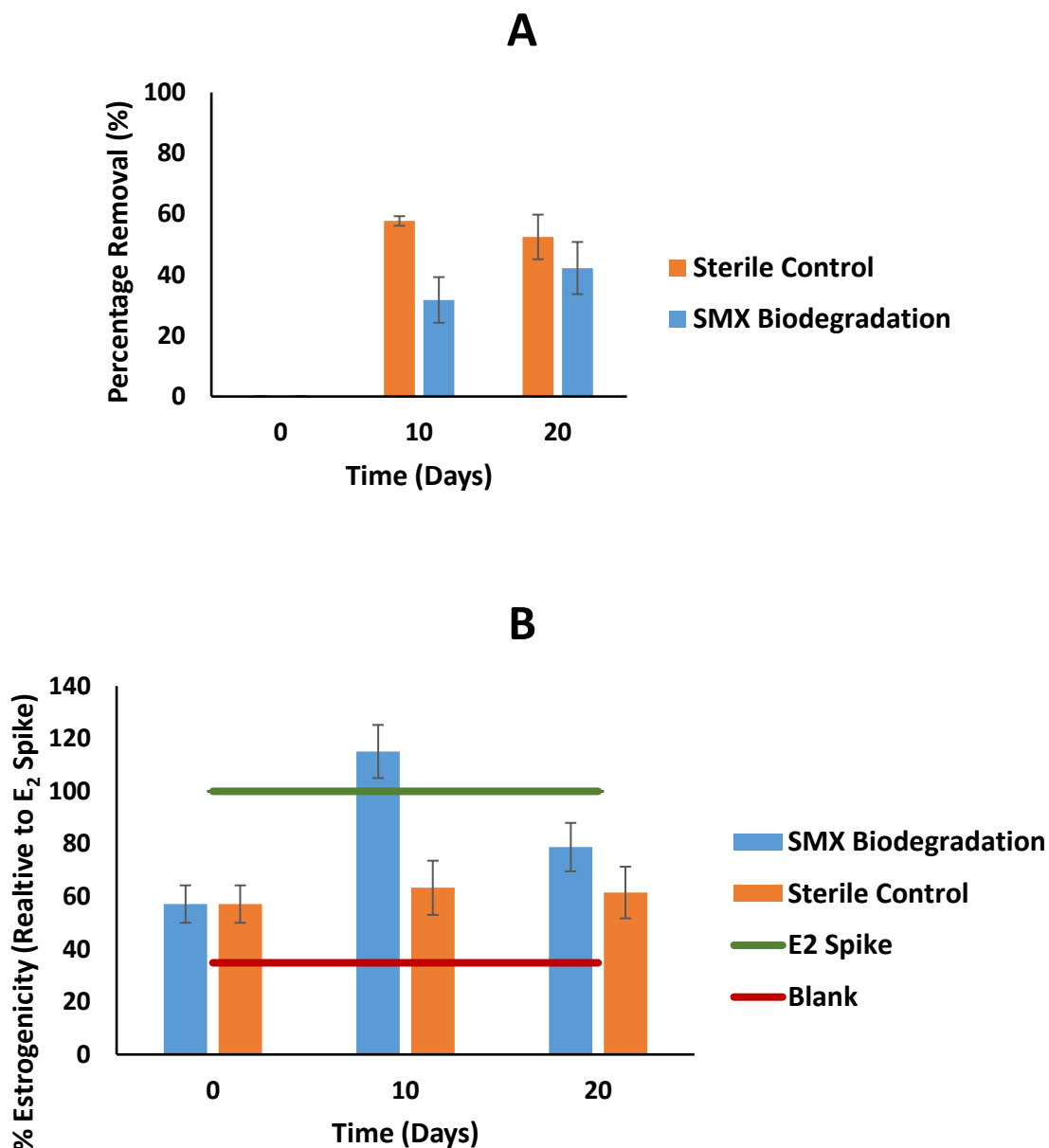
Figure 4-4: A) Percentage removal of SMX and B) Estrogenic effect of SMX relative to an EC50 Estradiol spike, over a 20 day incubation period with an inoculum isolated from a returned activated sludge sample of a WWTW.

4.3.2 Biological degradation of CBZ and SMX by carbon-starved biofilms:

Following the results from the previous section where biodegradation of CBZ and SMX by planktonic cells in batch reactors seemed improbable, biofilms were grown up on M63* medium and only once matured, the continuously exposed CBZ and SMX concentrations were analyzed to determine the extent of degradation at that time, if any. Planktonic cells lack several advantages that biofilm have, such as increased resistance to antimicrobial

compounds, a wider metabolic range due to aerobic and anaerobic zones in the biofilm structure, inter-species cooperation and horizontal genetic transfer, as well as increased protection from physical forces (Stewart and William Costerton, 2001; Wolcott et al., 2013; Madsen et al., 2012). The three-dimensional structure of biofilms is also advantageous as channels and tunnels within the biofilm structure and sorption onto the EPS increases the system retention time, allowing for longer microbe-compound contact time. (Rice et al., 2005).

Once the biofilm experimental setup was inoculated, growth was monitored to establish when the biofilms reached maturity, and thus, potentially possess the required machinery to degrade the CBZ and SMX. Figure 4-5 shows the optical density of the individual biofilms along with their duplicates. The expected sigmoidal growth curve was not observed, but rather waves of increased OD followed by low OD readings was observed. The same trend was followed by all the biofilms and even though there was variation in times and intensity of OD increases between the duplicates, the same trend was observed for each. The OD of the sterile control remained constant at 0, thus we can assume the control remained sterile for the duration of the experiment.

Initially, in addition to the OD readings, plate counts were done on the biofilm effluents, but when the initial plate counts correlated to the OD data, it was decided to only measure OD due to the time, cost and logistical restrictions of an experiment of this scale and duration.
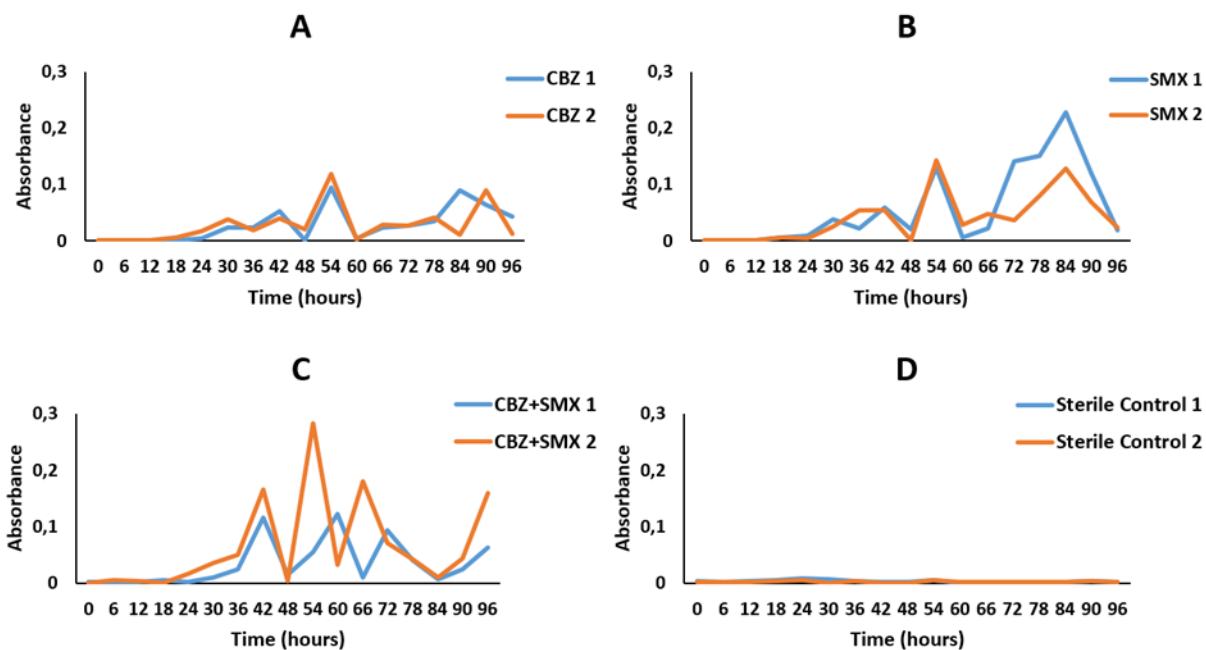
Figure 4-5: Growth curves of the A) CBZ, B) SMX, C) CBZ+SMX and D) sterile control biofilms from the planktonic cell release of each biofilm.

The observed fluctuations in the biofilm effluent OD can be explained by frequent dispersal events as seen in figure 6. Biomass would accumulate in the flowcell (Figure 4-6 A) for a few hours and within a few minutes the entire visual biofilm would detach and be carried to the effluent by the flow (Figure 4-6 B and Figure 4-6 C). This is in contrast to the standard biofilm growth curve on carbon-rich media like TSB (Bester et al., 2009) and can be explained as a response to the low nutrient environment, where the biofilm would accumulate biomass and at a threshold detach in search of a less nutrient-stressed habitat. Rice et al. (2005) found similar results when growing *Serratia marcescens* at low nutrient conditions and reported that biofilms use quorum sensing to facilitate synchronized sloughing of biomass on nutrient cues.

This recurring phenomenon made it difficult to quantify biofilm maturity, but when the recurring pattern was observed, the biofilm was considered to be at steady-state and. The biofilms were allowed a further 14 days to ensure well-adapted, mature biofilms, micropollutant degradation was analysed.

Figure 4-6: Biofilm biomass sloughing event. A) Biofilm biomass in flowcell, B) the same flowcell 5 min later and C) the same biofilm biomass in the effluent tubing 5 min after A).

The biofilms grown up on CBZ were able to metabolize 28% of the total CBZ molecules exposed to the biofilm (Figure 4-7 A). This is significantly better than the 0% of CBZ degradation by the suspended cell culture (Figure 4-3 A) of the same inoculum (one-tailed T-test, $P < 0.05$). There is variation between the removal efficiencies of the two duplicate biofilms grown up in the same conditions. The second CBZ biofilm only degraded about 6% of the CBZ, however, the frequent sloughing events of the biomass as seen in Figure 4-6 might explain the difference in removal efficiencies. During such an event, the biofilm loses most of the structure, biomass and EPS that potentially aid in CBZ degradation. A future study could test the removal efficiency during high biomass and low biomass presentation, during the sloughing/deposition cycles, as well as accumulation within the biomass.

There was a significant percentage increase in CBZ degradation when exposed to the biofilm grown on the media supplemented with the combination of CBZ and SMX (100 µg/L each) when compared to the biofilm grown up on the media supplemented with only CBZ (200 µg/L) (one-tailed T-test, $P < 0.05$). The duplicates of the combined biofilms removed 56% and 70% of the CBZ, whereas the best performing CBZ biofilm only removed 28%. Although variation between the duplicates is significant, it can be concluded that the combined biofilm is more efficient in CBZ removal, and SMX potentially provide some stimulus for another relevant metabolic pathway that also contribute to CBZ degradation. The variation between these duplicate biofilms can be explained by unsynchronized sloughing events, leading to inconsistent biofilm biomass, and consequently to inconsistent levels of CBZ degradation efficiency.

The same effect is seen in the removal of SMX by the biofilms (Figure 4-7 B). The SMX biofilms have a significantly lower removal efficiency of SMX when compared to the biofilms grown on both micropollutants ($P < 0.05$). The variation in SMX removal by the two duplicate combined biofilms is significant but might be due to a recent sloughing event as proposed above.

The influence of these regular sloughing events cannot be overlooked as an explanation for the observed lower removal efficiencies of CBZ and SMX by their respective biofilms, in comparison to the combined biofilms. To determine this, finer measurement intervals that follow the various points in the sloughing cycles are necessary. However, several factors might account for the possible better performance of the combined biofilms. The higher concentration of CBZ and SMX in the single compound biofilms media might have led to some inhibitory effect on the biofilm, resulting in a less active biofilm compared to the combined ones. For the SMX biofilm this can be expected as SMX is an antibiotic, but the minimum inhibitory concentration (MIC) of SMX is 25 mg/L, well above the 200 µg/L in the SMX biofilm medium, therefore an inhibitory effect on the biofilm is improbable (Smego, 1983). However, MIC's are determined based on cell death, and metabolic impact might nevertheless present at lower concentrations. At the same time, the increased pressure from the combined effect of CBZ and SMX may have led to several stress responses that aided in metabolism. As a stress response, biofilms are known to have an increased metabolic rate, higher biomass production; and in the case of nutrient deprived stress, produce more EPS and quorum sensing molecules, as well as altered gene expression (Sunago et al., 2015; Singh et al., 2017; Talagrand-Reboul et al., 2017; Williams et al., 2007).

Contradictory results exist on the efficiency of biofilms to remove CBZ and SMX. Various studies have reported that CBZ shows recalcitrance towards microbial degradation (Casas et al., 2015; Tang et al., 2017; Zhang et al., 2019), however, Hellauer et al. (2017) found that with the addition of a labile carbon source, that a previously nutrient deprived biofilm showed increased removal of CBZ; which could be due to co-metabolism, or simply more cells producing the required enzymes. This might explain why the combined biofilm was more effective in removing CBZ and SMX, as SMX is known to be more easily degraded by biofilms (Casas et al., 2015), thus acting as a nutrient source and facilitating the removal of CBZ. In a similar study by Zhang et al. (2019), where the addition of low levels of acetate to the system enhanced micropollutant degradation, adding carbon in excess reduced the removal efficiency. When in excess, the added carbon source lowered the dissolved oxygen content, resulting in redox potential changes incapable of maintaining the favoured metabolic state. It is proposed that the degradation of various compounds are co-metabolism dependant (Zhang et al., 2019). This theory is supported by the increased removal of both SMX and CBZ in the combined biofilm.
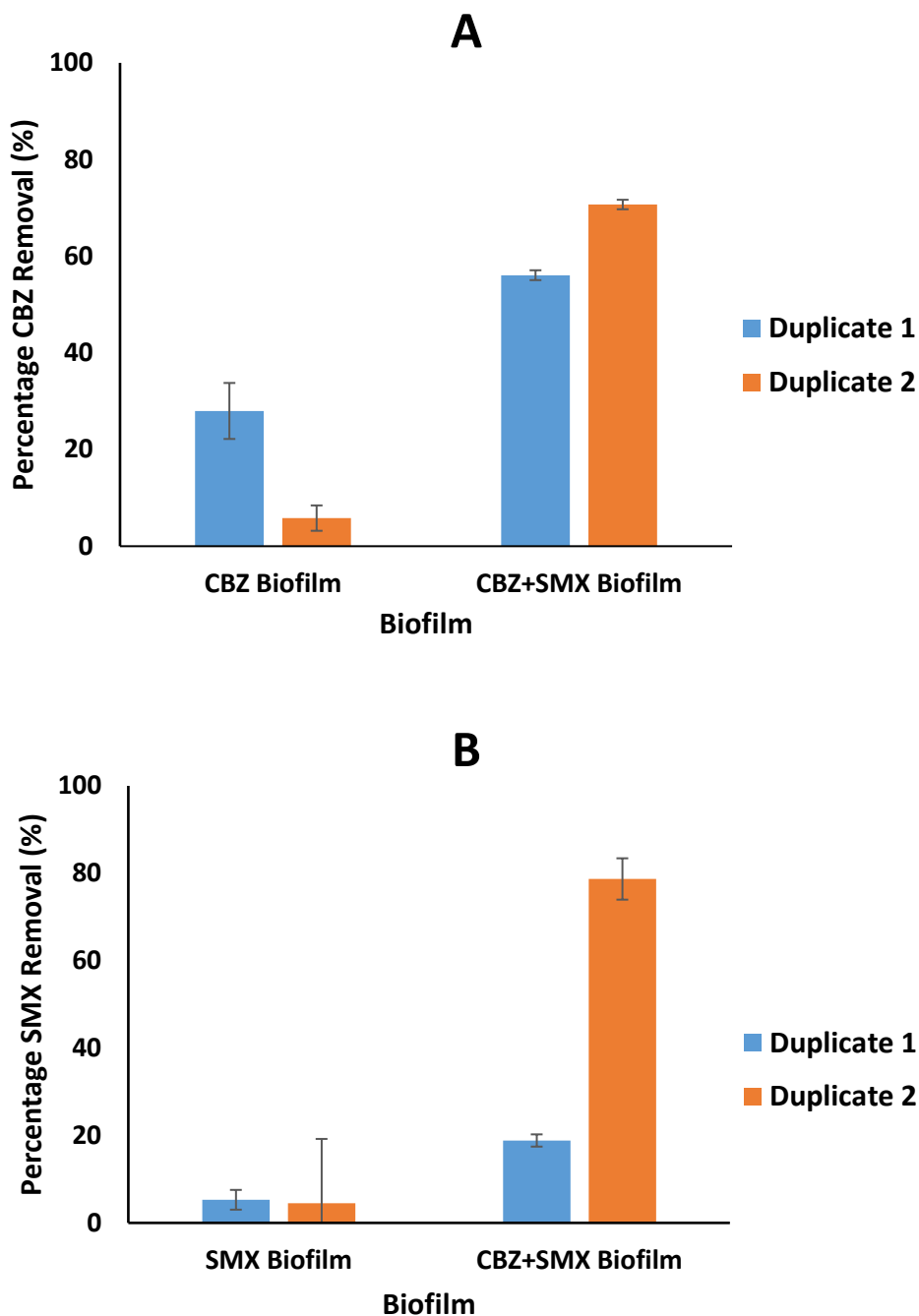
Figure 4-7: A) Percentage CBZ removal by duplicate biofilms grown up on CBZ enriched media and CBZ+SMX enriched media and B) Percentage SMX removal by duplicate biofilms grown up on SMX enriched media and CBZ+SMX enriched media.

When CBZ and SMX was exposed to the yeast anti-estrogen screen, individually, at the lower concentrations (200 μg/L each) for growing up the biofilms over an extended period of time (in comparison to the suspended cell culture reactors), neither of the compounds shows any effect towards estrogenicity/anti-estrogenicity before metabolism (Figure 4-8). SMX shows a minor addition to estrogenicity relative to the E_2 spike, however from previous results (Figure 4-4 A) it is known that SMX is a strong antagonist for E_2 binding to the hER,

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therefore the minor estrogenicity observed here can be disregarded. The effluent samples of the CBZ and the SMX biofilms shows no significant change in estrogenicity/anti-estrogenicity ($P < 0.05$). This no-observed effect can be expected as the concentrations of each is below the detection limit of the assay as well as the fact that CBZ and SMX shows minimal removal by the single compound biofilms (Figure 4-7), thus the formation of possibly active metabolites seems improbable. However, the combined biofilm effluent shows a drastic increase in estrogenicity in the sample with both CBZ and SMX. The sharp increase in estrogenicity can be ascribed to biological degradation, as from Figure 4-7 A and B it is evident that the combined biofilm removed a significant percentage of the CBZ and SMX exposed to the biofilm. Some of the main metabolites of CBZ and SMX, carbamazepine-10,11-epoxide and 4-OH-sulfamethoxazole respectively, have been reported to be more potent than the parent compounds (Russell, Spiller and Baker, 2015; Majewsky et al., 2014). The sterile control, fed with identical media as the combined biofilm, also shows an increase in estrogenicity, possibly due to estrogenic active products formed by association between CBZ and SMX, but is unclear as to why it did not show an estrogenic response in the control influent. Even though the sterile control effluent showed an increase in estrogenicity, the combined biofilm effluent shows a significantly stronger estrogenic response, resulting from the metabolites formed during biological degradation.
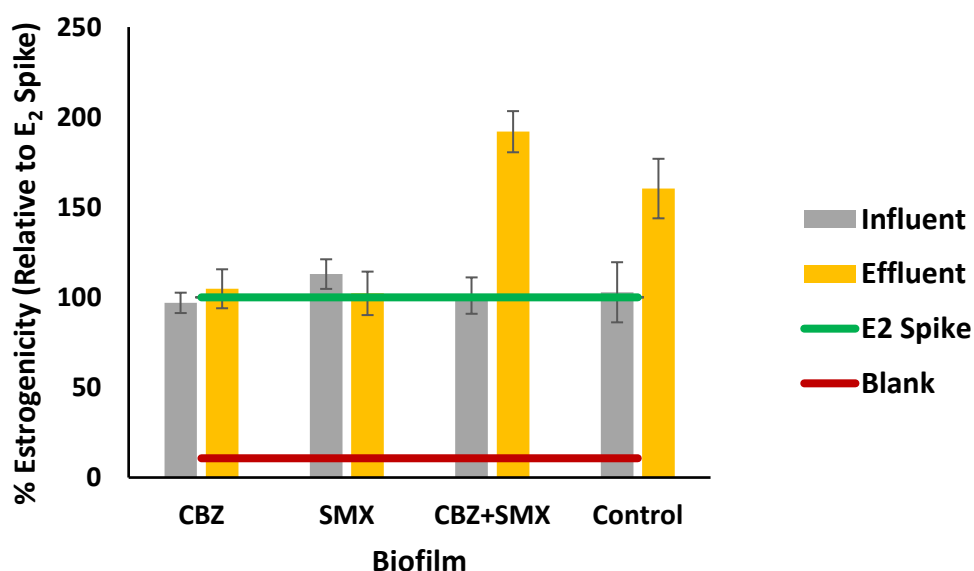

Figure 4-8: Estrogenic effect of CBZ, SMX and a combination of CBZ+SMX relative to an EC50 Estradiol spike, before and after exposure to biofilms grown up on the respective micropollutant enriched media.

4.4 Conclusions:

In conclusion, microbial degradation shows some potential for the abatement of micropollutants from the environment, especially when mixed communities associate to form biofilms with various advantages with regards to metabolic range. However, only under nutrient limited conditions, which isn't the case of any waste water effluent. In this study, biofilms performed better in the removal of CBZ and SMX than planktonic cell cultures of the same inoculum; various biofilm attributes described in the literature, such as biofilm structure, physiology, genetic variance, intra-community interaction and improved resilience to disturbances (Stewart and William Costerton, 2001; Wolcott et al., 2013; Madsen et al., 2012) could have played a role in the observed improved performance. The comparison between the ability of planktonic cells and biofilms to degrade micropollutants cannot directly be made without the consideration of a mass balance comparison of cell concentration. However, the total biomass present in a biofilm is not representative of the total active cells within the biofilm, and therefore a direct comparison is not possible when comparing total biomass. Biofilms grown up on a combination of CBZ and SMX enriched media, had higher removal rates of each compound than the single compound enriched media counterparts. These findings promote the influence of co-metabolism, as proposed by Zhang et al. (2019), and could potentially be further enhanced with the addition of a more complex suite of micropollutants. However, with the metabolism of parent compounds, active transformation products are formed and an additional treatment step might be needed to eradicate the estrogenicity/anti-estrogenicity footprint of the metabolized compounds. Residual organisms present in biologically treated water could easily be disinfected with various existing treatment options, such as ozonation, UV irradiation and chlorination.

An important consideration with regards of success in microbial metabolism for the abatement of CBZ and SMX, is that this was a controlled laboratory experiment. CBZ and SMX were removed by biofilms specific to these micropollutants at very low nutrient conditions, essentially forcing the biofilms to degrade the micropollutants, present at high concentrations relative to environmental and waste water concentrations. In conventional waste water treatment works (WWTWs) it is known that these compounds are not degraded efficiently (Archer et al., 2017). One reason could be the low concentrations of the micropollutants in real-world scenarios, resulting in a low K_m value in the Michaelis-Menten model. Low substrate concentrations results in a low binding affinity of the substrate to the degrading enzymes, which leads to a decreased maximum rate of reaction for micropollutant

degradation. Waste water and polluted environmental waters usually have a high organic matter content, reducing the dissolved oxygen content, thus the microbial communities in conventional WWTWs are not environments conducive to driving the metabolism of micropollutants. The implementation of a biological treatment system for micropollutant removal will have to be implemented as a polishing step in waste water treatment when the total organic content has been reduced to a minimum by other steps in the waste water treatment chain.

It is recommended for future studies that collection of biofilm effluent be synchronized with each individual biofilm's sloughing events to minimize variation in duplicate samples. It should also be explored to focus on longer cultivation periods for the micropollutant degrading biofilms, allowing up to three months for maturation. Micropollutant removal by a porous medium with sorption ability, such as a biofilm, is driven by two main processes: sorption to the biofilm, as well as biodegradation (Martínez-Hernández et al., 2016). For future studies it is recommended that the biomass of the biofilms be harvested from the flowcell system and the biomass quantified and disrupted by a physical method such as sonication, to extract any micropollutants from the biofilm.

As with ozonation, the potential of microbial degradation to remove micropollutants from water source is promising, but the eco-toxicological footprint seems to be more informative of the quality of the water. Additional treatment processes should be considered, as the parent compounds are degraded, that the metabolites often retain activity and requires longer treatment times. Ozonation and the consequent free radicals and energized breakdown of micropollutants would increase the endocrine activity of the breakdown compounds, whereas biological degradation might decrease it. A multi-pronged approach, harnessing various treatment types, might be feasible for complete removal of emerging contaminants, as well as their transformation products.

Chapter 5

General conclusions

5.1 General conclusions:

One of the primary objectives of this study was to design and optimise a laboratory prototype of the CabECO technology, for optimal ozone production within the design parameters of the technology. It was found that the number of CabECO cells, flowrate, current and system pressure influenced the amount of ozone generated by the CabECO prototype. The highest ozone concentration was reached with 2 CabECO cell in series, minimal flowrate (60 L/h), maximum current (5 Amp) and 2 bar system pressure, resulting in an ozone concentration of 5.2 ± 0.49 mg/L. However, to preserve the integrity of the system and to reduce the energy demand, the CabECO prototype was run at 3 Amp, 1 bar and 60 L/h, resulting in an ozone concentration of 2.85 ± 0.08 mg/L, which is sufficient for disinfection and the oxidation of organic pollutants (Khadre et al., 2001; Nishiki et al., 2011). The ozone concentrations measured during this study was less than the theoretical ozone concentration expected, resulting in a lower than expected electron transfer efficiency.

The CabECO technology showed good disinfection potential with various types of environmental water samples, however, high loads of organic matter present in the water proved problematic as the generated ozone was scavenged by the non-target pollutants, leaving less free ozone available for disinfection. The pre-treatment steps required to remove these non-target pollutants, such as coagulation and ultra- and nano-filtration, are complex and expensive; the type of water to be treated should be carefully considered before implementing the CabECO system for water treatment. In the case of high levels of non-target pollutants, even with reduced levels of ozone, all types of environmental water samples had the microbial load reduced by more than 97% (Figure 3-10), however, several orders of magnitude of CFU/mL still remained in the water. Scanning electron microscopy showed that the cell morphology of microbes were disrupted when exposed to ozone and the free radicals generated by electrochemical oxidation. The CabECO prototype also showed promising results for breaking down two selected micropollutants, CBZ and SMX, reaching removal efficiencies of more than 96% for both compounds after only 1 min exposure time in a controlled environment (Figure 3-15). However, when environmental water samples were analysed for micropollutant removal post CabECO treatment, it was found that some compounds proved recalcitrant to oxidation by ozone.

The potential of CBZ and SMX to interfere with the human endocrine activity was also investigated as an indication of environmental impact, and it was found that CabECO treatment significantly reduced the anti-estrogenicity and estrogenicity of CBZ and SMX.

Despite this reduction, it required much longer treatment times for hER interference than the 1 min needed to degrade most of the parent compounds. This is due to the formation of ozonation transformation products that retain some activity and require longer exposure to ozone for adequate degradation. The endocrine disrupting effect of environmental water samples was more significantly reduced in the less polluted Eerste River than in the severely polluted Plankenbrug River, probably due to the higher load of non-target pollutants scavenging the generated ozone.

It was found that ozone is an effective treatment option for water treatment (Khadre et al., 2001), however, several challenges still remain for the implication of ozone with regards to application, economic feasibility and its lack of residual disinfection capabilities. The electrochemical generation of ozone opened up new possibilities for water treatment applications, compared to the conventional generation of ozone from air and then mixed into water. The direct generation of ozone in the aqueous phase, as in the case with the CabECO technology, allows for increased concentrations of ozone as well as increased contact time with target pollutants. Although CabECO has some disinfection capacity, it has sufficient removal efficiencies of only selective micropollutants and should ideally be implemented with another treatment technology, such as microbial degradation or as a module in an advanced oxidation system.

Microbial degradation showed some promise for the abatement of CBZ and SMX when the compounds were exposed to carbon-starved biofilms, however, planktonic cells in liquid culture showed no removal for CBZ and only minor removal of SMX compared to the sterile control. The improved removal of CBZ and SMX by the carbon-starved biofilms could be attributed to the physical and physiological characteristics of biofilms. The biofilm structure, physiology, genetic variance, intra-community interaction and improved resilience to disturbances could all have been contributing factors that resulted in better removal of CBZ and SMX. However, the stochastic nature of biofilms could prove problematic when microbial degradation is implemented for micropollutant abatement, as removal efficiencies will vary with sloughing events observed in this study. Low substrate concentration, in this case micropollutants, will also result in a slow reaction rate in the proposed Michaelis-Menten reaction. Due to these regular sloughing events associated with nutrient limited biofilms, long retention times will have to be incorporated in microbial water treatment system designs.

Microbial degradation had very little effect on the estrogenic and anti-estrogenic activity of the two compounds. The concentrations of CBZ and SMX used in the medium of the biofilm experiment were too low to show an effect on the yeast anti-estrogen screen, due to the high cost of CBZ and SMX standards and the large volumes of medium that had to be made to grow up the biofilms. When CBZ and SMX were combined, better removal was found for both than when in isolation, indicating co-metabolism. This was also seen in the yeast anti-estrogen screen, demonstrating greater estrogenicity in the combined CBZ-SMX medium, post-metabolism. This could be due to the metabolites formed by the biofilm that retain some activity and, in some cases, become more potent. An additional treatment step would be necessary to completely eradicate the endocrine disrupting effect, perhaps one such as ozonation.

5.2 Considerations for future studies:

Due to the enormous and broad suite of micropollutants complicating the pollutant milieu faced by water treatment works, there is a growing need for future studies in the field of contaminant abatement using the electrochemical generation of ozone and more importantly, the direct production of free hydroxyl radicals, and microbial degradation of micropollutants. Due to the variety and recalcitrance of micropollutants, hybrid treatment provides the most promising potential for abatement, implementing both ozonation and microbial degradation and other technologies, to target a wider range of compounds for degradation. Synergistic effects, where one process is capable of completely degrading the other's persistent transformation products, may provide the solution to the complexity of the problem, by creating multipronged approaches (Grandclément et al., 2017).

More emphasis should be turned towards investigating the eco-toxicological footprint of environmental samples when investigating treatment technologies, rather than quantifying an ever growing list of emerging contaminants and their transformation products at immense costs. Eco-toxicological assays can provide an environmental and human impact assessment of the cocktail of contaminants present in environmental samples, giving a more informative indication of the quality of the water, in comparison to quantitative data on occurrence and persistence of a predetermined list of emerging contaminants. Once the environmental and human health impact is determined, focus should shift to identifying the responsible compounds, as well as the possible transformation products that from during the investigated treatment processes.

It is clear that much controversy exists in the scientific field regarding different technologies available for micropollutant abatement and the efficiencies thereof. There remains similar controversy in risk assessment, the detection of environmental micropollutants and the eco-toxicological footprint of low level chronic environmental exposure to parent and transformation by-products. Despite all the resources and funding applied to the field of micropollutant detection, abatement and risk assessment, we are nowhere near a thorough and complete list of compounds of emerging concern (CECs), nor do we understand their individual environmental and human health impacts individually, let alone the impacts of CEC mixtures. Based on the enormity of this task and the rate at which environmental crises are escalating due to population growth it remains critical to divert as much funding and focus towards the regulation and policy of the distribution and use of CECs. Aiming to reduce the anthropogenic chemical footprint in environmental waters, which is often currently creating a suite of potentially more toxic transformation products when treated. We will never be free of micropollutants, and further research must continue to attempt abatement, however, regulation at point-of-use and point-of-production would have greater impact.

As little as 1% of all water sources is fresh water accessible for use (Zaman and Sizemore, 2017), therefore it is crucial to apply resources and energy to both the preservation and reclamation thereof, not only to satisfy consumerism and ever-expanding population, but to ensure the survival of all lifeforms on Earth.

References/Bibliography

Al-Ahmad, A., Daschner, F. and Kümmerer, K. (1999). Biodegradability of Cefotiam, Ciprofloxacin, Meropenem, Penicillin G, and Sulfamethoxazole and inhibition of waste water bacteria. *Archives of Environmental Contamination and Toxicology*, 37(2), pp.158-163.

Angelakis, A. and Zheng, X. (2015). Evolution of water supply, sanitation, wastewater, and stormwater technologies globally. *Water*, 7(12), pp.455-463.

Archer, E., Petrie, B., Kasprzyk-Hordern, B. and Wolfaardt, G. (2017). The fate of pharmaceuticals and personal care products (PPCPs), endocrine disrupting contaminants (EDCs), metabolites and illicit drugs in a WWTW and environmental waters. *Chemosphere*, 174, pp.437-446.

Archer, E., Wolfaardt, G.M., Tucker, K.E., Bröcker, J.H.L., (expected publish date January 2020). Micropollutants and Endocrine-Disrupting Contaminants (EDCs) in wastewater treatment systems: Approach towards effective water reclamation and development of an adverse outcome pathway network for risk assessment. WRC report no. K5/2733//3. Water Research Commission, Pretoria, South Africa.

Bahlmann, A., Brack, W., Schneider, R. and Krauss, M. (2014). Carbamazepine and its metabolites in wastewater: Analytical pitfalls and occurrence in Germany and Portugal. *Water Research*, 57, pp.104-114.

Baker, D. and Kasprzyk-Hordern, B. (2011). Critical evaluation of methodology commonly used in sample collection, storage and preparation for the analysis of pharmaceuticals and illicit drugs in surface water and wastewater by solid phase extraction and liquid chromatography–mass spectrometry. *Journal of Chromatography A*, 1218(44), pp.8036-8059.

Benner, J., Helbling, D., Kohler, H., Wittebol, J., Kaiser, E., Prasse, C., Ternes, T., Albers, C., Aamand, J., Horemans, B., Springael, D., Walravens, E. and Boon, N. (2013). Is biological treatment a viable alternative for micropollutant removal in drinking water treatment processes? *Water Research*, 47(16), pp.5955-5976.

Carabin, A., Drogui, P. and Robert, D. (2015). Photo-degradation of carbamazepine using TiO2 suspended photocatalysts. *Journal of the Taiwan Institute of Chemical Engineers*, 54, pp.109-117.

Celiz, M., Tso, J. and Aga, D. (2009). Pharmaceutical metabolites in the environment: Analytical challenges and ecological risks. *Environmental Toxicology and Chemistry*, 28(12), p.2473.

César, J., Sumita, T., Junqueira, J., Jorge, A. and do Rego, M. (2012). Antimicrobial effects of ozonated water on the sanitization of dental instruments contaminated with E. coli, S. aureus, C. albicans, or the spores of B. atrophaeus. *Journal of Infection and Public Health*, 5(4), pp.269-274.

Chen, H., Zha, J., Liang, X., Li, J. and Wang, Z. (2014). Effects of the human antiepileptic drug carbamazepine on the behavior, biomarkers, and heat shock proteins in the Asian clam Corbicula fluminea. *Aquatic Toxicology*, 155, pp.1-8.

Christensen, P., Yonar, T. and Zakaria, K. (2013). The electrochemical generation of ozone: A review. *Ozone: Science & Engineering*, 35(3), pp.149-167.

Clara, M., Strenn, B., Gans, O., Martinez, E., Kreuzinger, N. and Kroiss, H. (2005). Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane bioreactor and conventional wastewater treatment plants. *Water Research*, 39(19), pp.4797-4807.

Data.worldbank.org. (2019). *Population, total | Data*. [online] Available at: https://data.worldbank.org/indicator/SP.POP.TOTL?name_desc=false [Accessed 8 Jan. 2019].

Death, A., McGrath, K. and Handelsman, D. (2005). Valproate is an anti-androgen and anti-progestin. *Steroids*, 70(14), pp.946-953.

Department of Water Affairs and Forestry, 1996. South African Water Quality Guidelines (second edition). Volume 1: Domestic Use.Dosti, B., Guzel-Seydim, Z. and Greene, A. (2005). Effectiveness of ozone, heat and chlorine for destroying common food spoilage bacteria in synthetic media and biofilms+. *International Journal of Dairy Technology*, 58(1), pp.19-24.

Du, B., Price, A., Scott, W., Kristofco, L., Ramirez, A., Chambliss, C., Yelderman, J. and Brooks, B. (2014). Comparison of contaminants of emerging concern removal, discharge, and water quality hazards among centralized and on-site wastewater treatment system

effluents receiving common wastewater influent. *Science of The Total Environment*, 466-467, pp.976-984.

Echa.europa.eu. (2019). *EC Inventory - ECHA*. [Online] Available at: https://echa.europa.eu/information-on-chemicals/ec-inventory [Accessed 2 Oct. 2019].

Falås, P., Longrée, P., la Cour Jansen, J., Siegrist, H., Hollender, J. and Joss, A. (2013). Micropollutant removal by attached and suspended growth in a hybrid biofilm-activated sludge process. *Water Research*, 47(13), pp.4498-4506.

Falås, P., Wick, A., Castronovo, S., Habermacher, J., Ternes, T. and Joss, A. (2016). Tracing the limits of organic micropollutant removal in biological wastewater treatment. *Water Research*, 95, pp.240-249.

Fryda, M., Schäfer, L. and Tröster, I. (2001). Doped diamond – A new material for industrial electrochemistry. *Recent research developments in electrochemistry,* 4, p.85-97.

Göbel, A., Thomsen, A., McArdell, C., Joss, A. and Giger, W. (2005). Occurrence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sludge treatment. *Environmental Science & Technology*, 39(11), pp.3981-3989.

Gómez-Ramos, M., Mezcua, M., Agüera, A., Fernández-Alba, A., Gonzalo, S., Rodríguez, A. and Rosal, R. (2011). Chemical and toxicological evolution of the antibiotic sulfamethoxazole under ozone treatment in water solution. *Journal of Hazardous Materials*.

Grandclément, C., Seyssiecq, I., Piram, A., Wong-Wah-Chung, P., Vanot, G., Tiliacos, N., Roche, N. and Doumenq, P. (2017). From the conventional biological wastewater treatment to hybrid processes, the evaluation of organic micropollutant removal: A review. *Water Research*, 111, pp.297-317.

Guo, Y., Wang, H., Wang, B., Deng, S., Huang, J., Yu, G. and Wang, Y. (2018). Prediction of micropollutant abatement during homogeneous catalytic ozonation by a chemical kinetic model. *Water Research*, 142, pp.383-395.

Guzzella, L., Novati, S., Casatta, N., Roscioli, C., Valsecchi, L., Binelli, A., Parolini, M., Solcà, N., Bettinetti, R., Manca, M., Mazzoni, M., Piscia, R., Volta, P., Marchetto, A., Lami, A. and Marziali, L. (2018). Spatial and temporal trends of target organic and inorganic

micropollutants in Lake Maggiore and Lake Lugano (Italian-Swiss water bodies): contamination in sediments and biota. *Hydrobiologia*, 824(1), pp.271-290.

Guzzella, L., Poma, G., De Paolis, A., Roscioli, C. and Viviano, G. (2011). Organic persistent toxic substances in soils, waters and sediments along an altitudinal gradient at Mt. Sagarmatha, Himalayas, Nepal. *Environmental Pollution*, 159(10), pp.2552-2564.

Ha, H., Mahanty, B., Yoon, S. and Kim, C. (2016). Degradation of the long-resistant pharmaceutical compounds carbamazepine and diatrizoate using mixed microbial culture. *Journal of Environmental Science and Health, Part A*, 51(6), pp.467-471.

Haghi, R., Chapoy, A., Peirera, L., Yang, J. and Tohidi, B. (2017). pH of CO 2 saturated water and CO 2 saturated brines: Experimental measurements and modelling. *International Journal of Greenhouse Gas Control*, 66, pp.190-203.

Heberer, T. (2002). Tracking persistent pharmaceutical residues from municipal sewage to drinking water. *Journal of Hydrology*, 266(3-4), pp.175-189.

Hellauer, K., Mergel, D., Ruhl, A., Filter, J., Hübner, U., Jekel, M. and Drewes, J. (2017). Advancing Sequential Managed Aquifer Recharge Technology (SMART) using different intermediate oxidation processes. *Water*, 9(3), p.221.

Henriques, A., Jenkins, R., Burton, N. and Cooper, R. (2009). The intracellular effects of manuka honey on Staphylococcus aureus. *European Journal of Clinical Microbiology & Infectious Diseases*, 29(1), pp.45-50.

Hoigné, J., Bader, H., Haag, W. and Staehelin, J. (1985). Rate constants of reactions of ozone with organic and inorganic compounds in water—III. Inorganic compounds and radicals. *Water Research*, 19(8), pp.993-1004.

Jankunaite, D., Tichonovas, M., Buivydiene, D., Radziuniene, I., Racys, V. and Krugly, E. (2017). Removal of Diclofenac, Ketoprofen, and Carbamazepine from simulated drinking water by advanced oxidation in a model reactor. *Water, Air, & Soil Pollution*, 228(9).

Jelic, A., Cruz-Morató, C., Marco-Urrea, E., Sarrà, M., Perez, S., Vicent, T., Petrović, M. and Barcelo, D. (2012). Degradation of carbamazepine by Trametes versicolor in an air pulsed fluidized bed bioreactor and identification of intermediates. *Water Research*, 46(4), pp.955-964.

Jones, O., Voulvoulis, N. and Lester, J. (2002). Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. *Water Research*, 36(20), pp.5013-5022.

Joubert, L., Ferreira, J., Stevens, D., Nazik, H. and Cegelski, L. (2017). Visualization of *Aspergillus fumigatus* biofilms with Scanning Electron Microscopy and Variable Pressure-Scanning Electron Microscopy: A comparison of processing techniques. *Journal of Microbiological Methods*, 132, pp.46-55.

Kaplan, J. (2010). Biofilm Dispersal: mechanisms, clinical implications, and potential therapeutic uses. *Journal of Dental Research*, 89(3), pp.205-218.

Khadre, M., Yousef, A. and Kim, J. (2001). Microbiological aspects of ozone applications in food: A review. *Journal of Food Science*, 66(9), pp.1242-1252.

Klavarioti, M., Mantzavinos, D. and Kassinos, D. (2009). Removal of residual pharmaceuticals from aqueous systems by advanced oxidation processes. *Environment International*, 35(2), pp.402-417.

Krulwich, T., Sachs, G. and Padan, E. (2011). Molecular aspects of bacterial pH sensing and homeostasis. *Nature Reviews Microbiology*, 9(5), pp.330-343.

Kuiper, G. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor. *Endocrinology*, 139(10), pp.4252-4263.

Kümmerer, K. (2009). Pharmaceuticals in the environment: sources, fate, effects and risks. *Environmental Science and Pollution Research*, 17(2), pp.519-521.

Kunkel, U. and Radke, M. (2012). Fate of pharmaceuticals in rivers: Deriving a benchmark dataset at favorable attenuation conditions. *Water Research*, 46(17), pp.5551-5565.

Lee, M., Blum, L., Schmid, E., Fenner, K. and von Gunten, U. (2017). A computer-based prediction platform for the reaction of ozone with organic compounds in aqueous solution: kinetics and mechanisms. *Environ. Sci.: Processes Impacts*, 19(3), pp.465-476.

Loos, R., Carvalho, R., António, D., Comero, S., Locoro, G., Tavazzi, S., Paracchini, B., Ghiani, M., Lettieri, T., Blaha, L., Jarosova, B., Voorspoels, S., Servaes, K., Haglund, P., Fick, J., Lindberg, R., Schwesig, D. and Gawlik, B. (2013). EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents. *Water Research*, 47(17), pp.6475-6487.

Luo, Y., Guo, W., Ngo, H., Nghiem, L., Hai, F., Zhang, J., Liang, S. and Wang, X. (2014). A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Science of The Total Environment*, 473-474, pp.619-641.

Madsen, J., Burmølle, M., Hansen, L. and Sørensen, S. (2012). The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunology & Medical Microbiology*, 65(2), pp.183-195.

Majewsky, M., Wagner, D., Delay, M., Bräse, S., Yargeau, V. and Horn, H. (2014). Antibacterial activity of sulfamethoxazole transformation products (TPs): General Relevance for Sulfonamide TPs Modified at the para Position. *Chemical Research in Toxicology*, 27(10), pp.1821-1828.

Martínez-Hernández, V., Meffe, R., Herrera López, S. and de Bustamante, I. (2016). The role of sorption and biodegradation in the removal of acetaminophen, carbamazepine, caffeine, naproxen and sulfamethoxazole during soil contact: A kinetics study. *Science of The Total Environment*, 559, pp.232-241.

McDowell, D., Huber, M., Wagner, M., von Gunten, U. and Ternes, T. (2005). Ozonation of carbamazepine in drinking water: Identification and kinetic study of major oxidation products. *Environmental Science & Technology*, 39(20), pp.8014-8022.

Miralles-Cuevas, S., Oller, I., Pérez, J. and Malato, S. (2014). Removal of pharmaceuticals from MWTP effluent by nanofiltration and solar photo-Fenton using two different iron complexes at neutral pH. *Water Research*, 64, pp.23-31.

Mulla, S., Hu, A., Sun, Q., Li, J., Suanon, F., Ashfaq, M. and Yu, C. (2018). Biodegradation of sulfamethoxazole in bacteria from three different origins. *Journal of Environmental Management*, 206, pp.93-102.

Nasuhoglu, D., Yargeau, V. and Berk, D. (2011). Photo-removal of sulfamethoxazole (SMX) by photolytic and photocatalytic processes in a batch reactor under UV-C radiation (λmax=254nm). *Journal of Hazardous Materials*, 186(1), pp.67-75.

Newman, S. (2004). Disinfecting irrigation water for disease management. In: *20th Annual Conference on Pest Management on Ornamentals*. [Online] San Jose, California: Society of American Florists. Available at: http://www.nurserycropscience.info/water/filtration-

disinfection/other-references/newman-2004-disinfecting-irrigation-water-paper.pdf [Accessed 3 Oct. 2019].

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., McKay, G., Siehnel, R., Schafhauser, J., Wang, Y., Britigan, B. and Singh, P. (2011). Active Starvation Responses Mediate Antibiotic Tolerance in Biofilms and Nutrient-Limited Bacteria. *Science*, 334(6058), pp.982-986.

Nishiki, Y., Kitaori, N. and Nakamuro, K. (2011). Performances of small-sized generator of ozone-dissolved water using boron-doped diamond electrodes. *Ozone: Science & Engineering*, 33(2), pp.114-120.

Osunmakinde, C.S., Tshabalala, O.S., Dube, S., Nindi, M.M., 2013. Verification and validation of analytical methods for testing the levels of PPHCPs (pharmaceutical & personal health care products) in treated drinking water and sewage. Water Research Commission (WRC), Pretoria, South Africa. Report no. 2094/1/13.

Oxidationtech.com. (2019). *Ozone equipment manufacturer and ozone system integrators ozone solubility in liquid ozone integration experts*. [Online] Available at: https://www.oxidationtech.com/ozone/solubility.html [Accessed 1 Jul. 2019].

Petrovic, M. (2014). Methodological challenges of multi-residue analysis of pharmaceuticals in environmental samples. *Trends in Environmental Analytical Chemistry*, 1, pp.e25-e33.

Radke, M., Lauwigi, C., Heinkele, G., Mürdter, T. and Letzel, M. (2009). Fate of the Antibiotic Sulfamethoxazole and Its Two Major Human Metabolites in a Water Sediment Test. *Environmental Science & Technology*, 43(9), pp.3135-3141.

Raeke, J., Lechtenfeld, O., Seiwert, B., Meier, T., Riemenschneider, C. and Reemtsma, T. (2017). Photochemically induced bound residue formation of carbamazepine with dissolved organic matter. *Environmental Science & Technology*, 51(10), pp.5523-5530.

Rice, S., Koh, K., Queck, S., Labbate, M., Lam, K. and Kjelleberg, S. (2005). Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. *Journal of Bacteriology*, 187(10), pp.3477-3485.

Russell, J., Spiller, H. and Baker, D. (2015). Markedly elevated carbamazepine-10,11-epoxide/carbamazepine ratio in a fatal carbamazepine ingestion. *Case Reports in Medicine*, 2015, pp.1-4.

Schwarzenbach, R. (2006). The Challenge of Micropollutants in Aquatic Systems. *Science*, 313(5790), pp.1072-1077.

Sharma, S. (2011). Endocrine disruptors environmental oxygen epigenetics and pregnancy. *Frontiers in Bioscience*, E3 (2), pp.690-700.

Sijimol, M., Jyothy, S., Pradeepkumar, A., Chandran, M., Ghouse, S. and Mohan, M. (2015). Review on fate, toxicity, and remediation of perchlorate. *Environmental Forensics*, 16(2), pp.125-134.

Singh, S., Singh, S., Chowdhury, I. and Singh, R. (2017). Understanding the mechanism of bacterial biofilms resistance to antimicrobial agents. *The Open Microbiology Journal*, 11(1), pp.53-62.

Smego, R. (1983). Trimethoprim-sulfamethoxazole therapy for Nocardia infections. *Archives of Internal Medicine*, 143(4), pp.711-718.

Sonnenschein, C. and Soto, A. (1998). An updated review of environmental estrogen and androgen mimics and antagonists. *The Journal of Steroid Biochemistry and Molecular Biology*, 65(1-6), pp.143-150.

Sotelo, J., Beltrán, F., Benitez, F. and Beltrán-Heredia, J. (1989). Henry's law constant for the ozone-water system. *Water Research*, 23(10), pp.1239-1246.

Soto, A. and Sonnenschein, C. (2010). Environmental causes of cancer: endocrine disruptors as carcinogens. *Nature Reviews Endocrinology*, 6(7), pp.363-370.

Stewart, P. and William Costerton, J. (2001). Antibiotic resistance of bacteria in biofilms. *The Lancet*, 358(9276), pp.135-138.

Stone W. (2017) Deliverable 1.3. SafeWaterAfrica. Self-sustaining cleaning technology for safe water supply and management in rural African areas. EU Grant No. 689925.

Sugano, M., Morisaki, H., Negishi, Y., Endo-Takahashi, Y., Kuwata, H., Miyazaki, T. and Yamamoto, M. (2015). Potential effect of cationic liposomes on interactions with oral bacterial cells and biofilms. *Journal of Liposome Research*, pp.1-7.

Sumpter, J. (1998). Xenoendocrine disrupters — environmental impacts. *Toxicology Letters*, 102-103, pp.337-342.

Suri, R., Singh, T. and Chimchirian, R. (2011). Effect of process conditions on the analysis of free and conjugated estrogen hormones by solid-phase extraction–gas chromatography/mass spectrometry (SPE–GC/MS). *Environmental Monitoring and Assessment*, 184(3), pp.1657-1669.

Swart, J., Pool, E. and van Wyk, J. (2011). The implementation of a battery of in vivo and in vitro bioassays to assess river water for estrogenic endocrine disrupting chemicals. *Ecotoxicology and Environmental Safety*, 74(1), pp.138-143.

Swart, N. and Pool, E. (2007). Rapid detection of selected steroid hormones from sewage effluents using an ELISA in the Kuils River water catchment area, South Africa. *Journal of Immunoassay and Immunochemistry*, 28(4), pp.395-408.

Talagrand-Reboul, E., Jumas-Bilak, E. and Lamy, B. (2017). The Social Life of *Aeromonas* through biofilm and quorum sensing systems. *Frontiers in Microbiology*, 8.

Tang, K., Escola Casas, M., Ooi, G., Kaarsholm, K., Bester, K. and Andersen, H. (2017). Influence of humic acid addition on the degradation of pharmaceuticals by biofilms in effluent wastewater. *International Journal of Hygiene and Environmental Health*, 220(3), pp.604-610.

Thanomsub, B., Anupunpisit, V., Chanphetch, S., Watcharachaipong, T., Poonkhum, R. and Srisukonth, C. (2002). Effects of ozone treatment on cell growth and ultrastructural changes in bacteria. *The Journal of General and Applied Microbiology*, 48(4), pp.193-199.

Torresi, E., Polesel, F., Bester, K., Christensson, M., Smets, B., Trapp, S., Andersen, H. and Plósz, B. (2017). Diffusion and sorption of organic micropollutants in biofilms with varying thicknesses. *Water Research*, 123, pp.388-400.

Vanderford, B., Mawhinney, D., Trenholm, R., Zeigler-Holady, J. and Snyder, S. (2011). Assessment of sample preservation techniques for pharmaceuticals, personal care

products, and steroids in surface and drinking water. *Analytical and Bioanalytical Chemistry*, 399(6), pp.2227-2234.

Wang, J. and Wang, S. (2018). Microbial degradation of sulfamethoxazole in the environment. *Applied Microbiology and Biotechnology*, 102(8), pp.3573-3582.

Williams, P., Winzer, K., Chan, W. and Cámara, M. (2007). Look who's talking: communication and quorum sensing in the bacterial world. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 362(1483), pp.1119-1134.

Wolcott, R., Costerton, J., Raoult, D. and Cutler, S. (2013). The polymicrobial nature of biofilm infection. *Clinical Microbiology and Infection*, 19(2), pp.107-112.

Xu, B., Mao, D., Luo, Y. and Xu, L. (2011). Sulfamethoxazole biodegradation and biotransformation in the water–sediment system of a natural river. *Bioresource Technology*, 102(14), pp.7069-7076.

Yu, Y., Liu, Y. and Wu, L. (2013). Sorption and degradation of pharmaceuticals and personal care products (PPCPs) in soils. *Environmental Science and Pollution Research*, 20(6), pp.4261-4267.

Zaman, M. and Sizemore, R. (2017). Freshwater resources could become the most critical factor in the future of the earth. *Journal of the Mississippi Academy of Sciences*, 62(4), pp.348-352.

Zhang, L., Carvalho, P., Bollmann, U., EI-taliawy, H., Brix, H. and Bester, K. (2019). Enhanced removal of pharmaceuticals in a biofilter: Effects of manipulating co-degradation by carbon feeding. *Chemosphere*, 236, p.124303.

Zheng, M., Xu, G., Pei, J., He, X., Xu, P., Liu, N. and Wu, M. (2014). EB-radiolysis of carbamazepine: in pure-water with different ions and in surface water. *Journal of Radioanalytical and Nuclear Chemistry*, 302(1), pp.139-147.