Interaction Between Micropollutants and Degradative Biofilm Communities: Basis for a Biomimetic Approach to Water Treatment

by

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An original thesis presented in fulfilment of the requirements for the degree *Magister Scientiae* in Microbiology in the Department of Microbiology, in the Faculty of Science at Stellenbosch University

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Table of Contents

List of Tables

List of Figures

List of Terms

adhesin = a component found on bacterial cell surfaces that facilitate adhesion; often a virulence factor

adsorption = the adhesion of atoms or molecules to a surface from an aqueous solution or gas

agonism = the binding of a ligand (called the agonist) to a receptor that elicits a response or cascade (antonym: antagonism)

anabolism = the building of more complex molecules using simpler molecules, energy and enzymes

antagonism = the binding of a ligand (called the antagonist) to a receptor that elicits no response, because it occupies the receptor which prevents binding of the native, agonistic, ligand.

arabinose = a five-carbon monosaccharide containing an aldehyde group (a sugar aldehyde); used as a carbon or energy source in some microbial organisms;

assimilate = the uptake of exogenous compounds by an organism to be used as substrates in anabolic or catabolic processes, or for other physiological requirements (e.g. vitamins as enzyme cofactors)

auto-inducer = a molecule that is recognised by certain bacteria, and acts as a regulatory molecule at threshold concentrations in these organisms; a mediator of quorum sensing; often a virulence factor

benzodiazepine = a type of pharmaceutical compound that acts upon gamma-aminobutyric acid (GABA) receptors; used as an anti-convulsant and a tranquiliser; see carbamazepine

biofilm = a stationary amalgamation of microbial cells surrounded by a matrix

biomimicry = the adaptation or emulation of processes or structures found in nature in order to solve problems of an anthropogenic nature

bioremediation = the use of microbes, or other organisms, to alleviate pollution events, or polluted resources

carbamazepine = a pharmaceutical of the benzodiazepine group of drugs, used as an antiepileptic;

catabolism = the breakdown of molecules, within the cell, to simpler metabolites in order to release energy

catabolite repression = a genetic pathway in bacterial organisms that turns off the expression of sets of enzymes involved in utilising certain sugars in the presence of glucose

constructed wetland = a biomimetic construct, that mimics a wetland ecosystem, through which wastewater is remediated

endocrine-disrupting compound (EDC) = a compound capable of interacting with endocrine systems in higher vertebrates, either through direct receptor interactions or through interference with components in hormonal pathways

extracellular matrix (ECM) = substances produced by cells that have structural purpose in the formation of the biofilm

extrapolymeric substance (EPS) = synonymous with ECM

glucose = a hexose sugar (monosaccharide) which is the priority compound in catabolite repression, and a labile carbon source

labile = term referring to a compound that is readily transformed through biological activity

lactose = a disaccharide consisting of glucose and galactose joined by a glycosidic linkage

life's principles = the set of guidelines on how to mimic natural systems; used in biomimetic design

methylparaben = a member of the paraben group, formed by the esterification of parahydroxybenzoic acid and methanol; one of the main micropollutants studied in this thesis

microenvironment = the structure and set of physiochemical conditions created by the formation of EPS around a group of sessile cells

micropollutant = compounds that are biologically active (elicit physiological responses) in organisms and persistent in the environment

mineralisation = breakdown or decomposition of compounds, releasing energy, using small molecules as final reducing agents

N-acyl homoserine lactone (AHL) = a type of auto-inducer recognised and used by gram negative bacteria

nephrostomy tube = a catheter used to drain the kidneys through the skin

paraben = an ester of parahydroxybenzoic acid and an alcohol, the type of alcohol determines the type of paraben that results from esterification

quorum sensing = gene regulation mediated by population density; the presence of auto-inducers is proportional to the cell population size and turns on the expression of certain genes once the auto-inducers reach a threshold concentration in the immediate environment

sessile = stationary; immobilised; refers to cells in a sedentary state (biofilms) within this thesis

wastewater treatment plant (WWTP) = a construct used to remove contaminants from wastewater to allow safe release of the water into the environment

xenobiotic = a compound that is foreign to an organism, but can elicit physiological responses within that organism

Declaration

By submitting this thesis electronically, I, Kirsten Kenchenten, declare that the entirety of the work contained therein is my own, original work and that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Kirsten Kenchenten

March 2017

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Master of Science/*Magister Scientiae*, 2017

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Abstract

The remediation and reclamation of water has become a critically important issue, as the use of water as a conduit of waste has resulted in the contamination of a resource that life on Earth cannot function without. Research has shown that pollutants at trace levels, or micropollutants, are not being removed adequately from treated water and, in some cases, the micropollutant concentrations actually increase following treatment. Microbial aggregates, such as biofilms, are important constituent in wastewater treatment, in both conventional wastewater treatment plants (WWTPs) and in new-generation alternatives such as biomimicry-based constructed wetlands. This project was conducted in the paradigm of biomimicry, the concept of using inspiration from nature to provide innovative solutions to anthropogenic problems. The aim of the study was two-fold; namely 1) to investigate the laboratory-based degradation of selected micropollutants by biofilms, with microbial activity as the key driver of biodegradation and, 2) to extrapolate these findings to gain understanding of the microbial processes responsible for the functioning of biomimicry-based water treatment systems. Of particular interest was the potential consequence of labile nutrient availability on the degradation of micropollutants in the treatment systems. The results showed that, while labile carbon sources (such as glucose) do affect the removal of the tested micropollutants, methylparaben and carbamazepine, the degree of this effect is not significant enough to explain the lack of micropollutant removal. In contrast, these two compounds had a significant effect on biofilm structure, even when applied at low concentrations (1000 $ng.L^{-1}$ and 600 $ng.L^{-1}$ for methylparaben and carbamazepine, respectively), as revealed by scanning confocal laser microscopy. Microbial biofilms execute degradative functions with flexibility and are furthermore able to efficiently adapt in a manner that humans have yet to replicate. Since our dependency on natural processes such as bio-utilisation and bio-assistance of microbes in our constructs cannot be denied or replaced, biomimicry shows promise as a framework for the design of wastewater treatment systems that replicate natural processes.

Opsomming

Die remediëring en herwinning van water het 'n krities belangrike saak geword, as gevolg van water wat gebruik word as 'n watervoor vir afval. Dit het gelei na die besoedeling van ‘n hulpbron waarsonder lewe op aarde nie kan funksioneer nie. Navorsing het bewys dat besoedeling in baie klein hoeveelhede as ook mikrobesoedeling nie doeltreffend vanuit behandelde water verwyder word nie en in seker gevalle selfs verhoog word na behandeling van die water. Mikrobiese aggregate soos biofilms, is 'n belangrike bydrae tot afvalwater behandeling in beide tradisionele rioolsuiweringswerke asook in nuwe-generasie alternatiewe soos biomimiek-gebaseerde opgerigte vleilande. Hierdie projek was gedoen in die paradigma van biomimiek, die konsep om inspirasie vanuit die natuur te verkry om innoverende oplossings te vind vir antropogeniese probleme. Die doel van die studie was tweeledig; naamlik 1) om die laboratorium-gebaseerde degradasie van geselekteerde mikrobesoedeling te bestudeer deur die gebruik van biofilms met mikrobiese aktiwiteite as die sleutel dryfpunt van die biodegradasie, asook die ekstrapolering van hierdie bevindings om kennis te bekom van die mikrobiese proses wat verantwoordelik is vir die funksionering van biomimiek-gebaseerde waterbehandeling sisteme. Van spesifieke belang was die potensiële gevolg wat labiele voedingstof beskikbaarheid op die degradasie van mikrobesoedeling in behandeling sisteme het. Die resultate het gewys dat labiele koolstof bronne (bv. glukose) wel 'n bydrae lewer om die getoetsde mikrobesoedeling, methylparaben en carbamazepine, te verwyder, maar ook dat dit nie beduidend genoeg was om die tekort van mikrobesoedeling te verduidelik nie. In kontras het hierdie twee verbindings het 'n beduidende effek op biofilmstrukture gehad, selfs waneer dit in klein konsentrasies (1000 $ng.L^{-1}$ en 600 $ng.L^{-1}$ vir methylparaben en carbamazepine, onderskeidelik) toegedien was, soos bewys deur middel van 'n skandering konvokale laser mikroskopie. Mikrobiese biofilms voer degradering uit met buigsaamheid en is daardeur in staat om aan te pas in ‘n manier wat mensdom nog moet naboots. Omdat ons afhanklikheid op natuurlike prosesse soos bio-benutting en bio-bystand nie ontken of vervang kan word nie, lyk biomimiek belowend as 'n raamwerk vir die ontwerp van afvalwaterbehandeling sisteme wat natuurlike prosesse naboots.

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"It is our choices, Harry, that show what we truly are, far more than our abilities."

– J.K. Rowling, *Harry Potter and the Chamber of Secrets*

Chapter 1: Introduction

Fresh water comprises less than 1% of the total volume of water on Earth, which must be shared by upwards of 7 billion humans (Dimick, 2014) as well as every other living organism residing on the planet. This implies that water is an extremely scarce resource that needs to support a vast number of individuals in the recent future. The manner in which water has been managed thus far is therefore relatively alarming considering that our survival is heavily dependent on its' availability: humans use water as a conduit of waste. Since the introduction of aqueducts by the Babylonians (750 B.C.) and thereafter the Romans (312 B.C.) (AncientHistoryEncylopaedia, 2016), water has been used to transport elimination waste away from our homes; to wash our clothes, bodies and various household items. Coupled with this, the sudden and vast advances in medical and scientific technology have resulted in the development and production of thousands of pharmaceutical drugs, cosmetics and personal care products, which end up in the surface water systems. These different substances contain at least one, if not many, chemical compounds that have detrimental effects on the health of the environment and the organisms within it, including that of humans (Boxall, 2004).

Technology has allowed for the implementation of wastewater treatment (WWT) methods that all aim to remove solid and dissolved waste (both organic and inorganic) as well as pathogens, heavy metals and some of these abovementioned chemical pollutants, in order to enable the recycling of the once heavily-sullied and mistreated resource without which life cannot exist. These WWT plants (WWTPs) vary, from the conventional sewage treatment plants to the newer, more ecologically-directed, sustainable, eco-friendly alternatives (constructed wetlands, soil aquifer treatments (SATs), etc.). Despite the variation in the detailed design parameters and engineered intricacies of these different WWTPs, the intention for their construction remains the same: the adequate treatment of wastewater.

In most countries, access to adequate WWT is something widely taken for granted, but in developing countries some communities do not have access to such crucial amenities. In South Africa, for example, only those residing within planned residential areas and farm plots have access to indoor plumbing and WWT. Many individuals living in rural settlements obtain water from communal taps and dispose of the wastewater in makeshift conduits that run the water, untreated, directly (or indirectly) into the surface water systems, causing serious pollution. Government intervention is not likely in the near future and therefore one of these communities, Langrug in the Franschhoek area of the Western Cape, was chosen to receive an Eco-Machine™, designed by John Todd Ecological Design, to treat the wastewater before it enters, and contaminates, the Berg River. Essentially this Eco-Machine, also referred to as a "Living Machine", comprises of a series of cells that each contain a variety of plants, animals, microorganisms and associated microbial biofilms. The water to be treated runs through these cells, while the organisms within degrade the organic and inorganic components to use as an energy source. The construct is biomimetic; as it is based on wetland biomes found in nature. More detail regarding the function of these constructed wetlands is given in the literature review.

Research has shown, especially in the case of conventional WWTPs, that the aforementioned anthropogenic chemicals are not completely removed during the wastewater treatment process (Daughton and Ternes, 1999). In a controlled environment, such as a laboratory, microbial biofilms have been shown to elicit degradation or sequestration (bioaccumulation) of these compounds (Bathe et al., 2009), through adaptation or recombinant DNA technology. However, the same removal efficiency has not been achieved when these microbes were applied to real-life environments or to environments that simulate the actual conditions (Goldstein et al., 1985). It is possible that the reason for this lowered removal efficacy is due to the increased availability of more labile (energetically-preferred) compounds in the environment. Microbes are energy efficient and will not degrade complex pollutants that may require specialised catabolic pathways with additional, energy-expensive enzymes when a more energy-efficient nutrient source is available (Snyder and Champness, 2007).

1.1) Research Aims and Hypotheses

The first aim of this thesis was the investigation of the competition between labile carbon sources, requiring very little energy investment on the part of the microbe, and micropollutants, which are relatively more recalcitrant to microbial degradation. This was achieved by constructing a novel biomimicry-based biofilm reactor that allows for exposure of a biofilm to a bulk aqueous flow with both a labile carbon source and a micropollutant. The intent is to allow depletion of the labile carbon source until the biofilm downstream is forced to use the remaining micropollutant as a carbon source. It is anticipated that the outcome of this study will guide future studies considering modifications to wastewater treatment processes aimed specifically at the removal of these compounds. The hypothesis was that the presence of a more labile nutrient would reduce or completely inhibit the degradation of the micropollutant. The reasoning behind this postulate was that while the more labile carbon source is present, the microbes will use it as a primary carbon and/or energy source simply because it produces a high energy output to input ratio. Utilisation of the micropollutant would likely require different metabolic pathways than the labile carbon source, and therefore the microbes within the biofilm would have to produce a different set of enzymes, requiring the initiation of further anabolic activities (which uses energy). Therefore, the utilisation of the micropollutant by the biofilm, in terms of its' net energy yield, would be inefficient relative to the labile source.

The second aim of this research was to ascertain the effects of the selected micropollutants (methylparaben and carbamazepine) on microbes, namely the effect on the structure of biofilms and the effect on recombinant yeast expressing human endocrine receptors. The effect of micropollutants (at environmental levels) was hypothesised to affect the structure of the biofilms but the extent to which the effects would be evident was not known. The recombinant yeast served to test the effects of both micropollutants on the yeast cell, as well as the effect on the endocrine receptors. Therefore, the purpose was two-fold: to test the effects on yeast (potential cytotoxicity) and the potential endocrine-disrupting

effects on humans. The hypothesis of this second aim was that methylparaben would show endocrine-disrupting effects on the receptors, and lethal effects on yeast and pure bacterial cultures, but that there would be a low inhibitory effect on the mixed microbial cultures. Carbamazepine was also hypothesised to have these same effects.

The final aim was to evaluate the potential of biomimetic constructs, such as constructed wetlands (Eco-Machines™) for removal of pollutants from wastewater and whether their use is viable in a real-world application. The hypothesis is that they will be advantageous in certain situations but that their use would be limited, since they are based on an ecosystem, which will always have carrying capacities and limitations.

The **main aims** for this research are **summarised** as follows:

1) To investigate the competitive effects of labile carbon sources on the metabolism of two selected micropollutants in the presence of degradative biofilms.

2) To investigate the effects of the selected micropollutants on the structure of biofilms to which they are exposed, as well as the endocrine effects of these same micropollutants as determined using transgenic yeast expressing human endocrine receptors.

3) To evaluate the potential of biomimicry in the design of systems for the removal of micropollutants from wastewater

Chapter 2: Review of Literature

2.1) Challenges for Modern Water Treatment Systems

Humans, compared to other species inhabiting the earth, are regarded as the most destructive. Ecosystems provide a cyclic, harmonious relationship between organisms, where each organism has a function within a niche and contributes to maintaining overall homeostasis. Humans often do not comply with this rule of nature, removing natural resources and mostly offering little but unusable, and often toxic, waste products. Water is one of these resources. Less than 1% of the water on the surface of the earth is potable (Lenntech, 2015; Dimick, 2014).

The advance in modern technologies has allowed for the production and use of a large array of synthetic compounds for both medical and non-medical use. Many of these compounds are readily available, are used in large quantities, and are prevalent in the water as a consequence. While personal-care products (PCPs) are not intended for ingestion, they are topically applied or are used as cleaning agents and are therefore washed out *talis qualis* into grey water. In the case of pharmaceuticals, some of the compounds in question are not completely metabolised in the body which, in turn, results in the excretion of un-metabolised, potentially active drugs (Jjemba, 2006) that eventually end up in the sewage system (WWTPs) (Gros et al., 2007; Kasprzyk-Hordern et al., 2009).

Research conducted by Kasprzyk-Hordern et al. (2009) involved the monitoring of rivers in South Wales, and revealed that treated effluents from WWTPs were the primary contributor to pharmaceuticals and personal-care products (PPCPs) in these rivers. The question that then arises from this information is to what degree PPCP accumulation is occurring in the river or surface water systems around the world? The next question that arises is why the wastewater treatment processes currently in use are inadequate for complete removal of these PPCPs?

There are currently two types of WWTPs in use: conventional WWTPs (in both municipal and industrial systems), and treatment systems that are designed with the aim to be more eco-friendly (natural wetland-based waste-water treatment systems, for example). The former is discussed in detail in this section, while the latter is covered in more detail in the section pertaining to biomimicry (section 2.4). Conventional treatment of sewage, occurring in conventional WWTPs, begins with transportation of the wastewater through the underground sewage system, until it flows into a wastewater treatment plant. Once there, the water is processed to remove organic pollutants and pathogenic microbes, and is subsequently returned into the surface water system.

The majority of global conventional WWTPs consist of 3 to 4 processes: preliminary treatment, primary treatment, biological treatment and tertiary treatment (Bitton, 1994). Preliminary treatment depends on the nature of the incoming sewage, but consists of grit chambers and screens to trap large contaminants

or articles of trash (Bitton, 1994). Primary treatment involves primary sedimentation, where both organic and inorganic solids (ones that settle) are removed (Pescod, 1992), after which the wastewater goes into biological treatment (secondary treatment). This consists of aeration and sedimentation (trickling filters, activated sludge, etc). The aeration serves to provide the ideal environment for biodegradation by aerobic microorganisms, and the microbes degrade the larger compounds in the sewage into small inorganic products, such as ammonia (NH_3), water (H_2O) and carbon dioxide (CO_2) (Pescod, 1992). This stage is where the majority of organic contaminants (including pharmaceuticals and personal-care products (PPCPs) are removed. The tertiary treatment consists of flocculation, oxidation and adsorption processes (Bitton, 1994; Zwiener and Frimmel, 2000), and is an adapted form of secondary treatment. This is the step in which the removal of residual constituent contaminants (PPCPs, heavy metals, phosphorus and other low concentration contaminants), not removed in secondary treatment, occurs (Pescod, 1992). Release of water from the plant into the surface water system occurs after the tertiary treatment. Even though PPCPs do undergo some form of metabolism and removal in the WWTP systems, some of them have been detected in surface water systems around the world (Gros et al., 2007; Kasprzyk-Hordern et al., 2009) and their removal is incomplete (Heberer, 2002). This incomplete removal does not support the release of treated effluent water into surface water. The relative concentrations of these substances in both influent and effluent water from WWTPs are indicative of not only the types, but also the concentrations of PPCPs flowing into and being degraded within these systems. Potential interactions between PPCPs may additionally influence removal efficiency in ways that are not well understood.

The following section of the literature review will focus on the significance of increasing population density and chemical advancements, due to increasing medical technology, in the direct and indirect contamination of water with PPCPs. The associated physiological effects of these PPCPs on non-target organisms is also discussed as it is of medical significance for humans, especially where potential effects of combined low-dosage and long-term exposure are concerned. Lastly, the current potential of WWTPs for the removal of PPCPs is discussed, including a summary of the efficacy of both WWTPs and Eco-machines in the removal of PPCPs, based on collected research data obtained from the available literature.

2.1.1) Dense Populations and Chemical Advancements

Humans have inhabited the earth for little over 200 000 years, with the emergence of human civilisations under 6000 years ago (Howell, 2015) according to the earliest records, dating back to Mesopotamia in 3500 B.C. (Seton, 1978; Ascalone and Schultz, 2007) and the Ancient Egyptians in 3000 B.C. (Grimal, 1992). Humans have come a long way from early roots, and have made remarkable advances in technology, science and engineering and, consequently, have successfully ensured dominance over the planet. The human population is estimated to be in excess of 7 billion people (Dimick, 2014), with each individual producing between 0.3 and 2.0 kg waste/capita/day (UNEP, 2005), depending on the level of development of the area, with more developed areas resulting in more

waste produced per capita per day, and even greater volumes of industrial waste. Also, according to research conducted by Kasprzyk-Hordern et al. (2009), the raw sewage collected from the municipalities in their study amounted to as much as 10 kg/day of pharmaceuticals and personal-care products for the Cilfynydd WWTP alone, which served 111 000 people at the time of publication. This is a concerning mass considering the small amounts that are used per person. It is therefore evident from the aforementioned information that the need to remove the vast amounts of waste products efficiently is significant.

Advances in medicine and science have enabled the production of an enormous array of pharmaceutical drugs and potentially toxic chemicals present in detergents, cleaning solutions and other personal-care products. In the literature these compounds are collectively grouped and referred to as pharmaceuticals and personal-care products (PPCPs) and micropollutants, and will be henceforth referred to as the latter. Examples of pharmaceutical drugs used in abundance include those of both an acute and chronic nature. The most common drugs for acute usage (taken for the duration of a few days to a week) that end up in the water systems include most antibiotics (β-lactam antibiotics, macrolides, sulphonamides, etc.), non-steroidal anti-inflammatories (NSAIDs) such as acetaminophen, diclofenac, paracetamol, tramadol, etc. and corticosteroids. Chronically-used drugs include β-blockers (Petrović et al., 2005), psychiatric drugs such as carbamazepine (Petrović et al., 2005), fluoxetine and diazepam, angiotensin inhibitors and synthetic hormonal contraceptives (17α-ethynylestradiol (Aris et al., 2014). In South Africa, drugs used for the treatment of diseases prevalent due to lower socioeconomic status are also relatively widespread, including anti-retrovirals for the treatment of HIV/Aids, and isoniazid and rifampin for the treatment of extensively drug-resistant tuberculosis (XDR TB) (CDC, 2013). These drugs are used for prolonged periods and their presence in effluent and surface water systems is therefore arguably more of a concern than that of most acutely-used drugs (with the exception of antibiotics) since they are used, and therefore excreted, over a longer period of time. Examples of common micropollutants (more specifically the aforementioned PCPs, rather than pharmaceuticals) include antimicrobial preservatives such as the parabens, other antimicrobials such as triclocarban, triclosan and other chemicals such as lauric acid and silicones (dimethicone, cyclomethicone, etc.) found in toothpastes, household detergents, shampoos and cosmetics (Boxall et al., 2012).

Many of these micropollutants, in their native (unaltered) form, have already been shown to occur in surface water systems into which effluent is released (Daughton and Ternes, 1999; Heberer, 2002). These compounds remain bioactive and can therefore induce intended, as well as unintended, physiological effects on non-target biological organisms after release into the environment. The retained naïve chemical structure of these substances indicates that they are still in their intended active form and, via logical deduction, may be able to elicit physiological responses in biological systems for which the pharmaceutical effects were not intended. Unfortunately, the problem does not end with

breaking down these parent compounds into daughter metabolites, as these are not only potentially biologically-active but can also reconstitute into their original parent compounds (Gros et al., 2007) when the effluent in which they are contained is released into surface water. This reversion was seen with 17-α ethynylestradiol (Aris et al., 2014). That being said, producing inactive metabolites, or metabolites with less extensive physiological activity than their parent counterparts, provides compelling reason to find solutions for the complete mineralization of these parent micropollutants. In the case where the micropollutants in question have daughter metabolites with greater biological activity than the parent compound, finding a means with which to break down the compound into metabolites is only the first step in the solution to removal of micropollutants. The next step would be to remove the daughter compounds via further breakdown into biologically inactive metabolites, or uptake or sequestration of these compounds by organisms such as bacteria, fungi or plants.

2.1.2) Chemical Loads in Municipal and Environmental Water

The aforementioned presence of micropollutants in surface water systems, into which effluent from wastewater treatment plants is released, is indicative of the inadequate, and clearly selective, removal of anthropogenic micropollutants. The respective micropollutant removal efficiencies also vary from plant to plant, depending on the types of processes applied (Ramirez et al., 2009), the initial levels and chemical properties of the water, as well as the chemical properties of the micropollutants themselves. The levels of the latter are determined by the usage of micropollutants by the population from whence the water is sourced. The relative concentrations of micropollutants in wastewater are of concern with systems using microbes for contaminant removal, where the type and relative abundances of certain micropollutants result in some being removed with better efficiency than others (Onesios-Barry et al., 2014). This, in turn, results in preferential removal of some micropollutants over others (as was seen in the research by Onesios-Barry et al (2014)), or perhaps not at all in the presence of carbon sources that are preferentially metabolised.

Kasprzyk-Hordern et al. (2009) came to the conclusion, from their research in South Wales, that effluents from sewage treatment plants are the largest contributors of micropollutants in natural river water systems, which in South Wales amounted to 3 kg of micropollutants released per day from the Cilfynydd WWTP. This amount is specific to South Wales and doesn't reflect the mass of micropollutants in other localities, but it does provide a sobering figure to the amount of these compounds that may be released into natural aquatic systems. As mentioned in the previous section, these same authors recorded an approximation of 10 kg of micropollutants per day in influent wastewater to the Cilfynydd WWTP. It could therefore be estimated that the WWTPs were responsible for the removal of 7 kg of the detected micropollutants. The Cilfynydd WWTP processed between 21 and 51 million litres in a 24 hour period (Kasprzyk-Hordern et al., 2009), (which means the concentration of micropollutants discharges was between 60 and 140 $\mu g.L^{-1}$ per day (using a rough

calculation based on flow rates of the WWTP). While this degree of removal may seem relatively effective, it means that some measure (approximately 3 kg in the case of the aforementioned research) of micropollutants, however minimal, are still being released into the environment. Some micropollutants are bioactive at low levels ($ng.L^{-1}$ and $\mu g.L^{-1}$) and since many of these compounds function at the receptor level, they can potentially still elicit physiological responses in mammals, including humans. Not much research has been conducted on the effects of micropollutants on humans *in vivo* due to the ethical limitations involved in this type of experimentation. Some adverse effects have been found to occur in mammals, such as rats, and lower level vertebrates, such as fish and amphibians. Experiments have also been conducted on human cancer cell lines and yeast expressing human receptors, such as the research done by Wróbel and Gregoraszczuk (2014) for the former and Routledge and Sumpter, (1996) for the latter. The effects of micropollutants on these types of organisms and cell lines are covered in the subsequent section.

The presence and relative abundance of micropollutants in aquatic environments around the world would depend on the nature and usage of products in the surrounding areas. The relative levels in influent and effluent wastewater in South Africa are shown in Table 2.1. These levels are accompanied by values for the U.K., as a comparison. The effluent wastewater values for the micropollutants are the values discharged into the surface waters. This data may only apply to specific treatment plants, but it gives an indication of the efficiency of WWTPs and the levels of micropollutants being discharged into surface waters. Methylparaben is removed with an efficiency of greater than 90%, whereas carbamazepine has a relatively low efficiency of removal and even appears to increase in concentration when compared to the influent.

Table 2.1: WWTP (influent vs effluent) concentrations of selected micropollutants
The data shows micropollutant concentrations in sewage influent and treated sewage effluent from a WWTP in both South African (indicated in bold) and international (United Kingdom) (*italics*) contexts. The removal efficiency data and concentration data are reported as is from the given literature.

Micropollutant (Type)	Concentration of Micropollutant in Water Source (ug/L)		Removal Efficiency (%)	Reference
	Influent of WWTP	Effluent of WWTP		
Carbamazepine (Anti-Tremorigenic)	**0.8** | *0.95*	**0.7** | *0.83*	**<10%** | *-24%*	**(Archer et al., 2017)** *(Kasprzyk-Hordern et al., 2009)*
Methylparaben (Preservative)	**200** |*11 601*	**0.1** | *9*	**>90%** | *>90%*	
Paracetamol (NSAID)	*178*	*0.35*	*>90%*	
Diclofenac (NSAID)	**6** | *0.26*	**4.5** | *0.18*	**40-50%**| *-33%*	
Trimethoprim (Antibiotic)	**9-10** | *2.9*	**2** | *0.876*	**70-80%**| *>70%*	

A concern is that many studies being conducted on surface water in the environment focus on those rivers into which significant volumes of WWTP effluent are released. This may lead to an overestimation and inaccurate conclusions on the state of surface waters as a whole, due to the fact

that these types of surface waters may show a much higher concentration than would be seen downstream where dilution, sequestration, dissipation or further biodegradation of the micropollutants may occur. Taking samples from various zones in the river systems would provide a more inclusive, and less biased, dataset of contaminant levels.

2.2) Physiological Effects of Micropollutants on Biological Systems

The presence of micropollutants in WWTP effluents released into surface waters has been reported. The cause of environmental contamination of micropollutants is thus attributed to inadequate removal during treatment (Ternes, 1998; Kasprzyk-Hordern et al., 2009). The significance of this, however, lies in whether these micropollutants have deleterious effects on biological organisms that live in/off these water sources and, if this is the case, what effects will be seen in higher mammalian organisms in the future. Compounds that have the ability of eliciting physiological or pharmacological effects in organisms to which the compounds are not native, are referred to as xenobiotics (Studdert et al., 2012; Katzung and Trevor, 2015). Based on this definition many, but not all, micropollutants are therefore classified as xenobiotics. For the purposes of maintaining clinical relevance, the focus of this research study was xenobiotic micropollutants. It is more pertinent that micropollutants, capable of causing any type of physiological effect on individuals, for which the compound was not intended, are removed from the contaminated source. This is because contaminating micropollutants can potentially elicit physiological responses in non-target organisms that are exposed to the aforementioned contaminated source (water, in this case).

The number of reports being published regarding the physiological effects of micropollutants in non-target vertebrates and invertebrates is increasing. Studies have been conducted that investigate effects of micropollutants (both pharmaceuticals as well as PCPs) (the specific references are mentioned in this chapter). Many micropollutants have been shown to elicit physiological effects on organisms and cell lines and, for the purposes of relevance and simplicity, only a few compounds will be discussed in detail. These compounds of relevance, used in the experimental portion of this research project, include the paraben group (particularly methyl- and propylparaben and psychoactive benzodiazepines (such as carbamazepine)), the latter of which is inefficiently removed during conventional treatment processes and both of which are not completely removed. Research conducted on the accumulation of micropollutants in animal populations exposed to the natural contaminated sources will also be discussed as they are of significance in linking the presence of micropollutants in environmental water sources with the adverse effects caused in organisms.

The aforementioned deleterious physiological endocrine effects bear no significance if it cannot be shown that the same effects occur with environmental concentrations of micropollutants. A study conducted by Brooks and colleagues in 2005 was the first published documentation on the accumulation of

anthropogenic pharmaceuticals in the liver, muscle and brain tissues of fish in aquatic river environments where effluent is known to be released (Brooks et al., 2005; Ramirez et al., 2009). This study however, used only post-mortem chemical and histological analyses to show physical accumulation of micropollutants and do not show what active effects these compounds had, if any, on living fish. Recording accumulation of micropollutants post-mortem in tissues of aquatic animals exposed to micropollutant-contaminated effluent water does not illustrate what deleterious effects occur as a result of bioaccumulation in the living animal, and therefore an accurate conclusion concerning the threat that micropollutants pose in the environment and to humans cannot be made with certainty. In response to these concerns, further cases were investigated to provide more information regarding the physiological effects of micropollutants on lower vertebrates and mammals: research conducted by Schultz et al. (2012) included the investigation of anatomical, physiological and behavioural responses of Fathead minnows (*Pimephales promelas*) to triclocarban (TCC) and triclosan (TCS), while the research conducted by Zenobio *et al.* in 2014 tested the effects of TCC, as well as a combination of TCC and TCS with other micropollutants. This research presents a dataset that expands on a literature gap where effects of single micropollutants do not show the same response when those same micropollutants are mixed with others. This research also maintains environmental relevance, as the experiments are conducted using concentrations of micropollutants that are representative of those found in the environment. Micropollutants have been shown, through different studies, to have disruptive effects on vertebrate endocrine systems, and have therefore been attributed the collective term endocrine-disrupting chemicals (EDCs). Some micropollutants have been shown to have estrogenic effects within mammalian systems, and are aptly termed xenoestrogens, examples of which include substances like bisphenol A (BPA) (Spengler et al., 2001) and the parabens, especially propylparaben.

2.2.1) Parabens

"Paraben" is the term used to describe the esters of ρ-hydroxybenzoic acid. The nature of the alkyl group attached to the carboxyl end of the acid determines the type of paraben (Jonkers et al., 2010), where a methyl, ethyl or propyl group indicates methylparaben, ethylparaben and propylparaben, respectively (Figure 2.1). Parabens are widely used as antimicrobial preservatives in cosmetics, PCPs and even food (Lemini et al., 2003). Propyl and/or methylparaben are present in at least 48% of PCPs and cosmetics (Núñez et al., 2008; Błędzka et al., 2014), and parabens are the second-most common ingredient found in cosmetics, second only to water (Cashman and Warshaw, 2005; Janjua et al., 2007). The concentration of parabens in most PCPs is around 0.3% w/v (Soni et al., 2002) or 0.4% (SCCP, 2008) for individual parabens, or up to 0.8% for parabens included in combination (more than one paraben) (SCCP, 2008), which amounts to concentrations of about 3-4 $g.L^{-1}$ and 8 $g.L^{-1}$, respectively. Parabens are primarily included in PCPs because they show broad-spectrum antimicrobial activity against bacteria, yeasts and moulds (Błędzka et al., 2014) Their specific mechanism of action is not well understood, but research has shown that parabens could interfere

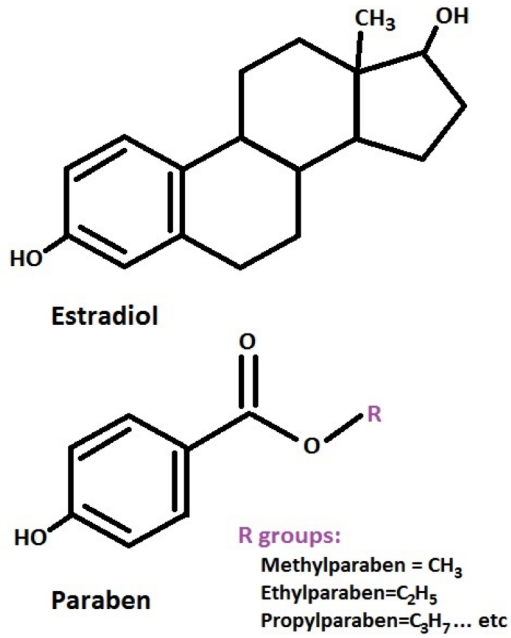


Figure 2.1: Schematic showing a structural comparison of estradiol (E_2) with the paraben group

The structure shown is common to all parabens, with exception of the R group. The R group, indicative of the alkyl group, varies according to the different types of parabens.

with mechano-sensitive channels in *Escherichia coli*, ultimately causing lethal changes in osmolarity due to disruption of osmoregulation (Nguyen et al., 2014). In addition to their antimicrobial properties, the use of parabens is popular because these compounds are inert, exhibit no noticeable colour or odour and possess low levels of systemic toxicity (Błędzka et al., 2014). The acceptable daily intake for methylparaben (over a decade ago) was 55 $mg.kg^{-1}.day^{-1}$ (Soni et al., 2002). In 2004, the European Food Safety Authority then declared the acceptable daily intake was between 0 and 10 mg/kg for methyl- and ethylparaben only (ESFA, 2004). Exposure occurs not only through ingestion (if in food) or through dermal absorption (in cosmetics and PCPs) but research shows that parabens can also be inhaled in households where paraben-containing products are used (Rudel et al., 2003, Canosa et al., 2007). Methylparaben is estimated to be present in household indoor air at average concentrations of 21 $ng.m^{-3}$ (Błędzka et al., 2014) and the nebulised intake was determined to be around 13.8 ng.kg body $weight^{-1}.day^{-1}$ and 0.547 ng.kg body $weight^{-1}.day^{-1}$ inhaled by children and adults, respectively, based on average respiratory rates and respiratory volumes for these two groups (Błędzka et al., 2014).

Parabens were considered safe by the FDA over a decade ago (Soni et al., 2001). However, research has shown that parabens induce estrogenic effects similar to those induced by estradiol, (Routledge et al., 1998; Wróbel and Gregoraszczuk, 2014). In contrast, a study done by Aubert *et al* in 2012 showed that parabens are converted to para-hydroxybenzoic acids (PHBAs) in blood plasma and they concluded that there appeared to be negligible effects in rats. They also commented on general levels of parabens in the human population and declared that parabens demonstrate a negligible effect on humans. This study, however, did not take into account long term effects of so-called "negligible" doses, nor did they consider investigating effects at the receptor level. Other research done on breast cancer cell lines MCF-7 and MCF-10A, showed that parabens (at 20nM test concentrations) stimulate estrogen receptor 1 gene expression (ESR1) in both cell lines (except for butylparaben, which did not affect ESR1 in the MCF-10A cell line) and ESR2 gene expression in MCF-7 cells (Wróbel and Gregoraszczuk, 2014). Since these cell lines are used to determine what compounds

contribute to the progression of breast cancer (in both males and females), it is very likely that long term exposure of parabens can contribute to estrogen-related cancers (Wróbel and Gregoraszczuk, 2014).

A more simplified approach to assess the direct effects of parabens on the human estrogen receptor in a transgenic yeast organism was described by Routledge and Sumpter in 1996. To supplement the contradicting information in the literature, this approach was utilised in this research to determine whether parabens are estrogenic, and therefore whether they can be considered potentially dangerous. The advantage of using the yeast assay is that it very elegantly shows the direct effects of estrogenic compounds at the receptor level in a simple, colorimetric assay. This recombinant yeast strain contains the human estrogen receptor (hER) gene within the genomic DNA in the nucleus, and an estrogen receptor element (ERE) on an exogenous expression vector within the cytoplasm (Routledge and Sumpter, 1996). A hybrid PGK promoter is inserted upstream of the ERE and a lacZ gene downstream of the ERE ((Routledge and Sumpter, 1996). Since the hER gene is located on the genome of the yeast, the receptor is always present in the yeast cell in proportion to the level of metabolism and expression of the genes on the chromosome. In the presence of estrogen, or an estrogenic compound, the estrogen ligand binds to the hER. Upon binding, the activated hER protein product acts as a DNA regulatory protein and binds to the ERE on the expression vector, which results in upregulating the expression of the lacZ gene downstream of the promoter. The lacZ gene encodes β-galactosidase, an enzyme that ordinarily would catabolise lactose and other disaccharides into monosaccharides, such as glucose and galactose, through cleavage of β-glycosidic linkages (Li et al., 2012). Chlorophenol red-β-D-galactopyranoside (CPRG), a structural analogue of β-galactose included in the assay medium, acts as a substrate for β-galactosidase (assembled by the yeast using the lacZ gene product). The product of the hydrolysis of CPRG is red/purple and quantitatively alters the medium colour. The degree of colour change can be measured spectrophotometrically and is indicative of the degree to which the test compound bound to the hER (degree of estrogenicity). This assay was conducted using methylparaben in the experimental design of this research project to demonstrate its effect on human estrogen receptors. A second yeast strain has also been developed, which contains the human androgen receptor (hAR) instead of the hER. The expressed hAR protein functions similarly to that of hER as they both are transcription factors that, upon activation by the binding of their native ligands, migrate to the nucleus to upregulate the genes to which they bind (Mooradian et al., 1987). Known native ligands that recognise, and bind to, the androgen receptor *in vivo* are testosterone and dihydrotestosterone (DHT) (Roy et al., 1999). The androgen receptor is closely related in structure to that of the progesterone receptor and therefore progestins or, indeed, progestin-mimics can bind competitively to the androgen receptor thereby blocking its function (Bardin et al., 1983, Raudrant and Rabe, 2003).

The apparent estrogenic activity exhibited by the parabens is not surprising, considering their structural similarity to estrogen at the site of the molecule where receptor-recognition occurs (see Figure 2.1). This structural similarity allows the paraben group of chemicals to act as estrogen receptor (ER) agonists, and indeed, parabens have been shown to induce estrogenic-like responses in yeast cells that exhibit this ER attached to downstream reporter genes. Estrogenic activity, through agonistic action of the ER, is noted in these transgenic yeasts through the upregulation of the reporter gene product in response to paraben exposure. Recent research by Wróbel and Gregoraszczuk in 2014 showed estrogen receptor activity in MCF-7 breast cancer cells, with the upregulation of mRNA and protein products triggered by both ESR1 and ESR2 (Estrogen receptors 1 and 2) agonistic activity. The evidence shows that the endocrine-disrupting activity of the parabens occurs and they are, consequently, not actually as benign as previously considered.

2.2.2) Carbamazepine

Carbamazepine is a pharmacological, anti-epileptic substance belonging to the benzodiazepine class of drugs (Katzung and Trevor, 2015). Carbamazepine is one of the many types of benzodiazepines available on the market. It is a scheduled medication sold under various proprietary names, such as Tegretol™ and Epitol™ (Drugs.com, 2015) prescribed for individuals with seizures (epilepsy). The drug is available in 100 -200 mg doses, taken at least once a day (DrugBank, 2005). Benzodiazepines function at the neuronal level by enhancing the activity of gamma-aminobutyric acid (GABA) and carbamazepine, specifically, works by inhibiting glutamate release at the glutamatergic synapse, stabilising the inactivated state of voltage-gated sodium receptor channels (Katzung and Trevor, 2015). Benzodiazepines are surrounded by a relatively large degree of controversy, especially in chronic long-term usage. The reasons for this controversy are numerous, but the most clinically significant is likely the adverse effects that a patient will experience as a consequence of long term administration of benzodiazepines. Some of these long term adverse effects include iatrogenic (affliction resulting from clinical intervention) hypothyroidism due to the increased metabolism of triiodothyronine (T_3) and tetraiodothyronine (thyroxine, or T_4), and hypercholesterolæmia by increasing the levels of high density lipoproteins (HDLs) in the blood (Drugs.com, 2015). Carbamazepine also has a teratogenic effect, causing congenital malformation and birth defects (Novartis, 2015). Not only do benzodiazepines cause very undesirable long term effects, they also interact with a multitude of other chronic drugs. Carbamazepine interacts with Eltroxin™ (a synthetic medication for individuals suffering from hypothyroidism), oral contraceptives and hormone replacement therapy (HRT), antibiotics and antifungals and cardiac medication, among many others (Drugs.com, 2015). Unintended pregnancies through the interaction of carbamazepine with hormonal contraceptives has been reported (Novartis, 2015). The smallest known lethal dose in adult humans is 3.2 g, with the oral LD_{50} determined to be between 1100 and 3750 $mg.kg^{-1}$ in rats (Novartis, 2015). The long list of contra-indications and side effects of carbamazepine becomes

significant when it is coupled to the fact that carbamazepine that enters the WWTP is removed with a very low efficiency (Zhang et al., 2008). In fact, it seems to actually accumulate in WWTPs in some cases, with effluent water containing more than the influent (Archer et al., 2017). This means these aforementioned interactive effects may be able to affect non-target individuals that merely consume water contaminated with carbamazepine, and other benzodiazepines.

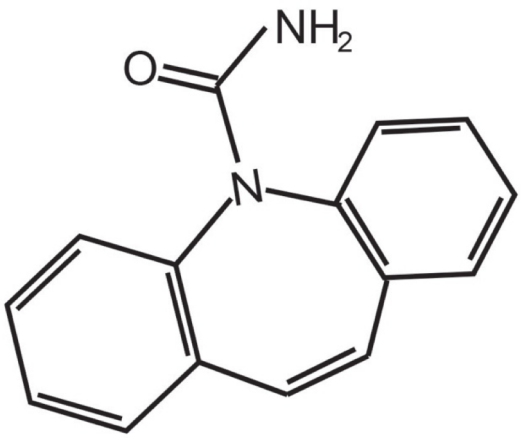


Figure 2.3: The structure of carbamazepine

A psychoactive anticonvulsant compound used in the treatment of epilepsy, that have shown thyrotropic effects in humans (Vainionpää et al., 2004).

In addition, carbamazepine has been shown to have endocrine disrupting effects specifically affecting thyroid hormone function. In a study done by Vainionpää et al. (2004), carbamazepine was shown to decrease the serum thyroid levels in young (prepubescent) girls. Carbamazepine induces degradation of tetraiodothyronine, or T_4 (one of the hormones involved in thyroid function) and also may prevent the release of this hormone into the blood (ThyroidUK, 2015). In other words, it reduces plasma levels of T_4. This is alarming, considering the thyroid is responsible for general growth and involuntary nerve functions, which will affect epileptic children who need to take this drug chronically. In the aforementioned study by Vainionpää et al., the effects on the serum thyroid levels was stated to appear reversible after withdrawal. While this may be the case, the long term effects of this pharmaceutical could not be assessed. Carbamazepine also appeared to cause lowered semen quality and decreased libido in men undergoing therapy (Reis et al., 2013), which suggests an endocrine disrupting effect on testosterone activity. The individuals who are undergoing chronic benzodiazepine therapy contribute to the levels of this class of drugs in the water system, due to the fact that 28% is excreted unchanged (RxList, 2015). Therefore, this 28% has the potential to indirectly affect other organisms coming into contact with the water in which the drug is present.

Carbamazepine is found to be present in wastewater all over South Africa ranging in concentration from less than 100 ng.L-1 in some regions (Odendaal et al., 2015) to over 800 ng.L-1 in others (Archer et al., 2017), and is considered one of the three major contaminants of emerging concern in South Africa (Odendaal et al., 2015). Concentrations of carbamazepine in South African WWTPs have been described in Table 2.1 above.

2.3) Biofilms

Microbial aggregations, such as biofilms, represent a major component of pollutant removal in the secondary stage of WWTPs (Pescod, 1992). Biofilms are a stationary amalgamation of living microbial cells surrounded by a matrix of extracellular polymeric substances (EPS) (Figure 2.3, in section 2.3.1). This is the growth form of bacteria that predominates in the environment (Henrici, 1933; Costerton et al., 1999; Hall-Stoodley et al., 2004; Romeo, 2008), and serves as a protective barrier against detrimental environmental conditions while also providing a mutualistic environment whereby many species undergo modular processes that fit together in one nested biofilm. Biofilm systems are extremely complex in their structure and function. The biofilm may also provide different pockets, or micro-environments, of anaerobic zones interspersed with aerobic zones. The different modular parts provide different degrees of stress which, in turn, provides an environment conducive to genetic mutation and reshuffling (Boles and Singh, 2008).

2.3.1) Structure

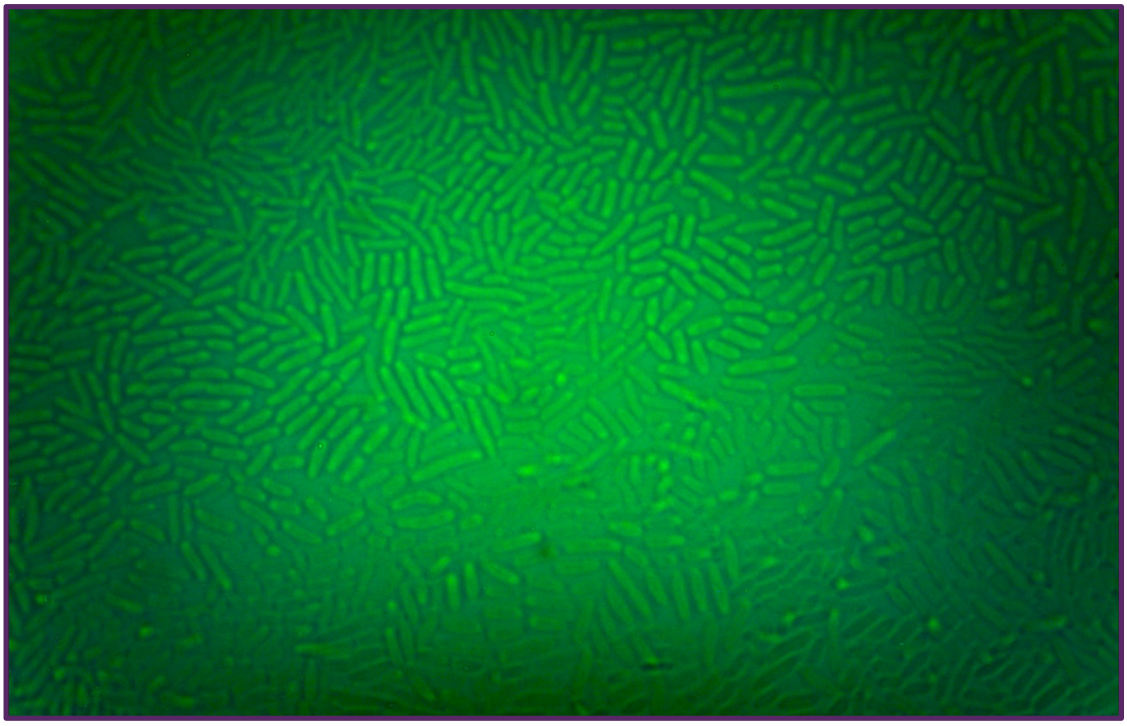


Figure 2.3 Basal layer of a biofilm viewed via light microscopy (1000X)

The image was obtained from real-time biofilms grown in flow cells using the reactor for the experimental design of this research. The image was taken with 100X oil immersion objective using a 2MP CMOS chip microscope camera.

Biofilms consist of a complex, intricate combination of the resident microbial cells and a gelatinous, protective matrix in which the former are enveloped (Lasa, 2006; Romeo, 2008). Together, the two form the living and non-living components of the biofilm, respectively, and the two components function together to create an extremely adaptable and optimally functional miniature ecosystem. Biofilms provide an alternative living state for microbial organisms, enabling many metabolic and

physiological abilities of which the individual, planktonic cells may not be capable of executing (Allison, 2003; Gilbert et al., 1997).

The defining characteristic of the biofilm is widely considered to be the extracellular polymeric substances, or EPS. It is often used interchangeably with the term "matrix", or "extracellular matrix" (ECM), to describe the entire non-cellular portion of the biofilm. A combination of polysaccharides, proteins and lipids form the matrix, of which there are two types: the capsular and slime types (Allison, 2003). The former refers to the part of the matrix that is intimately associated with individual cells, while the latter refers to that which connects the capsular parts of the matrix together. This part of the matrix is what is ultimately responsible for the formation of the multiple microenvironments within the biofilm which, in turn, is an important factor that allows for microbial members of varying environmental requirements to function together as a community. Each biofilm is unique in the microbial species present, the relative abundance of these species, as well as the types of microenvironments created within the biofilm.

2.3.2) Formation

The formation of the biofilm is a complex, multifaceted process involving many physiological cascades and communicative interactions between the organisms making up the biofilm. The currently accepted school of thought is that biofilm formation follows five concurrent and genetically-distinct phases (Donlan, 2002; Dunne, 2002; Davey and O'toole, 2000; P. Stoodley et al., 2002). Understanding the fundamental molecular mechanisms of biofilm formation was determined through a series of studies done based on biofilm assays conducted using mutant phenotypes of gram-negative Proteobacteria (O'Toole and Kolter, 1998a; O'Toole and Kolter, 1998b; Pratt and Kolter, 1998; Watnick et al., 2001; Watnick and Kolter, 1998). The five phases are (as summarised by Romeo (2008)): surface attachment, monolayer formation, migration (formation of multiple layers), EPS production and maturation. The stages of major significance that will be discussed are surface attachment and ECM production.

Surface Attachment

Surface attachment differs in motile and non-motile bacteria, largely because the surface components and appendages they exhibit are different. It is in these components/appendages that the mechanism of attachment to surfaces is determined.

Non-motile species express surface adhesins, and the expression of these adhesins is upregulated in the presence of a suitable attachment surface (Gotz, 2002). These adhesins essentially allow the individual bacterial cell to attach more readily to other neighbouring bacterial cells and to the nearby surface, by increasing their overall "stickiness" (Romeo, 2008;

Gotz, 2002). The molecular mechanism seems to involve the Biofilm-associated proteins (Bap), or their related homologs, in multiple bacterial species. The Bap related surface proteins were found in *Staphylococcus aureus* (Lasa and Penadés, 2006), while other, genetically-related proteins have been characterised in other bacterial species (Latasa et al., 2006). These homologous proteins serve the same function: attachment, and ultimately, are responsible for biofilm formation. The mechanism through which these Bap-related proteins mediate biofilm formation is largely unknown, but due to their large amino-acid repeat domains, it is likely the mediation of adherence is due to homophilic interactions between the Bap proteins themselves (Latasa et al., 2006), where the association of Bap proteins may serve to bind bacterial clusters (Latasa et al., 2005).

Motile species use a markedly different mechanism for surface attachment, by actively swimming and aggregating to the surface onto which adherence is desirable (Romeo, 2008). They use their motility organelles (pili, curli or flagella) to migrate to the surface after which a physiological cascade ensues, causing a drastic structural change resulting in attachment and entrance into a lifestyle switch (Romeo, 2008). This lifestyle switch is characterised by a change from motility into sessility, with the downregulation and ultimate cessation of the expression of the genes that code for motility organelles. Studies conducted by Pratt and Kolter (1998) showed that mutants deficient in flagella production were markedly defective in the formation of biofilms. Before permanent adherence occurs, the bacteria first adhere transiently. The transient period of adherence results in either stable association with the surface, entering into permanent sessility, or in a return to the planktonic state. Once the bacterial cells enter into a sessile state, they begin to produce the extracellular matrix that ultimately forms the structural backbone of the biofilm.

The switch from motility to a sessile, biofilm state involves complex molecular interactions. These interactions have been extensively studied in the model bacteria, *Bacillus subtilis*. It essentially involves the activity of a regulator repressor protein which, during times favouring biofilm formation, will cease active repression of the genes involved in matrix formation through an antagonistic process that inhibits the repressor protein (Branda et al., 2006). In *Bacillus subtilis*, this repressor protein is called SinR. It is constitutively produced and therefore ensures the bacteria remain in a motile phase. The SinI and YlbF/YmcA proteins serve to antagonise SinR activity in the case where biofilm formation becomes favourable. SinI activity is closely linked to Spo0A concentration (Romeo, 2008), a protein also associated with sporulation in periods of extreme stress such as nutrient depletion (Snyder and Champness, 2007).

Extracellular Matrix (ECM) Production

The production of the extracellular matrix (ECM), or the EPS, by the biofilm occurs through the transcription of genes encoding EPS components, called the *eps* operon (Kearns et al., 2005), along with other protein components, that are assembled together. Different species of bacteria produce different types of protein components. These EPS components are produced with varying levels of efficacy, but it is assumed that bacteria producing more EPS components still serve to aid other species in adhering to this matrix, regardless of the amounts of EPS components that they produce. In other words, mixed microbial biofilms may be more effective at forming biofilms than pure species due to the synergistic effects of the different types of EPS components on matrix formation. The EPS is involved with how the different species arrange within the biofilm, resulting in the formation of different microenvironments (Costerton et al., 1999). It must be noted that EPS, and subsequent microenvironment formation is unique to each biofilm that forms: not one biofilm will have the same abundance and arrangement of microenvironments as another.

Critical Evaluation of this Model

The five stages of biofilm development as described above is extremely oversimplified and arguably, temporally inaccurate, because the above five steps do not occur sequentially but instead overlap. Bester et al. (2009) showed that biofilms continually and consistently release planktonic cells during the entire developmental process, from as early as 6 hours after initial introduction of inoculum and attachment (Bester et al., 2009). Prior to this, Tolker-Nielsen et al. (2000) concluded that planktonic cells are released in multiple stages of the *Pseudomonas* biofilm formation, not just in the final one. In addition, the number of planktonic cells released is also indicative of the level of development of the biofilm. Biofilms release increasing amounts of planktonic cells into the bulk flow over time, eventually reaching a steady-state where the maximum amount of cells is released continuously.

2.3.3) Genetics

Sessile (biofilm) cells differ markedly from free-living planktonic cells at the molecular level, for instance in terms of the diversity of mutants generated in the respective growth mechanisms (Boles et al., 2004). This is because biofilms show an increased capacity for generating mutants in the absence of external mutagens or stress (Boles and Singh, 2008). Endogenous oxidative stress has been postulated as the reason behind the increased mutation rate in biofilms. Studies conducted by Boles and Singh in 2008 determined, through gene knockouts of *recA* and *katA* (encode for double-stranded DNA repair enzymes and catalase, respectively), that a combination of oxidative stress, lack of antioxidant processes, and functional double-stranded DNA break repair are required to produce phenotypic diversity in a pure culture biofilm (Boles and Singh, 2008). The presence of

microenvironments in the biofilm expose the microbes to different degrees of environmental conditions which, in turn, selects for certain advantageous mutants over time. It is postulated that the higher diversity of mutants generated in a biofilm serves as "insurance", ensuring that at least some of the population survives in adverse environmental changes due to the presence of many mutant phenotypes.

2.3.4) Function

The true function of a biofilm is under contention, where some research tends towards biofilms as purely a survival mechanism, some towards biofilms as a mechanism for proliferation, and some concluding that it is both. Biofilms do seem to function as a survival mechanism. In the medical field, biofilms are seen as a mechanism through which bacteria evade removal from catheters, acetabulum (hip) replacements and even heart valves. In clinical treatment, bacterial biofilms protect cells deep in the biofilm from therapeutic levels of antibiotics (Suci et al., 1994; Lewis, 2001). The biofilm also creates a physical barrier that enables evasion of contained cells from the host immune responses, both innate (complement-mediated lysis and general phagocytosis (Kindt et al., 2007)) and adaptive (opsonisation) (Janeway et al., 2001; Kindt et al., 2007; Aslam, 2008)). The presence of the EPS in the biofilm protects the bacterial cells deeply enveloped within, and therefore an immune response, innate or adaptive, can be hindered from removing the cells since these mechanisms rely on the recognition of certain extracellular ligands (Major Histocompatibility Complexes in the case of innate immunity, and antigens in the case of adaptive immunity (Kindt et al., 2007)) which are inaccessible when covered by EPS. A similar effect occurs with antibiotics, which require direct access to the target bacterial cell. The EPS serves as a defensive barrier, and the antibiotic has to diffuse through the EPS matrix to reach the target cells. In this way the EPS matrix and the biofilm serve as a dilution system: the antibiotics diffuse into the biofilm matrix, each antibiotic at different degrees (Aslam, 2008), but not all the molecules reach a target, essentially "diluting" the antibiotic below its' therapeutic dose in the biofilm (Stewart, 2003). Not only does the biofilm hinder antibiotic access to the cells, but some cells within the biofilms undergo metabolic changes, resulting in either dormancy or persister cell status, which also reduces the efficacy of antibiotics (Lewis, 2005). This is because cells that have entered dormant or persister cell status reduce, or altogether switch off, some metabolic processes such as protein synthesis or DNA replication, the enzymes of which are targets of the antibiotics. This essentially renders the antibiotic ineffective, since the target is not available for attack. Biofilms may also release large numbers of planktonic cells in response to severely detrimental environmental changes (nutrient depletion etc.) (Aslam, 2008), which also appears to be a survival mechanism, ensuring the progeny cells are able to establish a new biofilm elsewhere should the existing one not survive. In these ways, biofilms are intended as a survival mechanism.

There is also evidence in the literature of biofilms as a proliferative mechanism, although these are not as numerous as the evidence for biofilms as a survival mechanism. This may be purely because the biofilm as a proliferative mechanism is not clinically relevant, while the recalcitrance (through survival mechanisms) is of great clinical relevance and is therefore researched and reported upon more often. Evidence of biofilms as a means of proliferation and growth is remarked upon more often in mixed microbial biofilms. It appears that a biofilm consisting of multiple species functions almost as a higher organism, with mutualistic and synergistic activities between the different microbial species. It was found that the formation of *Escherichia coli* PHL565 biofilms was improved and enhanced by the presence of *Pseudomonas putida* (Castonguay et al., 2006), since the former showed very poor biofilm-forming ability in a pure culture (Castonguay et al., 2006). Research by Hassan et al. (2004) showed that the establishment of *Listeria monocytogenes* (a pathogenic microbe) is enhanced when in the presence of mixed *Pseudomonas putida* biofilms. Biofilms also serve as a site for horizontal gene transfer between organisms, both via intraspecies and interspecies interactions, and therefore allows microbes to acquire genes that are advantageous. Research conducted by Weigel et al. (2007) on a patient with a nephrostomy tube showed that there is potential for antibiotic resistance genes to be transferred between bacteria in the microenvironments created by the formation of biofilms. Metabolic interactions also occur in biofilms, whereby a compound is degraded by more than one species. This phenomenon, called co-metabolism, allows microbes to degrade a substrate that would otherwise be non-degradable in pure cultures. The process involves sequential degradation of a compound, whereby the product of the first step of degradation (conducted by one microbial species) becomes the substrate for degradation by another species of microbe (Biology-Online, 2005). It was shown that a mixed culture of multiple bacterial strains was able to mineralise significantly more of the pharmaceutical compound paracetamol, than pure cultures of the separate microbial strains (Zhang et al., 2013). This same research group also showed that the three microbial strains were all required in order for paracetamol to be completely degraded (Zhang et al., 2013). This interaction shows the potential of co-metabolism between bacterial strains in batch culture, and therefore shows that mutualistic interactions between bacteria do exist. The existence of mutualistic activity between microbes in batch culture shows that biofilms, by extension, may also serve to provide the environment to allow different species to interact, and cometabolise substrates, with each other.

The arguments can be put to rest, however, by assuming that biofilms are not only for survival or for proliferation, but rather that they are meant for both functions, and that the function they perform depends on what is necessary at the time. This shows the true degree of functional plasticity that a biofilm possesses. The biofilm appears to function as a very flexible system made up of different microcolonies of interacting microbes. The way that microbes function in a mixed microbial biofilm may be akin to multiple tissue and organ systems that work together to make up a fully-functional organism, capable of adapting to changes in the environment. It must be appreciated that biofilms

are more than just a way for microbes to survive or produce progeny: it is a system of interacting microbes, like a city of people, that function in an intricate way to achieve superior purposes that would be impossible were they to act individually.

Bacteria, not necessarily only when confined to the biofilm state of growth, possess a system known as catabolite repression. This is a molecular-based process that involves the inhibition of certain gene products for the metabolism of other carbon sources, such as lactose and arabinose, in the presence of glucose (Snyder and Champness, 2007). Microbes are therefore able to switch off energy and resource-expensive pathways in the presence of a carbon source that is more energy efficient. In the presence of glucose, and its metabolites, the cell produces repressors that bind to the operons of genes for metabolism of other carbon sources (Snyder and Champness, 2007). In the absence of glucose, or if glucose is depleted, the cell will then change the state of the repressor proteins. The type of carbon source present in the immediate environment of the cell determines which operon is activated. For example, if lactose is present then the *lac* operon is transcribed. The molecular processes are quite intricate and there is more than one type of catabolite repression, therefore the specifics will not be discussed here. The presence of a mechanism like catabolite repression in the bacterial genome shows the energy-efficiency with which bacteria function, further emphasising their plasticity (Snyder and Champness, 2007).

2.3.5) Effects of Micropollutants on Biofilms

There is a fair amount of literature on effects of biofilms on micropollutants, while the amount of data and research available on the effects of micropollutants on biofilms is very limited. This is partially because the effect microbes have on the pollutants (the removal thereof) is understandably of more significance than the effect the compounds may have on the biofilms. Knowledge of how micropollutants affect biofilms may provide insight into how it interacts with the cells or the EPS. This knowledge can then be used to manipulate and increase the removal of the compound by modifying the biofilm via the alteration of its' physiochemical environment, by changing the existing WWTP to include a stage that is designed to enhance micropollutant removal via changing the physiochemical environment of the biofilms. The gap in knowledge on how the pollutants physically and biochemically affect biofilms is large. Therefore, the effects of some micropollutants on biofilms will be investigated within the experimental design of this thesis, in order to determine the extent of effects, if any, these compounds have on biofilms. The greater the degree of effects on biofilms will result in more significance being placed on investigating these effects.

2.4) Biomimicry as a Conceptual Framework for the Study of Biofilms in the Context of Bioremediation and Micropollutant Removal

Bioremediation has received increased recognition, with the general change in mindset towards more ecologically-orientated and sustainable solutions, especially when it comes to the removal of pollutants from the environment. Bioremediation is essentially the introduction of microbes to a site with the specific intention of exploiting their metabolic processes in order to remove pollutants in that area, or to harness the metabolic potential of the resident microbial communities for this purpose (Anderson, 1995). Bioremediation has been used in the environment in some instances with great success, such as the use of dispersal to facilitate the cleanup of the Deepwater Horizon oil spill that occurred in the Gulf of Mexico in April of 2010. The remediation involved the use of dispersing agents, which served to emulsify the oils into smaller droplets, which facilitated faster microbial degradation through increased surface area exposure (Biello, 2010). The dispersal agent also served as a food source for the microbes (natural microbes living in the ocean), thereby increasing their numbers and further accelerating the bioremediative process (Biello, 2010).

In the laboratory environment, it was shown that biofilms of *Pseudomonas aeruginosa* N6P6 were capable of degrading polycyclic aromatic hydrocarbons (PAHs), with an increase in the degradation occurring in proportion to the increased expression of the *lasI* gene (Passador et al., 1993, Mangwani et al., 2015). The product of the *lasI* gene is involved in the synthesis of the N-acyl-homoserine lactone (AHL) auto-inducer which is involved in quorum sensing (Mangwani et al., 2015). Quorum sensing is a mechanism of regulating expression of certain genes in response to changes in the size of a cell population (Miller and Bassler, 2001). The implication of this finding is that biofilms are capable of degrading certain compounds, like PAHs, when cells that recognise and produce auto-inducers are present. Benzene, a carcinogenic environmental pollutant (Dean, 1985), has been shown to be degraded by microbial consortia in both aerobic (Gibson and Subramanian, 1984, Smith, 1990) and anaerobic conditions (Edwards and Grbić-Galić, 1992). The range of the given examples shows at least 30 years' worth of research on the biodegradative abilities of microbes. Therefore, it would seem an obvious solution to look to microbes for the degradation of the micropollutants that are becoming a problem in water sources, especially those that appear to be recalcitrant. The extreme plasticity and adaptability of microbial biofilms provide the best opportunity for selecting for consortia that are adapted to degrade one or more of these micropollutants. Since mixed microbial biofilms appear to have the greater potential for flexibility, due to the large variety of microbes and associated genetic and metabolic functions, the focus should arguably be on these mixed consortia for remediation of water containing low concentrations of micropollutants. These biofilm consortia may be found in natural water sources (rivers, lakes and dams), which is often an added advantage, since these microbes have already been exposed to the micropollutants in the water systems in which they reside. The key is to find a natural system that enables maximising the exposure of a microbial consortium to the polluted water source.

Despite the demonstrated micropollutant removal potential of microbes, the use of these mechanisms to remediate contaminated environments often fail. This is because the remediative processes, that work in a pilot or laboratory-scale study, are difficult to apply to the scale of the larger, real environment due to the presence of variables that did not exist in the closed laboratory (or pilot) environment. To solve that problem, natural systems may be used as inspiration, in order to create a system that would allow for these optimal exposure conditions, mimicking a natural system that already conforms to these criteria. Biomimicry offers a potentially powerful approach in the search for solutions to the removal of recalcitrant micropollutants and other compounds of concern (Lurie-Luke., 2014).

Biomimicry has become somewhat of a catchphrase since the term was introduced by Benyus in 1997, leading many to think it is a relatively new concept. However, this is not the case. Biomimicry has been around since, at least, the time of the Ancient Greeks. Biomimicry has multiple, arbitrary, definitions but in essence it is the use of a structure, process or design that already exists in the natural environment, and adapting it to perform in an industrial setting in an innovative way, in order to solve the challenges of humans (TheBiomimicryInstitute, 2015).

It must be noted that there are also many other biological terms that appear to be related to biomimicry, but are not. Examples are the terms bio-utilisation and bio-assistance, which refer to the harvesting of an organism or product and the domestication or modification of an existing organism, respectively (Benyus, 2009). Biomimicry is different in that it involves the creation of an apparatus that emulates what its muse does, without the presence of said muse.

One of the earliest recorded examples of biomimicry is that of Icarus and Daedalus and, although it is part of Greek Mythology, it still serves as an elegant example of humans employing biomimetic principles. The mythological tale follows Daedalus and his son, Icarus who were imprisoned in the Labyrinth by Minos (Kindberg and Turner, 2010; GreekMyths, 2016). Daedalus, having built the Labyrinth for Minos, knew how intricate it was and he knew the coast of Crete was littered with guards (GreekMyths, 2016). Therefore, they could escape by neither land nor sea. Daedalus realised that they would need another way out and saw the flight of birds that came and went as they pleased. He then fashioned working wings out of branches and wax, and he and his son Icarus managed to escape the Labyrinth through flight. The rest of the story is very well known among popular culture: Icarus, in his excitement, flew too close to the sun despite his fathers' warnings. He then drowned in the sea (later called the Icarian Sea (GreekMyths, 2016)). The point of the story, in the context of biomimicry, is that Icarus and Daedalus observed the flight of birds and were able to copy the structure and function that the wings served the birds. Through innovation (using limited resources), the two men were able to mimic the wings and leave the Labyrinth. This may be just a myth, but the story serves as a very elegant example of what biomimicry is, and illustrates just how long the concept has been around.

Other, more recent, biomimetic processes have since been created. One of the most overlooked examples is that of Velcro, the material that comes in two complementary strips that serve to attach two parts or surfaces together upon application of pressure. The concept was inspired by burrs, which have tiny hook-like protrusions extending from the seed capsule, that attached themselves to clothing and animal fur. One side of the Velcro strip has the same hook-like protrusions and the complementary side of the Velcro is a soft, brushed textile to which the hooks attach (shown in Figure 2.4). This example shows how biomimicry can be applied in a very simplistic, elegant way. The mimicry of the attachment of burrs to animal fur is simply structural biomimicry, but there are other facets of biomimicry, making it a concept that is much more complex than its definition belies. Another example of biomimicry is that of the 'gecko pad', a handheld pad that was designed based on the pad of a geckos' foot, by Biomimetics and Dextrous Manipulation Lab. The apparatus is designed such that it can support large weights upon application of the pressure to the pads held against a surface (Cutkosky, 2016). It also has the added feature that it can exhibit the same adhesive force regardless of its size (Cutkosky, 2016). These two examples serve as a very elegant and remarkable application of biomimicry in technology, although there are many other examples of biomimetic constructs.

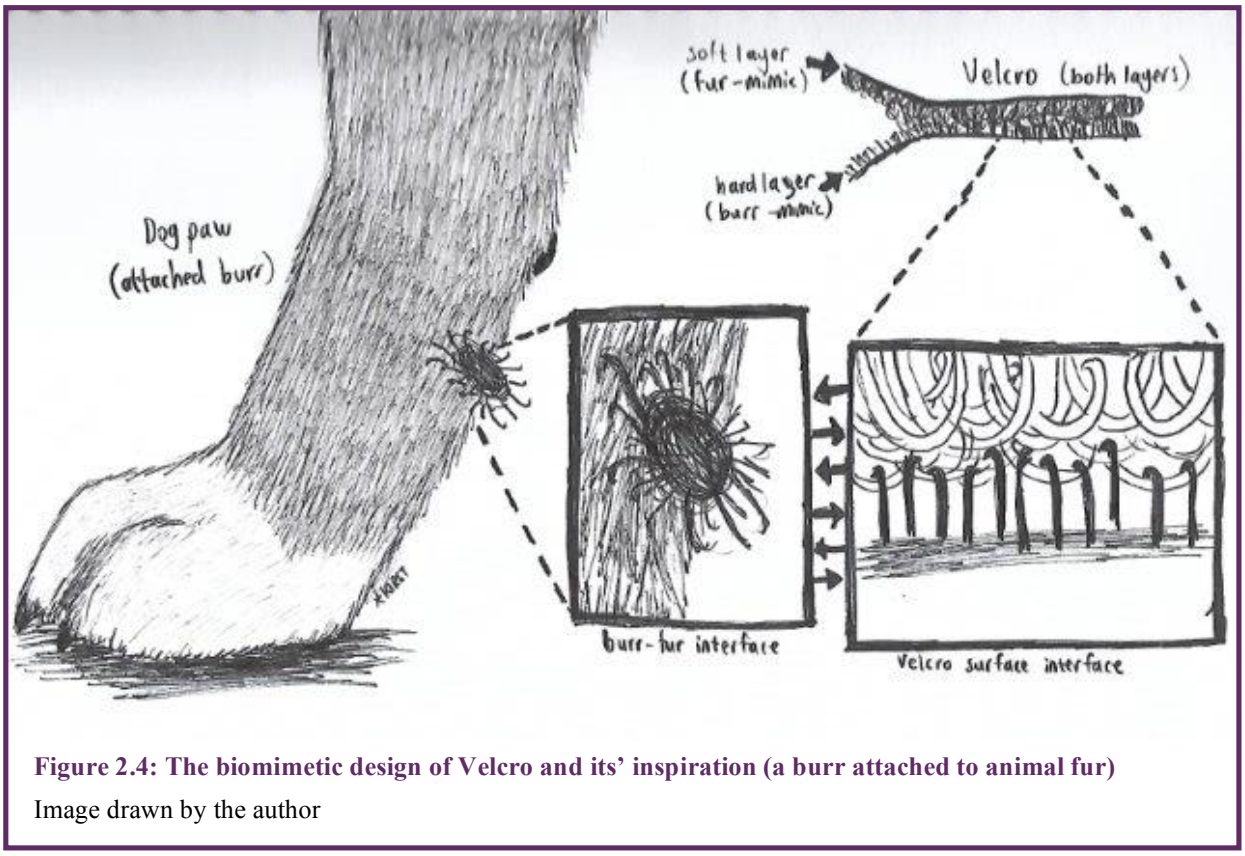


Figure 2.4: The biomimetic design of Velcro and its' inspiration (a burr attached to animal fur)
Image drawn by the author

The diagram in Figure 2.5 shows the principles of biomimetic designs, or "lessons to be learned from nature" (Biomimicry3.8, 2013) colloquially called "life's principles" by the Biomimicry Group. The "Life's Principles" pie-chart shows the main principles a design should incorporate in order to mimic nature as closely as possible. For example, the story of Icarus and Daedalus mimicking the wings of birds to escape the Labyrinth is an example of the application of the "resource efficient", "locally-

attuned and responsive" and the "evolve to survive" principles. Daedalus "fit form to function" by designing the wings in order to facilitate flight for him and his son. They used resources that were readily available to them within the Labyrinth, and therefore had to be inventive in order to achieve their goal. The "evolve to survive" was applied quite literally by replicating the birds' wings ("strategies that work") to a form that they could use to fly. These principles may be applied to other biomimetic ideas to ensure they remain as nature-inspired as possible.

Figure 2.5: "Life's Principles".

These are the conditions under which nature operates, and which should be applicable to biomimetic constructs. Image reproduced from Biomimicry3.8© 2013 Biomimicry3.8, The Biomimicry Group (image is licensed under creative commons)

The Eco-Machine™, created and developed by John Todd Ecological Design, is another example of a nature-inspired (biomimetic) process. It was one of the initial aims of this thesis to evaluate an Eco-Machine™ to be built in a township called Langrug, in Franschoek in the Western Cape, for its water treatment efficiency. Although this aim was not included in this thesis due to extended delays in its

construction, the investigation of the removal efficiency of these constructs in the paradigm of biomimicry still applied to this research. The Eco-Machine™, also referred to as a Living Machine, is essentially a man-made, or mimicked, assembled wetland ecosystem (Woolley-Barker, 2013). The construct consists of a network of interconnected, consecutive tanks through which the wastewater moves during treatment. The components of these Living Machines include primary and equalisation tanks, where solids settle and water levels are buffered, respectively (LivingMachine, 2012), followed by the wetland cells. These wetland cells are numerous and each contain a selection of organisms from various kingdoms: Plantae, Animalia, Fungi, Protists, etc. The cells each contain a combination of plants, with associated root and sediment biofilms, algae, fungi and other detritivores, as well as fish and other animals. The components all function together as a closed ecosystem, through which the wastewater is treated. The members of each cell of the ecosystem remove the organic contaminants from the water by either incorporating these contaminants into their own biomass or catabolism of the larger organic contaminants, gaining energy as a result, into building blocks and simple compounds (nitrates, carbon dioxide and water, etc.) that are used as final electron acceptors, thereby removing them from the water. In return for breaking down the contaminants from the water, the plants and associated organisms grow in order to produce an aesthetically-pleasing , yet functional, "garden" effect.

The Living Machine, as described above, as well as the Eco-Machine™, like the one that is still to be built at Langrug, is not purely biomimetic but rather a combination of bio-assistance, bio-utilisation and biomimicry (Woolley-Barker, 2013). The biomimetic aspect involves the mimicry of a wetland ecosystem in nature. The components of the system, however, are obtained from nature as is and are incorporated into the construct, which is bio-utilisation. The combination of different organisms as separate components into each cell is bio-assistance, where the combination of the organisms allows humans to achieve a specific goal (water treatment, in this case). The efficacy of constructed wetlands in the removal of pollutants from wastewater has been well researched. A study conducted by Hijosa-Valsero (Hijosa-Valsero et al., 2010a) showed that a constructed wetland/pond hybrid shows comparable removal efficiencies of micropollutants such as naproxen, ibuprofen and caffeine to that of conventional WWTPs. They also postulated that the presence of plants improves the removal efficiencies of some micropollutants (Hijosa-Valsero et al., 2010a). Constructed wetlands also showed more promise in the removal of micropollutants than did a conventional WWTP receiving the same influent wastewater (Hijosa-Valsero et al., 2010b). This is especially true for the more recalcitrant compounds, such as carbamazepine, which was better removed by the constructed wetlands than by conventional WWTP (Hijosa-Valsero et al., 2010a; 2010b).

While biomimicry is advantageous in inspiring humans to design eco-friendly, sustainable and nature-inspired systems that do not contribute to pollution and environmental damage, there are certainly limitations. Firstly, humans are limited to what they can build with synthetic materials. In other words, in order for something to be biomimetic, it must be a synthetic construct that is bio-inspired but does

not actually use the inspired organism (Benyus, 2009). This limits biomimicry to the applications that can be mimicked. There are limits to what humans can build, but nature has made some uniquely-functioning systems which humans can manipulate, but not emulate. Bacteria are an example: single-celled organisms that respond with plasticity to their environment through cues. They contain genetic material that encode millions of different functions. They are capable of expressing these genes in an energy-efficient manner, expressing only those they require, while managing to adapt to environmental changes. All of this is controlled by nothing more than their chromosomal (and extrachromosomal) material. Humans, while currently unable to emulate such a prototype, can manipulate and alter these functions to harness and commandeer this efficient bacterial machinery for solving wastewater treatment problems (through bio-utilisation and bio-assistance). Biomimicry (as well as bio-assistance and bio-utilisation) can provide inspiration as means to achieve this.

Another consideration, which pertains specifically to the wetland-mimicking constructs, is that because it is a natural system, it is limited by the systems' carrying capacities. The system uses living organisms that work together, but these organisms are limited with regard to the volumes of water that they can process. In other words, the amount of water that can be processed adequately in a specific time frame may be limited within these constructed wetlands. If the treatment of larger volumes of water is required, the wetland-mimic must be increased in physical size, in order to handle larger volumes without a reduction in efficacy. This was also reported in the literature, where it was stated that constructed wetlands work best for small populations, due to the large surface area required per individual, or rather, the smaller volumes that can be handled by these systems (Kadlec and Wallace, 2008, Hijosa-Valsero et al., 2010a). Therefore, it is essential to thoroughly assess the abiotic and biotic thresholds of these systems in order to optimally incorporate them into the environment and ensure no further contamination of surface waters occurs as a result of exceeding these maximum capacities.

Biomimicry is an excellent concept that challenges companies, as well as the innovative individual, to create solutions to man-made problems by adhering to the rules (“life’s principles”) that are found in nature. The purpose of biomimicry is to create sustainable solutions that are eco-friendly, and therefore do not further contribute to the problems that need solving, while sourcing the answers from natural systems (Benyus, 2009). While the concept does have its advantages, its limitations cannot be ignored. Humans are still reliant on the activities of microorganisms, the genius of which cannot yet be synthetically reproduced. So, while the concept of biomimicry is a great way to invent new bio-inspired constructs and products, the merit of using bio-assistive and bio-utilisation processes and ideas should not be overlooked, but rather included in biomimetic designs.

Chapter 3: Materials and Methodology

3.1) Preparation of the Inocula for Experimentation

3.1.1) Pure Culture Inoculum

Pseudomonas sp. strain CT07 (GenBank Accession number DQ 777633; (Bester et al., 2005)), isolated from a cooling tower, was used for pure culture studies. Strain CT07 was cultured in 3 $g.L^{-1}$ tryptone soy broth (TSB) overnight at 26°C. It was then streaked out onto tryptone soy agar (TSA: 3 $g.L^{-1}$ TSB (Biolab, Merck); 12 $g.L^{-1}$ bacteriological agar (Biolab, Merck)) to make a working stock from which overnight cultures were inoculated. The remainder of this culture was then aliquotted into sterile cryogenic tubes with 80% glycerol (uniLAB®, Merck) to a final concentration of 40% glycerol. These cultures were stored at -80°C and used as inoculum in all subsequent experimentation.

A calibration curve to establish the relationship between optical density at 600nm and cell concentration was obtained in order to standardize inoculum size for minimum inhibitory concentration (MIC) and minimum biofilm inhibitory concentration (MBIC) determinations. In essence this was achieved by preparing an overnight culture of CT07 in 3 $g.L^{-1}$ TSB supplemented with 1% glucose, and then making 2mL volume dilutions (using 0.9 % saline as the diluent) of this overnight culture in ratios of saline to overnight culture ranging from 1:1 to 79:1.

Each dilution was measured with a spectrophotometer (Spectroquant® Pharo 300, Merck™) at a wavelength of 600 nm, after a blank calibration with 0.9% saline, using 1 mL of sample in 10 mm cuvettes. Each of the different dilutions were subjected to a separate 10-fold dilution series in sterile 0.9 % saline and plated out onto TSA agar plates, supplemented with 0.06% glucose. The plates were incubated for 48 hours at 26°C and the respective colony forming units were counted. The data obtained was used to construct a curve displaying optical density against the logarithm of cell concentration.

3.1.2) Mixed Culture Inoculum

3.1.2.1) Cultivation of Mixed Culture

Since mixed cultures are inconsistent with respect to species composition and relative abundances, a single sample was used to generate all inocula to be used for subsequent experimentation. To achieve this, a simple perfusion column was constructed to allow for the biofilm-forming organisms to colonize the surface of 25 mL 3mm glass beads packed in a 60 mL syringe, for subsequent use as the source of inoculum (Figure 3.1).

The perfusion column was positioned in the opening of an Erlenmeyer flask (see Figure 3.1), and attached to a peristaltic pump (Watson-Marlow® 205S, Watson-Marlow Limited) via silicone tubing

(2 mm inner and 3 mm outer diameter). The apparatus was then sterilised with 20% NaOCl (ChlorGuard®, Prime Cleaning Supplies) for an hour. Sterile reverse osmosis (RO) water was allowed to drip through for 24 hours to dilute the spent NaOCl out of the apparatus. Sterile TSB (3 $g.L^{-1}$) was flushed into the system to displace the water. Subsequently, a sample of activated sludge from the Stellenbosch Municipality WWTP (previously collected in a 1.5mL Eppendorf® tube), of approximately 10 µL was added to the column. The flow rate of growth medium supplied by the peristaltic pump was adjusted to 15 $mL.h^{-1}$ and left to continually perfuse over the beads for 7 days. The column was gently agitated daily to allow the culture to distribute evenly between the beads. The beads were then aseptically poured into a sterile petri dish and distributed into 1.5 mL Eppendorf® tubes containing 500 µL of 40% glycerol. Some samples were frozen at -80°C for long term storage and the remainder, used for working stocks, were stored at -20°C.

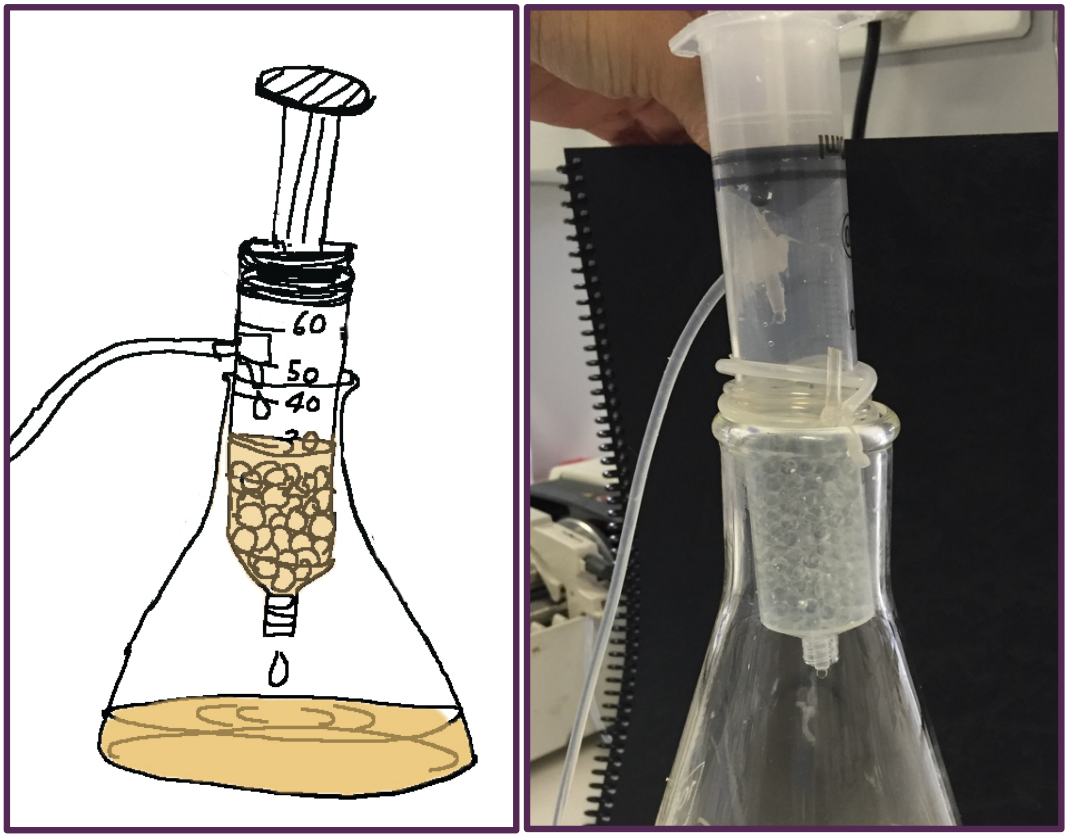


Figure 3.1: Bead inoculum perfusion column

Design of the perfusion column (left) and the apparatus as constructed in the laboratory (before addition of any medium or inoculum) on the right.

3.1.2.2) Reproducibility of Growth Patterns

A calibration curve for optical density vs. cell concentration was performed for the mixed microbial inoculum to ensure consistent cell concentrations for MIC determinations. This was accomplished by aseptically dropping 3 biofilm-covered glass beads (created according to the protocol in 3.1.2.1) into

triplicate sterile test tube containing 10 mL TSB (3 $g.L^{-1}$) supplemented with 1% D-glucose and incubating overnight at 26°C. This overnight cultures were then used to create a range of dilutions.

The optical density of each dilution was measured at 600 nm and simultaneously plated out on TSA after performing a 10-fold dilution series, as was conducted for the CT07 pure culture in section 3.1.1. The data was recorded in a table and a linear curve obtained, the mathematical equation of which was used to determine the approximate concentration of the culture in the subsequent experiments, using only the optical density.

The accuracy of the mathematical function was validated by substituting the optical density of diluted overnight cultures and calculating the expected cell concentration. To establish the real cell concentration and validate the equation, the culture was also plated out on TSA after serial dilution. This was carried out each time mixed microbial overnight culture was used for an experiment and the data recorded. The % accuracy of the curve was then determined to ensure that the function was sufficiently accurate for use in MIC experimentation.

3.1.3) Yeast Culture Inoculum

The recombinant yeast cultures hAR and hER were used in yeast screening assays. The two strains contain genes encoding the human androgen and human estrogen receptors, respectively. The presence of androgens (in the case of hAR) and estrogens (in the case of hER) result in binding of these compounds to the androgen or estrogen receptor which, in turn, results in the increased expression of β-galactosidase. The increased levels of this enzyme result in a colour change that can be quantified. The optical density of the yeast (at 600 nm) can also be compared with controls to determine the dose of methylparaben or carbamazepine that are cytotoxic to the yeast. The molecular mechanisms of this assay were discussed in greater detail in the literature review.

A curve correlating the logarithm of optical density at 600 nm and the cell concentration was constructed, for both the hER and hAR strains of *Saccharomyces cerevisiae*, as was done for the CT07 pure bacterial culture above. The yeast medium was prepared, as described by Routledge and Sumpter (1996), by preparing 45 mL minimal medium (13.61 g KH_2PO_4; 1.98 g $(NH_4)_2SO_4$, 4,2 g KOH, 0.2 g $MgSO_4$; 1 mL 0.5 $mg.mL^{-1}$ $Fe_2(SO_4)_3$; 50 mg L-histidine; 50 mg adenine; 20 mg L-arginine-HCl; 20 mg L-methionine; 30 mg L-isoleucine; 30 mg L-lysine-HCl; 25 mg L-phenylalanine; 100 mg L-glutamate; 150 mg L-valine; 375 mg L-serine; added to 1 L reverse osmosis water, autoclaved at 121°C for 15 minutes) in a sterile 50 mL Erlenmeyer flask. To this, 5 mL 20% w/v glucose, 1.25 mL 4 $mg.mL^{-1}$ aspartic acid, 400 µL 24 $mg.mL^{-1}$ threonine, 125 µL 20 mM copper sulphate solution and 500 µL vitamin solution was added. The vitamin solution comprised 4.44% (w/v) thiamine; 4.44% pyridoxine; 4.44% pantothenic acid; 22.22% inositol; 0.4% biotin dissolved in reverse osmosis water and filter sterilised. To this medium, 125 µL

yeast stock was added and incubated at 26°C in a shaking incubator overnight. Fresh medium was prepared the next day, and 1 mL of the yeast overnight culture was added for a second overnight incubation at 26°C. The yeast culture was diluted in the same manner as done for the CT07 strain and the dilutions were plated out and grown on solid yeast extract peptone dextrose (YPD) (Biolab, Merck) medium to obtain countable numbers.

3.2) Effects of Micropollutants on Microbial Cultures

The effects of carbamazepine and methylparaben on both the mixed microbial culture and CT07 strain were evaluated using minimum inhibitory concentrations (MICs). The effects of these micropollutants on recombinant yeast was also evaluated. The yeast assays evaluate both the endocrine disrupting potential of carbamazepine and methylparaben, as well as any potential lethal effects of these compounds on the yeast.

3.2.1) Minimum Inhibitory Concentration Determinations

MICs are assays that are used to determine the effects of potential antimicrobial compounds on a chosen bacterial strain. Varying concentrations of a chosen compound are introduced to a consistent amount of bacterial cells in a series of test-tubes or wells to determine the concentrations at which the bacterial growth is inhibited. MIC assays were conducted in order to determine the inhibitory effects, if any, of carbamazepine and methylparaben on the mixed culture. The pure culture was used to determine a baseline effect of these compounds on a single strain, since the mixed culture contains multiple species and it was postulated that the mixed culture would show increased resistance to the effects of the compounds.

3.2.1.1) Preparation of Micropollutant Dilutions

The minimum inhibitory concentrations (MICs) of both carbamazepine and methylparaben were determined for both the mixed culture and pure culture (CT07), using an adaptation of the method described by Sabaeifard et al. (2014). Stock solutions of methylparaben (>99.0% purity, Sigma-Aldrich®) and carbamazepine (>98% purity, Sigma-Aldrich®) were made at concentrations of 80 $g.L^{-1}$ and 40 $g.L^{-1}$, respectively by dissolving the powdered forms in methanol. The concentration ranges tested were 74.5 $ng.L^{-1}$ to 10 $g.L^{-1}$, for both methylparaben and carbamazepine. A two-fold dilution series was set up across 28 wells in a 96-well microtitre plate in triplicate. The concentration range was 74.5 $ng.L^{-1}$ to 10 $g.L^{-1}$ for both methylparaben and carbamazepine dilutions. Methylparaben dilutions were set up by placing 50 µL of methylparaben stock in the first well, and 25 µL absolute methanol in the subsequent 27 wells, transferring 25 µL of the methylparaben stock into the neighbouring well and mixing thoroughly until all 28 wells were diluted. Carbamazepine dilutions were set up in the same manner as was done for methylparaben, except the initial stock and methanol

volumes were doubled to 100 and 50 µL, respectively (transferring 50 µL across the wells) due to the fact that the carbamazepine stock was more dilute than that of methylparaben (due to lower solubility of carbamazepine in methanol than methylparaben). The microtitre plates were left under the sterile flow of the biological safety cabinet, with the lights off, until the methanol had completely evaporated, leaving only the dry compound in the wells.

3.2.1.2) MICs

Overnight cultures of the mixed and pure cultures were measured spectrophotometrically at 600nm using the Spectroquant® Pharo 300 spectrophotometer (Merck). The optical density reading was substituted into the respective equation, and the f(x) value was calculated to determine the estimated cell concentration. For example, for diluted CT07 overnight culture, the optical density of 0.052 at 600 nm was measured and the value substituted for x:

$F(x) = 0.4955\ln(x) + 9.4385$

$= 0.4955\ln(0.052) + 9.4385$

$= 7.9735 \rightarrow 10^{7.9735}$

$= 9.41 \times 10^{7}$ CFU.mL^{-1}

The volume of this culture required to make a 1 x 10^{6} CFU.mL^{-1} in the final well volume of 200 µL was calculated to be 2 µL. The inoculum was also plated out and was determined to contain 2.9 x 10^{6} CFU.mL^{-1}.

A 10 g.L^{-1} sterile solution of TSB supplemented with 0.2% glucose was prepared and added to each well, with 200 µL in the negative control wells and 200 µL less the volume of overnight culture to the other wells. To the positive controls, only overnight culture was added to a final volume of 200 µL. The overnight culture was then added to each well, the plates sealed with oxygen-permeable sterile film and incubated at 26°C in a shaking incubator for approximately 20 hours. The optical density of the plates was measured at 600 nm using an xMark™ Microplate Spectrophotometer (BioRad). Figure 3.2 demonstrates the placement of samples in the microtitre plates.

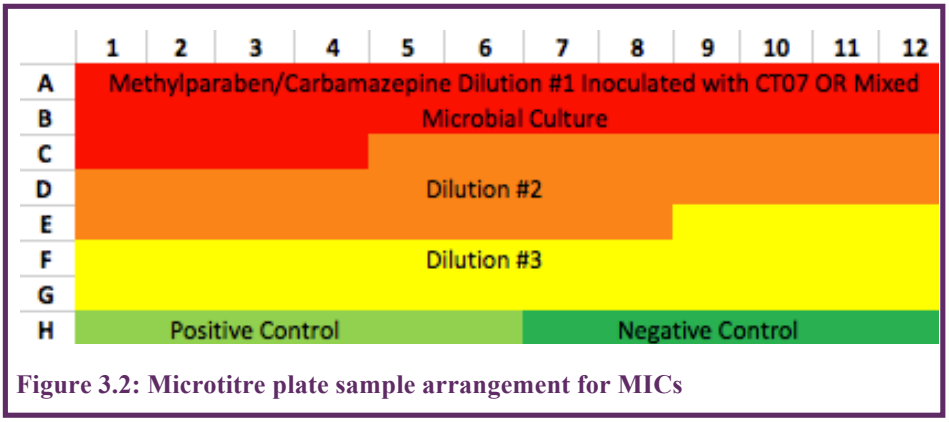


Figure 3.2: Microtitre plate sample arrangement for MICs

3.2.1.3) Data Analysis

The raw MIC data was analysed by subtracting the mean of the negative control optical densities from the mean of the optical densities for each concentration of the micropollutant being tested. The concentration range (in log $ng.L^{-1}$) was calculated and plotted against the means of the differences between the optical density for each concentration less the negative control. This was done for both the carbamazepine and methylparaben MIC data.

The standard error bars displayed at each point, were calculated using the formula:

$$\sigma_M = \frac{\sigma}{\sqrt{N}}$$

where σ_M is the standard error of the mean; σ is the standard deviation of the data set and N is the number of data points in the data set (alternatively, the number of measurements in the population in the statistical vernacular). The standard error of the mean measures the degree of uncertainty, or variation, of the data around the mean (Samuels and Whitmer, 2003), in other words, it measures the degree of sampling error.

3.2.2) Effect of Micropollutants on Recombinant Yeast

Yeast screen assays, described by Routledge and Sumpter (1996), make use of recombinant yeast strains that possess a human receptor gene, as well as the receptor element the receptor binds to. In this case, the hER and hAR strains were used, the former possesses an estrogen receptor gene (the product of which is a regulatory protein that binds estrogen) as well as the element to which an estrogen receptor binds to upregulate beta galactosidase expression, which is present downstream of the element. The latter strain, hAR, is the same as hER except it contains an androgen receptor and its associated element. These assays, as described in detail in Section 2.2.1, quantitatively measure the amount of estrogen or androgen-induced colour change, as a result of the presence of estrogenic or androgenic compounds in the growth medium. Therefore, they are used to quantify the endocrine disrupting potential of a test compound on the receptor the yeast expresses. The androgen assay was modified to create an anti-androgenic assay. This was done by adding a consistent concentration of an androgen (dihydrotestosterone (DHT)) to a range of carbamazepine concentrations, and measuring the lack of colour change (no colour change indicated a positive result: antagonism of the androgen receptor, or anti-androgenicity). The potential inhibitory effect of carbamazepine for the binding of androgens to the receptor was then evaluated.

3.2.2.1) Methylparaben Estrogenicity

In order to establish the degree of estrogenic effect of methylparaben, a standard curve was established to correlate the degree of estrogenicity to a known estrogenic compound, 17β estradiol (E_2). An

overnight culture of the hER strain was prepared the day before the assay, using the method discussed in section 3.2.1.

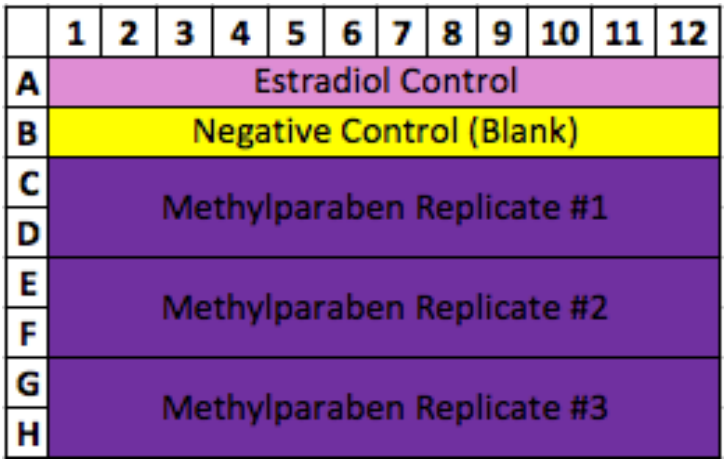


Figure 3.3: Yeast assay sample arrangement for methylparaben standard

Flat-bottomed 96-well plastic microtitre plates were used for the yeast estrogen assay. A stock solution of methylparaben (100 $g.L^{-1}$) was used to make a two-fold serial dilution in a separate microtitre plate from the ones used for the assay. The concentration range was 596 $ng.L^{-1}$ to 5 $g.L^{-1}$. This was done by adding 200 µL of the methylparaben stock solution to the first well of a row within the microtitre plate. To the subsequent 23 wells, 100 µL of HPLC-grade methanol (Sigma-Aldrich®) was added. Thereafter, 100 µL of the 200 µL methylparaben stock was transferred over to the well immediately adjacent to it. 100 µL was transferred across the wells until the dilution series was completed. 10 µL of the 24-well dilution series was transferred to wells C1-D12, E1-F12 and G1-H12 of duplicate microtitre plates. A similar dilution series was made for E_2 using only 12 wells and 30 µL of methanol diluent. 10 µL of this dilution series was transferred to wells A1-12 of both microtitre plates. 10 µL methanol was added to wells B1-12 of both plates, for the negative control. Figure 3.3 is a schematic representation of the plate arrangement. The plates were placed in a biosafety cabinet, with the light off, until the methanol had evaporated completely.

The yeast medium was then prepared by adding 4 mL 20% w/v glucose, 1 mL 4 $mg.mL^{-1}$ aspartic acid, 400 µL vitamin solution (see section 3.1.3), 320 µL 24 $mg.mL^{-1}$ L-threonine, 100 µL 20 mM copper sulphate solution and 400 µL CPRG stock to 36 mL minimal medium (as described previously in section 3.1.3). The medium was filter sterilised to ensure complete removal of any possible contaminant microorganisms.

The optical density (at 600 nm) of the hER overnight culture was measured and an appropriate volume of cell suspension added to seed it with 4.0 $x10^7$ cells. The seeded medium was then mixed thoroughly and a multichannel pipette was used to add 200 µL of the mixture to each well in both microtitre plates (aseptically, in a biological safety cabinet). The plates were covered and the sides sealed with tape to

prevent evaporation of the liquid medium during the incubation period. The plates were incubated at 30°C until the colour change was sufficient (typically about 5 days). The optical density was measured at 540, 575 and 620 nm, after mixing through agitation, and the data analysed as shown in section 3.2.2.3.

3.2.2.2) Carbamazepine Anti-Androgenicity

Carbamazepine, a recalcitrant compound, is suspected of exhibiting an anti-androgenic effect in higher organisms. In order to explore this, a standard curve was established to correlate the degree of anti-androgenicity of flutamide (a pharmaceutical with known anti-androgenic effects) with the suspected anti-androgenic compound (carbamazepine).

An overnight culture of the strain was prepared as described in section 3.2.1. The carbamazepine anti-androgen assay was done in a similar manner as the methylparaben estrogen assay, with slight modifications: 10 µL 16.67 µM dihydrotestosterone (DHT) solution (200 µL 100 µM DHT stock to 1000 µL methanol) was added to every well in the microtitre plates except for the negative control wells (B1-6), as the test androgen which carbamazepine would antagonise. Then, a carbamazepine dilution series (596 $ng.L^{-1}$ to 5 $g.L^{-1}$ concentration range) was set up, using 50 $g.L^{-1}$ stock. A volume of 20 µL 50 $g.L^{-1}$ stock was added to the first wells (C1, E1 and G1) of both microtitre plates and a 23-well doubling dilution series was prepared in a separate microtitre plate, in the same manner as for the methylparaben dilution above. A volume of 10 µL of the 23-well dilution series was added to the yeast microtitre plates (wells C2-D12, E2-F12 and G2-H12). A flutamide dilution series was made in exactly the same manner as the E2 dilution series above, and 10 µL from each well was added to wells A1-12 of both microtitre plates. Then, 10 µL methanol was added to wells B1-6 as a negative control (Figure 3.4). The plates were left under the flow of a biological safety cabinet until the methanol in each well was completely evaporated. The yeast assay medium was prepared as above, except that 1.6 mL hAR overnight culture (OD_{600} = 0.8) was added to the assay medium. After the addition of 200 µL seeded medium, the plates were covered and incubated at 30°C for 3 days. Readings of the plates were taken at 540, 575 and 620 nm. Data analysis is shown in section 3.2.5.

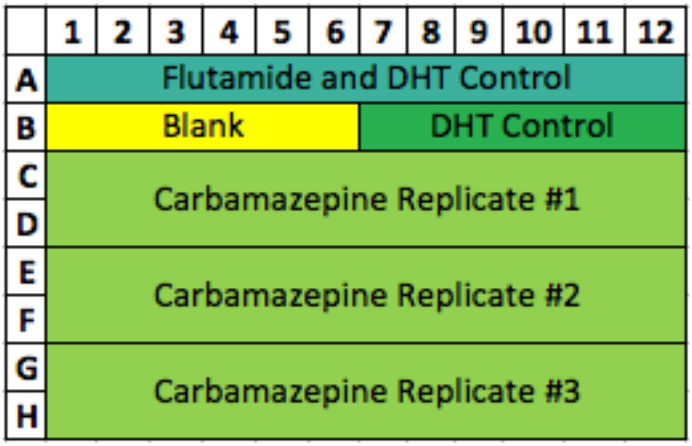


Figure 3.4: Yeast assay sample arrangement for carbamazepine standard

3.2.2.3) Data Handling to Obtain Standard Curves

The readings at 540 nm for each concentration must be subtracted from the reading at 620 nm for that same concentration, less the average for the blank:

$$A_{corrected} = A_{540} - (A_{620} - A_{blank}),$$

Where A represents the absorbance reading, and A_{blank} is the average of the blank readings. The negative control values were averaged for hER, and the positive control (without DHT, yellow wells) values were averaged for hAR, and these averages were used as the $A_{blank,}$. The calculations were done separately for individual concentrations for the separate replicates and then were ultimately averaged, to reflect each of the 6 separate data sets as one data set. There were 3 sets of technical repeats per plate, and two biological repeats (2 plates) per micropollutant. These analyses were done for methylparaben, carbamazepine and the two comparative standards, flutamide and estradiol. The standard error bars displayed at each point, were calculated using the formula:

$$\sigma_M = \frac{\sigma}{\sqrt{N}}$$

3.3) Flow Cell Reactor

3.3.1) Design and Construction

A flow cell reactor was designed, the intention of which was to test whether the consumption of labile carbon sources by biofilm-bound microorganisms would lead to the metabolism of the remaining, recalcitrant carbon sources. This was achieved by extending the length of the reactor, by the insertion of silicone tubing (2 mm inner diameter) between two perspex flow cells (Wolfaardt et al., 1994), in order to increase both temporal and spatial exposure of sessile cells to the medium in the bulk aqueous phase. Next, sampling points were inserted at various positions of the reactor to obtain aqueous phase samples from. The original design used three-way stopcock connectors and silicon tubing with an inner diameter

of 10 mm to facilitate ease of sampling, but required too much medium and too frequent medium changes to be feasible. The reactor sampling points were modified using small plastic T-pieces (2 mm inner diameter, 38 mm length and 21 mm height) (Cole-Parmer®) which were constructed from various plastic barbed connectors to serve as collection points. Silicone tubing with a smaller inner diameter (2 mm) was used to connect all the collection points. Silicone tubing is readily permeable to both oxygen and carbon dioxide (Kroukamp and Wolfaardt, 2009) thus allowing sufficient gaseous exchange to ensure that nutrient concentration be the dominant limiting factor and not oxygen availability.

Flow cells (Wolfaardt et al., 1994) were incorporated into the system at the upstream and the downstream ends of the tube reactor, in order to visually evaluate the difference in the biofilms at either end of the reactor. Each flow cell channel was 31 mm long, 4.0 mm wide and 2.2 mm in depth (Bester, 2005). The completely constructed biofilm reactor, attached to the peristaltic pump is shown in Figure 3.5. The flow rate in the system was 27 $ml.h^{-1}$, leading to the displacement of the internal volume of each flow cell chamber (approximately 300 µL) every 40 seconds, and the entire reactor (3.5 mL) approximately every 7 minutes (the times increased slightly proportional to the amount of biomass in the system).

The **samples tested in the reactor** included either glucose or a micropollutants, or a combination of both. The sample types tested were as follows:

(1) Glucose only (**GLC**), a control
(2) Glucose and methylparaben (**GMP**)
(3) Glucose and carbamazepine (**GCBZ**)
(4) Methylparaben only (**MP**)
(5) Carbamazepine only (**CBZ**)

The samples, when referred to directly, will have been referred to as the abbreviated names in the rest of this thesis. The full names will sometimes have been used where the contents of the sample were referred to, and not the sample name itself.

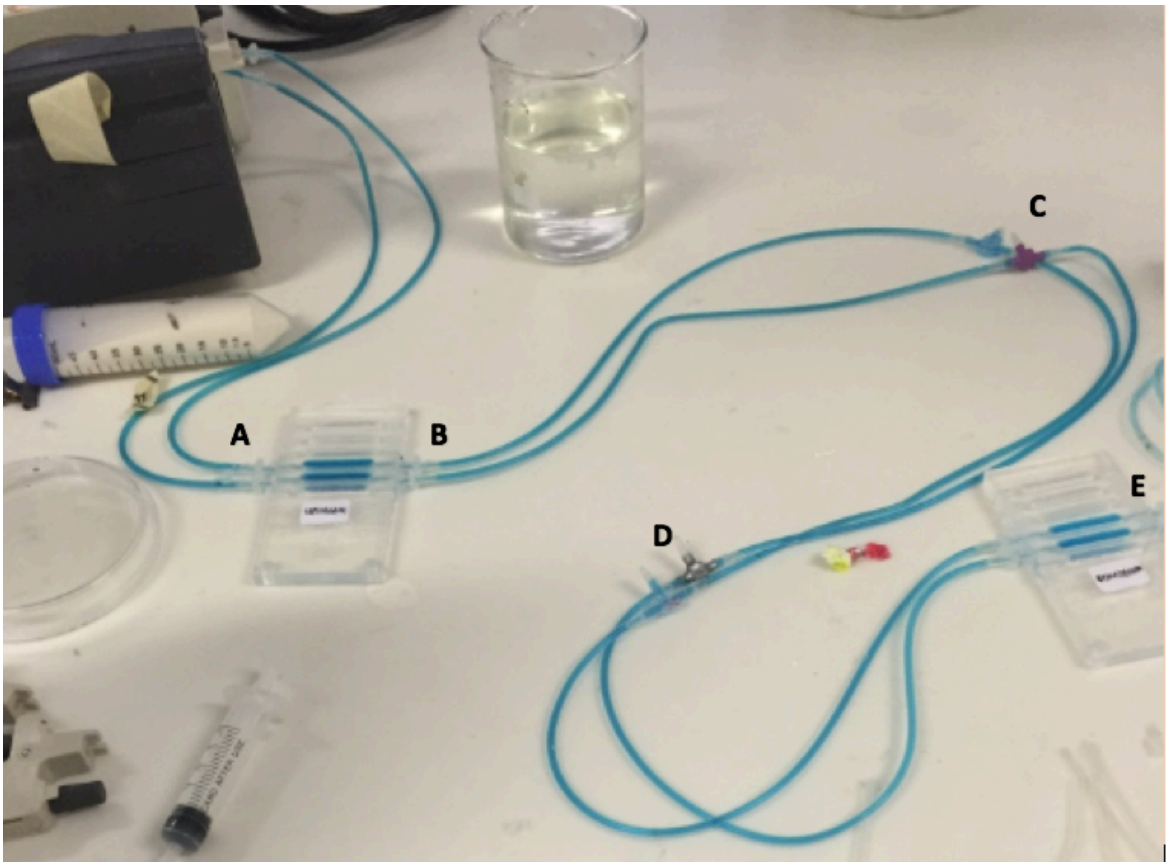


Figure 3.5: Flow cell reactor

The peristaltic pump (top left) pumped the blue fluid (used to enhance the visibility of the tubing) through the entire system from the beginning of the reactor (**A**) to the end of the reactor (**E**) and out of the system into a large Erlenmeyer flask used for waste collection (not pictured). The 4 collection points from which samples were collected throughout experimentation are labelled **B**, **C**, **D** and **E** for collection points 1, 2, 3, and 4, respectively. The lengths of tubing between collection points 1 and 2, 2 and 3, and 3 and 4 were each 30 cm.

3.3.2) Optimisation

The reactor was optimised according to the following final requirements: tubing diameter of 3mm and flow rate of 27 $mL.h^{-1}$ that prevented excessive biomass accumulation (which caused large portions of biomass to detach from the biofilm, stopping the flow) without frequent medium changes (a 5 L flask of medium replaced every 3 days for duplicate samples). Numerous aspects of the physical system were altered, as well as the experimental conditions, over the optimisation process. The length, width and other physical parameters of the reactor were altered several times throughout the experimental process, in order to optimise the data collection and consumption of resources. Optimisation of experimental conditions included varying the flow rates, the types of growth medium and the types and concentrations of primary (labile) carbon sources. An initial flow rate of 15 $mL.h^{-1}$ was chosen because it was the rate used for the perfusion column, but this was increased and set at 27$mL.h^{-1}$ for the final experimentation parameters. M9 minimal medium was selected to ensure control over the type and concentration of

carbon source applied to the biofilms. The primary carbon sources were originally both glucose and lactose, because both could be enzymatically quantified and would support a wider range of microbes than just glucose alone. However, lactose was eventually removed in order to simplify the experiment as much as possible (using glucose only). Synthetic wastewater was tested as a growth medium, but since it contained amino acids which could be utilised as carbon and energy sources and resulted in blockages within the reactor due to the accumulation of large amounts of biomass, it it was not pursued in subsequent experimentation. The concentrations of the various carbon sources were chosen to reflect that which appears in the actual wastewater treatment system.

The diagram of the entire reactor as well as the components, shown in Figure 3.5, represents the final (optimised) reactor system that was used.

3.3.3) Operation of the Reactor

Overnight cultures were prepared by dropping 3 biofilm-covered beads into 10 mL sterile glucose-supplemented TSB and incubating at 26^{o}C overnight. The reactor was sterilised by running a 20% NaOCl solution through the entire system for an hour. Sterile reverse osmosis water was then run through the system overnight. The next day, the M9 minimal medium (0.680% $NaHPO_4$; 0.300% KH_2PO_4; 0.050% NaCl; 0.100% NH_4Cl; 0.024% $MgSO_4$ and 0.001% $CaCl_2$) was prepared and the appropriate nutrient sources were added, depending on the test being performed: 0.06% glucose; 1000 ng.L^{-1} methylparaben and 600 ng.L^{-1} carbamazepine, to reflect those concentrations found in environmental samples. The medium was then pumped through the reactor to rinse out the water and then the pump was stopped.

The optical density of the overnight culture was measured and subsequently diluted, immediately before inoculation, in saline in order to adjust it to OD_{600} of 0.1. The diluted inoculum was injected into the reactor at the upstream end of the reactor (collection point 1 in Figure 3.5), using a syringe and 22-gauge needle, until the system was completely filled (about 3.5 mL). The inoculum was left to adhere for 30 minutes, after which the peristaltic pump was switched on. The entire reactor was then kept in the dark, to prevent growth of algae and other phototrophs, for the duration of experimentation, which was 6 days.

3.4) Reactor Experimentation

3.4.1) Microscopy

3.4.1.1) Light Microscopy

The biofilms attached to the glass coverslips of the flow cells were observed, in real-time, using a UB202i light microscope (UOP™) fitted with a 5.0 megapixel (MP) DCM500 USB 2.0 CMOS chip camera. The ScopePhoto x64 (Scopetek) freeware was used to capture real time images and video

footage of the biofilms. The biofilms were mostly observed, photographed and filmed at the 100x oil immersion objective magnification.

3.4.1.2) Confocal Laser Scanning Microscopy (CLSM)

Preparation of Samples

After the 6-day incubation period, the two flow cells at the upstream and downstream ends were sacrificed for CLSM analysis. The peristaltic pump was turned off and 300 µL of a Baclight™ Live/Dead (ThermoFisher Scientific) dye solution (0.2% SYTO®9; 0.2% propidium iodide) was injected slowly into the flow cell chambers using a sterile 1 mL syringe and a 26-gauge sterile needle. The flow cells were then incubated in dark conditions for up to 45 minutes, depending on the thickness of the biofilm. The pump was switched back on (set to the same flow rate) for 10 to 15 minutes to allow the residual dye to be completely flushed from the system. The pump was then switched off again, and the connectors for each chamber from the downstream side were clamped off and removed carefully, and the silicone tubing within the outlets were obstructed (using marine silicone (Bostik®)) to retain the fluid inside the channel of the flow cells. The tubing upstream of the flow cell chambers were then clamped and removed for each chamber, tilting the open side of the flow cell upwards to prevent loss of the fluid within the chambers. These inlets were then also sealed, and the flow cells were cleaned with 70% ethanol, wrapped in aluminium foil and placed in a dark container for transport to the microscope.

Obtaining Image Stacks

The CLSM data was obtained in the form of Z-image stacks, which are a type of file containing multiple images. The file type in which the stacks were saved was an LSM 5 file with the extension ".lsm". The images were obtained using an LSM780 with ELYRA S.1 Superresolution Platform Confocal Microscope (Zeiss), with lasers set to 2 tracks: 488 and 561 nm corresponding to green and red fluorescence of the BacLight components, respectively. A total of 9 image stacks were obtained in the Z-direction of duplicate flow cell channels for each variable (GLC, GMP, etc.) using an oil-immersion 40X objective, starting at the biofilm attached to the glass coverslip and extending into the biofilm. The resolution of each image in the stack was 1024 x 1024 pixels, saved in dual channels (red and green). The dimensions of the stacks were 212.55 µm square (x and y). The depth of the stack varied per sample, as the thickness of each Z-stack was adjusted according to the thickness of the biofilm in each sample.

Data Analysis

Biofilm analysis was done using the COMSTAT script (Heydorn et al., 2000) written for the Matrix Laboratory (MATLAB®) software (The MathWorks Inc.), while qualitative image analyses were done using ImageJ software or Adobe® Photoshop.

The MATLAB® software requires the image stacks to be in a specific format. Each image stack must be separated into the separate image layers, in .TIF format. Each .lsm file (containing the image stack) was converted in to its constituent images using Zen Lite 2012 software (Zeiss™). Each .lsm file was exported from Zen Lite and each image slice saved as separate .TIF files within an appropriately named folder. The image (and supporting info files) data analysis was done according to the protocol provided in Heydorn et al. (2000) as well as the operational manual provided with COMSTAT (upon request to the author, Arné Heydorn).

Each image set (stack of images) was analysed and converted into 6 separate data sets:

(1) average biofilm thickness (μm)
(2) maximum biofilm thickness (μm)
(3) biomass ($\mu m^3/\mu m^2$)
(4) substratum coverage (%)
(5) roughness coefficient (Ra*)
(6) surface area to biovolume ratio ($\mu m^2/\mu m^3$)

The term 'biomass' describes the volume of space occupied by the biofilm compared to the surface area covered. Substratum coverage refers to the proportion of the glass surface that was covered with biofilm within the visible field of the microscope image. Roughness coefficient (Ra*) is a measure of heterogeneity of the biofilm (Heydorn et al., 2000). It is a dimensionless measurement that was suggested by Murga et al., (1995), and it describes the surface topography of the biofilm. Surface area to biovolume ratio, simply put, describes the amount of surface area per unit volume. What it actually describes is the absorptive potential of the object, based on its surface area and volume (Blamire, 2001). A larger ratio means a greater absorptive potential of the surface of the biofilm, which is of importance in nutrient availability.

The data was analysed using one-way analysis of variance (ANOVA), with a P value of 0.05. ANOVA tests whether one population (or data set) is the same as another: this is called the null hypothesis. The ANOVA analysis returns an F value and a P value. The F value must be higher than the F_{crit} value (provided during the analysis), and the P value returned must be smaller than 0.05 for the null hypothesis to be rejected. Statistical programs were available for ANOVA analysis, but it was preferred to analyse the data in Microsoft Excel® using a plugin (installed

separately) that specifically allowed ANOVA analyses. There were 9 data points per data set, where one data set was one category of one sample type (i.e. glucose upstream average thickness was one data set, while glucose downstream average thickness was a second data set, glucose upstream maximum thickness a third, etc.). Each category was only compared with other samples within the same category and the sample types were only compared when they had one carbon source in common. Upstream categories were only compared with upstream categories, and downstream with only downstream. For example, the average thicknesses of GLC upstream and GMP upstream were compared, but not the average thicknesses of GLC upstream and GMP downstream, or GLC upstream and MP upstream.

3.4.2) Secondary Carbon Source (Micropollutant) Analysis

Liquid chromatography mass spectrometry (LCMS) was performed on samples from the reactor, as well as the medium that was being pumped into the reactor, in order to determine if, and to what degree, the micropollutants were being degraded. The samples containing glucose and those that did not were compared to determine if the presence of glucose affected the degradation of the secondary carbon sources (the micropollutants: methylparaben and carbamazepine).

3.4.2.1) Fate of Secondary Carbon Sources (Micropollutants) in the Reactor

Raw samples were extracted from the reactor after the 6-day incubation period and concentrated using solid phase extraction (SPE). Samples from the downstream end of the reactor (collection point 4 in Figure 3.5) were collected in 20 mL volumes, in sterile 50 mL Falcon tubes, using the same flow rate as what is used to run the medium during normal incubation. With a flow rate of 27 $mL.h^{-1}$, collection of at least 20 mL for solid phase extraction took approximately 45 minutes. The longer waiting period for the sample to reach sufficient volume necessitated keeping the samples on ice to reduce further bacterial degradation of the micropollutant. Once enough of the sample was collected, the samples were centrifuged at 3 000 rpm for 10 minutes to pellet the biomass. The supernatant was collected and kept on ice until processing.

A 12 slot manifold (Supelco™, Sigma) was set up with a -5 mmHg vacuum (1 $mL.min^{-1}$ flow rate) and, for each sample to be extracted, a 3 mL (60 mg) hydrophillic-lipophillic balance (HLB) SPE column (Supelco™ Sigma-Aldrich®) was placed into one of the 12 manifold slots and labelled according to the sample to be extracted. The exterior of each of the columns was wrapped in aluminium foil to prevent UV degradation of the samples during processing. A volume of 3 mL HPLC-grade methanol (Sigma-Aldrich®) was drawn through each column, and thereafter 3 mL Millipore water. Care was taken to close the taps of the manifold so that a very small meniscus of water was still covering the adsorptive material within the columns. This served to prevent drying of

the material after conditioning, which would have rendered the adsorptive material less effective in binding the compounds for analysis.

Each of the samples, kept on ice in the interim, was pushed through a 0.22 µM cellulose-acetate syringe filter (Amtast™) to remove all cellular material and polysaccharides before being drawn through their respective HLB columns at 1 $mL.min^{-1}$. Once the samples had completely passed through the columns, the HLB adsorptive materials were completely dried out, wrapped in foil and frozen in airtight package at -20°C until a week before sample submission, in order to preserve the structure of the compounds contained within them.

The frozen columns were thawed and eluted into HPLC-grade (99.9% purity) methanol (Sigma-Aldrich®). Once thawed, the columns were placed into the slots in the manifold. The samples were eluted out of the column using 3 mL HPLC-grade methanol, per sample, in pyrolysed, borosilicate glass test tubes. The methanol in each tube was evaporated completely, in the dark using nitrogen gas with clean borosilicate glass Pasteur pipettes fixed onto the gas distribution head. Once each tube was completely dry, the sample was re-suspended in 200 µL HPLC-grade methanol and vortexed rigorously to ensure the highest amount of resuspension possible. Each sample, now concentrated to 200 µL, was transferred to a 200 µL pyrolysed borosilicate glass vial insert (Stargate Scientific) placed within a 2 mL amber glass vial and sealed tightly with a polypropylene cap possessing a PTFE seal. The samples were stored at -20°C until submission for analysis, or for use in the yeast screens.

Each time samples were submitted for mass spectrometry, a set of standard concentrations for each analyte was prepared. These standards ranged in concentration from 1ppm ($mg.L^{-1}$) to 10 ppt ($ng.L^{-1}$). The analysis was done using an Acquity UPLC® Mass Spectrometer using 0.1% formic acid and 9:1 solution of water and acetonitrile.

The data acquired from the mass-spectrometer were analyses using ANOVA, with a P value of 0.05. The influent and effluent concentrations were also included in Table 4.2, along with the standard error (of the mean). The percentage removal (referred to as removal efficiency in the table) was also calculated and included in Table 4.2. ANOVA and standard error calculations were also conducted on the removal efficiencies (shown in Table 4.2).

3.4.2.2) Endocrine-Disrupting Effects of Reactor Samples

The samples prepared for mass spectrometry were used in yeast assays to determine if the concentration of the micropollutants in the reactor, taken on day 6 of the reactor runs, (concentrations corresponding to those reported for WWTPs: methylparaben at 1000 $ng.L^{-1}$ and carbamazepine at 600$ng.L^{-1}$ (Archer et al., 2017) were capable of eliciting any endocrinologic effects on the receptors

the recombinant yeast. The concentrated samples which contained carbamazepine were subject to an anti-androgenic assay (using hAR strain) while those which contained methylparaben were subject to an estrogen assay (using hER strain).

Samples were collected from the downstream end of the flow cell reactor (collection point 4 in Figure 3.5) supplied with M9 minimal medium supplemented with 1000 $ng.L^{-1}$ methylparaben or 600 $ng.L^{-1}$ carbamazepine (with or without glucose, depending on the sample type) and concentrated 100X (as described in section 3.4.3.1). The estrogen yeast assay was prepared by making an E_2 dilution series, as described in section 3.2.2.1. The concentrated samples were placed into the microtitre plates in 20, 10 and 5 µL volumes (10X, 5X and 2.5X concentrations of the original 100X concentrated samples) in duplicate for both the estrogen and anti-androgen assay. The samples were allowed to evaporate completely before the addition of 200 µL assay medium, seeded with the appropriate strain of yeast (hER for estrogen assay and hAR for anti-androgen assay, respectively). The plates were incubated at 30°C until undergoing an adequate degree of colour change. Readings were taken at 540 and 620 nm and the data processed as described in section 3.2.2.3.

3.4.3) Primary Carbon Source (Glucose) Analysis

Glucose analysis was conducted on the reactor samples in order to determine the degree of glucose depletion due to microbial metabolism at various spatial distributions across the reactor. Glucose was the primary (more labile) nutrient source and it was postulated that the depletion of glucose would occur at a certain point within the reactor to the degree where the microbes downstream would be forced to use the micropollutant as a carbon source.

Approximately 1 mL of bulk-liquid was sampled from each collection point and the samples were centrifuged at 8 500 rpm for 15 minutes in a table top microfuge. The supernatant was aliquotted into sterile Eppendorf tubes (approximately 1 mL volume per sample). Standard glucose solutions of 500 $mg.L^{-1}$ and 800 $mg.L^{-1}$ were made for each set of samples. The tubes were then kept frozen at -20°C until submission for enzymatic analysis. Duplicates of replicate test were collected and the standard error (of the mean) was calculated for each set of replicates (data point on the graph), using the equation provided earlier.

3.4.4) Planktonic Cell Release

Planktonic cell counts were performed on samples collected daily from the downstream effluent end of the reactor (collection point 4 in Figure 3.5), until a steady-state was reached (consistent cell concentrations in the collected effluent samples). Thereafter, cell counts were taken from each of the 4 collection points, on day 6, in duplicate. Cell count data was obtained by collecting 1 mL of effluent in a

sterile 1.5 mL Eppendorf tube from the respective sampling points, after clamping off the part of the reactor directly downstream of the point from which effluent was taken. The clamp was removed and 100 µL of the effluent in the tube was subjected to a 10-fold dilution series in sterile saline and plated onto solid M9 minimal medium (0.06% glucose, 1.2% agar). The plates were incubated for 48 hours at 26^{o}C, after which the colonies were enumerated and recorded.

Chapter 4: Results

4.1) Microbe-Micropollutant Interactions

The effects of methylparaben and carbamazepine on microbial growth and biofilm formation were investigated. The possible endocrine-disruption potential of these two compounds on yeast expressing human estrogen and androgen receptors was also determined. Lastly, the effects of these compounds on physical structure of the biofilm was investigated in order to ascertain how the presence of these compounds can alter biofilm structure (and perhaps function, by extension).

4.1.1) Microbial Inhibitory Effects of Methylparaben and Carbamazepine

The inhibitory effects of methylparaben on the growth of the CT07 culture and the mixed microbial culture are shown in Figure 4.1. The graph shows the optical density of the culture with increasing concentrations of methylparaben (in log ng.L^{-1}). The functions for best-fit trend lines as well as the the R^2 values are shown below the curves. Due to the erratic nature of the mixed MIC curve, a best-fit curve was included (called MIC modified) in order to mathematically estimate the MIC value (since the concentration range was not high enough in the experimental design for it to be represented on the original curve.

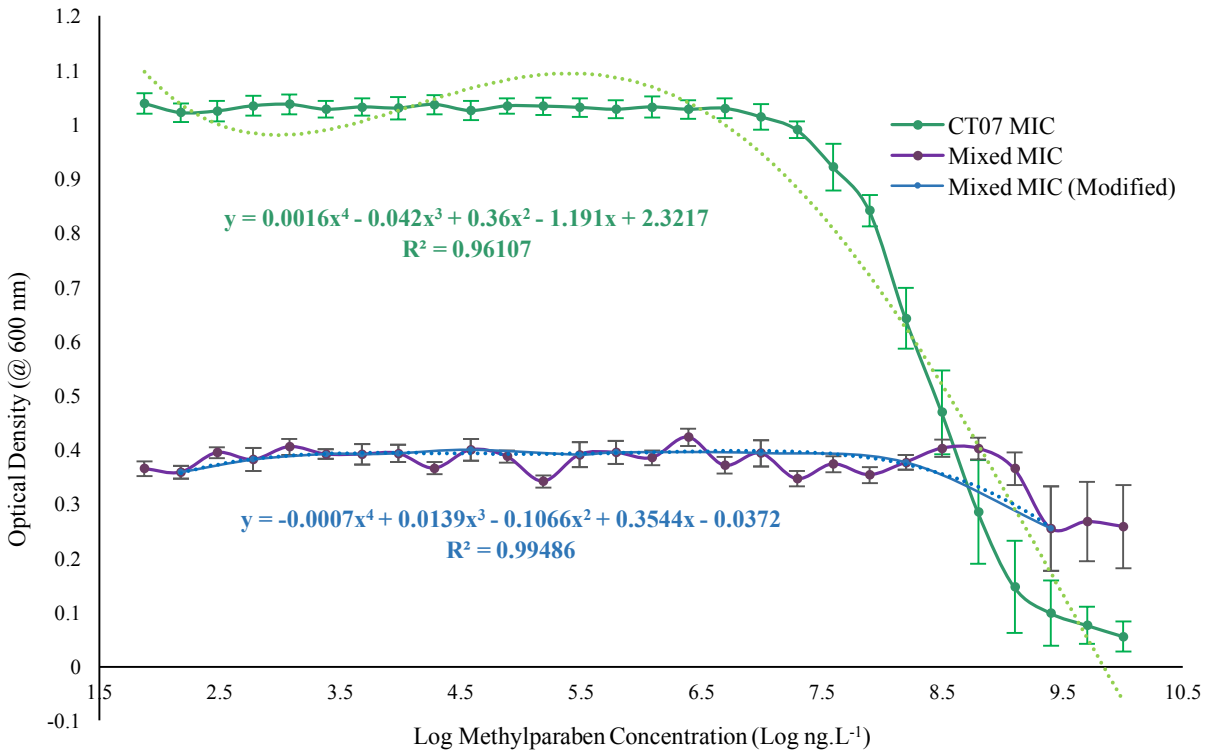


Figure 4.1: MIC of methylparaben for both CT07 and mixed microbial cultures

The MIC of methylparaben for CT07 and the mixed microbial culture are represented by the green and purple curves, respectively. Best-fit curves are represented by the dotted lines (green for CT07 and blue for the mixed microbial culture), with their R^2 values and equations shown in the corresponding colour. The MICs were measured at an optical density of 600 nm. The error bars represent the standard error of the mean for each point.

It must also be pointed out that the mixed microbial culture did not reach the same maximum optical density within 20 hours as the pure culture did. In order to maintain consistency, however, the same experimental parameters (incubation time) had to be applied to both CT07 and the mixed culture. The optical density of the CT07 and mixed microbial cultures after 20 hours was 1.06 and 0.42, respectively. The optical density of the negative control was 0.052 for both cultures.

The optical density of the CT07 culture at log 10 ng.L^{-1} (10 g.L^{-1}) was 0.055, which indicated 95% growth inhibition. The **MIC of methylparaben for CT07** was therefore slightly higher than 10 g.L^{-1}, and was mathematically determined to be **10.6 g.L^{-1}** by extrapolating the curve. The optical density at 10 g.L^{-1} (0.055) and the optical density required for complete growth inhibition (0.052) were used to estimate the concentration of methylparaben at 0.052 using a simple ratio equation. The optical density of the mixed microbial culture at 10 g.L^{-1} was 0.258: only 44% growth inhibition. Therefore, the **MIC of methylparaben for the mixed microbial culture** was notably higher than 10 g.L^{-1}, and was mathematically estimated to be around **256.7 g.L^{-1}**, based on the equation:

$$M = \frac{y_2 - y_1}{x_2 - x_1}$$

where M is the gradient of the curve and the x and y values refer to two coordinates on the curve. The last two points on the MIC (Modified) curve (blue curve) were used to calculate the gradient of that part of the curve and this gradient was then used to calculate the x value when y was 0.052 (the optical density at which the MIC occurred) if the curve had been extrapolated to cross the x-axis.

The MIC data of carbamazepine for both the CT07 and mixed microbial cultures is presented in Figure 4.2. Differences in maximum optical density were also seen with carbamazepine MIC assay (1.08 and 0.38 for CT07 and the mixed culture, respectively). The negative control for both cultures was 0.051.

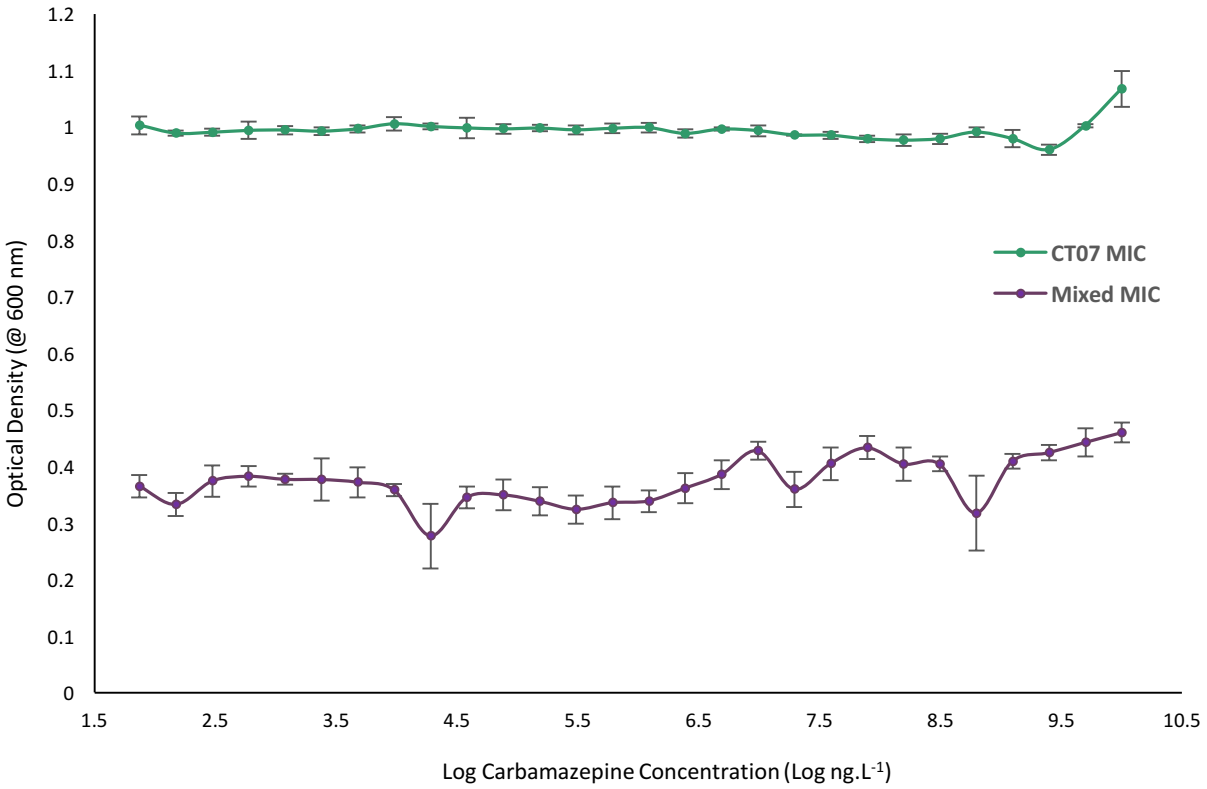


Figure 4.2: MIC of carbamazepine for both CT07 and mixed microbial cultures

The MIC of carbamazepine for CT07 and the mixed microbial culture are represented by the green and purple curves, respectively. The MICs were measured at an optical density of 600 nm and the error bars represented the standard error of the mean for each concentration of carbamazepine.

4.1.2) Potential Endocrine-Disrupting Effects of Methylparaben and Carbamazepine

Two recombinant *Saccharomyces cerevisiae* strains were used to reveal possible physiological responses, caused by methylparaben and carbamazepine, in human endocrine systems. Figure 4.3 contains the data for the yeast estrogen assay, while Figure 4.4 contains the data for the yeast anti-androgen assay. Due to the fact that the anti-androgen assay was a reversal of the normal estrogen/ androgen assay, the negative and positive controls were reversed, since the yellow medium in the anti-androgenicity assay would have indicated a positive response (inhibition of the androgen) while red/purple medium indicated a lack of anti-androgenic effect. The colour of the medium for the positive control in the anti-androgen assay and the negative control in the estrogen assay remained the same and each was used to calculate the corrected absorbance values seen in the y-axis of the curves in Figures 4.3 and 4.4.

Figure 4.3 shows the standard curve for estradiol, used as the estrogenic standard against which methylparaben was compared. The ED_{50} marker represents the point at which 50% of the maximum estrogenic effect is seen. The results show that **methylparaben** has a low-to-moderate potent estrogenic effect, as the **estrogen receptor agonism** occurs only at methylparaben concentrations upwards of **19.5 mg.L^{-1}**. The portion of the curve in figure 4.3 that intersected with the negative

control line was the point at which the yeast cells were completely killed by methylparaben (1.58 g.L^{-1}) Figure 4.4 shows the results of the anti-androgenic yeast assay with the hAR strain. Flutamide, the standard, showed the expected sigmoidal shape. The **carbamazepine** curve showed that anti-androgenic effects, or **androgen receptor antagonism**, occurred at concentrations above **776 µg.L^{-1}**. The carbamazepine curve fluctuated after the initial exponential decrease. It was expected that the curve would continue in an exponential decrease, much like that of flutamide, but the curve inflected and fluctuated to an eventual, but gradual, decrease. The anti-androgenic point was taken to be where the initial decrease occurred: 6.087 on the x-axis (776 µg.L^{-1}**)**.

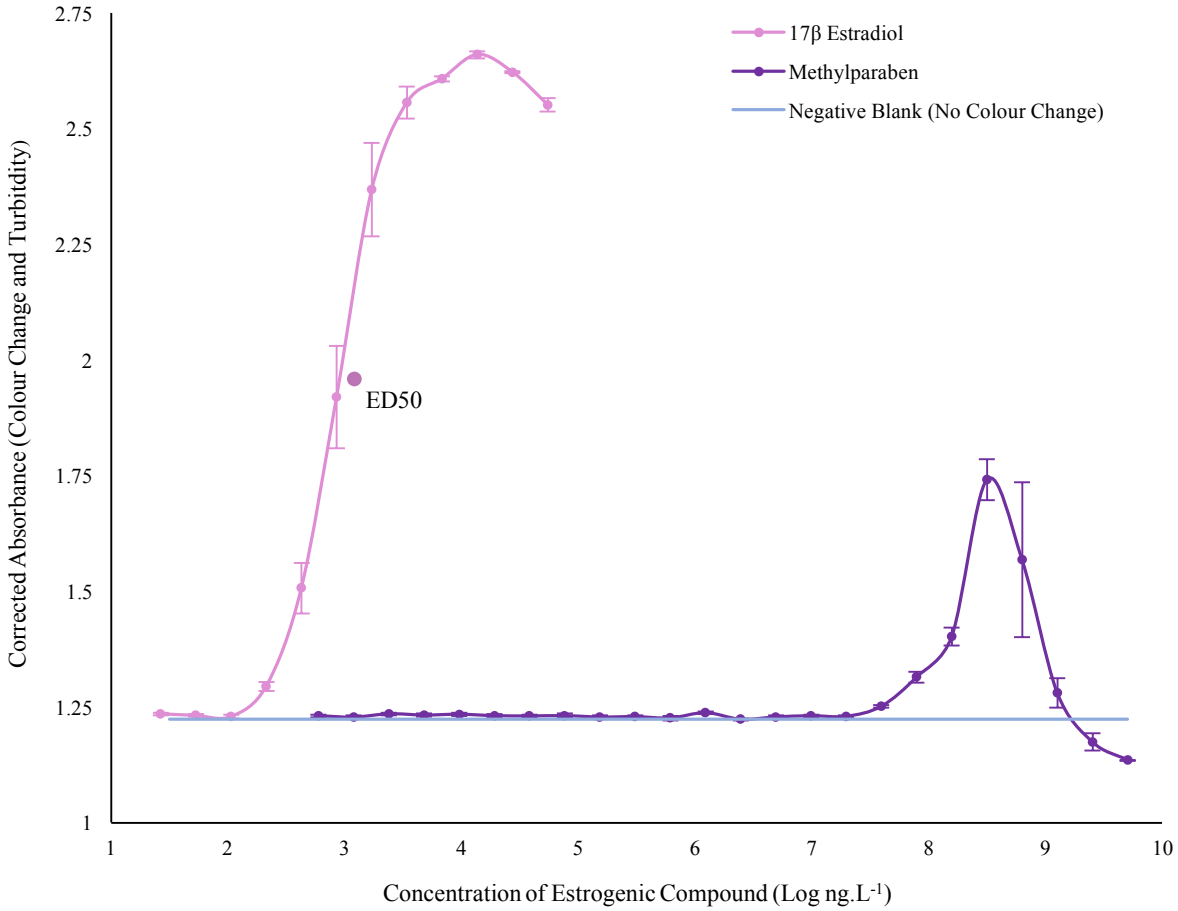


Figure 4.3: Estrogenicity data for methylparaben for the hER strain of yeast

The figure depicts the estrogenicity curves for both estradiol and methylparaben. The blank reading for the assay has been depicted as the line y = 1.223. The error bars indicate the standard error of the mean for each concentration of either methylparaben or estradiol.

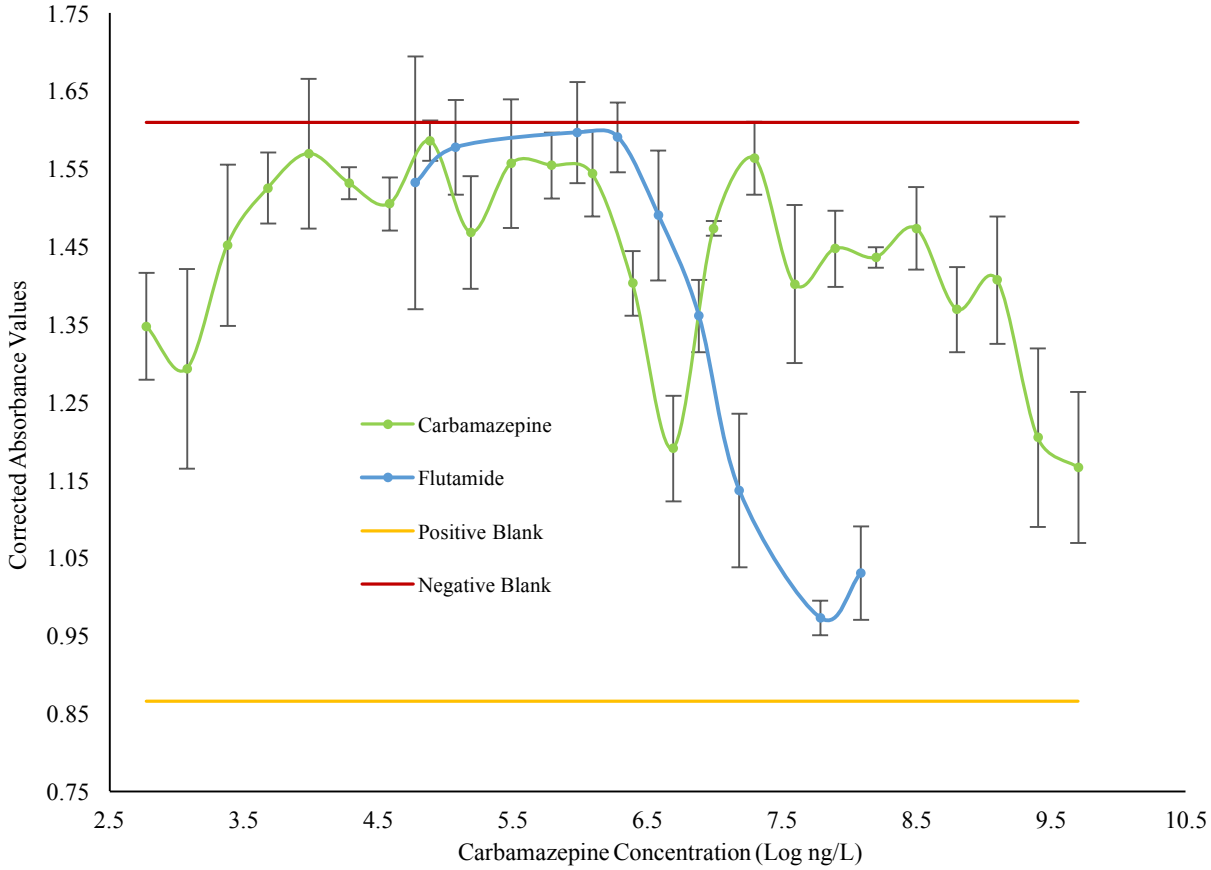


Figure 4.4: Anti-androgenicity data for carbamazepine for the hAR strain of yeast

The positive and negative blank reading for the assay have been represented as the lines y = 0.866 and y = 1.609, respectively. The latter represents colour change as a result of androgenicity of DHT, while the former represents the result of complete inhibition of the androgen. The error bars indicate the standard error of the mean for each concentration of either carbamazepine or flutamide.

4.1.3) Effects of Micropollutants on Biofilm Structure

4.1.3.1) Light Microscopy

The flow cells, situated in the upstream and downstream ends of the reactor, provided an ideal means to evaluate the development of the biofilm in real-time. Light microscopy was conducted by viewing the glass-covered surface of the flow cells, both upstream and downstream of the reactor, at 1000X (using a 100X oil-immersion lens). Still images were taken of the biofilm using the attached microscope camera (2 megapixels), which allowed for qualitative analysis of the biofilm in real-time, in order to ascertain what effects, if any, occur within the biofilm as a result of position (upstream vs downstream) and presence/absence of the compound. Figure 4.5 shows a colour plate of with representative images of the biofilm of each sample (20+ images for each sample GLC, GMP. MP. GCBZ and CBZ).

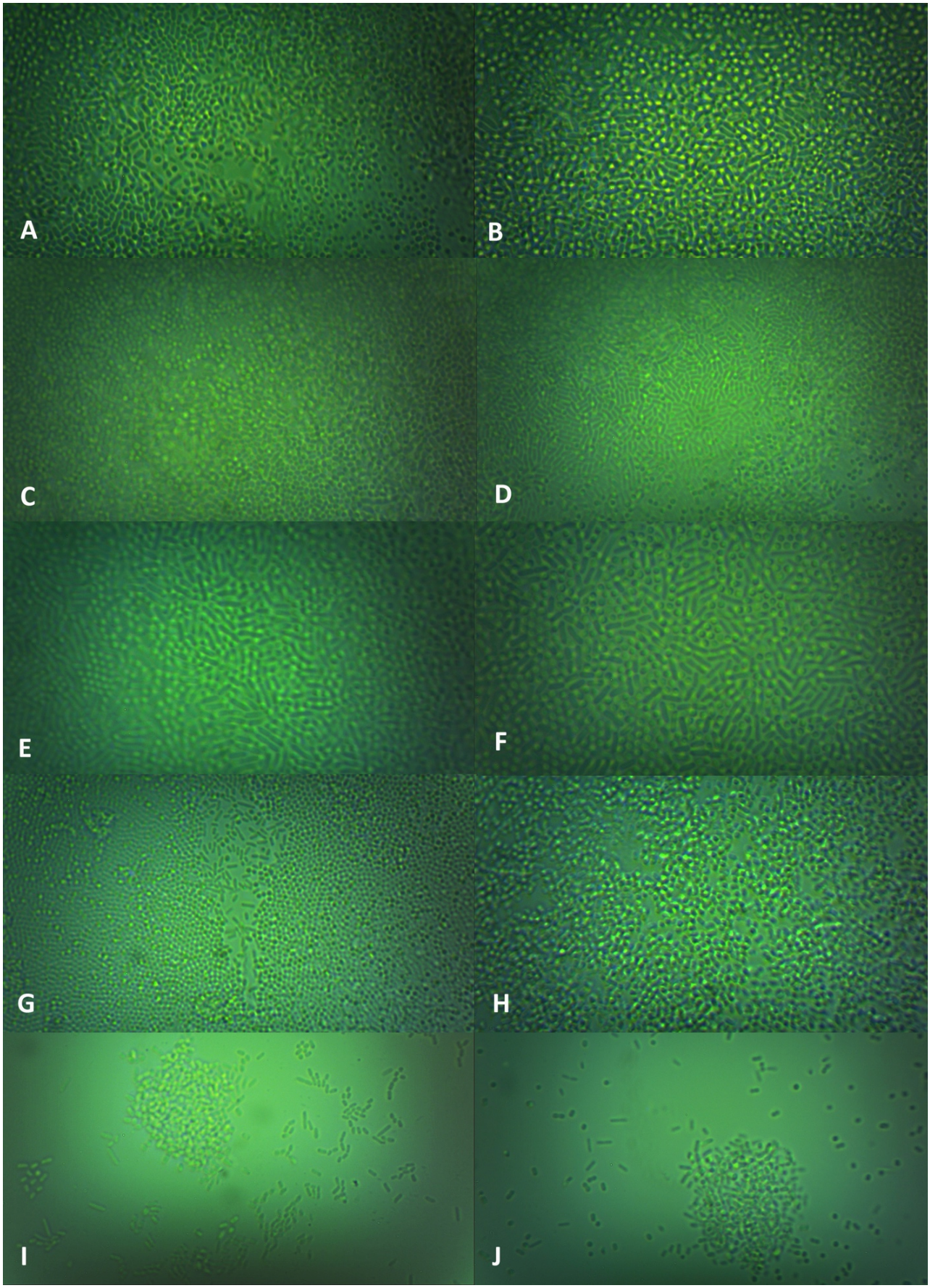


Figure 4.5: Light micrographs taken of the respective biofilms attached to the glass surface in the flow cells.

The images on the left depict the biofilms from the flow cell located upstream of the reactor while the images on the right depict the biofilms downstream. **A-B**: Glucose only (GLC); **C-D**: Glucose and methylparaben (GMP); **E-F**: Glucose and carbamazepine (GCBZ); **G-H**: Methylparaben only (MP); **I-J**: Carbamazepine only (CBZ).

4.1.3.2) Confocal Laser Scanning Microscopy (CLSM)

The image stacks were processed an analysed as described in the methods section. The physical characteristics of the biofilms are shown in Figure 4.6 and 4.7, with the corresponding ANOVA results and T-Test analyses presented in Table 4.1.

The images obtained from the image stacks were layered over one another using Adobe Photoshop, to obtain a topographical view of each biofilm. For illustration, these images are shown (for each sample type) in Figure 4.8. The MATLAB® analysis yielded thematic maps of the biofilm, where the degree of colour indicated the depth of the biofilm. These maps are shown in Figure 4.9.

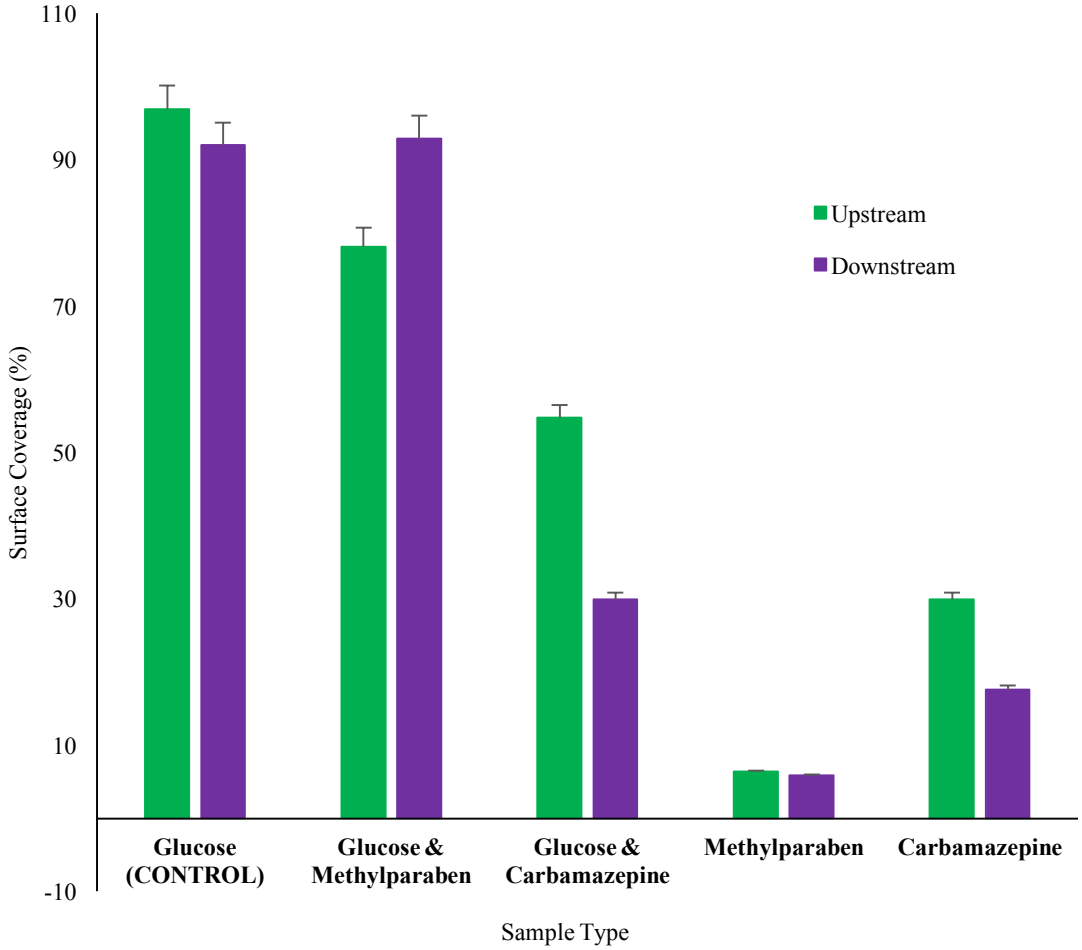


Figure 4.6: Surface coverage of the biofilms of different samples for confocal data (in %)

The substratum (surface) coverage of the glass coverslips of the biofilm is shown in percentage surface coverage. The error bars represent the standard error of the mean, calculated for the mean of each sample type. Data for the upstream flow cell is shown in green, while that of the downstream is shown in purple.

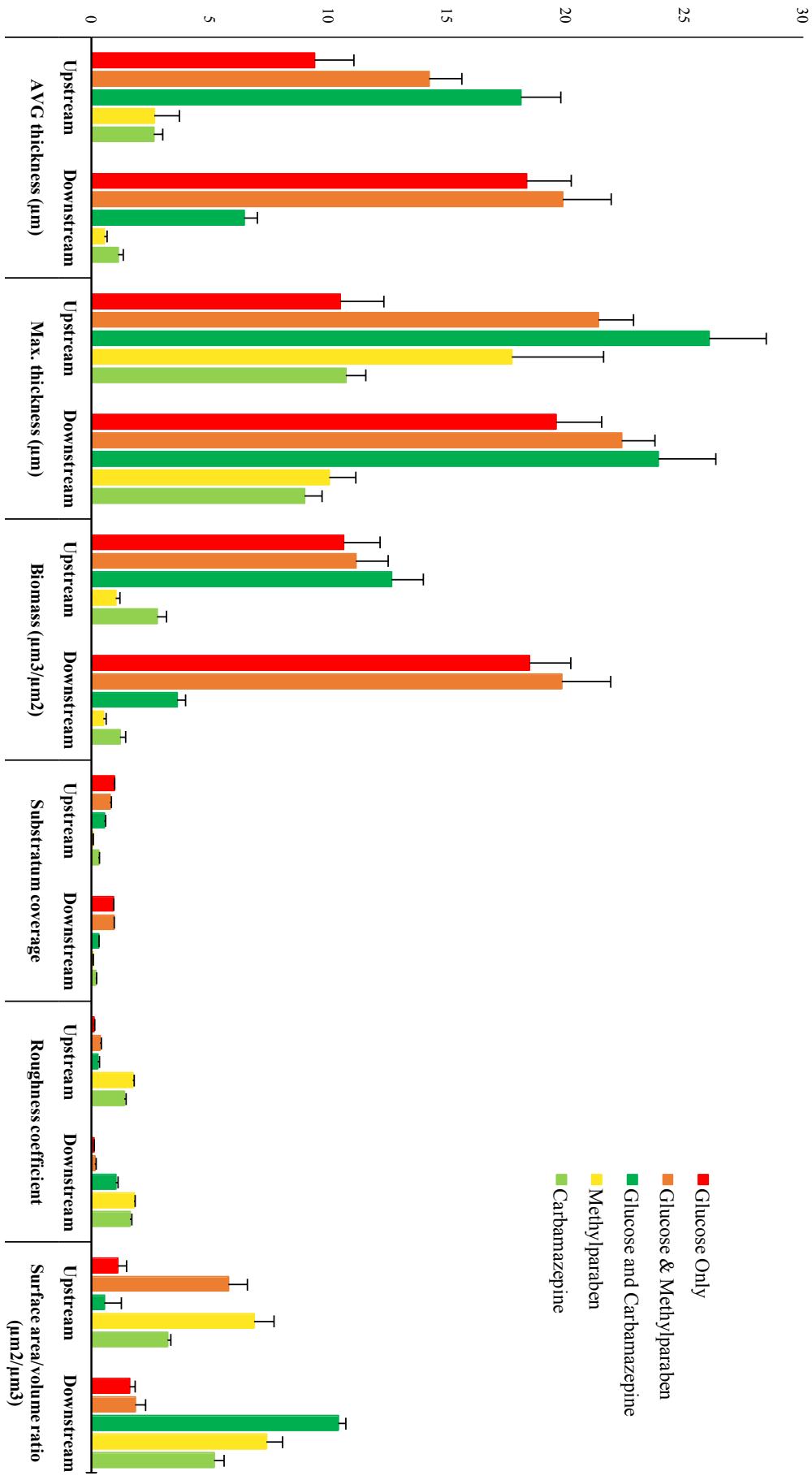
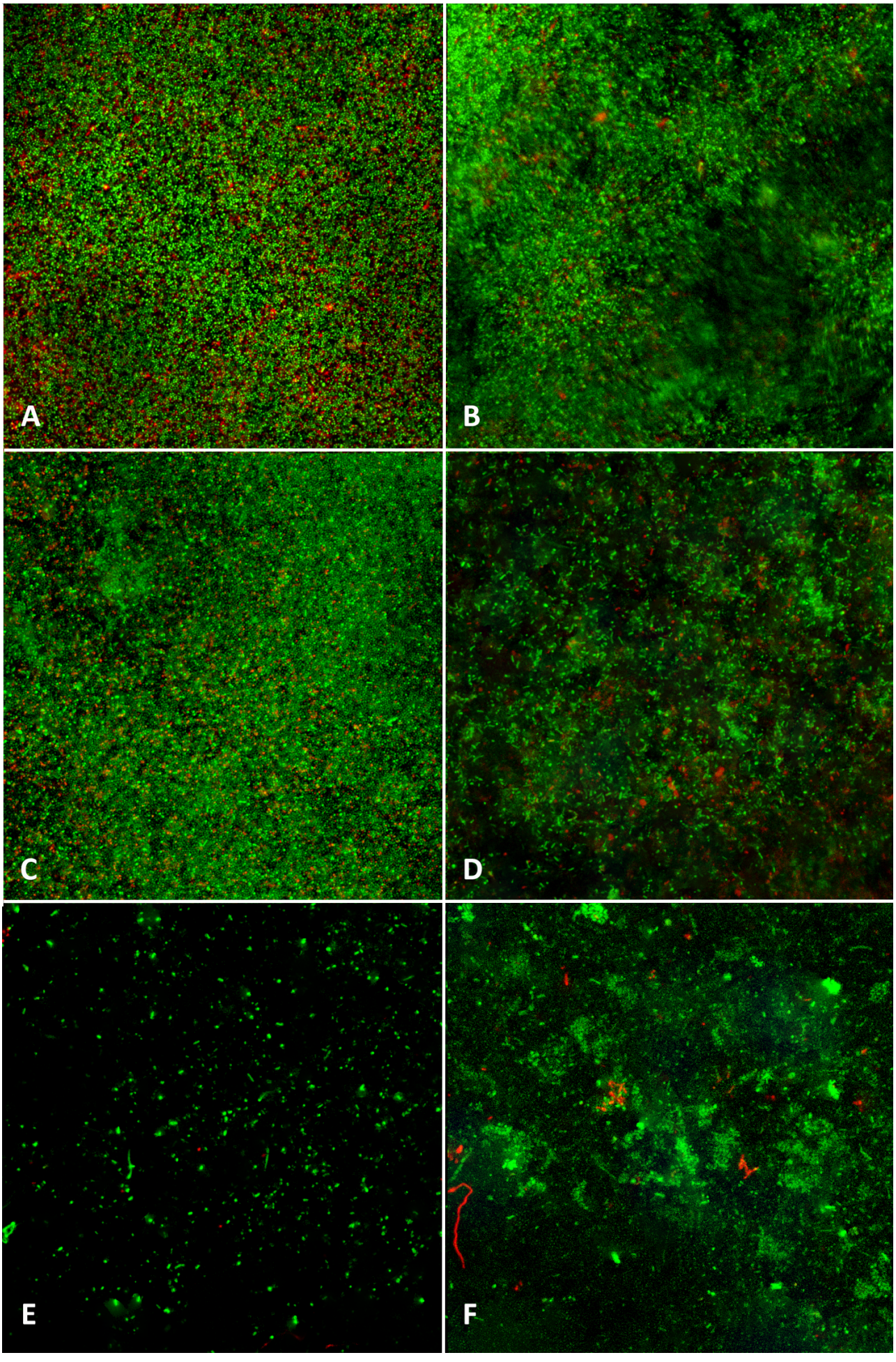


Figure 4.7 Mean Within Sample Groups for Processed Confocal Data in the Categories of Thickness (Average and Maximum), Biomass, Substratum Coverage, Roughness Coefficient and Surface Area to Volume Ratio

Table 4.1 ANOVA analysis of the confocal data

The column on the left shows the samples that are compared with the samples on the top of the table. A "O" indicates no rejection (the two samples compared were statistically similar). An "X" indicates that the null hypothesis is rejected (the two samples are considered statistically different.

Test Sample Parameter (ANOVA / T-Test)		Glucose Only		Glucose and Methylparaben		Methylparaben		Glucose and Carbamazepine		Carbamazepine		ALL	
		Up stream	Down stream	Up stream	Down stream	Up stream	Down stream	Up stream	Down stream	Up stream	Down stream	Up stream	Down stream
Average Thickness (µm)													
Glucose Only	UP			O				O				X	X
	DOWN				O				X				
Glucose and MP	UP	O				X		O					
	DOWN		O				X		X				
MP	UP			X						O			
	DOWN				X						X		
Glucose and CBZ	UP	X		O						X			
	DOWN		X		X						X		
CBZ	UP					O		X					
	DOWN						X		X				
Maximum Thickness (µm)													
Glucose Only	UP			O				X				X	X
	DOWN				O				X				
Glucose and MP	UP	O				X		O					
	DOWN		O				X		X				
MP	UP			X						O			
	DOWN				X						X		
Glucose and CBZ	UP	X		O						X			
	DOWN		X		X						X		
CBZ	UP					O		X					
	DOWN						X		X				
Biomass													
Glucose Only	UP			O				O				X	X
	DOWN				O				X				
Glucose and MP	UP	O				X		O					
	DOWN		O				X		X				
MP	UP			X						X			
	DOWN				X						X		
Glucose and CBZ	UP	O		O						X			
	DOWN		X		X						X		
CBZ	UP					X		X					
	DOWN						X		X				
Roughness Coefficient													
Glucose Only	UP			X				O				X	X
	DOWN				O				X				
Glucose and MP	UP	X				X		O					
	DOWN		O				X		X				
MP	UP			X						X			
	DOWN				X						X		
Glucose and CBZ	UP	O		O						X			
	DOWN		X		X						X		
CBZ	UP					X		X					
	DOWN						X		X				
Substratum Coverage													
Glucose Only	UP			X				X				X	X
	DOWN				O				X				
Glucose and MP	UP	X				X		X					
	DOWN		O				X		X				
MP	UP			X						X			
	DOWN				X						X		
Glucose and CBZ	UP	X		X						X			
	DOWN		X		X						X		
CBZ	UP					X		X					
	DOWN						X		X				
Surface Area to Volume Ratio													
Glucose Only	UP			X				X				X	X
	DOWN				O				X				
Glucose and MP	UP	X				O		O					
	DOWN		O				X		X				
MP	UP			O						X			
	DOWN				X						X		
Glucose and CBZ	UP	X		O						X			
	DOWN		X		X						X		
CBZ	UP					X		X					
	DOWN						X		X				



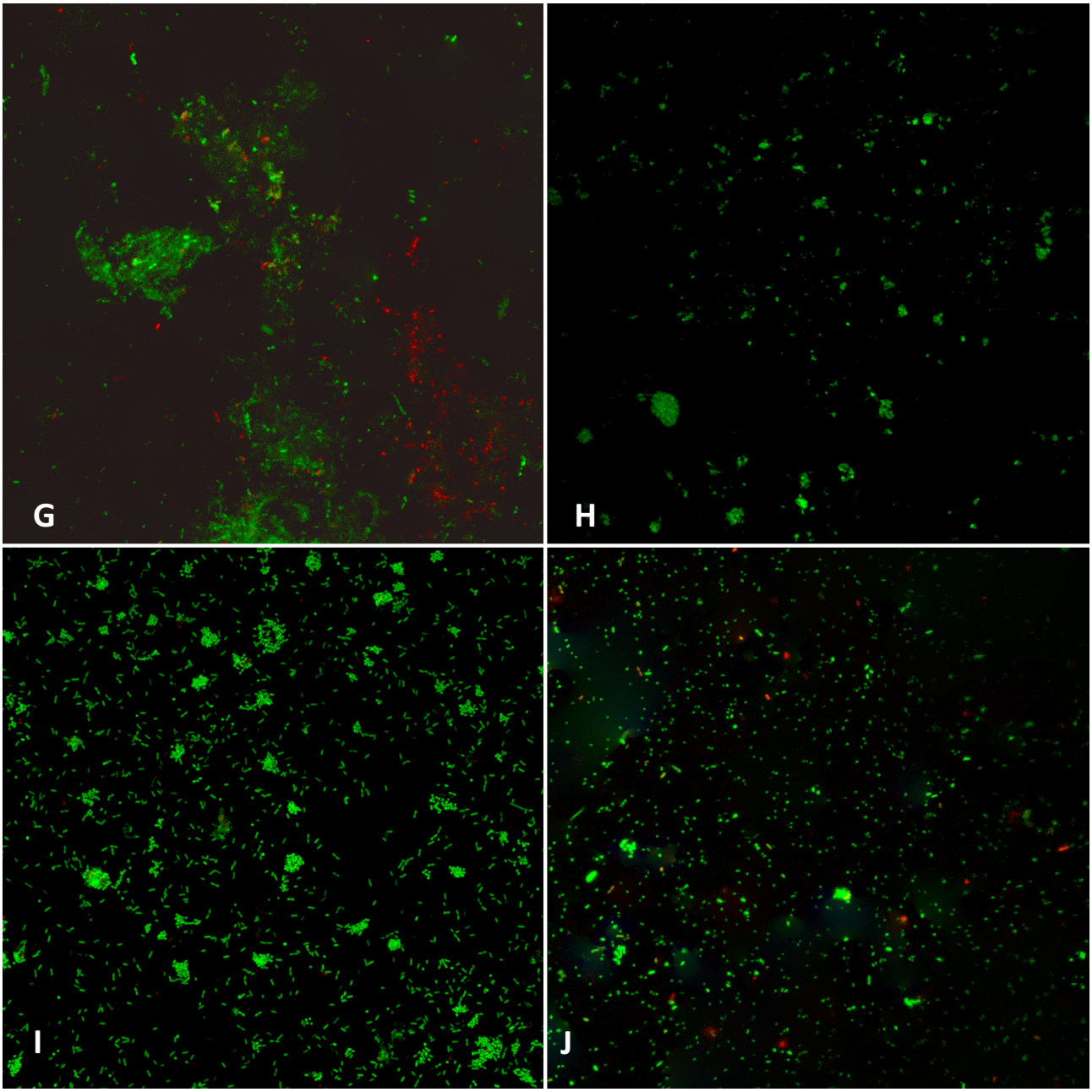


Figure 4.8: Confocal images (in RGB colour) created by slice overlaying (this page and previous page)

The images, starting from A and ending at J, are all image slices that were layered over one another to form the full stack. In other words these photos are 3D (x, y and z axes). Images on the left show the upstream flow cell, while images on the right show the downstream flow cell. **A-B** = glucose only samples (control samples); **C-D** = glucose and methylparaben samples; **E-F** = glucose and carbamazepine samples; **G-H** = methylparaben only samples, **I-J** = carbamazepine only samples. All images are at 63X magnification, with no optical zoom.

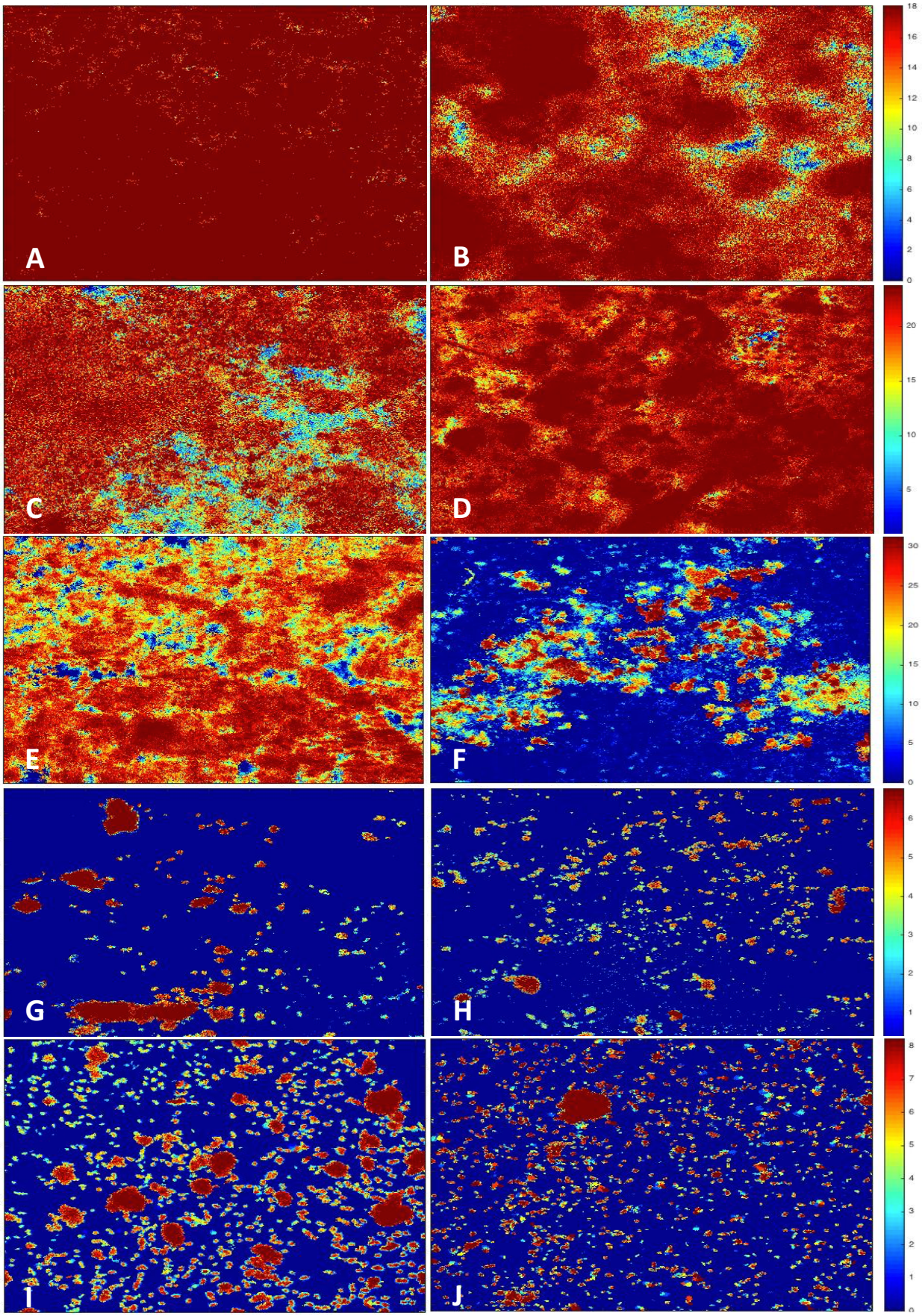


Figure 4.9: Colour intensity (thematic) maps for the biofilms of the confocal data of the different samples
The images in this plate represent the topographical structure of the biofilms of the various samples where **A-B** = glucose only; **C-D** = glucose and methylparaben; **E-F** = glucose and carbamazepine**; G-H** = methylparaben only; **I-J** = carbamazepine only. The colour variation is indicative of the thickness of the biofilm. Colour intensity keys are provided on the right, and are measured in µm.

4.2) Influence of Flow Cell Reactor Design on the Assimilation of Carbon Sources and Adhered Biofilms

4.2.1) Spatial Gradient of Carbon Sources

The concentrations of the carbon sources were measured with distance through the reactor. Figure 4.10 shows the depletion of glucose throughout the reactor (measured at the different collection points of the reactor). The concentrations of the secondary carbon sources (methylparaben and carbamazepine) were evaluated using LCMS. The standards and blanks of the LCMS data were not accurate, often returning values much higher than what was added, and the blank samples (with only pure methanol) would return with high amounts of micropollutant. Therefore, only the differences between influent and effluent are shown (relative concentration change). Table 4.2 shows the standard error of the influent and effluent concentrations and removal efficiencies of both methylparaben and carbamazepine-containing samples in the flow cell reactor.

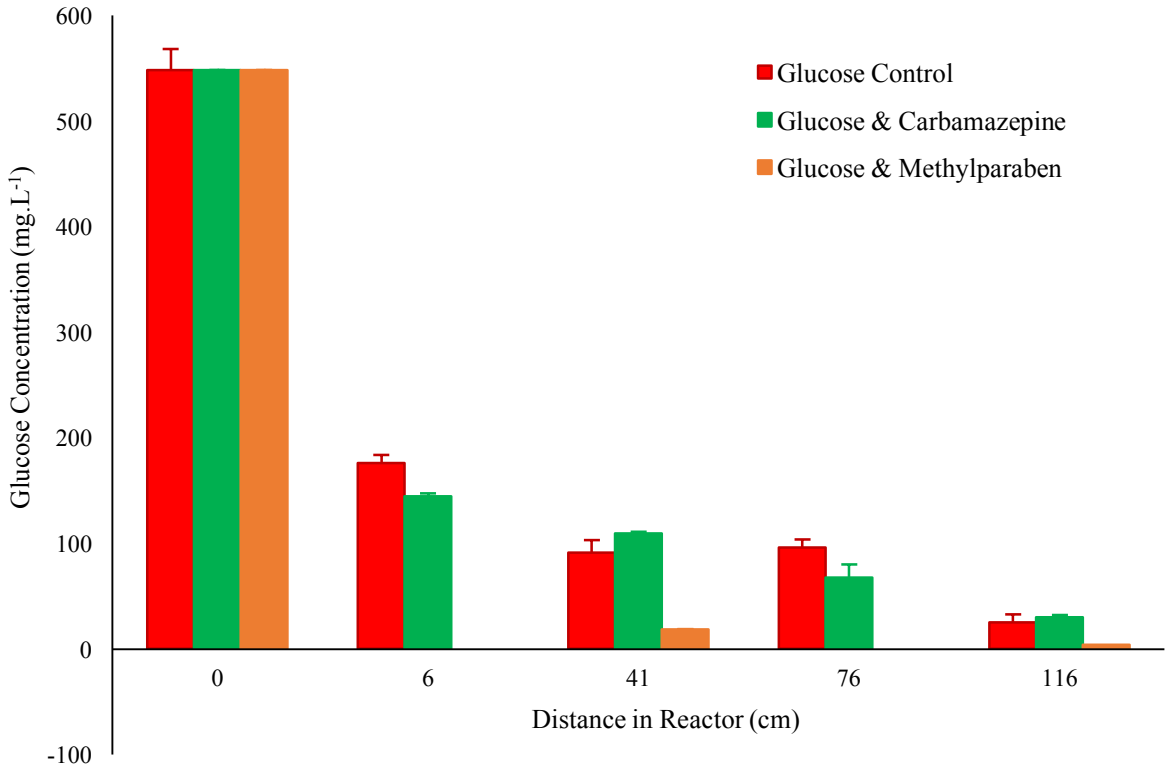


Figure 4.10: Spatial reduction of glucose throughout the reactor

The graph represents the reduction of glucose concentration for the samples that contained glucose (GLC, GMP and GCBZ). The error bars represent the standard errors of the mean (for each data point of which there were 3 or 4 replicates, depending on the usable data).

Table 4.2: Mass-spectrometry data (and statistical analysis) of secondary carbon sources

Sample		GMP	MP	GCBZ	CBZ
Compound		Methylparaben	Methylparaben	Carbamazepine	Carbamazepine
Removal Efficiency	Mean (%)	59.89	59.32	10.37	-2.58
Influent ($ng.L^{-1}$)	Std Error	1.38	4.6	0.83	0.83
Effluent ($ng.L^{-1}$)	Std Error	1.14	2.25	0.15	2.03

4.2.2) Endocrine-Disrupting Potential of Effluent from the Reactor

The results of the yeast screen tests are shown in Table 4.3. The samples contained levels of micropollutants that are representative of what is present in the environment (in WWTPs and effluent from WWTPs). The data is displayed in a qualitative manner, where a visible colour change are represented by a "+"and no colour change by a "-". The names of the control samples are accompanied by an asterisk (*).

Table 4.3: Qualitative results for yeast sssays of concentrated reactor samples

Sample Name		Concentration			Sample Name		Concentration		
		10X	5X	2.5X			10X	5X	2.5X
Glucose & MP #1	Rep 1	-	-	-	Glucose & CBZ #1	Rep 1	-	-	-
	Rep 2	-	-	-		Rep 2	-	-	-
Glucose & MP #2	Rep 1	-	-	-	Glucose & CBZ #2	Rep 1	-	-	-
	Rep 2	-	-	-		Rep 2	-	-	-
MP #1	Rep 1	-	-	-	CBZ #1	Rep 1	-	-	-
	Rep 2	-	-	-		Rep 2	-	-	-
MP #2	Rep 1	-	-	-	CBZ #2	Rep 1	-	-	-
	Rep 2	-	-	-		Rep 2	-	-	-
Glucose & MP Medium	Rep 1	-	-	-	Glucose & CBZ Medium	Rep 1	-	-	-
	Rep 2	-	-	-		Rep 2	-	-	-
MP Medium	Rep 1	-	-	-	CBZ Medium	Rep 1	-	-	-
	Rep 2	-	-	-		Rep 2	-	-	-
MP 100 $g.L^{-1}$ *	Rep 1	-	-	+	CBZ $g.L^{-1}$ *	Rep 1	-	-	-
	Rep 2	-	-	+		Rep 2	-	-	-
MP 100 $ug.L^{-1}$ *	Rep 1	-	-	-	CBZ 100 $ug.L^{-1}$ *	Rep 1	-	-	-
	Rep 2	-	-	-		Rep 2	-	-	-
MP 10 $ug.L^{-1}$ *	Rep 1	-	-	-	CBZ 10 $ug.L^{-1}$ *	Rep 1	-	-	-
	Rep 2	-	-	-		Rep 2	-	-	-

4.2.3) Planktonic Cell Release and the Presence of Carbon Sources

Effluent cell numbers were obtained every 24 hours for a 4 day incubation period (Figure 4.11). Figure 4.12 shows the planktonic cell numbers obtained from each of the 4 collection points of the reactor during the steady-state of the biofilms. This state was characterised by maximum and consistent planktonic cell numbers released from the biofilm into the bulk flow.

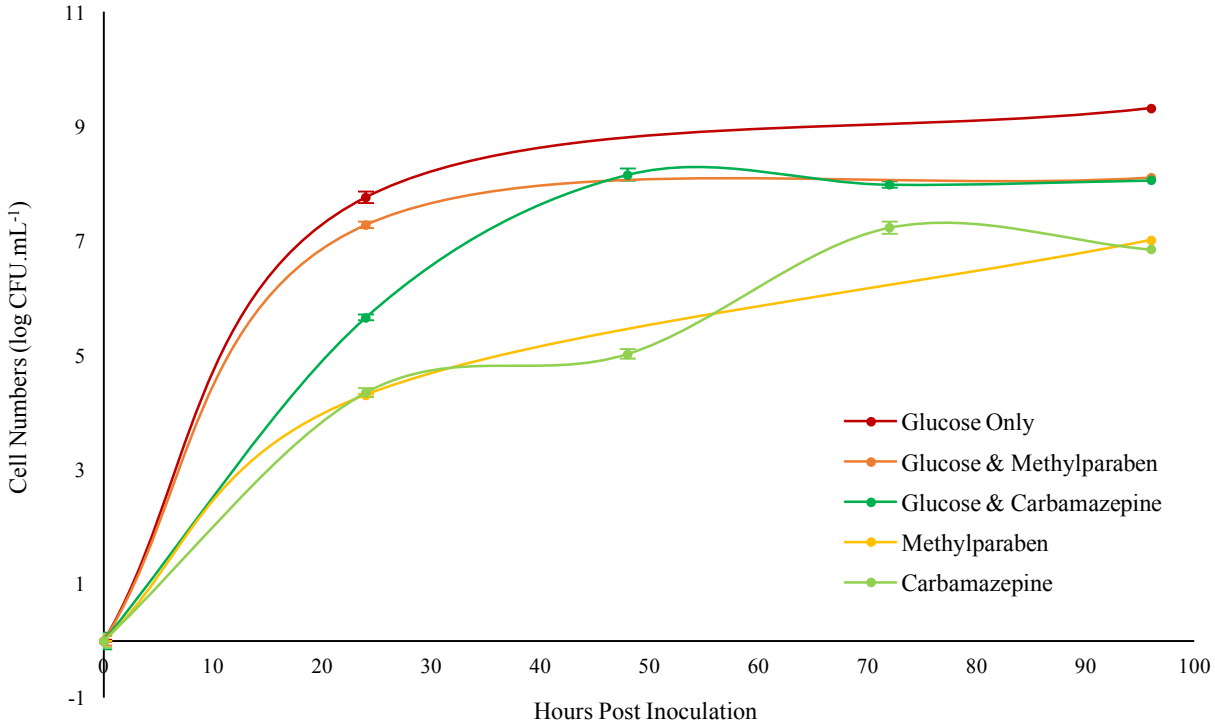


Figure 4.11: Planktonic cell numbers from the flow cell reactor for the duration of the incubation period

The graph shows the logarithm of culturable cell concentrations over a 4-day incubation period. The standard errors were calculated using the standard deviation of the 4 replicates for the logarithm of the cell concentration.

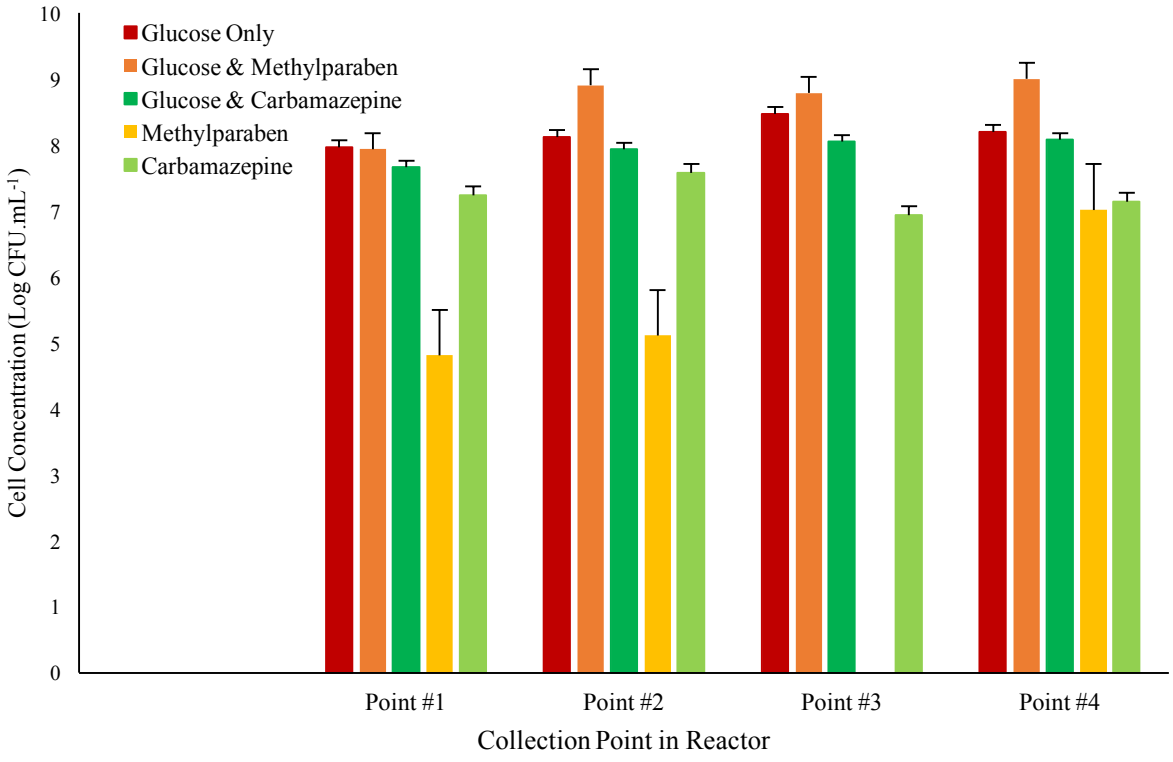


Figure 4.12: Planktonic cell numbers from the collection points within the reactor

The graph shows the logarithm of culturable cell concentrations over the 4-day incubation period. The standard errors were calculated using the standard deviation of the 4 replicates for the logarithm of the cell concentration.

Chapter 5: Discussion

Water is one of the most important, if not the most important, resources supporting life on Earth. Human behaviour over the last few centuries has polluted and sullied this key resource. Water is regarded as a renewable resource, provided we can remove these pollutants to detoxify the water. However, considering the degree to which it has been polluted, the renewal or recycling thereof may not be as simple as originally thought. The remediation of water is going to be an essential key to our continued survival on this planet. The natural environment has always evolved ingenious ways of maintaining homeostasis after disturbances to an equilibrium and, now that our water resource is reaching a critical state (in terms of both quantity and quality), it is pertinent that humans look to nature for solutions to a problem we have not yet adequately solved.

This research was conducted against this background; firstly, to consider what the potential effect of polluting compounds are on higher organisms, and secondly, the interaction between these compounds and microorganisms, the latter of which, through their metabolic processes, ultimately are of major importance in the removal of these compounds from our water sources. Two prominent micropollutants, namely methylparaben and carbamazepine, were selected and their potential effect on human endocrine functionality was investigated. The estrogenic effect of methylparaben on the human estrogen receptor was evaluated, which has been shown in the literature(Lemini et al., 2003, Błędzka et al., 2014, Wróbel and Gregoraszczuk, 2014) and that of carbamazepine for its inhibitory effects on the human androgen receptor, which were seen in men undergoing anti-epileptic therapy (Reis et al., 2013). Therefore, there was evidence of endocrine disrupting potential of these compounds, but the intention of the experimentation in this research was to not only confirm these effects, but to ascertain the effects of these two compounds on microbes, both in batch culture (planktonic cells) and in biofilms (adhered/ sessile cells).

This project was part of an initiative to further the concept of Biomimicry in South Africa. While the biomimicry-inspired Eco-Machine™ at Langrug was not installed in time for analysis in this research, the focus was nevertheless on a key component of Eco-Machines, namely microbial metabolism and this potential in biomimetic constructs for wastewater treatment. The data is thus presented and considered within the framework of Biomimicry.

5.1) The Effect of Micropollutants on Microbial Cultures

Methylparaben was the first compound investigated in this study. Its inhibitory effects on microbial cultures were established through both MIC and recombinant yeast assays. The MIC assay tested the effects of a concentration range of methylparaben (74.5 ng.L-^{1} to 10 g.L-^{1}) on the growth potential of both a pure bacterial culture (CT07) and a mixed microbial culture. The estimated MIC for the mixed culture (256.27 g.L^{-1}) was much higher than that for CT07 (10.6 g.L^{-1}). The curve for the mixed culture MIC (Figure 4.1) shows fluctuations of the optical density around the maximum optical density, while the curve for CT07 showed a more consistent line. The reason for the fluctuations was most likely due to the presence of multiple

microbial species in the mixed culture. The varying concentrations of methylparaben potentially selected for the organisms that could survive and grow in the presence of methylparaben at the particular concentration in the well. The MIC curve for the mixed culture did not show a decrease like that which was seen for CT07. This was very likely due to the source of the culture: a WWTP through which methylparaben was degraded. The large number of different microbial species present ensured at least one or two of those organisms capable of tolerating methylparaben survived in the wells at which high concentrations of methylparaben were present, resulting in some degree of growth (and therefore a lower than maximum, but measurable, optical density). An improvement to the experimental method would have been to repeat these assays with a higher maximum methylparaben concentration in order to accurately determine the true MIC, instead of mathematically estimating the MIC (since a concentration of 256 $g.L^{-1}$ should not be possible to obtain with methylparaben in an aqueous solution). The remarkable difference in these MIC values between the two cultures shows the large degree of resilience that mixed microbial cultures have against antimicrobial preservative compounds such as methylparaben.

There were notable differences in the optical density reached by the two cultures in the 20-hour incubation period: 1.06 for CT07 and 0.42 for the mixed microbial culture. These differences in optical density can be explained by the presence of different microbial species in the mixed culture, compared to only one species in the CT07 culture. The generation time for bacterial species is generally much shorter than that of yeasts and other fungi. The presence of yeasts and fungi (confirmed presence in the culture through viewing under the microscope), as well as other microbes with varying generation times within the mixed microbial culture, probably increased the time taken for the culture to reach stationary phase, meaning the optical density would have taken a greater amount of time to reach the same optical density that CT07 culture did overnight.

The effect of methylparaben on recombinant yeast was also determined using a yeast estrogen assay. The purpose of this assay was two-fold: it determined the lethal dose of methylparaben for yeast, as well as to determine the degree of estrogenicity of methylparaben on the human estrogen receptors that were expressed by the yeast recombinant. Methylparaben showed no estrogenicity up to 19.5 $mg.L^{-1}$, after which there was an exponential increase in estrogenicity until 316 $mg.L^{-1}$ after which there was a rapid decrease in measured response. This decrease was indicative of a cytotoxic effect and the cytotoxic effects occurred before the complete saturation of estrogen receptor binding by methylparaben (maximum estrogenicity). In other words, the yeast cells were killed by the high doses of methylparaben but that the agonistic effects on the estrogen receptor would have continued to increase if the cytotoxic effects did not exist, such as would occur in mammalian (especially human) cells, to which the estrogen receptor is native. The data means that methylparaben is estrogenic, albeit to a low degree (>19.5 $mg.L^{-1}$), and that it is also cytotoxic to yeast. This is congruent with the literature review, where it was explained that methylparaben is used as an antimicrobial preservative in products, especially against fungi (yeasts) (Błędzka et al., 2014) and also that estrogen receptor agonism has been recorded in cancer cell lines that express estrogen receptors (Wróbel and Gregoraszczuk, 2014). The low degree of cytotoxicity of methylparaben against the yeast cells means that methylparaben must be included at concentrations equal to, or higher than, the lethal concentration, in order

to function as an adequate antimicrobial preservative in personal-care products. This is congruent with the literature review, were the concentration of methylparaben in most cosmetic (personal-care) products is around 0.3 – 0.4% (Soni et al., 2002, SCCP, 2008)(or 3 – 4 $g.L^{-1}$). Based on the data, this concentration is sufficient to induce estrogen receptor agonism in humans. Therefore, the concentrations at which methylparaben is included in products could also induce endocrine disrupting effects in humans, especially in products that are applied directly to the skin (and not washed off) and products that are inhaled or ingested. In the study conducted in 2014 by Wróbel and Gregoraszczuk, concentrations of methylparaben above 3.04 $\mu g.L^{-1}$ (20 nM) could induce agonism of estrogen receptors in breast cancer cell lines (more sensitive to estrogen due to increased receptors in these cells). Relevant products contain upwards of 4 $g.L^{-1}$ (26.3 mM) methylparaben and tissue concentration of methylparaben need only reach 3.04 $\mu g.L^{-1}$ (20 nM) for estrogenic effects to occur in breast cells. Therefore, it is possible that products like antiperspirants and body lotions, applied in close proximity to breast tissue, can be absorbed into breast tissue. This could possibly promote, or perhaps even initiate, estrogen-induced breast cancer especially in long term use of methylparaben (and other paraben) -containing products. The relevance of this data to water treatment, lies in the degree to which parabens are used (and released into wastewater) and the degree of removal that occurs in the WWTP. According to the data in the literature review, methylparaben is removed at an efficacy of greater than 90% (Kasprzyk-Hordern et al., 2009, Archer et al., 2017) and while that seems adequate, the potential for accumulation of parabens exists. The residual parabens will be present in recycled water, and the continuous use of paraben-containing products, especially wash-off products, may result in a slow accumulation concentrations of parabens in surface water (into which WWTP effluent is dispensed).

Carbamazepine, the second compound that was investigated in this study, was also evaluated for its potential effects on microbial cultures. The data in Figure 4.2 show the effects of increasing carbamazepine concentrations on the growth (optical density) of both CT07 and mixed microbial cultures. No MIC for carbamazepine could be determined as it exhibited no inhibitory effects on either of the cultures. In addition, it appeared (from the data in Figure 4.2) that the optical density increases at the highest tested concentrations of carbamazepine, in both cultures. This data implies that the increased concentration of carbamazepine enhanced growth of the microbes. The other, more likely explanation is that the increased concentrations of carbamazepine resulted in supersaturation of the solution, resulting in excess carbamazepine precipitating out of the solution. This could have proportionally increased the optical density with increased carbamazepine concentrations, which resulted in the apparent proportional increase in optical density that was seen in Figure 4.2. The latter explanation is more likely, based on the low solubility of carbamazepine in water (O'Neil, 2001), approximately 17.7 $mg.L^{-1}$ at 25^{o}C (DrugBank, 2005). This implies that the apparent lack of growth inhibition seen in the data could have actually been the result of a low, consistent concentration of carbamazepine in all the concentrations where more carbamazepine was added than was able to dissolve (saturation of the solution). This implies that carbamazepine, if it is toxic to microbes at higher concentrations, would not be able to adequately dissolve in an aqueous solution to elicit any inhibitory effects, and therefore the MIC of carbamazepine cannot be determined in a water-based solution. It is also possible that carbamazepine has no inhibitory effects on microbes, based on its structure and function:

carbamazepine is a neurotropic pharmaceutical compound that mimics neurotransmitters found in mammalian systems. Therefore, it is possible that carbamazepine has no inhibitory effect because microbes have no nervous system and therefore a molecular target may not actually exist in single-celled organisms. An MIC could perhaps be established for microbes that are able to grow in an oil-based solution where carbamazepine, a highly lipophilic compound, can dissolve with a higher maximum solubility. The same fluctuations in optical density were seen in the mixed culture with carbamazepine as was seen with methylparaben. The explanation for these findings is the same for carbamazepine as it was for methylparaben. This test was not useless, however, since the concentrations that carbamazepine is found in water would be below this solubility level. However, the presence of solvents, soaps and other dispersing agents (emulsifiers) in wastewater may increase its solubility in water.

The endocrine-disrupting effects of carbamazepine were also investigated. There have been findings that showed potential anti-androgenic effects in male humans (Reis et al., 2013). Despite these findings, the assay was conducted in this research in order to confirm these effects, and the concentration range at which these effects begin to occur and whether the effects occur at levels lower than the therapeutic dose. The latter is especially significant when referring to levels that occur in water being dispensed into environmental surface water by the WWTPs. The data from these anti-androgenic assays (Figure 4.4) showed that carbamazepine did exhibit inhibition of the binding of DHT to the androgen receptor. The anti-androgenic effects occurred at carbamazepine concentrations upwards of 776 $\mu g.L^{-1}$. Therefore, while carbamazepine has a low potency, it still has the potential to interact with the binding of androgens to the androgen receptor, thereby interfering with normal hormonal processes. Carbamazepine, sold under various proprietary names, is usually administered to patients at daily doses of 100 – 200 mg (DrugBank, 2005), depending on the manufacturer. It has been stated previously that 28% of the total plasma concentration is not metabolised by the body and is excreted as the active parent compound. This 28% then ends up in the WWTP which removes this compound with low efficacy. The degree of removal ranges from 20% to low negative values (Kasprzyk-Hordern et al., 2009, Archer et al., 2017), which implies that carbamazepine concentrations undergo an apparent increase as the compound travels through the WWTP, possibly through re-assembling of metabolites by microbial activity. Carbamazepine is mainly used for epilepsy, therefore the use of this drug cannot be discontinued, as epilepsy is a serious medical condition. As a consequence, the use of carbamazepine will be prevalent in the years to come. Considering the low degree of removal (and increase in concentration in some cases) in WWTPs, it has the potential to persist in the meagre 1% of the total surface water available for use, with no currently available effective means with which to remove it. If this is considered, along with the large degree of interactions carbamazepine has with other drugs (discussed in detail in the literature review), the potential for non-target effects on both animals and humans exists. This may not be a current concern, since the levels at which carbamazepine is present in WWTP effluent is still only at the nanogram per litre level, but if accumulation of the compound is a possibility then the levels may rise, over time, to be conducive for pharmacological effects in non-target individuals. The effects of carbamazepine on normal hormonal processes in the body, such as the anti-androgenic effects already mentioned, could also be a potential risk resulting from accumulation of the compound in water systems.

The reactor samples, concentrated during solid-phase extraction for LCMS analysis, that were subject to recombinant yeast screens did not show any qualitative effects on the recombinant yeast (Table 4.2). This was not surprising, considering that endocrine-disrupting effects occur at concentrations above 19.5 $mg.L^{-1}$ for methylparaben and 776 $ug.L^{-1}$ for carbamazepine, and the concentration of these compounds in the samples were lower than this threshold. The assay was conducted in order to confirm the lack of endocrine-disrupting effects in environmental samples. This implies that environmental concentrations of methylparaben and carbamazepine should not elicit any endocrine-disrupting effects, but some mammalian cells may be more sensitive to these effects than the recombinant yeast (such as was seen in the research conducted by Wróbel and Gregoraszczuk in 2014). Lack of colour change in these samples does not mean they are definitely safe, as there may be other physiological factors, involved in the endocrine-disrupting potential of these compounds, that may not have been present in yeast cells. However, the results do show that there is no direct effect on the estrogen and androgen receptors in the recombinant yeasts.

All the deleterious effects of both of these compounds on mammalian systems, coupled with their inadequate removal by conventional WWTPs, highlights the significance of finding more effective strategies to be implemented in wastewater treatment.

5.2) The Effect of Micropollutants on Microbial Biofilms

Although the MIC experiments showed minimal effects on the microbial cultures (in the case of carbamazepine), or effects at high concentrations only (in the case of methylparaben), the effects of these compounds on microbial biofilms were investigated. This was conducted in order to determine if environmental levels of these two compounds have any effect on the structure of the biofilms to which they are exposed. This was achieved by the use of the flow cell reactor. The extended length of the reactor allowed increased temporal exposure of either one or two different energy sources (carbon sources) (either the micropollutant and/or glucose) to the biofilm. The motivation behind the extended length was that the biofilms upstream would use up the primary carbon source (glucose) first, leaving only the secondary carbon source (micropollutant) behind and that the biofilms would use the micropollutant due to lack of another suitable energy source. The increased length also increased the time to which the micropollutants were exposed to the biofilms, allowing increased opportunity to metabolise the micropollutants, if any catabolic potential existed in the biofilm. The inclusion of glucose was to determine if catabolite repression, or a mechanism similar in function, would have had an inhibitory effect on metabolism of the micropollutant added together with glucose. The premise was that the microbes would metabolise glucose first, due to the high energy (ATP) output compared to the energy required to express the enzymes required for catabolism (higher net ATP yields). The pathways that the microbes would require to metabolise the micropollutant were assumed to be lower in net ATP yields than the metabolism of glucose, therefore presence of glucose would result in a decrease in metabolism of the micropollutant compared to the degree of metabolism of the

micropollutant alone. This hypothesis, and the hypothesis that environmental levels of micropollutants would be sufficient to alter the structure of biofilms, were both tested using the reactor.

The differences in biofilm structure were evaluated using a COMSTAT script in MATLAB®, where image stacks obtained with CLSM were converted into qualitative data. The data is shown in Table 4.1. The concentration of methylparaben included in the medium (1 $\mu g.L^{-1}$) was found to be sufficient to cause measurable changes in various structural aspects of the biofilm. Where methylparaben was included with glucose in the medium, the biofilms showed statistically significant increases in roughness coefficient and surface-area-to-biovolume ratio and a statistically significant decrease in percentage of substratum coverage, i.e. biofilm footprint area (Figure 4.7). The decreased substratum coverage can also be seen in the comparison of Figure 4.9A and 4.9C. The thicknesses of the biofilm (both average and maximum) were increased with the addition of methylparaben into the medium, although the differences were not large enough to be considered statistically significant (Figure 4.7 and Table 4.1) with a P value of 0.05. The increased thickness could have been due to the presence of an additional source of energy and carbon, and that more cells were present as a result. The depletion of methylparaben in the reactor does suggest that the cells were capable of metabolising it, and perhaps the microbes utilised an inherent esterase to utilise the oxygen and the methyl group (Martinez-Martinez et al., 2014). This significant depletion of methylparaben (59.89% with glucose and 59.32% without; Table 4.2) implies that the partial metabolism of methylparaben allowed for an increased source of carbon and energy that would explain the increased thickness of the GMP biofilms compared to glucose alone. However, this explanation was not consistent with the planktonic cell numbers that were obtained from the GMP samples in the reactor (Figure 4.11), since the numbers were lower than that of glucose only, and the degree of glucose depletion shown in Figure 4.10 was almost the same with GMP, GCBZ and glucose only. Therefore, the logical explanation would be that the amount of biomass was increased, but that it was due to more deposits of EPS and not an increase in cell numbers. The cause is not known, but it is possible that the presence of methylparaben promotes increased expression of EPS components, in an attempt to increase protection of the cells. Methylparaben was suggested to act on cell osmoregulation (Nguyen et al., 2014) and therefore an increase in the production of EPS may have served to protect the cells from these osmoregulatory effects. The cause of the decreased substratum coverage percentage is not certain, but it could possibly have been caused by the adsorption of methylparaben to the glass surface, which may have reduced the amount of surface area available for interaction with pili for surface attachment. Adsorption results in the binding of molecules to a surface (Brown et al., 2006), which creates a film over the aforementioned surface. Methylparaben is slightly polar and lipophilic, thus the adsorption of the paraben molecules to the glass during bacterial adhesion may have caused decreased surface coverage by bacterial cells due to the reduction of physical space. Another explanation may be that an increased need for interaction between species to metabolise the more complex molecule was required, which could have caused increased cell clustering and formation of more distinct micro-colonies with EPS-filled spaces between them. The biofilms were thicker downstream than upstream in the GMP and glucose only. The reason for this is not known as, logically, the depletion of nutrients in the reactor should have resulted in less potential for growth downstream, and a thinner biofilm as a result. It is

possible that the planktonic cells, released from the upstream parts of the biofilm, become stressed at the low levels of nutrients in the downstream parts of the biofilm and revert to a sessile stage and adhere to the biofilm, also with the development of protrusions into the bulk liquid phase to increase the surface area for nutrient capture. This would cause an increase in biofilm thickness without a drop in total planktonic cells released. Figure 4.12 shows that planktonic cell numbers from the second, third and fourth collection points were not increased, since the cell numbers between the three points were more or less the same. It would be expected that the cell numbers would increase as the bulk flow travels further down the reactor, since the amount of cells released should be cumulative. However, there was no significant drop or increase in cell numbers in the biofilms between the three last collection points. There was a small decrease between the cells numbers obtained from point #1 and point #2 (Figure 4.12), and this could be where cells reverted to the sessile stage and joined the biofilm. The lack of significant increase or decrease in cell numbers in the biofilm implies a net zero effect between planktonic cell release (detachment) and reversion of sessility (reattachment). It is likely the cells were released in similar numbers to those that were added back into the biofilm in the more downstream parts of the reactor, causing an apparent lack of the expected increase in release of planktonic cells. This shows the remarkable responsiveness of biofilm communities to their physical environment (the nutrients contained within the bulk flow, in this case), where cells are constantly released into the environment in order to ensure survival and/or proliferation of the biofilm (Bester et al., 2009).

The samples in which both glucose and carbamazepine were present (GCBZ), compared to glucose alone (GLC), also showed statistically significant differences in various structural parameters of the biofilm. These differences were more pronounced than in the GMP samples. The average thickness of GCBZ biofilms was higher than glucose alone only in the upstream flow cells (Figure 4.7), while the GCBZ biofilms were thinner than glucose alone in the downstream flow cell. This decrease in thickness was statistically significant (Table 4.1). However, the maximum thicknesses of both upstream and downstream GCBZ biofilms were significantly thicker than the upstream and downstream biofilms for glucose only (GLC). The downstream flow cell biofilms that were exposed to both glucose and carbamazepine were thinner, on average, than the downstream flow cell with glucose only but the maximum thickness was larger than glucose only. This implies that the biofilms with both glucose and carbamazepine were more varied in surface topography, as in the surface was more convoluted, but that the overall thickness was lower than in the GLC samples. The roughness coefficient of the GCBZ downstream biofilm was consistent with this statement, as it was larger in the GCBZ downstream samples, and the larger the roughness coefficient the greater the variation in height of the surface of the biofilm (Heydorn et al., 2000). The amount of biomass also reflects this, as it is significantly lower than the biofilm exposed to glucose alone (Figure 4.7). The substratum (surface of the glass) coverage for the GCBZ samples was lower in both upstream and downstream flow cells compared to GLC and GMP samples. This is seen most significantly in Figure 4.9F. The cause of this was most likely due to the adsorption of carbamazepine to the glass, as carbamazepine is very lipophilic, and has a low solubility in water (O'Neil, 2001, DrugBank, 2005) more lipophilic than methylparaben, which may have resulted in the larger degree of adsorption of the molecules to the glass surface. Adsorption may have created

a film that prevented attachment of the microbial cells to a larger percentage of the glass flow cell surface than with GMP or GLC samples. Another explanation is that the increased need for cometabolism caused clustering of cells, reducing the surface area covered. The reason for the lower biomass and thickness of GCBZ downstream compared to upstream is likely due to the depletion of glucose through the reactor, which resulted in less potential for cell growth and EPS production in the downstream portion of the reactor. This was also suggested for the GLC and GMP samples, but the lack of increased average biofilm thickness implies that the cells did not reattach to the biofilm, as was suggested in the former two sample types. The lack of increased biomass (Figure 4.7), the significantly increased maximum thickness (Figure 4.7) and the lack of increased cell counts (Figure 4.12) would suggest that the cells reattached to the biofilm but that the reattachment occurred in only specific, limited parts of the biofilm, such as in discrete micro-colonies. This would explain the significant increased variation in the surface topography of the biofilm, but the lack of increased biomass or cell counts. The cause for limited spatial adhesion in the biofilm could very likely have been due adsorption of carbamazepine onto the biofilm and the adsorption onto the glass surface at the attachment stage of incubation, mentioned earlier. Adsorption of carbamazepine has been reported to occur to a degree, although not extensively, in agricultural soils (Calisto and Esteves, 2012), and therefore it is possible that adsorption was responsible for the findings described above. The other explanation is that the cells aggregated into these longer, protruding cell clusters in order to aid increased capture of nutrients from the bulk flow (essentially increasing the surface area exposed to the bulk flow) in response to the depletion of nutrients. This is consistent with the increase in roughness coefficient seen with the GCBZ downstream compared to upstream.

The carbamazepine was not used as a carbon or energy source, based on the LCMS data where carbamazepine levels actually increased while the bulk flow travelled through the reactor (effluent levels are higher than influent levels). The influent levels for both GBZ and CBZ samples were the same, but the effluent carbamazepine levels were increased in both samples of effluent: -10.37% in GCBZ and -2.58% in CBZ. This is consistent with what was stated in the literature review, where carbamazepine increases in concentration during water treatment in some cases (Kasprzyk-Hordern et al. 2009), although it can be expected that the breakdown products that contribute to reconstitution in wastewater were absent in this study. However, it is possible that carbamazepine molecules within the medium flasks broke down, through an unknown mechanism, during the incubation period which would cause a lower than expected concentration in the influent. This would, in turn, cause an apparent increase in carbamazepine in the effluent if these metabolites were to be reconstituted back into carbamazepine within the reactor. The removal efficiencies of the two micropollutants were not significantly influenced by the presence of glucose (Table 4.2) but the presence of glucose seems to have increased the degree to which carbamazepine accumulated in the reactor (degree of increased carbamazepine was larger in GCBZ than in CBZ). The statistical analysis of the mass spectrometry results (Table 4.2) suggests that the results are reliable and have low errors, but there were not enough replicates. Four samples were taken for the influent and effluent of each micropollutant (both with and without glucose) but the mass spectrometer used was very sensitive (maximum of 0.9 $g.L^{-1}$) and the machine was communal. It was possible that previous users had not regarded

this maximum concentration limit because some of the readings showed large amounts of micropollutants in the blanks (HPLC-grade methanol only), which only occurs when carry-over from one sample to the next takes place during the sample reading in the mass-spectrometer. There were also compounds present in the samples that were not added. Since reverse osmosis water was used, the presence of these contaminating compounds was likely from carry-over in the machine, or from residual compounds in the laboratory glassware. The presence of micropollutants of interest (methylparaben and carbamazepine) in the blanks reduced the reliability of the quantitative influent and effluent concentration data, and this was why only the relative change in concentration was reported. Since the influent and effluent concentrations were measured using the same analysis, they could still be used to calculate the relative change in concentration, but not to report actual concentration in the medium. Due to the difficulty encountered with standardising the LCMS data, only two samples per GMP, MP, GCBZ and CBZ were presented in this research. This is not a sufficient number of replicates to ensure that the results were precise, therefore an improvement would have been to increase the number of samples and submissions. This was not possible in the scope of this research, because of budget constraints. Therefore, a standard protocol and mass spectrometry methods should be established so that any and all possible experimental errors could be removed to avoid wasting time and money on repeated LCMS sample extractions and submissions. There was not sufficient time to do this within the scope of this research.

The samples in which methylparaben (MP) or carbamazepine (CBZ) was added, showed significant differences in the topography and aggregation of the biofilms. The maximum thicknesses of the biofilms in the MP samples was significantly larger than those in the CBZ samples, in the upstream flow cells, and larger to a lesser degree in the downstream flow cells. However, these differences were both statistically significant in the ANOVA/T-Test calculations with a P value of 0.05. The reason why the methylparaben-exposed biofilms were significantly thicker than carbamazepine-exposed biofilms was likely due to partial catabolism of the compound via the inherent esterases, mentioned earlier, which would result in the release of methyl alcohol (methanol). The result of the hydrolysis may have yielded energy for increased metabolic activity, either growth of cells or increased EPS production. The other possibility is that the methanol released during esterification could have been a source of carbon and energy. Although methanol is toxic (Skrzydlewska, 2003), it can be assimilated by bacteria, such as Alpha Protetobacteria (Radajewski et al., 2000; Madsen, 2008), which may have been present in the biofilm. The LCMS data showed that methylparaben was degraded (metabolised) to an extent (about 60%, Table. 4.2) and if it was assimilated, then this would explain why the thickness was increased in the MP samples than it was in the CBZ samples. The roughness coefficient was larger overall in the CBZ samples than the MP samples.

Based on the findings of this research it is evident that micropollutants do affect the structure of the biofilms to which they are exposed, thereby achieving the first aim of this research. The hypothesis that small concentrations of micropollutants (1000 $ng.L^{-1}$ for methylparaben and 600 $ng.L^{-1}$ for carbamazepine) would have an effect on biofilm structure was therefore not rejected. It is still unsettling, however, that concentrations at the microgram and nanogram per litre level can affect bacterial biofilms to a measurable

degree. These findings bring to the fore that micropollutants, even at infinitesimal concentrations, may have a more pronounced effect on bacterial biofilms than would have been thought possible.

5.3) Potential of Biomimetic Concepts for Adequate Micropollutant Removal

Biomimicry is not a new concept, but it is being more widely accepted as a means to use inspiration from nature to solve anthropogenic problems. Based on the incomplete removal of micropollutants by conventional WWTPs already discussed in this research, another, more effective, method is required if micropollutants are to be removed with a larger degree of efficacy. It is possible that biomimicry may provide the means with which to acquire the solution to incomplete micropollutant removal.

The potential of biomimetic designs in the treatment of wastewater, and greater removal efficiency of micropollutants, may lie in the nature of microorganisms themselves. While biomimetic constructs, such as Eco-Machines™ and constructed wetlands, mimic a natural system, they make use of existing organisms and their metabolic capabilities. It has been argued that the use of microbes to solve wastewater problems is not biomimicry, but is rather classified as bio-assistance and bio-utilisation. These definitions essentially prevent the use of these organisms in novel systems, if the status of "biomimicry" is to be achieved. Therefore, the two processes of bio-assistance and bio-utilisation should be considered part of the biomimetic design process, allowing the consideration of microbes as agents to biomimetic designs.

Microbial biofilms, from the findings in the previous sections, follow many of the guidelines included in the "lifes' principles" paradigm (Figure 2.5), used in the design of biomimetic constructs, further contributing to the merit of using microbes in biomimetic designs. It was suggested that planktonic cells, released by the more upstream part of the biofilm, reattach to the downstream biofilm in response to the drop in nutrient concentration. Biofilms (a sessile living state) offer a situation more conducive to low nutrient density, since the cells can revert to cometabolism (living with other microbes) or even enter a state of dormancy until an environmental change occurs that is more suitable for growth (increased nutrient density). This adaptation to changing conditions, through environmental cues, is one of the key aspects of "lifes' principles". The planktonic cells "integrate the unexpected" and "adapt to changing conditions" (Figure 2.5), by reverting to a sessile living state when nutrients are lacking. The biofilm creates an environment where microbes can utilise resources efficiently ("be resource efficient", Figure 2.5), through cometabolism or through dormancy. The change in biofilm thickness in the presence of micropollutants, especially with methylparaben, would suggest that the microbes within the biofilm use the micropollutants to build up biomass, whether they use the compound (or its metabolite) as a building block or they produce more EPS in its' presence. Either way, the presence of micropollutants, even at low concentrations, can trigger responses in the biofilm. Metabolism of methylparaben (seen in the research) is an example of how microbes can adapt to metabolising compounds that may not be natural (synthetic) or that the biofilm would not ordinarily be exposed to. This shows how biofilms can elegantly execute "lifes' principles" to "use readily available materials and energy" (Figure 2.5). The change in overall structure of the biofilm in the presence

of micropollutants, such as the formation of discrete microcolonies and significant changes in the topography (roughness coefficient and surface area to biovolume changes), also shows how microbial biofilms can change structure in response to their immediate environment. This complies with the principles of self-organisation and the incorporation of diversity (Figure 2.5) in order to optimally use the resources that are available, for survival and proliferation.

The above examples, linking the principles of biomimetic design to biofilms, illustrate just how much microbial biofilms adhere to "lifes' principles" (which they would, since they are directly from nature) and therefore would be prime candidates to explore in solving wastewater treatment challenges. Perhaps harnessing the abilities of microbes, taken from nature, and manipulating these abilities to solve wastewater treatment challenges is not entirely biomimetic (according to anthropogenically-assigned definitions). However, the fact that microbial biofilms closely follow the principles of biomimetic design shows how valuable they can be in wastewater treatment, especially considering their functions cannot yet be emulated by humans.

Other biomimetic constructs, such as constructed wetlands, have been shown to remove certain recalcitrant compounds, such as carbamazepine (Hijosa-Valsero et al., 2010a), with greater efficiency than conventional WWTPs, further emphasising the potential of biomimetic design for improved wastewater treatment and micropollutant removal. Based on the findings from the literature, coupled with the ability of microbial biofilms to adhere to the principles of biomimetic design and the fact that other biomimetic constructs (which make use of biofilms) have been shown to exhibit improved micropollutant removal shows that biomimicry is indeed a viable option in improved removal of micropollutants from wastewater. The fact that biomimetic designs make use of existing ideas in nature, also makes biomimetic design a more sustainable and potentially more eco-friendly option in the search for more efficient solutions to wastewater treatment.

Chapter 6: Conclusions

The remediation and reclamation of water has become a critically important issue. The use of water as a conduit of waste has resulted in the contamination of a resource that life on Earth cannot function without. Wastewater treatment data show that pollutants are often not being removed, to a degree considered adequate, from treated water and, in some cases, micropollutants are found at higher levels in treated water.

The first aim within this study was to determine the effect of a labile carbon source on the degradation of two micropollutants. Based on the findings the presence of glucose, a labile carbon source, does seem to have an effect on the removal of micropollutants, although under the conditions applied in the experimentation this effect was not significant.

The effects of two compounds were investigated in this study: methylparaben and carbamazepine, the former is a compound that has a high removal efficiency in existing wastewater treatment plants, while the latter is relatively recalcitrant. Methylparaben, as previously mentioned, is a compound widely used as a microbiological preservative in personal care products. The concentrations of this compound in wastewater effluent in South Africa can be as high as 1 $\mu g.L^{-1}$ (Archer et al., 2017). While conventional WWTPs appear to remove methylparaben with relative efficacy (100 $ng.L^{-1}$ in effluent (Archer et al., 2017)), the continued presence of these compounds in treated water remains a concern. The MIC of methylparaben (10.6 $g.L^{-1}$ for CT07, and estimated 256.7 $g.L^{-1}$ for the mixed culture), is alarming considering this compound is used as an antimicrobial preservative in personal care products and that the lowest inhibitory concentration to prevent growth of microbes is greater than the threshold concentration at which estrogenic effects occur. Considering that the final concentration of methylparaben is present in personal care products is between 0.3 and 0.4% (Soni et al., 2002, SCCP, 2008), this compound is included in cosmetics in dose high enough to prevent microbial growth, which happens to be the dose required to elicit estrogenic effects. The use of this compound in personal care products should therefore be questioned, as the deleterious effects could outweigh the benefits. There is evidence that this is already happening in the industry, where the number of "paraben-free" products available on the market has increased, and some brands have even removed parabens from their ingredient list.

Carbamazepine is an anti-epileptic pharmaceutical compound with a long list of deleterious endocrine effects. This compound is also extremely recalcitrant, with levels increasing during the water treatment process. The mechanisms behind this apparent increase are, as yet, not fully understood but the increase is alarming nonetheless.

The list of potential biologically-active micropollutants is long and the two compounds investigated here represent a mere fraction of these. The more these compounds are used and are continuously added into the water systems, while not being completely removed, means that these compound will continue to build up, albeit slowly, in our water systems. The implication being that these compounds, while perhaps not yet

harmful, may eventually reach levels whereby they may elicit deleterious effects in higher organisms, without a means with which to adequately remove them.

The second aim of this study was to determine the effects of the chosen micropollutants on the biofilms. The effects are arguably significant, considering the very small concentrations they were tested at. The effects that these compounds appear to have on the growth and ultimate physical appearance of biofilms is also something of a concern because it means these compounds, even at nanogram levels, have a measurable effect on the structure, and perhaps the metabolic activities of microorganisms and may, in turn, cause some level of deleterious effect on the metabolism of the biofilms. This may, in turn, actually hinder the removal of the compound causing the effect, or even of other compounds present in the bulk flow of the exposed biofilms.

Despite the growing body of evidence, there still seems to be inadequate concern for the imminent water crisis. In order to secure life on earth in the future, the water sources we have need to be reused and, in order to do that, the water needs to be sufficiently cleared of micropollutant contaminants. It appears that conventional wastewater treatment is often not sufficient to solve this problem, pointing to the need for improvement of existing technologies, or to find alternatives. A renewed recognition of nature's innovative and adaptable ways to address problems that humans could not arrive at themselves, and adoption of nature's solutions, or processes that can be harnessed, manipulated and enhanced, in order to solve pollutant removal problems should be encouraged. Using biomimetic principles may provide useful cues. The third aim to evaluate the potential of these systems in the treatment of water could not be achieved as the Eco-Machine™ has not yet been installed due to delays related to tender processes and government approval, therefore such evaluation remains an academic exercise. These systems do show promise, although their lower throughput levels should be considered in order to set realistic expectations.

Nature has developed its own solutions to handling problems, some of which are amazingly elegant in their simplicity, and perhaps humans need to look to nature for an improved water treatment solution. After all, the simplest solutions are often the most effective.

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