

Investigation of a microbial consortium able to degrade selected emerging pollutants with endocrine-disrupting capabilities from wastewater in Bloemfontein

by

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DECLARATION

I, **Lehlohonolo Benedict Qhanya**, _____, hereby declare that this research project, submitted to the Central University of Technology, Free State, for the Degree Doctor of Health Sciences in Biomedical Technology, is my independent work. This work complies with the Code of Academic Integrity as well as other relevant policies, procedures, rules, and regulations of the Central University of Technology, Free State. I declare that it has not been submitted before to any institution by me or any other person for the attainment of a qualification.

.....

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I certify that the above statement is correct.

.....

Prof. O de Smidt (Promoter)

DEDICATION

*To my family, whose love and encouragement have been my anchor and my compass
throughout this journey.*

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ACRONYMS AND ABBREVIATIONS

ASV	Amplicon sequence variant
ATZ	Atrazine
BOD	biochemical oxygen demand
BOD	Biochemical oxygen demand
bp	Base pairs
BPA	Bisphenol A
COD	Chemical oxygen demand
DO	Dissolved oxygen
EDCs	Endocrine-disrupting chemicals
EE2	17 α -ethinylestradiol
EPs	Emerging pollutants
HRT	Hydraulic retention time
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LC	Liquid chromatography
MIC	Minimum inhibitory concentrations

MS/MS	Tandem mass spectrometer
MSM	Minimal salts medium
NGS	Next-generation sequencing
PCoA	Principal Coordinate Analysis
PCR	Polymerase chain reaction
PICRUST	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
QIIME	Quantitative Insights into Microbial Ecology
rRNA	Ribosomal ribonucleic acid
SPME	Solid phase microextraction
TCS	Triclosan
TDS	Total dissolved solids
TSS	Total suspended solids
WSP	Wastewater stabilisation pond
WSPs	Wastewater stabilisation ponds
WWTPs	Wastewater treatment plants

ABSTRACT

Emerging pollutants (EPs) have become a growing environmental concern due to their persistence, ubiquity, and potential to cause adverse ecological and human health effects. Among these, endocrine-disrupting chemicals (EDCs) are of particular interest because of their ability to interfere with hormonal systems. Endocrine-disrupting chemicals (EDCs) such as atrazine (ATZ), bisphenol A (BPA), 17 α -ethinylestradiol (EE2), and triclosan (TCS) are known to be persistent in the environment and their presence is of global concern. These compounds are often detected in the final effluent of wastewater, indicating the incomplete removal of EDCs. Some wastewater treatment plants (WWTPs) utilise wastewater stabilisation ponds (WSPs) that use physical and biological processes to remove EPs. This research aimed to address the identified knowledge gap by investigating the occurrence and persistence of selected EDCs in a wastewater stabilisation system, particularly in two maturation ponds, in Bloemfontein, South Africa, and to determine their biodegradation by the indigenous microorganism consortium.

The concentrations of ATZ, BPA, EE2 and TCS in the inflow and outflow of two wastewater maturation ponds (the inflow and the outflow ponds) were quantified by LC-MS/MS. Microbial composition was characterised using next generation sequencing (NGS). The inhibitory effects of these EDCs on microbial community were also investigated by determining the minimum inhibitory concentrations (MIC). Laboratory enrichment experiments were conducted in mineral salts medium (MSM) inoculated with pond water, with each EDC supplemented at 10 mg/L, incubated at 26°C, and shaken for 35 days. Degradation was monitored over 35 days by LC-MS/MS using peak area (AUC) as a proxy for relative concentration, and microbial growth was tracked through optical density (OD) and colony-forming units (CFU).

All four target EDCs were detected in the maturation ponds at variable concentrations. The concentrations of EE2 in the inflow pond ranged from 0.0057 $\mu\text{g/mL}$ to 125.0 $\mu\text{g/mL}$. Physicochemical variables revealed that pH and TDS influenced the microbial communities in these ponds. Microbial

diversity investigated by 16S rRNA sequencing showed the presence of Proteobacteria, the most predominant phylum across the inflow and outflow samples. At the genus level, *Flavobacterium* and *Microcystis* were predominant, followed by *Dechloromonas* and *Pseudomonas* across the inflow and outflow pond samples. ITS analysis revealed unidentified phyla that accounted for 68.85% of all ASVs, followed by Ascomycota that occurred across the inflow and outflow samples. The inhibitory effects of EDCs on the microbial growth in the wastewater maturation ponds under study were evident. MIC assay results indicated that microbial growth was largely unaffected at lower concentrations (0.1 - 1 µg/mL), with inhibition becoming evident at higher doses (10 - 100 µg/mL). ATZ showed the lowest toxicity, sustaining microbial growth even at 100 µg/mL. BPA exhibited moderate inhibition, with growth declining significantly at 100 µg/mL. EE2 induced marked inhibition between 10 - 100 µg/mL, while TCS demonstrated the highest toxicity, suppressing microbial growth even at concentrations as low as 1 - 10 µg/mL.

Maximum optical density (OD_{600nm}) and colony forming units (CFU/mL) of $LogOD_{600nm}$ 0.387, and 1.23×10^7 CFU/mL were observed by Day 35. Microbial growth data confirmed active proliferation, suggesting a potential biological contribution to the reduction in the concentrations of EDCs. Study showed 90% signal reduction within 14 days for EE2 and BPA, while TCS showed only limited removal (66%) relative to controls. A spike in TCS control flasks on Day 35 and an increase in the experimental flasks suggested analytical challenges. A possible interference in MSM matrix with ionisation and recovery of TCS was possible.

This study demonstrated that the maturation ponds in the wastewater stabilisation system acted as reservoirs for diverse EDCs and harboured microbial communities with biodegradative potential. The integration of field monitoring, microbial characterisation, and laboratory biodegradation assays highlighted both the promise and challenges of EDC bioremediation. While BPA and EE2 were readily degraded, ATZ remained recalcitrant, and TCS posed both analytical and biological barriers to removal. These findings underscore the importance of coupling environmental assessments with laboratory

experiments to fully understand EDC. Future work should refine detection methods and media component interference for triclosan detection, investigate metabolic pathways, and explore the role of microbial consortia in enhancing pollutant removal.

Chapter 1

Introduction and Background

1.1. Introduction

Emerging pollutants (EPs) have become a growing environmental concern due to their persistence, ubiquity, and potential to cause adverse ecological and human health effects. Among these, endocrine-disrupting chemicals (EDCs) are of particular interest because of their ability to interfere with hormonal systems. The United States Environmental Protection Agency (USEPA, 1997, p.12) and Gore (2014, para. 3) define EDCs as “exogenous chemical substances or mixtures that alter the structure or function(s) of the endocrine system and cause adverse effects at the level of the organism, its progeny, populations, or subpopulations”.

Endocrine-disrupting chemicals (EDCs) include a wide array of synthetic compounds such as industrial chemicals, pharmaceuticals, pesticides, and personal care products. Common examples include nonylphenol, octylphenol, bisphenol A (BPA), atrazine, and triclosan. Many of these compounds are not only toxic but are also suspected carcinogens and contributors to antimicrobial resistance. Although their estrogenic activity is typically several magnitudes lower than that of natural estrogens, including 17 β -estradiol (E2), estrone (E1), synthetic estrogens such as 17 α -ethinylestradiol (EE2), they have been demonstrated to cause endocrine disruption in aquatic species (Chang et al., 2011; Routledge & Sumpter, 1996).

Endocrine-disrupting chemicals (EDCs) and other organic micropollutants have been consistently detected in surface and groundwater across various regions, including the United States (US), the United Kingdom (UK), Germany, China, and South Africa (Bester et al., 2008; Kolpin et al., 2002). Geological surveys conducted in the US Survey have reported widespread contamination caused by

pharmaceuticals, personal care products, plasticizers, detergents, hormones, and flame retardants (Barnes et al., 2008; Erickson, 2014; Focazio et al., 2008). Their detection at low nanogram-per-Liter (ng/L) concentrations underscores their resistance to environmental degradation.

The environmental fate and transport of these compounds are governed by a complex interplay of processes, including photolysis, sorption-desorption, advection, and microbial degradation. As water resources become increasingly constrained due to urban expansion, climate variability, and growing demand, reusing treated wastewater will become essential. This amplifies the need for a comprehensive understanding of how these pollutants behave in wastewater treatment and aquatic ecosystems.

Microorganisms play a vital role in the transformation and mineralization of organic pollutants. Biodegradation has been shown to occur both in engineered systems such as WWTPs and natural settings like rivers and aquifers. Laboratory and pilot-scale studies have demonstrated compound-specific removal efficiencies. For example, near-complete removal of sulfamethoxazole under mesophilic and thermophilic conditions has been reported, while compounds like carbamazepine have exhibited resistance to degradation regardless of treatment conditions (Carballa et al., 2006; Zhang et al., 2016). However, results from WWTPs cannot always be applied to natural systems like surface waters or maturation ponds, where environmental conditions and microbial communities are quite different (Mueller & Gehring, 2025). Field studies have reported wide variability in microbial degradation rates, with pharmaceutical half-lives ranging from a few days to several months depending on site-specific conditions (Benotti & Brownawell, 2009; Yamamoto et al., 2009). Despite rapid degradation in treatment systems, compounds such as caffeine may behave conservatively in natural waters, making them useful tracers of wastewater contamination (Buerge et al., 2003).

Numerous bacterial species have been isolated for their ability to degrade EDCs. For instance, *Novosphingobium* sp. has been shown to degrade estradiol (Fujii et al., 2002), while other strains such as *Pseudomonas* sp., *Arthrobacter* sp., *Nocardioides* sp., and *Sphingomonas* sp. are associated with the

degradation of BPA and atrazine (Gummadi et al., 2009; J. Zhang et al., 2009; W. Zhang et al., 2013). Despite these advances, most earlier studies focused on high-estrogenic compounds and laboratory conditions. However, little is known about microbial communities and their ability to metabolise a broad spectrum of EDCs, especially under changing environmental conditions. In addition, the microbial ecology remains underexplored in these aquatic ecosystems. Culture-independent techniques, such as NGS, can complement culture-based methods and provide a more complete picture of the structure and functions of microbial communities. These insights are essential to identify microbial with high bioremediation potential and to develop bioaugmentation strategies.

Conventional wastewater treatment plants (WWTPs) play a crucial role in removing organic carbon (chemical oxygen demand, COD), nutrients (N and P), and EDCs, but the processes they use are not always efficient. Advanced chemical oxidation technologies such as ozonation must be employed for better removal. For instance, removal of up to >90% for carbamazepine has been reported (Falås et al., 2016; Liu et al., 2021; Magdeburg et al., 2014). Even though the activated sludge treatment processes can be effective for some compounds, residual levels often get into water source and cause pollution that harms the environment. This raises concerns about human long-term exposure to partially treated effluents reaching drinking and irrigation sources (Dickin et al., 2016).

Wastewater stabilisation ponds (WSPs) are frequently utilised by most developing countries as a suitable alternative treatment of wastewater which can provide effective and low cost means of treatment. These systems are recommended for communities with a population less than 5 000 (Edokpayi et al., 2021; Mahapatra et al., 2022). Wastewater stabilisation ponds (WSPs) use natural physicochemical and biological mechanisms such as temperature, pH, dissolved oxygen (DO), light intensity, and hydraulic retention time (HRT), resulting in reduced levels of pollutants in wastewater (L. Liu et al., 2018). For rural and peri-urban areas, their simple design, low operational cost, and robustness against load fluctuations make them particularly suitable. However, the mechanisms underlying the attenuation of EPs, including EDCs, in these systems remain poorly understood.

1.2. Problem Statement

Although a substantial body of literature exists on EDC occurrence and degradation in conventional WWTPs, limited attention has been given to WSP systems, particularly in the context of microbial degradation. Understanding the microbial communities in these systems and their roles in EDC attenuation can contribute significantly to environmental risk assessments and sustainable wastewater management practices.

This study investigates the occurrence and persistence of selected EDCs in the wastewater stabilisation maturation ponds in Bloemfontein, South Africa, and evaluates their possible removal by indigenous microbial consortium.

1.3. Aims and Objectives

The aim of the study was to investigate the potential of indigenous microbial consortia in removing selected endocrine-disrupting chemicals, namely bisphenol A (BPA), atrazine, triclosan, and 17 α -ethinylestradiol (EE2), from the wastewater stabilisation ponds under study.

Objectives

The objectives of the study were to:

1. Quantify the occurrence and concentration of selected EDCs in maturation ponds in a wastewater stabilisation system.
2. Evaluate microbial compositions of wastewater sampled from two maturation ponds (the inflow and outflow ponds).
3. Identify bacterial consortia capable of degrading targeted EDCs.
4. Assess the biodegradation capacity of indigenous microorganisms under laboratory conditions.

1.4. Chapter Layout

This thesis is organised into six chapters:

Chapter 1: General introduction

This chapter provides an overview of emerging pollutants, particularly endocrine-disrupting chemicals (EDCs), their sources, environmental persistence, and limitations of current wastewater treatment systems, followed by the study's aims and objectives.

Chapter 2: Literature review

The chapter reviews existing research on the occurrence, environmental fate, and microbial degradation of EDCs. It covers key microbial species known for EDC degradation, bioremediation techniques, wastewater stabilisation ponds (WSPs), and current analytical methodologies. The chapter also identifies critical research gaps addressed in this study.

Chapter 3: Endocrine-disrupting chemicals (EDCs) and microbial diversity of the wastewater treatment maturation ponds

This chapter examines the occurrence, concentration, and distribution of selected EDCs sampled from wastewater in two maturation ponds of a selected wastewater treatment plant in Bloemfontein. It also investigates the microbial diversity present in the effluents, using the Illumina next-generation sequencing platform.

Chapter 4: Microbial consortium tolerance to selected endocrine-disrupting chemicals (EDCs)

The investigation assessed the tolerance levels of microbial consortia to various concentrations of selected EDCs. This chapter evaluates microbial growth in environments with increasing EDC concentrations to determine which concentrations of EDCs promoted increased microbial growth. The results identify the conditions under which indigenous microbial communities may have the highest resilience and potential for bioremediation applications.

Chapter 5: Enriching Indigenous microbial consortia for EDCs utilisation

This chapter discusses how indigenous microbial consortia from the WSP systems had been enriched and considers how they could grow in the presence of selected endocrine-disrupting chemicals (EDCs) as potential growth substrates. It focuses on experimental assay conducted under controlled laboratory

conditions and evaluates microbial community shift in the presence of bisphenol A (BPA), atrazine, triclosan, and 17 α -ethinylestradiol (EE2), providing insight into the consortium's biodegradation potential.

Chapter 6: General concluding remarks and future work

This chapter summarises the main findings of the study and examines its implications for sustainable wastewater management and microbial bioremediation strategies. It also discusses the limitations of the study and highlights future directions for research on similar applications and environmental risk assessment.

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Chapter 2

Literature review

2.1. Introduction

The presence of emerging pollutants (EPs) in aquatic systems has become a pressing global concern. Over time, human activity has led to the release of an increasing number of unregulated or recently regulated synthetic and natural chemicals into the environment. These substances, together with their biologically mediated metabolites and abiotically formed transformation products, pose significant risks to both human and environmental health (Tavengwa & Dalu, 2022). Examples of EPs include pharmaceuticals and personal care products (Adeleye et al., 2022; Madikizela, L. M., Muthwa, S. F., & Chimuka, L., 2014), illicit drugs (Ntshani & Tavengwa, 2022), flame retardants (Cristale, 2022), pesticides (Montagner et al., 2022), disinfectant by-products (Vizioli et al., 2022), surfactants, biocides, plasticizers, potentially toxic elements (Galhardi et al., 2022; Hashemi & Kaykhaii, 2022; Kudu et al., 2022), and nanomaterials (Yardy et al., 2022).

Over the past century, the production of synthetic chemicals has risen dramatically. In 1930, global production of synthetic chemicals amounted to 1 million tons, but by 2000 it had increased to 400 million tons (United Nations Environment Programme, 2019). Between 2000 and 2017, global chemical industry production capacity grew from 1.2 billion tons to 2.3 billion tons (United Nations Environment Programme, 2019). Presently, there are approximately 350,000 chemicals in use worldwide (Wang et al., 2020). However, the increasing reliance on synthetic chemicals has had significant consequences. Of the 345 million tonnes of synthetic chemicals consumed in the European Union (EU) in 2016, 22% posed environmental risks and 62% were deemed hazardous to human health (Eurostat, 2020). According to estimates by the World Health Organisation (WHO), chemicals caused over 1.6 million deaths globally in 2018 (Das & Horton, 2018; Landrigan et al., 2018).

Among emerging pollutants (EPs), endocrine-disrupting chemicals (EDCs) are among the most important and especially concerning. These compounds can block or mimic the natural hormones that regulate the endocrine system in both animals and humans (Kasonga et al., 2021; Vieira et al., 2020). Substances that exhibit estrogenic activity, such as steroid sex hormones, alkylphenols, polybrominated diphenyl ethers (PBDEs), and phthalates, are categorised as EDCs (Petrović et al., 2003). The worldwide growth in the production of synthetic chemicals has continued unabated even amidst growing knowledge of their adverse impacts. This chemical industry will be worth \$5 trillion in 2027, and it is estimated that it may ultimately double by 2030 (United Nations Environment Programme, 2019).

The global increase in synthetic chemical production has persisted despite growing awareness of their harmful effects. Sales of chemicals such as pharmaceuticals, and many others, are projected to exceed US\$5 trillion by 2030, which is nearly double the 2017 figure (United Nations Environment Programme, 2019). Globally, at least 2 million tonnes of sewage, industrial, and agricultural waste introduce chemicals into aquatic systems. A 2013 national assessment reported that fewer than 10% of South Africa's 824 wastewater treatment plants in 153 municipalities were compliant with regulatory standards, with approximately one-third classified as critical and requiring urgent rehabilitation. Consequently, it was estimated that up to 50 000 litres per second of untreated or inadequately treated wastewater were being discharged into receiving water bodies (DWA, 2013). At the global level, wastewater production is also considerable; the annual volume of wastewater generated worldwide has been estimated to be approximately six times greater than the volume of water contained in all the world's rivers (Corcoran, 2010). This comparison underscores the magnitude of wastewater generation relative to renewable surface freshwater resources, rather than suggesting that contaminated water exceeds total global freshwater availability.

Wastewater treatment could provide a potential solution to mitigate the existing problem of EPs in the aquatic environment. Treated wastewater offers a sustainable solution to water scarcity. In addition to supplying reusable water, treated wastewater contains residual organic matter and biogenic nutrients

such as nitrogen, phosphorus, and potassium, which can contribute to plant nutrition when appropriately managed (Al-Suhaibani et al., 2021). A common practice globally is the utilisation of treated wastewater for agricultural purposes, particularly irrigation (De Gisi et al., 2016; Senthil Kumar & Saravanan, 2018).

Wastewater stabilisation ponds (WSP) systems offer a cost effective, low energy, and natural method for treating wastewater. Traditionally, conventional WSPs consisted of different types of ponds, such as anaerobic ponds (APs), facultative ponds (FPs), and maturation ponds (MPs), each offering distinct treatment and design characteristics (Vaghee, 2021). The main features and functions of each pond type are summarised in Table 1.1.

Table 1.1: Main features and functions of anaerobic, facultative, and maturation ponds

Feature	Anaerobic ponds	Facultative ponds	Maturation ponds
Depth (m)	3–5	1.5–3	1–2
HRT (days)	10–50	5–20	5–10
OLR (kg BOD ₅ . Ha ⁻¹ . Day ⁻¹)	High, >300	Medium, 100–300	Low, <100
Microorganism responsible for BOD reduction	Anaerobic bacteria	Facultative bacteria and algae	Aerobic bacteria
Major functions	Pre-treatment and BOD reduction	BOD reduction and nutrient removal	Polishing, SS and pathogen reduction, and some nutrient removal
By-product formed	Biogas, methane, carbon dioxide	SS in the form of algal and bacterial cells, carbon dioxide	None

Key: HRT: hydraulic retention time; OLR: organic loading rate; BOD₅: 5-day biochemical oxygen demand; SS: suspended solids

Source: Adapted from Vaghee, 2021

These ponds' configurations, whether in series or parallel, play a crucial role in yielding high quality effluents. WSPs present the simplest method to treat wastewater that is widely used all over the world,

particularly in developing countries where sufficient land for treating domestic and industrial wastewaters exist (Ali & Üçüncü, 2023; Kilian et al., 2024; Mahapatra et al., 2022). Despite low concentrations of emerging pollutants (EPs) ranging from nanograms per litre (ng/L) to micrograms per litre (µg/L) in water and their effects on living organisms, they are associated with toxicological effects, endocrine disruption, and acute and chronic toxicity threats to human health and aquatic organisms (Geissen et al., 2015; Vasilachi et al., 2021). Rampant increase in xenobiotic chemicals and the increased presence of EDCs highlight the urgent need for effective strategies to mitigate their adverse effects on global health and environmental sustainability (Sanganyado & Kajau, 2022).

2.2. Emerging Pollutants

Emerging pollutants (EPs) present significant remediation challenges due to their physicochemical properties, which promote environmental persistence, mobility, and resistance to conventional treatment processes. Many EPs possess structural features that confer recalcitrance, including strong carbon-halogen bonds (e.g., C-F in perfluorinated compounds), aromatic ring stability, steric hindrance, and low susceptibility to enzymatic attack. For example, perfluorinated compounds (PFCs) contain carbon-fluorine bonds with bond dissociation energies exceeding $480 \text{ kJ}\cdot\text{mol}^{-1}$, rendering them highly resistant to thermal, chemical, and biological degradation (Bolan et al., 2021; Wackett, 2022; Zhou et al., 2024). Many EPs exhibit moderate to high octanol–water partition coefficients ($\log K_{ow}$), enhancing their bioaccumulation potential and sorption to organic matter (Karickhoff et al., 1979; Mackay & Fraser, 2000; Schwarzenbach et al., 2017). Others display high aqueous solubility and low distribution coefficients (K_d), increasing environmental mobility in aquatic systems (Joss et al., 2006; Vieno et al., 2007). The presence of ionisable functional groups results in pK_a -dependent speciation, which significantly influences membrane permeability, sorption behaviour, and removal efficiency in wastewater treatment systems (Petrović et al., 2003). Conventional biological treatment processes rely primarily on microbial enzymatic oxidation and co-metabolism; however, compounds exhibiting low biodegradation rate constants, limited

bioavailability, or toxicity toward microbial consortia often demonstrate poor removal efficiencies (Onesios et al., 2009).

Moreover, the structural diversity of EPs results in varied degradation pathways, incomplete mineralisation, and formation of stable transformation products that may retain endocrine activity or toxicity. These combined factors-chemical stability, resistance to microbial enzymatic cleavage, sorption-desorption, and transformation product persistence-contribute to the fastidious and recalcitrant nature of EPs in aquatic environments (Feng et al., 2021; Hussain et al., 2022; Yao et al., 2014).

The widespread use of EPs includes everyday household activities, protection of human and animal health, as well as industrial and agricultural production. Pharmaceuticals and personal care products (PPCP) are the most extensively utilised EPs on the globe. Brominated flame retardants, for example, are extensively used in electrical appliances, textiles, automobiles, and building materials due to their low cost, high performance, and minimal usage requirements, contributing significantly to their presence in the environment (Hörold, 2014; Morgan & Kilinc, 2021; Weil & Levchik, 2015). Endocrine-disrupting chemicals (EDCs) are another prominent group of EPs that are widely used in personal care products, detergents, thermal paper, and plastics. Their strong migratory nature allows them to spread far from their sources, with microplastics being detected in remote locations like the freshwater systems of Antarctica (Encarnação et al., 2019; González-Pleiter et al., 2020; Martín-Pozo et al., 2021). Emerging pollutants (EPs) are ubiquitous in the environment, including in the air, water, and soil, illustrating their far-reaching impact (Dumont & Tuzet, 2022; Enyoh et al., 2019; Yan et al., 2022). Figure 2.1 presents a schematic view of the routes by which some emerging pollutants enter various receptors, including groundwater, surface water, and consumers.

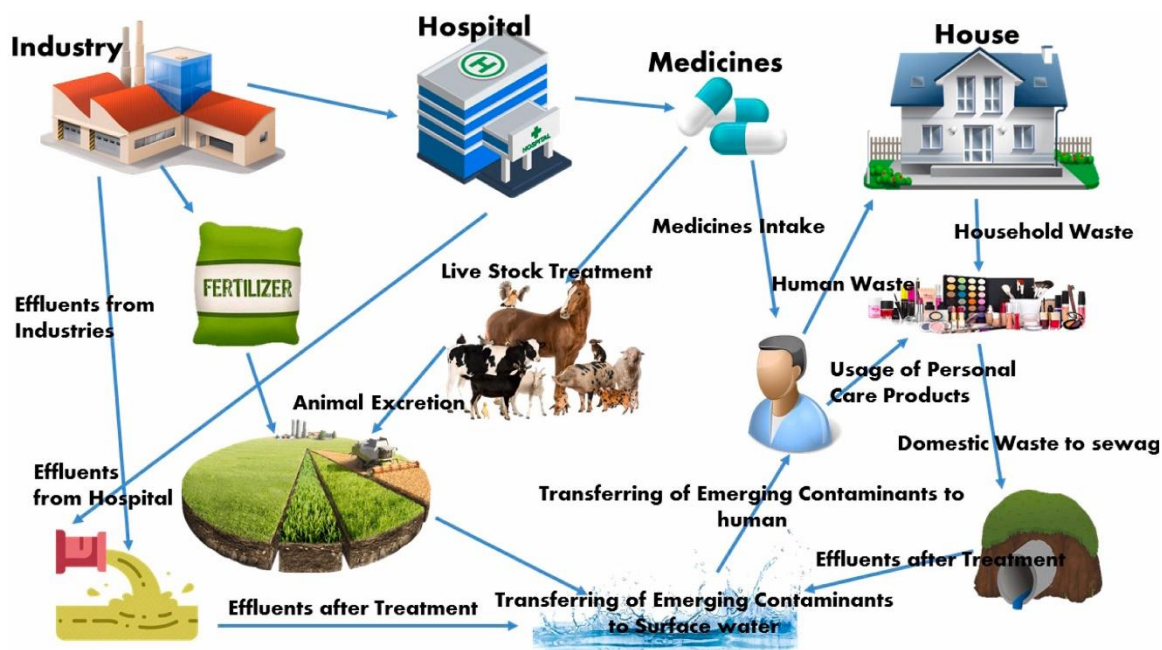


Figure 2.1: Environmental origins and distribution routes of emerging pollutants (EPs). Source: Adapted from Rathi et al., 2021.

Another key problem is the biological toxicity associated with EPs. These pollutants are linked with multiple health concerns, including toxicity of certain organs, neurotoxic effects, and reproductive as well as developmental toxicity (Li et al., 2022). In addition, EPs can bioaccumulate and can become toxic over time, posing significant threats to living organisms, ecosystems, and human health (Engwa et al., 2019; Fenton et al., 2021; Pereira et al., 2015). Moreover, EPs are associated with certain pollutants that have cumulative effects over time, which makes monitoring and intervention easier. Emerging pollutants (EPs) accumulate over time to harmful levels, which make them difficult to detect and to formulate interventions. This era of prolonged remediation efforts is a result of limitations in current monitoring technologies, which further exacerbates the issue (Zhang et al., 2019).

Over the years, EPs have gradually been detected in the marine ecosystem, often through inadequate or untreated discharge, whether intentional or accidental. These pollutants have had and will continue to have significant health risks and have widespread impacts on marine life and the marine ecosystem (Wilkinson et al., 2017). These pollutants originate from diverse sources, such as pharmaceuticals,

personal care products, fertilisers, pesticides, hormones, antibiotics, artificial sweeteners, surfactants, flame retardants, illegal drugs, antibacterial agents, and perfluorinated compounds (Cabeza et al., 2012; Richardson & Kimura, 2017). When these pollutants are released into the environment, especially polar organic compounds can be detected in surface waters, soils, sediments, and groundwater aquifers. These pollutants are transported through unsaturated soil zones and often reach deeper groundwater systems, contributing to their widespread distribution (Lapworth et al., 2012; Li, 2014; Wang et al., 2017; Weber et al., 2017). Sorption (attachment to soil or sediment particles), biodegradation (microbial breakdown), photolysis (degradation by sunlight), and abiotic transformations such as hydrolysis (Baena-Nogueras et al., 2017; Yamamoto et al., 2009) are some of the processes that determine the environmental fate and transport of these pollutants, and these processes play a critical role in the persistence, spread, and impact that EPs have on the environment.

The presence of EPs, such as pharmaceuticals, cosmetic products, nanoparticles, and perfluorinated compounds, poses a growing ecological challenge. These substances threaten the health and welfare of marine organisms and ecosystems as well as human health due to their bio-accumulative and persistent nature (Chaturvedi et al., 2023; Tijani et al., 2016). To effectively address EPs that are now a global issue, it will require significant advancement in treatment technologies, improved monitoring systems, and robust regulatory frameworks to mitigate their environmental and health impacts.

2.3. Endocrine-disrupting Chemicals

Substances with endocrine activity encompass a wide range of industrial and agricultural chemicals, including pesticides, plasticizers, organometals, long-chain polymers, natural estrogens and androgens, illicit drugs, personal care products, and pharmaceuticals used in clinical and veterinary applications. Many of these EPs, such as nonylphenol (NP), perfluorooctanoic acid (PFOA), and bisphenol A (BPA), are resistant to conventional wastewater treatment processes, leading to their persistence and potential

bioaccumulation in the environment (Ahmed et al., 2021; Deblonde et al., 2011). Endocrine-disrupting chemicals (EDCs) are known to cause endometriosis as well as prostate, testicular, and breast cancers and also give rise to severe complications in the reproductive health of humans and animals where they have been known to reduce sperm count, leading to the production of fragile eggs (Dutta et al., 2023). They are also known to compromise the immune systems of aquatic wildlife (Lauretta et al., 2019; Pesqueira et al., 2020; Pramanick, 2022; Rehman et al., 2018; Sifakis et al., 2017; Smarr et al., 2018; Street et al., 2018; Yadav et al., 2021). This broad class of compounds includes natural estrogens such as estrone (E1), 17 β -estradiol (E2), and estriol (E3) (Bao et al., 2006; Blair et al., 2000; Folmar et al., 2002; Nishihara et al., 2000), as well as natural androgens like testosterone (T), dihydrotestosterone (DHT), and androsterone (A) (Bauer et al., 2000; Fang et al., 2003). Synthetic hormones, such as 17 α -ethynylestradiol (EE2), Norgestrel (N), and Trenbolone (Tr), are also notable contributors to endocrine disruption in aquatic environments (Bauer et al., 2000; Blair et al., 2000). Additionally, phytoestrogens, including isoflavonoids and coumestrol that are derived from plant-based sources, are frequently detected in wastewater sewage treatment plants (Bacaloni et al., 2005; Stopper et al., 2005). Other industrial compounds, such as bisphenol A and nonylphenol, further exacerbate the issue due to their estrogenic activity and widespread usage (Gałazka & Jankiewicz, 2022; Katrina et al., 2024; Souza et al., 2024; Struk-Sokołowska et al., 2024). These chemicals can interfere with and disrupt the endocrine complex communication system and produce adverse effects in both humans and wildlife (Gore et al., 2015), while they also affect aquatic biodiversity (Kasonga et al., 2021).

The endocrine system functions in the control and coordination of various body functions, as depicted in Figure 2.2. This system comprises complex hormone-producing glands and receptors. These glands produce chemical messengers called hormones, such as corticosteroid, oestrogen, testosterone, adrenaline, androgen, insulin, triiodothyronine, and thyroxine, to regulate the body's development, growth, reproduction, metabolism, immunity, and behaviour (Kasonga et al., 2021; Sifakis et al., 2017; Tijani et al., 2016). The endocrine glands secrete hormones that circulate throughout the body via the

bloodstream to the receptors of target cells, which bind and modulate cellular or organ functions. The quantity of hormones produced by the body depends solely on the stimulus it receives. In fact, they can even operate in minute concentrations.

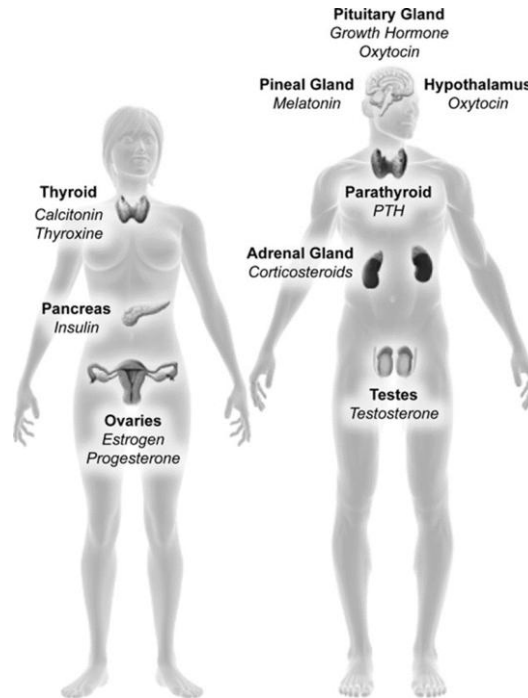


Figure 2.2: Diagram of major endocrine glands in the human body. The model illustrates that all major endocrine organs – including the hypothalamic-pituitary-adrenal (HPA) axis, reproductive organs, pancreas, and thyroid gland – are susceptible to disruption. EDCs also affect hormone-regulated metabolic systems and brain function. Source: Adapted from Schug et al., 2011.

The EDCs can disrupt the normal functioning of the endocrine system and alter hormonal functions in several ways, as illustrated in Figure 2.3 (Caliman & Gavrilescu, 2009).

- EDCs can mimic or antagonise the effects of naturally occurring hormones, potentially leading to overstimulation.
- EDCs can bind to the receptor within a cell and block the binding of endogenous hormones. The normal signal then fails to occur and, ultimately, the body fails to respond properly.
- EDCs can interfere with or block the production or action of natural hormones or their receptors.

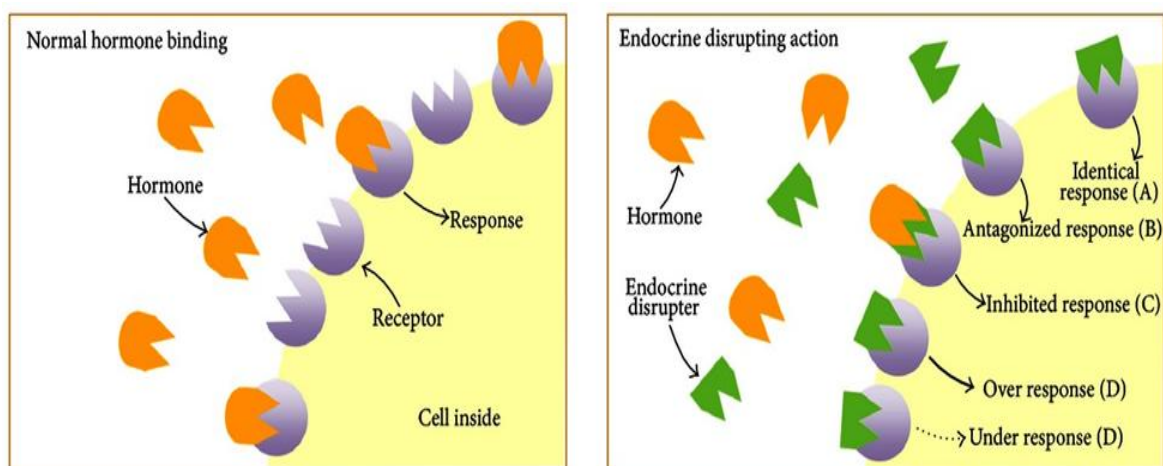


Figure 2.3: Illustrations of how EDCs that mimic hormones can alter normal hormone function in cells.
Source: Adapted from Sosa-Ferrera et al., 2013.

2.4. Endocrine-disrupting Chemicals in Wastewater and the Role of Wastewater Stabilisation Ponds (WSPs) in the Removal of EDCs

Over the last two decades, the pervasive presence of EDCs in surface waters, groundwater, and wastewater effluents has been extensively documented (Geissen et al., 2015; Luo et al., 2019). Several studies have documented the presence of EDCs in WWTPs across various regions. For instance, Yu et al. (2013) reported the detection of 14 EDCs—including pharmaceuticals such as paracetamol, naproxen, and ibuprofen—in five WWTPs located in southern California. In Greece, Stasinakis et al. (2008) investigated eight WWTPs and found substantial concentrations of 4-n-nonylphenol (4-n-NP), nonylphenol monoethoxylate (NP1EO), nonylphenol diethoxylate (NP2EO), triclosan (TCS), and bisphenol A (BPA) in raw influent, treated effluent, and sewage sludge. Similarly, Zhang & Zhou (2008) reported the occurrence of estrone and 17 β -estradiol in both influent and effluent samples from the Horsham WWTP in West Sussex, UK. In Rome, Italy, Spataro et al. (2019) detected EDCs such as nonylphenol monoethoxylate and nonylphenol diethoxylate in wastewater collected from the main WWTPs serving the metropolitan area.

Conventional waste stabilisation ponds (WSPs), commonly known as lagoons, are widely used wastewater treatment systems consisting of three interconnected pond types: anaerobic ponds (Aps), facultative ponds (FPs), and maturation ponds (MPs) (Kayombo, 2005; Mara, 2001, 2013; Sah et al., 2012). These ponds differ in geometry, hydraulic flow patterns, organic loading, and biochemical processes. Their efficiency in removing carbon, nutrients, and pathogens improves with an increasing number of ponds (Von Sperling, 2015b).

Over the past three decades, various biological, physical, and chemical wastewater treatment technologies have been developed (Barakat, 2011; Rathore & Bhardwaj, 2023). However, no single method has been identified as the most effective, as each system presents unique advantages and challenges in terms of operational complexity, environmental impact, sludge production, effectiveness, feasibility, practicability, and cost-efficiency. While chemical, biological, and physical techniques can eliminate many EPs, none are sufficiently capable of removing a wide range of EPs (Sharma & Sanghi, 2012).

Despite the widespread use of WSPs globally, research on their efficiency in removing EDCs remains limited. To date, only a limited number of studies have examined the removal of EDCs in WSPs, and out of the hundreds of identified EPs in wastewater, only a few have been studied in these systems (Coleman et al., 2010; Gruchlik et al., 2018). This lack of research exposes a critical knowledge gap, given the prevalence of WSP technology worldwide. Most of the compounds analysed in WSPs were derived from pharmaceuticals and personal care products (PPCPs). Studies have indicated that the removal efficiency of certain EPs in WSPs can be comparable to or even exceed that of conventional treatment systems, though removal performance varies widely (Alvarino et al., 2014; Gruchlik et al., 2018; Matamoros et al., 2016). Some recalcitrant compounds, such as carbamazepine, gemfibrozil, and propranolol, have shown removal rates below 30%, whereas easily degradable compounds like caffeine, ibuprofen, and paracetamol have exhibited removal rates exceeding 95% (Park et al., 2020; Silva et al., 2021). Generally, resistant EPs in conventional treatments (e.g., carbamazepine) are poorly removed in WSPs,

while compounds that are effectively treated in conventional systems (e.g., caffeine, paracetamol) tend to show similar removal efficiencies in WSPs (Gruchlik et al., 2018; Park et al., 2020). The performance of WSPs is influenced by multiple factors, including the type of wastewater treated, organic loading levels, pond geometry, and hydraulic behaviour (Mara, 2013; Sah et al., 2012; Shilton, 2015). Additionally, a variety of physical, chemical, and biological processes—such as sedimentation, adsorption, and biodegradation—operate within each pond and are affected by environmental factors like algal presence, light intensity, pH, and temperature (Sah et al., 2012; Shilton, 2015; Von Sperling, 2015b). A comprehensive understanding of these processes is detailed by Shilton (2015).

Although limited studies have examined the removal of EDCs in pond systems that treat domestic wastewater, existing research suggests that significant biodegradation can occur under optimal conditions (Katibi, Yunos, Man, Aris, Nor, et al., 2021a). Factors such as hydraulic retention time (HRT) and dissolved oxygen (DO) levels are crucial in enhancing biodegradation efficiency, highlighting the importance of proper pond design and operation. Furthermore, WSPs in regions with average temperatures between 15 – 20°C and above have shown improved removal of EPs via biodegradation compared to those in colder climates (below 10°C) (Gruchlik et al., 2018; Leusch et al., 2014; Matamoros & Salvadó, 2012; Wang et al., 2023). However, research on removing other significant EP classes from WSPs, such as pesticides, perfluorinated compounds, illicit drugs, and artificial sweeteners, remains extremely limited or non-existent. This gap underscores the urgent need for further studies to fully assess the capabilities and limitations of WSPs in treating a broad range of emerging pollutants.

2.5. Microbial Community Diversity in Wastewater Stabilization Ponds (WSPs)

Given the limitations of conventional water treatment techniques, biodegradation has emerged as a promising and sustainable approach for treating soils and wastewater contaminated with EDCs and other EPs (Chaturvedi et al., 2023; Gao et al., 2020; Rodríguez-Hernández et al., 2022). Biodegradation harnesses the metabolic activity of microorganisms or their enzymes to break down pollutants, offering

several economic and environmental advantages, including low input costs, broad applicability, long-term effectiveness, and minimal infrastructure requirements. This method is particularly well-suited for addressing pollution in natural water environments where conventional treatment systems often fall short (Gao et al., 2020; Gavrilesco et al., 2015; Sun et al., 2024; Werkneh et al., 2022).

Microbial remediation of EPs is recognised as an effective and economically viable approach as it utilises a diverse range of microorganisms to degrade pollutants while using them as carbon or nitrogen sources to sustain their metabolic activities (Mahmoud, 2016; Arora, 2019; Bhatt et al., 2021; Chen et al., 2013; Chen et al., 2015; Siles & Margesin, 2018; Zhan et al., 2018). Microorganisms are ubiquitous, thriving in diverse natural and extreme environments such as soil, water, the human gut, hydrothermal vents, acid mine runoff, and oil reservoirs (Aguinaga et al., 2018; Cycon & Piotrowska-Seget, 2016; Delegan et al., 2019; Ding et al., 2017; Jatowiecki et al., 2016; Arora, 2020; Shekhar et al., 2020; Wang et al., 2018; Zierer et al., 2018).

Due to their genetic diversity and metabolic adaptability, microbial populations hold great potential for remediating contaminated environments (Bastida & Jehmlich, 2016; Bhatt & Barh, 2018; Chen et al., 2015; Dangi et al., 2019). Studying microbial communities in polluted environments provides valuable insights into the specific microbial traits that enhance degradation efficiency, ultimately contributing to developing more effective and sustainable bioremediation strategies. Various microorganisms, including bacteria, fungi, yeasts, and algae, have demonstrated significant potential in the bioremediation of EPs and EDCs. Bacteria such as *Pseudomonas*, *Bacillus*, and *Rhodococcus* play a crucial role in pollutant degradation, while fungal species like *Aspergillus*, *Penicillium*, and *Trichoderma* have also shown high bioremediation capabilities (Bhatt et al., 2021; Nzila, 2013; Sathishkumar et al., 2008; Sunita et al., 2013; Zhao et al., 2017). Among fungi, white-rot fungi (WRF) have received considerable attention due to their ability to produce extracellular lignin-modifying enzymes that facilitate the breakdown of complex aromatic compounds. *Pleurotus ostreatus* and *Trametes versicolor*, for example, have been shown to effectively degrade EDCs such as bisphenol A (BPA) and triclosan (TCS), achieving degradation efficiencies greater

than 90% under laboratory conditions (Singh et al., 2019). Under laboratory conditions, non-ligninolytic fungi like *Umbelopsis isabellina* have been found to metabolize EDCs such as technical nonylphenol (tNP) and 4-t-octylphenol (4-t-OP) with high removal rates exceeding 90% within 12 hours at an initial concentration of 25 mg/L (Janicki et al., 2018). Yeasts such as *Candida rugopelliculosa* have also demonstrated the ability to utilise and degrade various phenolic pollutants, including BPA and 4-methylphenol, under aerobic conditions (Rajendran et al., 2017). In addition to bacteria, fungi and microalgae have shown promise in removing EDCs from aquatic ecosystems. Species such as *Chlorella vulgaris*, *Chlamydomonas reinhardtii*, and *Selenastrum capricornutum* have been observed to absorb and degrade hormones like β -estradiol (E2) and 17 α -ethynylestradiol (EE2) (Gao et al., 2020; Mishra et al., 2021). Experimental studies reporting 95-100% removal of these hormones within 7 days suggest that microalgae could serve as a low-cost and sustainable method for photooxidation and biodegradation of EDCs (Gao et al., 2020).

The efficiency of microbial biodegradation in aquatic environments is significantly influenced by various ecological factors, including water composition, salinity, temperature, carbon and nitrogen sources, moisture content, pH, and inoculum concentration (Bhatt et al., 2019; Megharaj et al., 2011; Wu et al., 2014). Microorganisms possess remarkable catabolic potential as they utilise specific genes, enzymes, and metabolic pathways to facilitate pollutant degradation. These mechanisms enhance remediation efficiency and drive the evolution of novel microbial traits and adaptive capabilities (Liu et al., 2020; Schöler et al., 2017; Widada et al., 2002).

2.6. Interactions between Microbial Communities and EDCs

The efficiency of contaminant removal or degradation from water depends on multiple factors, including the quality of the source water, the treatment processes used, and the intrinsic chemical properties of pollutants, such as aromatic carbon content, functional group composition, molecular weight, and relative

hydrophobicity (Y. Luo et al., 2014; Santos et al., 2013; Vieno et al., 2007). While several physical and chemical remediation techniques have been developed to address various environmental pollutants, none have been able to completely remove the pollutant load. This limitation has led to the need for more efficient, eco-friendly biological methods that utilise whole microorganisms, such as fungi, bacteria, algae, and yeasts—or their enzymatic metabolites (El-Sheekh & Mahmoud, 2017; Ahirwar et al., 2020). These biological processes offer advantages such as low energy requirements, reduced processing time, minimal environmental hazards, and cost-effectiveness (Monteil et al., 2019). Furthermore, microbial bioremediation transforms harmful contaminants into less toxic by-products, including carbon dioxide (CO₂), water (H₂O), inorganic salts, and microbial biomass (S. Das & Dash, 2014).

Microbial remediation enhances degradation efficiency through the secretion of extracellular and intracellular catalytic enzymes that accelerate the transformation of a wide range of toxic organic pollutants (Karigar & Rao, 2011; Saravanan et al., 2021). Extensive research have demonstrated the ability of bacterial and filamentous fungal strains to degrade EDCs such as 17 β -estradiol (E2), EE2, BPA, nonylphenol (NP), TCS, and ATZ, as well as other persistent organic pollutants including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and certain pharmaceuticals (Cajthaml et al., 2009; Roccuzzo et al., 2021; Schmidt et al., 2017). Among these microorganisms, white-rot fungi are particularly notable for their ability to degrade structurally complex and recalcitrant compounds.. Through the production of non-specific oxidative enzymes such as laccases, manganese peroxidases (MnP), and lignin peroxidases (LiP), white-rot fungi can oxidise phenolic and non-phenolic EDCs—including BPA, NP, EE2, and TCS—thereby reducing both their concentration and estrogenic activity (Cajthaml et al., 2009; Grelska & Noszczyńska, 2020; Zhuo & Fan, 2021). The high redox potential of these enzymatic systems enables the breakdown of complex and persistent organic pollutants that pose risks to human and animal health.

Bacteria also play a crucial role in removing organic and inorganic pollutants from the environment. Their ability to metabolize EDCs depends on key factors such as nutrient availability, electron donors,

acceptors, and essential growth ions (Ahmad et al., 2017; Aragaw, 2021). Many bacteria have evolved new catabolic pathways to utilise EDCs as sources of carbon, energy, or nutrients, or simply to detoxify them (Chakraborty & Das, 2016; Roccuzzo et al., 2021). However, bacterial remediation efficiency is strongly influenced by environmental pH, as most neutrophilic wastewater bacteria exhibit optimal growth and enzymatic activity within a narrow pH range (typically pH 6.5-8.0). Deviation from this optimal range can suppress cellular metabolism, reduce enzyme stability, and impair membrane transport processes, thereby lowering degradation rates (Madigan et al., 2018; Chen et al., 2015). For example, ATZ biodegradation is strongly influenced by environmental pH, as both microbial growth and the activity of ATZ-degrading enzymes (e.g., *AtzA–AtzC* involved in s-triazine ring hydrolysis) are optimal under near-neutral conditions. Deviations toward acidic pH have been reported to reduce degradation rates due to decreased enzyme stability and altered substrate availability (Mandelbaum et al., 1995; Udiković-Kolić et al., 2012).

Notable bacterial genera involved in the degradation of EDCs include *Acinetobacter*, *Pseudomonas*, and *Novosphingobium* (Zhang et al., 2016). For example, *Pseudomonas* and *Acinetobacter* species have been shown to degrade alkylphenols such as nonylphenol and octylphenol at concentrations ranging from 1 to 50 mg/L, achieving removal efficiencies exceeding 70% under aerobic conditions (Zhang et al., 2016). Similarly, *Novosphingobium* strains have been reported to metabolise bisphenol A (BPA) at initial concentrations of 5-20 mg/L through enzymatic ring-cleavage pathways, resulting in substantial reductions within days of incubation (Zhang et al., 2016). Algae and selected yeast strains have also demonstrated the ability to remove EDCs in experimental systems. Algal-mediated removal of compounds such as estrone, 17 β -estradiol, and BPA has been reported at initial concentration below 1 mg/L, with removal efficiencies ranging from 60% to near complete elimination depending on algal species, light intensity and cultivation conditions (Bai & Acharya, 2019). Algae can mitigate EDCs through a combination of photodegradation, intracellular biodegradation, and biosorption or bioaccumulation. However, several studies have shown that elevated BPA concentrations (>20 mg/L) can inhibit algal

growth, reduce photosynthetic activity, and consequently diminish remediation efficiency (Alazaiza et al., 2022; Gulnaz & Dincer, 2009).

Recent research has shown that combining bacterial and fungal consortia can enhance the biodegradation of emerging pollutants. A bacterial consortium consisting of *Pseudomonas umsongensis*, *Bacillus mycoides*, *Bacillus weihenstephanensis*, and *Bacillus subtilis*, when paired with a fungal consortium of *Mucor circinelloides*, *Penicillium daleae*, *Penicillium chrysogenum*, and *Aspergillus niger*, has been found to effectively eliminate the potential adverse effects of certain EPs such as monochlorobenzene (CB) (Bhatt et al., 2021; Cheng et al., 2017; Zaborowska et al., 2019). This synergistic approach enhances pollutant degradation by leveraging the diverse metabolic capabilities of both bacteria and fungi.

Additionally, the interaction between algal and bacterial communities in WSPs has gained significant scientific interest due to its impact on treatment efficiency (Cantera et al., 2021; L. Liu et al., 2020). Low water turbidity enhances light penetration in the water column, thereby promoting algal photosynthesis and increasing dissolved oxygen (DO) concentrations. Elevated DO levels support aerobic heterotrophic bacteria, which more efficiently degrade organic matter, reducing biochemical oxygen demand (BOD) and releasing inorganic nutrients that further stimulate algal growth. This synergistic interaction between algae and aerobic bacteria is a defining feature of well-functioning WSPs and has been widely reported in pond-based treatment systems under conditions of high solar irradiance and low suspended solids (Craggs et al., 2012; Mara, 2013; Shilton & Harrison, 2003). This symbiotic relationship enhances the performance of WSPs by promoting efficient organic matter decomposition and nutrient recycling (Srivastava et al., 2020). Algae rapidly proliferate, absorbing inorganic nitrogen and phosphorus while simultaneously supplying DO, which is essential for bacterial metabolism. This interaction contributes to the reduction of BOD and chemical oxygen demand (COD), improving the overall efficacy of WSP-based wastewater treatment systems (Chen et al., 2019; Mahapatra et al., 2022). The increasing body of research on microbial consortia, coupled with advancements in understanding microbial interactions in

WSPs, highlights the potential for bioremediation to serve as a sustainable and efficient alternative for managing emerging pollutants and EDCs in contaminated environments (Adamu et al., 2023; Chaturvedi et al., 2023; Shaheen et al., 2024; H. Zhang et al., 2021).

2.7. Environmental and Operational Factors Affecting EDC Removal in WSPs

The removal of EDCs in WSPs is a complex process that is influenced by multiple factors, including design parameters (e.g., sludge age, hydraulic retention time [HRT], organic loading rate [OLR], environmental conditions [sunlight, temperature, pH, toxic compounds], type of sludge, and operational conditions) (Clara et al., 2005). While several studies have assessed the behaviour of EPs in WSPs, published data on the concentrations and removal efficiency of EDCs in these systems remain limited (Brandt et al., 2013; Froehner et al., 2011; Queiroz et al., 2012; Ternes et al., 1999).

Temperature plays a critical role in the performance of WSPs, particularly in regulating the activity of algae and bacteria involved in micropollutant biodegradation. Studies have shown that removal rates of pharmaceuticals, personal care products (PPCPs), and EDCs tend to be higher during warmer months than during colder seasons (X. Li et al., 2013; Matamoros et al., 2015). For instance, Li et al. (2013) observed greater removal efficiency of PPCPs and EDCs in a US lagoon system during September (average temperature 20°C) compared to November (average temperature 4°C). Similarly, Matamoros et al. (2015) found that both biodegradable compounds (e.g., oxybenzone, naproxen, benzothiazole, methyl paraben, triphenyl phosphate) and moderately volatile compounds (e.g., galaxolide, tonalide) were more effectively removed in summer (26°C) than in winter (11°C). Biodegradation processes are generally optimised at the physiological temperature range of microorganisms, which promotes maximum enzymatic activity and microbial metabolism (Saravanan et al., 2021).

Light exposure significantly enhances the performance of WSPs by driving photosynthesis, which supports algal growth and oxygen production (Davies-Colley et al., 2005; Heaven et al., 2005). Sunlight also plays a crucial role in the removal of EPs via photodegradation, particularly through ultraviolet (UV)

radiation (200–400 nm), which can directly or indirectly degrade contaminants (Boreen et al., 2003; Hora et al., 2019; Mayer & Ryan, 2017; Rivera-Utrilla et al., 2013). Moreover, pH fluctuations in WSPs affect EDC removal. At neutral pH 7, acidic pharmaceuticals such as gemfibrozil, diclofenac, ibuprofen, and naproxen exist primarily as anions and remain in the dissolved phase, from which they are predominantly removed through biodegradation and photodegradation (Linge et al., 2021; S. Wang et al., 2021). The pH of WSPs varies diurnally and with depth due to algal photosynthesis and respiration, typically ranging from a pH of 7 to 9.4 (Kayombo et al., 2002; Liu et al., 2018). Low pH values (e.g., <7) can indicate incomplete CO₂ consumption by algae and may signal system overload, leading to reduced dissolved oxygen (DO) concentrations and odour production (Von Sperling, 2015a).

Hydraulic retention time (HRT), defined as the average time wastewater remains in the pond before discharge, plays a significant role in sedimentation and pollutant removal. Longer HRTs improve the settling of suspended particles and allow for extended microbial interactions, leading to better degradation of EPs (Hijosa-Valsero et al., 2010; Li et al., 2013). Research has highlighted the importance of long HRTs in achieving higher EDC removal rates (Gómez-Gutiérrez et al., 2007; Servos et al., 2005). In WSPs, pollutant removal typically occurs in the early stages of treatment, particularly in anaerobic or facultative ponds where microbial degradation is most active. Pond depth influences the retention period and affects sunlight penetration, which in turn impacts disinfection mechanisms in algal ponds. Liu et al. (2018) found that, while the reduction of faecal bacteria in wastewater increased with solar intensity and HRT, it decreased with depth. In colder climates, nutrient removal can be particularly challenging. Currently, there is a lack of sufficient research on the seasonal trends in wastewater lagoon mechanisms for nutrient (nitrogen and phosphorus) removal in cold regions, particularly in Canada. Environmental conditions, including seasonal variations, significantly affect EDC removal efficiency in WSPs (Smyth et al., 2018; D. Zhao et al., 2020). Seasonal changes impact temperature, sunlight duration, intensity, and biomass production, leading to variations in removal performance. The effects of seasonality are most

evident in colder climates where ambient temperatures drop to $\leq 4^{\circ}\text{C}$, compared to more temperate regions with winter temperatures $\geq 11^{\circ}\text{C}$ (Matamoros et al., 2016).

2.8. Advances in Monitoring and Analysing EDCs and Microbial Diversity

Recent global initiatives have been driving the adoption of sustainable technologies in analytical chemistry, particularly in sampling and sample preparation methods for detecting and characterising EPs. One of the most notable advancements is the solid-phase microextraction (SPME) chemical biopsy approach, which provides a high-throughput and flexible format for quantifying EPs (Gong et al., 2022). Solid-phase microextraction (SPME) is enhanced by matrix-compatible thin film coatings and balanced coverage phenomena, which allow for effective elimination of matrix effects while enabling the extraction of a broad range of compounds with varying physicochemical properties. This method is highly versatile and can be used with multiple analytical techniques, including gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS), and direct MS coupling, making it a valuable tool for comprehensive EC analysis (Meher & Zarouri, 2025; W. Wang et al., 2023). Depending on the target contaminants, a variety of instrument-detector combinations can be employed to optimise analytical results. Techniques such as GC/MS, High-Performance Liquid Chromatography-Mass Spectrometry (HPLC/MS), Liquid Chromatography-Ultraviolet (LC/UV), and GC/tandem mass spectrometry (MS/MS) provide highly accurate quantitative data. However, these sophisticated instruments require substantial capital investment and specialised personnel training, making them resource intensive (Briciu et al., 2009). Nonetheless, their ability to deliver precise and comprehensive quantitative results makes them indispensable in the field of analytical chemistry for the monitoring of EPs.

For decades, water contamination for drinking and recreational use has been primarily assessed using culture-based enumeration and detection of faecal indicator bacteria (FIB), such as *Escherichia coli*, total coliforms, and *Enterococci*. This approach has long been considered the 'gold standard' for evaluating

microbial water safety (José Figueras & Borrego, 2010). Traditional culture techniques rely on the isolation and characterisation of microorganisms using commercial growth media, such as Luria–Bertani (LB) medium, Nutrient Agar, and Tryptic Soy Agar (TSA) (Kirk et al., 2004). However, recent advancements in molecular methods and Next-generation sequencing (NGS) have transformed water quality assessment by enabling the analysis of entire waterborne microbial communities. Next-generation sequencing (NGS) is a high-throughput molecular technique that enables parallel sequencing of millions of DNA fragments, allowing detailed characterisation of microbial community composition and diversity from environmental samples (Tan et al., 2015; Wani et al., 2021). These innovations have facilitated the development of new microbial indicators and markers for source tracking and the study of microbially mediated processes. Earlier studies investigating the relationship between microbial communities and water quality predominantly used 454 pyrosequencing (Roche) due to its relatively long read lengths (initially 110 bp, now ~1000 bp) and optimised sequencing conditions with robust bioinformatic workflows (McLellan et al., 2010; Sergeant et al., 2012; Van Dijk et al., 2014; Vandewalle et al., 2012).

The introduction of the Illumina (Solexa) platform, initially designed for genome sequencing with shorter read lengths (35 bp), gradually gained prominence in environmental analysis as paired-end sequencing allowed read lengths comparable to 454 pyrosequencing (Van Dijk et al., 2014). More recently, the Illumina MiSeq platform has been widely adopted for shotgun amplicon sequencing of small subunit ribosomal RNA (SSU rRNA) (Newton et al., 2015). Studies have demonstrated that MiSeq outperforms 454 pyrosequencing and Ion Torrent in sequencing depth, coverage, detection sensitivity, and false positive rates (J. Z. Li et al., 2014; Loman et al., 2012; Sinclair et al., 2015). To complement these advances, several open-source bioinformatics platforms, including MOTHUR (Kozich et al., 2013) and QIIME (Caporaso et al., 2010), have been optimised for the analysis of Illumina-generated paired-end sequences, further improving microbial community characterisation in water quality research. These developments highlight the shift from culture-based methods to high-throughput molecular techniques, enabling more comprehensive and precise microbial assessments in aquatic environments.

2.9. Challenges and Knowledge Gaps

Despite the growing concern over contamination by EDCs and its adverse effects on human health and ecosystems, a comprehensive review of the impacts, removal strategies, and limitations of current treatment technologies remains lacking. Given the persistent nature of EDCs, understanding both their characteristics and the effectiveness of available treatment approaches, along with their advantages and limitations, is crucial for developing more efficient and sustainable removal processes (Katibi, Yunus, Man, Aris, bin Mohd Nor, et al., 2021b). Existing treatment technologies rely on physical, chemical, and physicochemical mechanisms, which are largely dictated by the physicochemical properties of EDCs. However, eliminating these contaminants using a single treatment method is often insufficient, necessitating the integration of multiple treatment techniques to overcome the limitations of individual approaches (Khan et al., 2022). In this context, the appropriate selection and optimisation of treatment technologies are pivotal in enhancing EDC removal from wastewater. Advanced hybrid treatment systems that combine biological, chemical, and physical processes offer promising solutions for improving treatment efficiency and addressing the persistent challenges posed by EDC contamination (Azizi et al., 2022).

The advent of NGS has transformed the study of microbial communities, enabling high-throughput identification and relative quantification of microorganisms. A widely used approach, namely metabarcoding, utilises sequencing of genes with both conserved and highly polymorphic regions, such as 16S rDNA, to classify microbial populations on a large scale (Caporaso et al., 2010, 2012). In WSPs, biodegradation plays a key role in pollutant removal where microorganisms—including bacteria, fungi, and algae—break down organic compounds into simpler structures, sometimes leading to complete mineralisation (Garcia-Rodríguez et al., 2014). This biologically mediated degradation occurs under aerobic and anaerobic conditions and is considered one of the major pollutant removal mechanisms in WSPs (Garcia-Rodríguez et al., 2014; Li et al., 2013). However, there remains a lack of clarity regarding the specific contribution of individual microorganisms versus microbial communities in the biodegradation

of EPs, such as pharmaceuticals and personal care products (PPCPs), particularly in activated sludge treatment (Wang & Wang, 2016). More critically, no studies have comprehensively examined the role of microbial communities in the removal of EPs in WSPs, highlighting a significant knowledge gap. Additionally, despite the well-documented effectiveness of pond systems in wastewater treatment, a comprehensive understanding of the research status and current challenges in WSP technology remains incomplete. Addressing these gaps was deemed essential for optimising WSPs as cost-effective and sustainable wastewater treatment solutions.

The presence of harmful chemicals and their emerging derivatives in the environment, particularly aquatic systems, has become a significant global environmental concern. In recent years, extensive efforts have been dedicated to developing treatment technologies aimed at removing EPs from aqueous solutions. However, a knowledge gap persists, highlighting the substantial challenge of ensuring the safety of reused water intended for human consumption. Relying on a single treatment method for the complete removal of emerging contaminants has proven insufficient, necessitating the integration of multiple treatment techniques to overcome the limitations of individual approaches (Ahmed et al., 2021; Rout et al., 2021). These techniques can still play a crucial role in concentration pre-treatment and can be progressively incorporated into advanced treatment systems to enhance the degradation of EPs in wastewater. A synergistic approach that combines and optimises stand-alone solutions for eliminating or degrading environmentally significant pollutants may prove to be a key strategy in the future. Further research should focus on identifying critical components and modification procedures to improve treatment efficiency and gain a deeper understanding of pollutant degradation mechanisms. An exciting avenue of research in the removal of EPs involves the development of innovative materials and nano-catalysts for biological treatment processes. These advancements align with green chemistry principles, offering the potential to design highly efficient and environmentally sustainable treatment methods.

2.10. Conclusion

The increasing presence of emerging pollutants (EPs), particularly endocrine-disrupting chemicals (EDCs) in aquatic environments presents a significant global challenge. These contaminants originate from diverse sources, including pharmaceuticals, personal care products, pesticides, industrial chemicals, and plasticizers, and are often resistant to conventional wastewater treatment methods. Their persistence in water bodies poses serious risks to human health, aquatic ecosystems, and biodiversity, necessitating urgent and effective mitigation strategies. Levels ranging from ng/L to µg/L can induce endocrine disruption. Compounds such as bisphenol A, nonylphenol, estrone, and 17β-estradiol have been reported to alter hormonal regulation, reproductive development, and population in fish and other aquatic species at concentrations below 1 µg/L, while chronic human exposure has been associated with metabolic and reproductive disorders. Conventional wastewater treatment processes typically achieve partial removal of EDCs, with reported removal efficiencies ranging from 20–70%, resulting in effluent concentrations that remain biologically active. This highlights the limitations of single-treatment approaches in effectively removing EPs and underscores the need for integrated treatment techniques that combine physical, chemical, and biological methods. Waste stabilization ponds (WSPs) have emerged as a promising natural and cost-effective treatment system that is capable of reducing concentrations of EPs through biodegradation, photodegradation, and adsorption with reported removal efficiencies ranging from 60% to over 90% depending on compound. However, the removal efficiency of WSPs is influenced by multiple environmental factors, including temperature, hydraulic retention time (HRT), pH, light intensity, and microbial activity. The role of microbial communities in the degradation of EDCs remains an area of limited research and is fraught with significant knowledge gaps. Microbial diversity studies suggest that bacteria, fungi, and algae play crucial roles in biodegradation processes, but the specific metabolic pathways and interactions involved require further investigation. The use of NGS has provided new insights into microbial community ecology in wastewater systems, yet there is still an inadequate understanding of microbial contributions to EDC removal in WSPs.

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Chapter 3

Monitoring endocrine-disrupting chemicals and microbial diversity in wastewater treatment maturation ponds

3.1. Introduction

In recent years, rapid industrialisation and urbanisation have released numerous unregulated chemical and biological substances into the environment. These substances are commonly known as emerging pollutants (EPs) (Petrie et al., 2014). These pollutants originate from various sources, including industrial processes, agricultural activities, pharmaceuticals, and urban activities. EPs encompass over 20 classes of compounds, including persistent organic pollutants (POPs), pharmaceuticals, personal care products (PPCPs), veterinary drugs such as antibiotics, endocrine-disrupting chemicals (EDCs), and nanomaterials such as micro- and nano-plastics. Despite their growing presence, much remains unknown about their environmental behaviour and potential impacts (Naghdi et al., 2018; Noguera-Oviedo & Aga, 2016). Endocrine-disrupting chemicals (EDCs) are frequently detected in water bodies, and particularly in the effluent, influent, and sludge of wastewater treatment plants (WWTPs). The sources of EDCs in the environment include municipal and household wastewater, building materials, agricultural runoff, mining waste, industrial emissions, and solid waste. These pollutants are often not effectively removed from wastewater treatment plants (WWTPs), resulting in their entry into water bodies where they are taken up by various organisms (Rogowska et al., 2020; Vega-Morales et al., 2013; Zhou et al., 2019). Synthetic hormones such as 17 α -ethinylestradiol (EE2) and other EDCs such as triclosan (TCS), atrazine (ATZ), and bisphenol A (BPA) enter the environment through wastewater (Dhillon et al., 2015). For instance, EE2 is a widely used contraceptive that is found in birth control pills (Evans & Sutton, 2015), while triclosan, a broad-spectrum antimicrobial, is a key component in many pharmaceuticals, personal care products, and household items such as bedding, dishwashing products, and sporting goods (Dhillon

et al., 2015). Atrazine, once the most widely used herbicide against broadleaf weeds, was banned in the USA and Europe in 2003 due to its detection in drinking water (Székács et al., 2015). Bisphenol A is another common EDC found in a wide range of plastic consumer products, including the linings of tin cans, food and water containers, medical devices, and toys (NIEHS, 2018). These compounds remain prevalent despite conventional treatment processes and pose a significant challenge to environmental and public health due to their potential to interfere with hormonal systems even at low concentrations (Khan et al., 2020; Ortúzar et al., 2022; Rout et al., 2021). Unlike traditional pollutants, EDCs tend to be persistent and stable even at low concentrations in the environment, particularly in water bodies (Vandenberg, 2021). For instance, EDCs were detected in four rivers in the Eastern Cape, South Africa, with average concentrations of 0.1471 ug/L (bisphenol A), 0.7334 ug/L (triclosan), 0.0285 ug/L (atrazine) (Farounbi & Ngqwala, 2020), and 0.004 ug/L (EE2) (Tang et al., 2021).

Emerging pollutants, including EDCs, have become a matter of global concern due to their presence in surface water and their potential adverse effects on human health and ecosystems (Vandenberg et al., 2009; 2013). The increase in endocrine-related diseases in female and male reproductive systems and other disorders such as cancers (particularly breast, endometrial, ovarian, cervical, prostate, and testicular cancers) has heightened public concern about the effects of EDCs on human health (Bräuner et al., 2021; Corpuz-Hilsabeck & Culty, 2023; Lin et al., 2022). For instance, BPA, which is a widespread EDC, is found in many consumer products and has been linked to an increased risk of contracting certain cancers (Seachrist et al., 2016). It has also been shown to negatively affect female reproductive health and fertility (Ziv-Gal & Flaws, 2016). EE2 has been linked to endocrine disruption in aquatic species, causing feminisation and reproductive issues, with some wastewater effluent concentrations exceeding safe levels for aquatic organisms (Tang et al., 2021). The prevalence of these pollutants in surface waters underscores the need for ongoing monitoring, comprehensive risk assessments, and effective mitigation strategies to protect human and environmental health (Eladak et al., 2015; Mersha et al., 2015; Robles-Molina et al., 2014; Rochester & Bolden, 2015). These contaminants often co-exist at polluted sites

(Atteia & Guillot, 2007; Delgado et al., 2012; Dyer, 2003; Skubal et al., 2001), and therefore developing methodologies for their simultaneous and efficient degradation is desirable. While chemical and physical remediation techniques can offer rapid results, microbial degradation is eco-friendly and cost-effective for removing EDCs from contaminated environments under anaerobic or aerobic conditions. However, reported removal efficiencies may vary widely depending on compound structure, microbial community composition, environmental conditions, and the pond system design. Furthermore, the mechanisms governing EDCs removal in complex systems such as wastewater stabilization ponds (WSPs) remain insufficiently characterised, particularly in relation to indigenous microbial consortia and different concentration exposures. These inconsistencies highlight the need for further investigations into microbial and functional degradation capacity (Azubuike et al., 2016).

Most earlier biodegradation studies primarily focused on isolated pure cultures (Cerqueira et al., 2011; Mikesková et al., 2012; Tuyen et al., 2022). Aerobic bacteria belonging to the Proteobacteria, Actinobacteria, and Firmicutes phyla were identified as efficient EDC degraders (Hussain et al., 2023). Among eukaryotes, EDC-degrading agents were found within genera such as *Phanerochaete chrysosporium*, *Trametes versicolor*, *Bjerkandera adjusta*, and *Pleurotus* sp. (Daâssi & Alharbi, 2023; Křesinová et al., 2018). Presently, more than 80 bacterial genera are classified as EDC-degraders, including species of *Bacillus*, *Virgibacillus*, *Novosphingobium*, *Rhodococcus*, *Acinetobacter*, *Pseudomonas*, *Sphingomonas*, *Enterobacter*, *Klebsiella*, *Aeromonas*, *Comamonas*, *Thauera*, *Deinococcus*, *Stenotrophomonas*, and *Mycobacterium* (Budeli et al., 2021; Wojcieszynska et al., 2020). However, these isolates may not be the dominant degraders within contaminated environments (Chen et al., 2023; Lin et al., 2023; Zhang et al., 2023). Microbial consortia, with their broad enzymatic capabilities, offer an advantage over individual microorganisms due to synergistic interactions within microbial communities, leading to more effective and robust degradation of contaminants (Salam, 2024). Recent advancements in high-throughput sequencing technologies have greatly enhanced the ability to

investigate microbial ecology in environments contaminated with EDCs and their metabolic potential in degrading these contaminants.

Recently, attention has grown around characterising enriched consortia capable of degrading various contaminants (Spini et al., 2018; Vaidya et al., 2018). Understanding the ecology and phylogeny of indigenous microbial consortia from diverse contaminated sites is essential for devising effective clean-up strategies. However, the use of enriched consortia for remediating mixed EDCs remains in its early stages. Questions remain about how major degraders vary when growing on different contaminants and how degradation efficiency shifts with various initial microbial sources. The present study examined the microbial community structure and metabolic potential associated with EDC degradation in wastewater collected from maturation ponds. The objectives were to: (i) gain an overview of the targeted wastewater pond system's physicochemical characteristics, (ii) quantify the concentrations of selected EDCs in the maturation ponds, (iii) identify the microbial composition within the pond system, and (iv) predict the functional potential of the indigenous microorganisms that could be responsible for EDC removal.

3.2. Materials and Methods

3.2.1. Sampling sites and sample collection

Six samples were collected in spring and summer from a wastewater stabilisation pond (WSP) system that consists of five maturation ponds (Figure 3.1), because it is the only facility in Bloemfontein, South Africa, that still operates a series of wastewater stabilization as part of its treatment system. The system is situated a few kilometres (~1.7 km) from the Bloemspuit wastewater treatment plant (WWTP) near Bloemfontein, South Africa. The system serves a population of approximately 2 834 714 people and receives 57,000 m³/d of wastewater per day (Sello & Oke, 2022). Samples from ponds 1 (inflow; influent) located at 29°07'32"S; 26°16'32"E and 5 (outflow; effluent) located at 29°07'32"S; 26°16'06"E were collected into 1 L pre-washed and sterilised amber (Simax) glass bottles and secured with screw caps. The bottles were rinsed twice with the respective pond samples before filling, and all the samples were

collected 1 meter from the surface using a grab sampling stick. Samples were stored in ice, transported to the laboratory for further analysis, and analysed within 12 hours after sampling.



Figure 3.1: The location and the configuration of the maturation ponds in Bloemspuit, South Africa.

3.2.2. Chemical analyses

3.2.2.1. Physicochemical parameter analysis

To determine the overall quality of the maturation ponds and assess the effectiveness of wastewater treatment, parameters such as temperature, pH, electrical conductivity (EC), and total dissolved solids (TDS) were measured using a portable multi-parameter field meter (HACH SENSION + MM150, USA). Dissolved oxygen (DO) and oxidative reduction potential (ORP) were measured on-site using a portable multi-parameter meter HACH HQ40D.

3.2.2.2. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) analysis

Method for EP concentration analysis using solid phase extraction (SPE)

High purity (>98%) chemical standards for bisphenol A (BPA), atrazine (ATZ), 17 α -ethinylestradiol (EE2), and triclosan (TCS) were purchased from Sigma Aldrich (St. Louis, MO, USA), while internal standards (positive mode: atrazine-d5 and negative mode: bisphenol A-d16) were provided by the Facility for Genomics and Proteomics, Microbial, Biochemical and Food, University of the Free State. The stock solution for each standard was prepared in methanol at a concentration of 1 μ g/L. High-performance

liquid chromatography (HPLC) graded methanol (MeOH), formic acid, and ammonium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO). Ultra-pure water (18 Ω) was prepared using a Milli-Q purification device (Millipore, Billerica, MA, USA) and was used in all experiments.

Internal standards (positive mode: atrazine-D5 and negative mode: bisphenol A-D16) were added to the 500 mL samples as received, then filtered through glass fibre filters and concentrated onto methanol-conditioned C18 6 mL solid-phase extraction cartridges (Strata, Phenomenex) at a flow rate of 5 mL/min. Bound analytes were slowly eluted from the dried cartridges using 2 mL of methanol and 2 mL of ethyl acetate. The eluant was vacuum-dried (Thermo Scientific Savant Speedvac) until almost dry and then reconstituted in 1 mL of H₂O with 10 mM ammonium acetate. A calibration curve was generated from purified water samples (500 mL) spiked with all analytes at concentrations of 0.0001 ppm, 0.0010 ppm, 0.0100 ppm, 0.1000 ppm, 1.0000 ppm, and 10.0000 ppm, along with a blank (no standards added), and extracted by SPE as previously described.

Targeted analyses of EPs were performed using 2 multiple reaction monitoring (MRM) transitions per analyte (Table 3.1). After generating the standard curve, the WSP (inflow and outflow) samples were subjected to LC-MS/MS analysis. The levels of bisphenol A (BPA), atrazine (ATZ), 17 α -ethinylestradiol (EE2), and triclosan (TCS) in the water samples were determined using an AB sciEX 4000 QTRAP hybrid triple quadrupole ion trap mass spectrometer with a Shimadzu HPLC stack as a front-end. Briefly, each extracted sample (20 μ L) was separated on a C18 (150 mm x 4.6 mm, Gemini NX, Phenomenex) column at a flow rate of 300 μ L min⁻¹ using a 5 min gradient in positive ionisation mode (5% solvent A [H₂O/0.1% formic acid] to 95% solvent B [MeOH/0.1% formic acid]) and in negative ionisation mode (5% solvent A [H₂O/10 mM NH₄OH] to 95% solvent B [MeOH/10 mM NH₄OH]) with a total run time of 15 min to allow for column re-equilibration. Eluting analytes were ionised by electrospray in the TurboV ion source at 500°C heat to evaporate excess solvent, 30 psi nebuliser gas, 30 psi heater gas, and 20 psi curtain gas. In positive ionisation mode, the ion spray voltage was set at 5500 V and -4500 V in negative ionisation mode. All data acquisition and processing were performed using Analyte 1.5 AB sciEX software.

Table 3.1: List of MRM transitions for each LC-MS/MS analyte.

Mode	Q1 (m/z)	Q3 (m/z)	ANALYTE
Positive ionisation	216.100	104.000	Atrazine 1
	216.100	174.000	Atrazine 2
	221.161	179.000	Internal standard
	221.161	161.000	Internal standard
Negative ionisation	214.116	223.200	Internal standard
	214.116	142.200	Internal standard
	286.900	35.000	Triclosan 1
	286.900	33.900	Triclosan 2
	295.230	144.900	17 α -ethinylestradiol 1
	295.223	159.000	17 α -ethinylestradiol 2

3.2.3. Microbial characterisation

3.2.3.1. Genomic DNA extraction, PCR amplification, and sequencing

Prior to the DNA extraction, 1 L of each sample was centrifuged in a centrifuge (Neofuge 15R) at 5 000 x g for 5 min, and the supernatant was discarded. The obtained pellets were transferred to a 2 mL microcentrifuge tube and genomic DNA extraction was performed using the procedure described in the NucleoSpin® DNA stool kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). DNA concentration and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, NanoDrop products, Wilmington, DE, United States) and 0.8% Agarose gel electrophoresis. Each DNA extract was standardised to 100 ng/L in nuclease-free water (Whitehead Scientific, Cape Town, South Africa). Genomic library preparation and sequencing were performed at the Agricultural Research Council (ARC), Onderstepoort Veterinary Institute in Pretoria, South Africa. A directed approach was used to obtain

bacterial 16S rRNA amplicon sequencing library by amplifying a ~550 bp region located in the hypervariable V3 and V4 region of the 16S rRNA gene using region of interest-specific primers with overhang Illumina adapter overhang nucleotide sequences (16S F 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG – 3' and 16S R 5' – GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC – 3'; (Klindworth et al., 2013). Eukaryotic community sequencing libraries were obtained by amplification of the internal transcribed spacer 1 (ITS1) region using ITS1 and ITS2 primers with Illumina adapter overhang nucleotide (ITS1 5'–TCGTCGGCAG-CGTCAGATGTGTATAAGAGACAGTCCGTAGGTGAACCTGCGG – 3' and ITS2 5'–GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTGCGTTCTTCATCGATGC – 3' (White et al., 1990). The amplicons were purified using the Agencourt AMPure XP bead clean-up kit (Beckman Coulter Genomics, Danvers, MA, United States), followed by a second amplification to attach dual indices and Illumina sequencing adapters using the Nextera XT Index kit (Illumina, San Diego, CA, United States). Final purification was performed using the AMPure XP bead clean-up kit (Beckman Coulter Genomics), followed by library quantification, normalisation, and pooling and denaturing before being subjected to 2 × 300-cycle sequencing on the Illumina MiSeq using the MiSeq v3 reagent kit (Illumina). Sequences obtained were deposited into the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database. The accession number for all bacterial and eukaryotic sequences deposited into the NCBI-SRA database is PRJNA1234587.

3.2.3.2. Data processing and statistical analysis

The raw sequences obtained were analysed using Quantitative Insights into Microbial Ecology v.2, QIIME2 (<https://qiime2.org>) (accessed August 12, 2023) (Caporaso et al., 2010). The paired end reads imported into QIIME2 were quality controlled and combined using DADA2. This resulted in amplicon sequence variants (ASVs) with a 99% similarity. The ASV table was built, and chimeras were removed. The taxonomy for the bacterial 16S rRNA and eukaryotic sequences was assigned using the SILVA (v132) (Quast et al., 2013) and UNITE (v8.20) (Abarenkov et al., 2024) databases. The alpha rarefaction

curve was constructed with 1 000 iterations, which showed that the diversity present in all samples had been adequately captured. To account for differences in sequencing depth among samples, all datasets were rarefied to the minimum sequencing depth across the dataset (51,682 reads per sample) prior to diversity analyses.

The R-Studio (Posit Team, 2025), an Integrated Development Environment (IDE) for programming the R language (R Core Team, 2024), was used to analyse the processed gene data further (Oksanen et al., 2019). Homogeneity of variances for physicochemical parameters among seasons was assessed using Bartlett's test ($p > 0.05$), confirming that the assumption of equal variances was met. Preliminary analyses indicated no significant differences in physicochemical parameters across seasons (ANOVA, $p > 0.05$). Therefore, seasonal samples were pooled and treated as replicates in subsequent analyses to increase statistical power. Paired t-tests were conducted to determine the significant differences in the physicochemical parameters between the inflow and outflow of the two maturation ponds under study. Alpha diversity indices (Shannon, Observed and Chao1 test) were calculated using the Vegan R package (Oksanen et al., 2020), and Bray-Curtis distance was used to generate a dissimilarity matrix. The microbial community structure was visualised using principal coordinate analysis (PCoA). The Adonis function in Vegan for the R package was used to perform a permutational analysis of variance (PERMANOVA) test to assess differences in composition between the ponds. Preliminary analyses indicated no significant differences in microbial diversity across seasons (PERMANOVA, $p > 0.05$). Therefore, seasonal samples were pooled and treated as replicates in subsequent analyses to increase statistical power. Differences within habitats/ponds were further tested using the permutational homogeneity test of multivariate dispersions (PERMDISP) with the betadisper function from the R package Vegan v2.4-1. Capscale function from the R package Vegan v2.4-1 was used to perform distance-based redundancy analysis (db-RDA) to summarise the variation in microbial community structure in response to physicochemical parameters and EDCs.

3.2.3.3. Functional analysis

To obtain insight into the functional potential of the bacterial communities and identify putative metabolic pathways related to energy metabolism as well as the degradation and metabolism of xenobiotic compounds, specifically drug metabolism pathways, taxon-based metabolic profiling using the PICRUSt2 pipeline (Langille et al., 2013) as implemented in QIIME2 was performed with default settings. As a prerequisite for PICRUSt2 analysis, taxonomy classification was redone using reference sequences from the Greengenes 13_8 99% database (McDonald et al., 2012). The functional metagenome prediction data were analysed using the “categorise by function” command (level 3) in the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. Only KEGG pathways in energy metabolism and the degradation and metabolism of xenobiotic compounds, specifically drug metabolism pathways, were considered in the analyses of the samples. The ASVs with the highest gene count contribution values for these pathways were determined for WSP (maturation pond) samples.

3.3. Results and Discussion

3.3.1. Physicochemical parameters of wastewater maturation ponds (inflow and outflow)

Physicochemical analyses during the sampling period of six months indicated alkaline (inflow pH: 7.85 and outflow pH: 9.45), oxic (inflow DO: 7.18 mg/L and outflow: 10.81 mg/L), mesophilic (~25°C and ~21°C for inflow and outflow ponds), and oxidative (ORP of 144.80 mV (inflow) and 129.81 mV (outflow) conditions (Figure 3.2 and Table 3.2). There was a significant difference in the physicochemical parameters of the inflow and outflow ponds ($p < 0.01$). There was a notable increase in pH and DO concentrations from the inflow to the outflow, while the ORP concentration and temperature of the inflow were higher than those of the outflow. The pH values obtained in this study were higher than those of 6.10 to 7.03 reported by Igbinosa et al. (2009) during the assessment of treated wastewater effluents in a rural South African community. The permissible pH values of effluent for discharge into the environment range from 5.5 to 9.5 (Department of Water Affairs, 2010). However, direct comparison between geographically and temporally distinct systems must be interpreted with caution, as pH in wastewater

effluents could strongly be influenced by site-specific operational conditions, influent characteristics, seasonal variation, and biological activity. The elevated pH observed in the effluent is most plausibly attributed to intensified photosynthetic activity by microalgae and cyanobacteria, whereby CO₂ uptake reduces carbonic acid concentrations and shifts the carbonate equilibrium toward increased hydroxyl ion (OH⁻) production (Wetzel, 2001; Metcalf & Eddy, 2014). Such effects are particularly pronounced in shallow pond systems with high light penetration (Craggs et al., 2003; Mara, 2013). Elevated DO concentrations further support this interpretation, as algal photosynthesis enhances oxygen saturation, particularly under low turbidity and improved light availability (Falkowski & Raven, 2013; Sen et al., 2013).

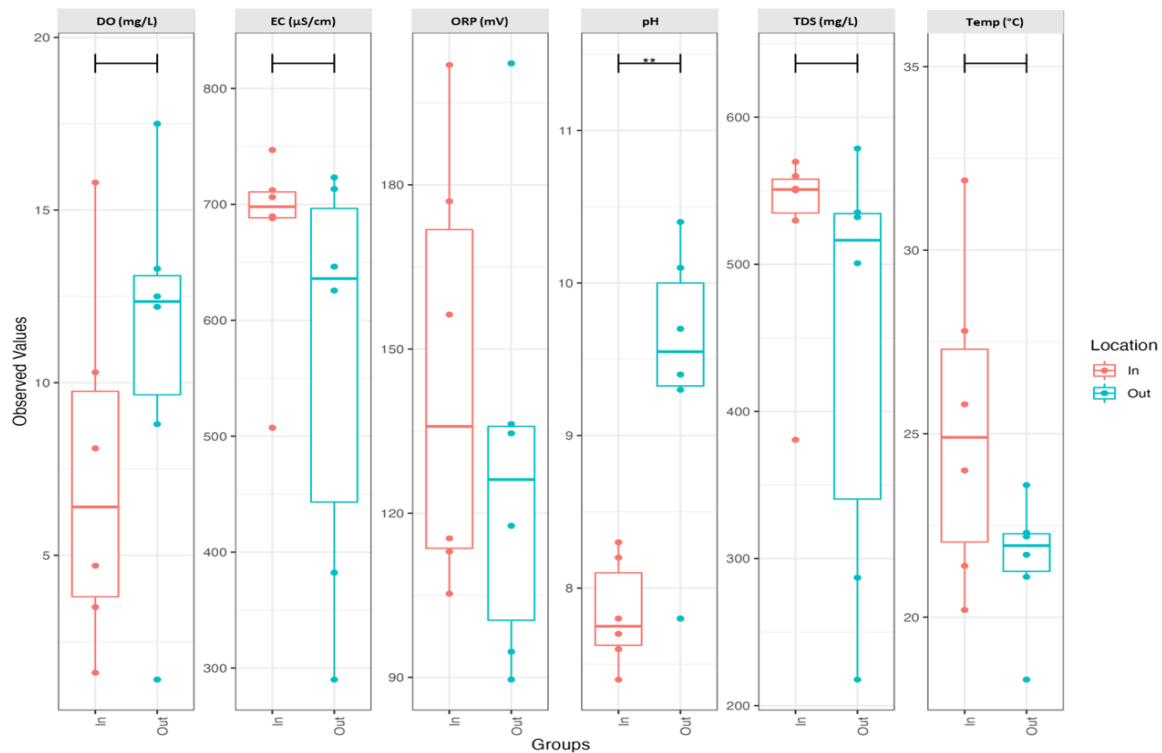


Figure 3.2: Boxplot of physicochemical parameters during sampling period between the inflow (In) and the outflow (Out) ponds of the maturation pond system (** $p < 0.01$). Average values $\pm$ SD ($n = 3$) for samples collected monthly over a six-month period. Data represent the mean of triplicate measurements at each time point to capture overall system trends.

The mean temperatures of the inflow and outflow ponds were $\sim 25.2^{\circ}\text{C}$ and $\sim 21.5^{\circ}\text{C}$, respectively ($p < 0.05$). Such a temperature range supports optimal microbial activity, including efficient decomposition

or degradation of chemical compounds (Alisawi, 2020). Theoretically, a linear relationship exists between EC and TDS, where high EC values typically correspond to high TDS. However, factors such as temperature and the presence of certain dissolved solids can influence the TDS and EC ratio. As such, EC and TDS were measured independently of each other. The electrical conductivity (EC) mean values were 675 $\mu\text{S}/\text{cm}$ (inflow) and 563 $\mu\text{S}/\text{cm}$ (outflow), reflecting moderate levels of dissolved ions typical of treated domestic wastewater effluent. These EC values fall within the permissible discharge limits stipulated in national wastewater discharge guidelines (South Africa, 2013:12).. The mean total dissolved solids (TDS) of the inflow were ~524 mg/L and ~442 mg/L (outflow). Given the proportional relationship between EC and TDS in treated effluents, the observed reduction in both parameters indicates decreased ionic strength across the treatment system. From an irrigation perspective, TDS concentrations below 700 mg/L are generally classified as posing low salinity risk to most crops under normal soil drainage conditions and are considered safe for irrigation (Alberta Agriculture and Irrigation, 2000).

Table 3.2: Physicochemical parameters of the maturation ponds (inflow and outflow).

Sampling sites	Seasons	Parameters (Mean $\pm$ SD)					
		pH	EC ($\mu\text{S}/\text{cm}$)	TDS (mg/L)	DO (mg/L)	ORP (mV)	Temperature ($^{\circ}\text{C}$)
Inflow Pond (P1)	Spring	7.40 $\pm$ 0.15	688.00 $\pm$ 0.82	550.30 $\pm$ 0.58	4.67 $\pm$ 0.02	177.00 $\pm$ 5.46	20.20 $\pm$ 0.11
		7.70 $\pm$ 0.13	712.30 $\pm$ 1.70	569.70 $\pm$ 1.53	1.59 $\pm$ 0.03	156.30 $\pm$ 5.06	21.40 $\pm$ 0.18
		7.60 $\pm$ 0.01	689.70 $\pm$ 2.36	551.30 $\pm$ 2.31	3.48 $\pm$ 0.59	201.90 $\pm$ 9.82	24.00 $\pm$ 0.23
	Summer	7.80 $\pm$ 0.10	747.00 $\pm$ 2.16	560.00 $\pm$ 2.65	8.11 $\pm$ 0.71	115.40 $\pm$ 4.09	25.80 $\pm$ 0.91
		8.30 $\pm$ 0.29	706.30 $\pm$ 6.94	529.70 $\pm$ 6.43	15.77 $\pm$ 2.14	113.00 $\pm$ 19.50	27.80 $\pm$ 0.49
		8.20 $\pm$ 0.12	507.30 $\pm$ 1.70	380.70 $\pm$ 1.53	10.31 $\pm$ 0.52	105.30 $\pm$ 6.50	31.90 $\pm$ 1.03
		7.80 $\pm$ 0.07	723.30 $\pm$ 1.70	578.70 $\pm$ 1.53	8.83 $\pm$ 0.06	202.20 $\pm$ 2.46	18.30 $\pm$ 0.10

Outflow Pond (P2)	Spring	9.70 ± 0.05	646.30 ± 0.47	532.00 ± 25.12	13.31 ± 0.26	134.60 ± 3.76	21.70 ± 0.15
		10.10 ± 0.00	625.70 ± 0.94	500.70 ± 1.15	17.49 ± 0.42	117.70 ± 5.64	22.20 ± 0.27
	Summer	9.30 ± 0.03	713.30 ± 8.99	535.30 ± 8.02	12.19 ± 0.44	89.60 ± 2.45	21.10 ± 0.22
		10.40 ± 0.09	382.30 ± 8.26	287.00 ± 7.81	12.54 ± 1.66	94.70 ± 7.63	23.60 ± 0.72
		9.40 ± 0.17	290.00 ± 2.94	217.70 ± 2.52	1.38 ± 0.42	136.30 ± 3.68	22.30 ± 0.26

3.3.2. LC-MS/MS analysis

During the sampling period, all four selected EDCs were detected regularly in both the inflow and outflow pond samples at varying concentrations (Figure 3.3). The BPA concentration in the inflow pond varied from 0.089 to 0.587 µg/L, while a range of 0.003 to 0.145 µg/L was observed in the outflow pond. A study conducted in and around Cape Town, South Africa, reported BPA in the range of 12.66 ± 0.81 µg/L from Bellville maturation pond effluents (Adeleye, 2016). This reported concentration is higher than those observed in both the inflow and outflow ponds of the present study. Regulatory limits for BPA vary across jurisdictions and environmental matrices. While the European Union and the United States have established restrictions and guideline values for BPA. Harmonised maximum contaminant levels for BPA in wastewater effluents and surface waters are not uniformly defined. In South Africa, specific regulatory thresholds for BPA in wastewater discharge or surface waters remain limited or are not explicitly stipulated in the national standards..

ATZ concentrations ranged from 0.009 µg/L to 0.134 µg/L between the inflow and outflow ponds. A comparable concentration of 0.0095 µg/L of ATZ in Uitenhage-treated effluents in the Eastern Cape, South Africa, was reported by Farounbi and Ngqwala (2020). In 2016, the Environmental Protection Agency (EPA) conducted an atrazine ecological risk assessment, determining that the scientifically

derived concentration of atrazine, measured as a 60-day average, adversely impacted the aquatic environment at 3.4 $\mu\text{g/L}$. However, considering the Scientific Advisory Panel's (SAP's) recommendation, EPA recalculated the level of concern for atrazine at 9.7 $\mu\text{g/L}$ as a 60-day average (USEPA, 2024). Although the use of atrazine was banned in Germany in 1991 (Vonberg et al., 2014), the EU has established Environmental Quality Standards (EQS) for atrazine, with an annual average of 0.6 $\mu\text{g/L}$ and a maximum allowable level of 2 $\mu\text{g/L}$ (Parlakidis et al., 2022). In the current study, atrazine concentrations in the inflow and outflow ponds were below the stipulated EU and US standards. The concentrations of EE2 in the inflow pond ranged from 0.0057 $\mu\text{g/L}$ to 125.0 $\mu\text{g/L}$ and a range of 0.009 $\mu\text{g/L}$ to 117.0 $\mu\text{g/L}$ was detected in the outflow pond. In comparison, a maximum EE2 concentration of 0.008 $\mu\text{g/L}$ in South African wastewater effluent, which is considerably lower than the values observed in the current study, was reported by Manickum and John (2014).

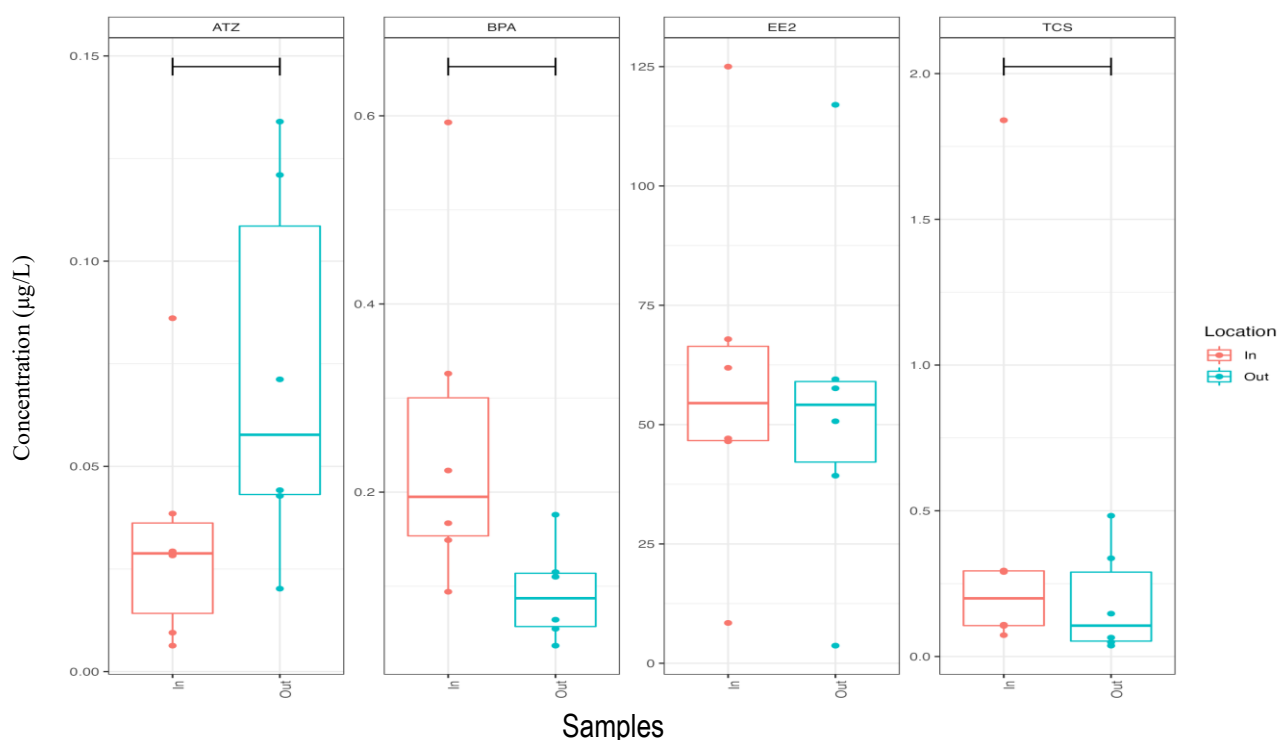


Figure 3.3: Box and whisker plots comparing EE2, TCS, BPA, and ATZ concentrations between the two WSPs. In: inflow pond; Out: outflow pond.

The mean concentration of TCS detected in the inflow pond was 0.316 µg/L, while it was 0.069 µg/L in the outflow pond. These observed concentrations were lower than the 0.09 ± 0.12 µg/L concentration in South African municipal wastewater as reported by Kanama et al. (2018). EE2 and TCS are listed on the EU watchlist, whereas the US and South Africa have not established standards for these compounds in water yet.

3.3.3. Bacterial diversity and abundance

A total of 5 177 bacterial ASVs, ranging from 190 to 705 per sample, were identified with >99% sequence identity. Of the total number of ASVs, 2 771 (representing 8.24% of the total number of sequences) were unique to inflow samples, 2 454 (7.17%) were unique to outflow samples, and 2 455 (84.58%) were shared between the two ponds under study.

The bacterial diversity of the inflow and outflow ponds, measured in terms of the number of observed ASVs per sample (richness) using the Simpson index (evenness) and the Shannon index, showed a significant difference in all tested alpha diversity matrices (Figure 3.4). High bacterial species richness was observed in the inflow pond compared to the outflow pond (Chao 1), whereas very high bacterial diversity was observed in the inflow and outflow ponds (each Shannon index (H) >3.50). However, species evenness (Simpson index, D) was slightly lower in the outflow pond than in the inflow pond. Higher Shannon and Simpson diversity indices in the inflow pond suggested a highly diverse bacterial community with relatively even species distribution. Very high Shannon and low Simpson indices of the outflow pond indicated high bacterial diversity and dominance by a few bacterial species.

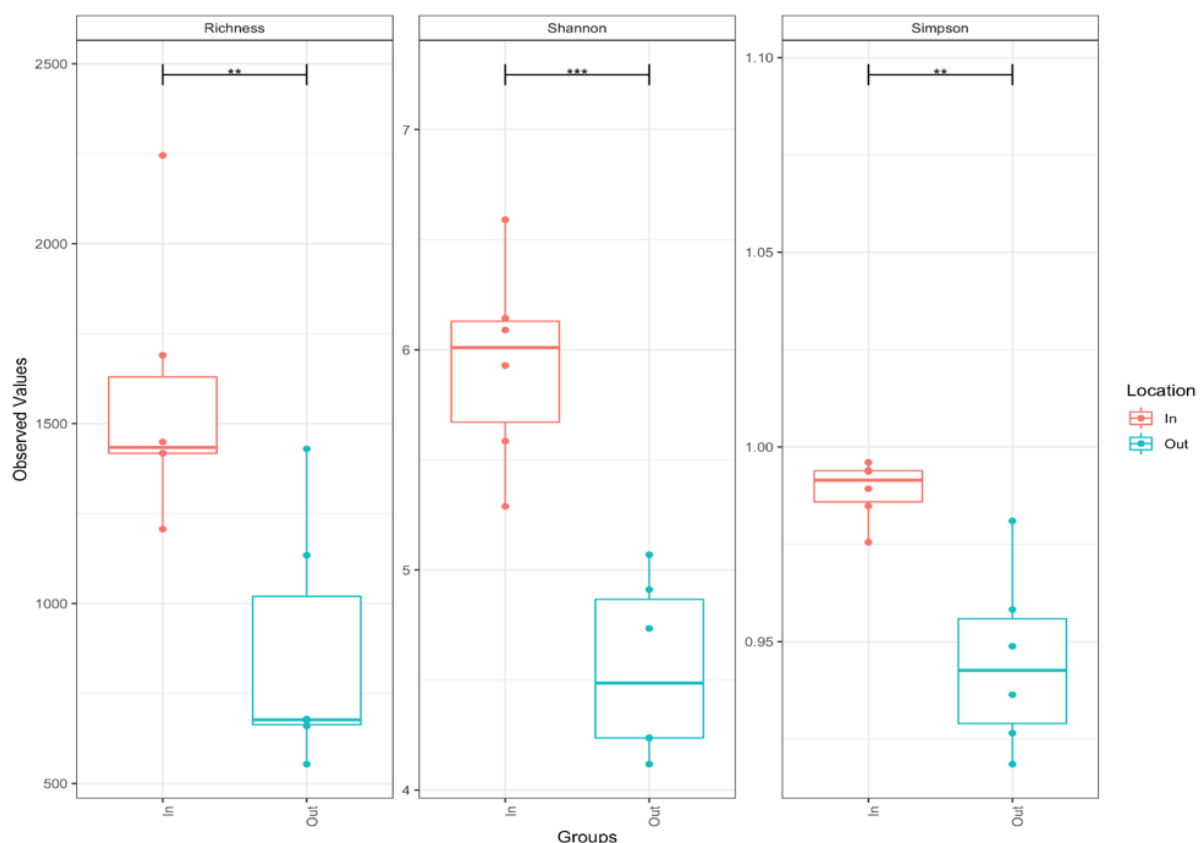


Figure 3.4: Bacterial alpha diversity measures (observed number of ASVs richness (Chao1) and Shannon diversity and Simpson index of inflow (In) and outflow (Out) ponds. The line inside each box represents the median while whiskers represent the lowest and highest levels within the 1.5 interquartile range (IQR). ** (p < 0.01) and *** (p < 0.001).

The relative abundance of bacteria at the phylum (>1%) level in all samples (n=12) showed the presence of 15 phyla, of which Proteobacteria (35.12% of ASVs) was the most predominant phylum across the inflow and outflow samples (Figure 3.5). This was followed by Bacteroidetes, Cyanobacteria, Planctomycetes, and Verrucomicrobia (18.50%, 16.13%, 7.33%, and 6.84% of ASVs, respectively). Proteobacteria was the dominant phylum as this is a fast-growing microbial community (Alotaibi et al., 2022). This concurred with a finding by Yang et al. (2020), who observed Proteobacteria as the most abundant phylum in WWTPs activated sludge (AS) samples from industrial and municipal zones in China, with the highest abundance noted in locations with human interference. Bacteroidetes, Cyanobacteria, Planctomycetes, and Verrucomicrobia were also detected in WSPs by Gómez-Acata et al. (2017). The higher diversity observed in the inlet is likely attributable to elevated organic loading and heterogeneous

substrate availability, which support a broader spectrum of heterotrophic bacteria. The influent wastewater typically contains diverse carbon sources, nutrients, and suspended solids that promote microbial coexistence and niche partitioning (Wu et al., 2019; Ju & Zhang, 2015). Conversely, as wastewater progresses through the treatment system, reductions in biodegradable organic matter, shifts in redox conditions, and increased DO, create selective conditions favouring metabolically specialised and stress-tolerant taxa (Chen et al., 2022; Saunders et al., 2016).

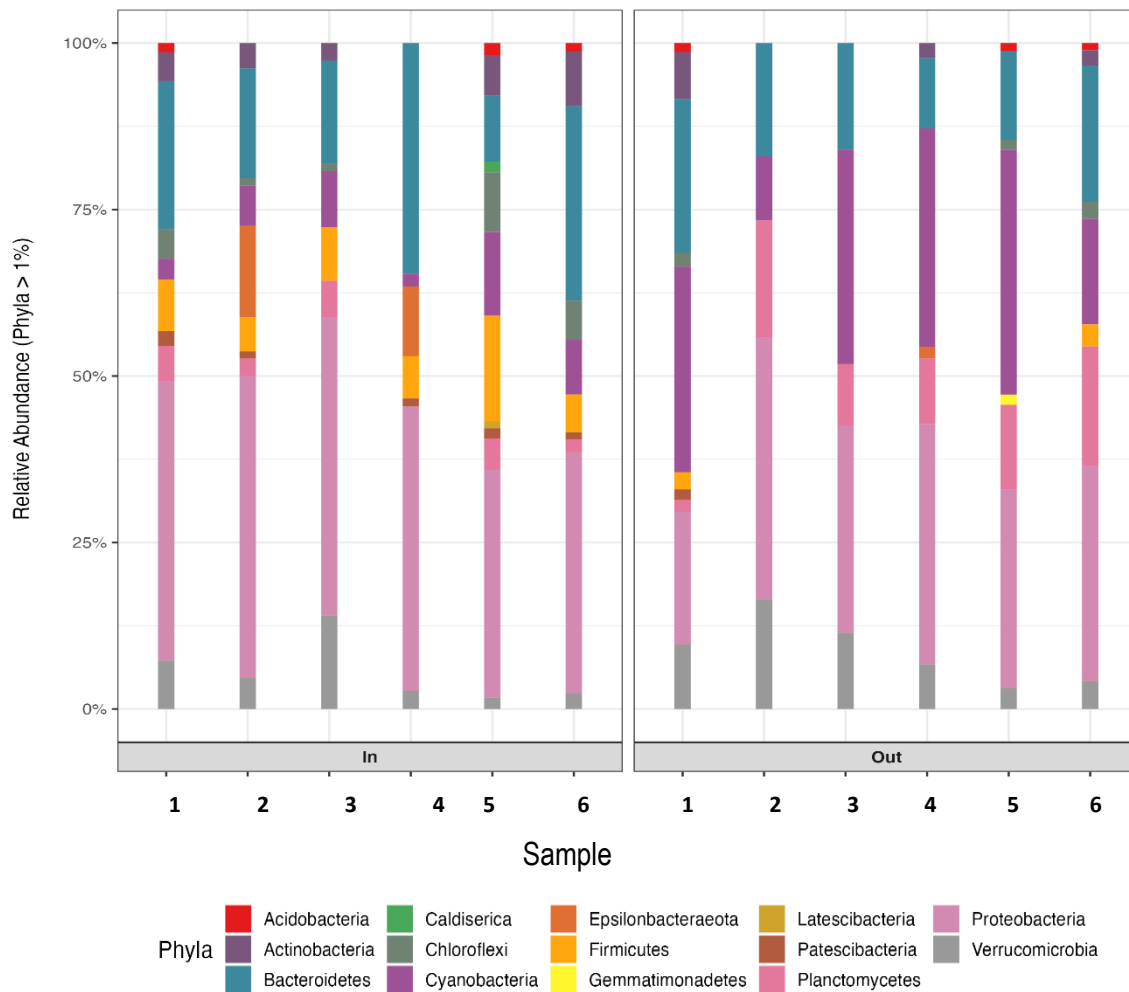


Figure 3.5: Relative abundance of bacterial taxa (phylum level) between the maturation (In: inflow and Out: outflow) pond samples. 1 to 6: inflow and outflow pond samples.

Bacteria belonging to the Bacteroidetes family are known to degrade organic compounds with high molecular weights (Kwon et al., 2019).

At the genus level, *Flavobacterium* and *Microcystis* were predominant (9.50% and 6.22% of ASVs, respectively), followed by *Dechloromonas* and *Pseudomonas* (4.19% and 3.56% of ASVs, respectively) across the inflow and outflow pond samples (Figure 3.6). There was a shift in the bacterial community of the WSP system. For instance, *Dechloromonas* and *Clostridium_sensu_stricto_1* were dominant (4.19% and 3.22% of ASVs, respectively) and were higher in the inflow pond, while *Microcystis_PCC-7914*, *Pirellula*, and *Coelastrella_sp._M60* (3.31%, 2.59% and 1.97% of ASVs, respectively) were higher in the outflow pond. *Flavobacterium* sp. was isolated from WWTPs in Korea by Ryu et al. (2007), and it reportedly degrades organic compounds in soil and water (Chaudhary et al., 2019; Saber & Crawford, 1985), while paracetamol was detected in municipal wastewater by Palma et al. (2018). However, while *Microcystis*, which is a genus of Cyanobacteria, is known to produce potent toxins, its role in degrading EPs is unknown. It has been averred that its physiological responses to such compounds can significantly influence its ecological role and the overall health of aquatic ecosystems (Wang et al., 2024). *Pseudomonas* sp. is known to degrade some emerging pollutants, including atrazine (Sene et al., 2010).

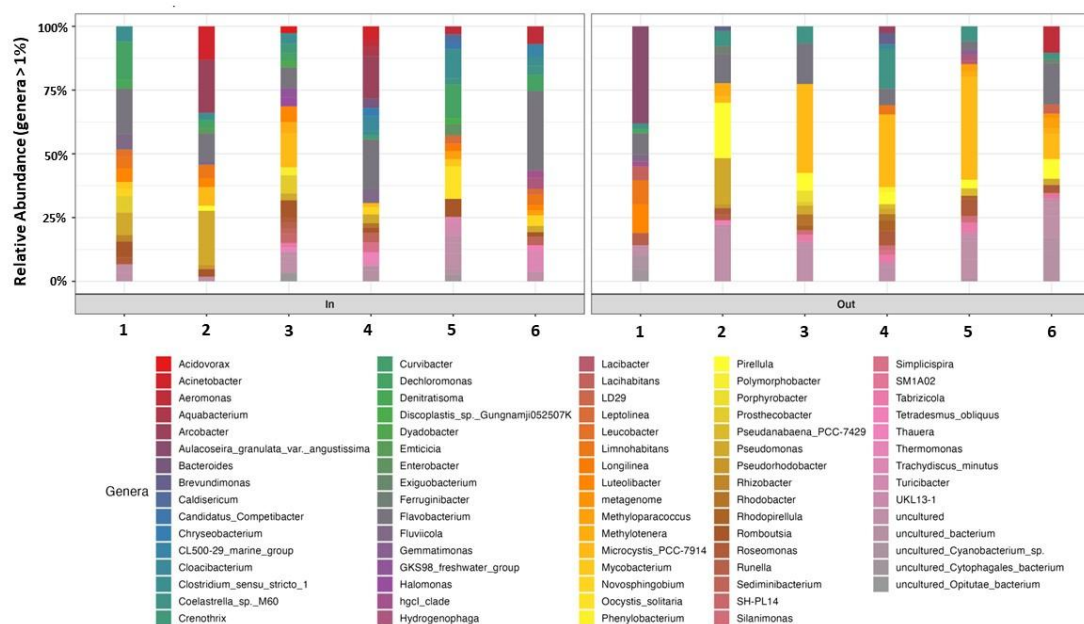


Figure 3.6: Relative abundance of bacterial taxa (genus level) between the maturation (In: inflow and Out: outflow) pond samples. 1 to 6: inflow and outflow pond samples.

Various researchers studied the core bacterial community communities present in activated sludge in wastewater treatment bioreactors or plants located in a variety of geographical locations (Nascimento et al., 2018; Wang et al., 2022; Zhang & Meng, 2021). Presently, there is limited knowledge about whether maturation ponds in different geographical locations share any commonalities in terms of bacterial communities. In the current study, it was found that a core set of bacteria existed in these ponds (Figure 3.7), indicating that the composition and diversity of bacterial communities in the partially treated wastewater found in the maturation ponds were similar even though the wastewater quality, , was significantly different among the communities, as proposed by Ragush et al. (2015). Similar core bacteria detected at phylum level (Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria) were also observed in activated sludge from five biological wastewater treatment reactors in China and the United States in microarray experiments (Xia et al., 2018). Also, it is important to note that the proportion of the phyla distribution between the current study and the earlier study showed similar trends, with Proteobacteria being the dominant phyla and Bacteroidetes, Firmicutes, and Actinobacteria present as the subdominant phyla. The dominance of Proteobacteria and Bacteroidetes detected in this study was also observed in treated wastewater in a tropical WSP study using the PCR-DGGE technique (Belila et al., 2013) and in activated sludge samples from 14 WWTPs in China using 454 pyrosequencing (Ding et al., 2013). Within the Proteobacteria community in wastewater samples, Gammaproteobacteria comprised the predominant group while the minor group contained Deltaproteobacteria. In treated wastewater from a tropical WSP, the largest group was Betaproteobacteria, and the second largest group was Gammaproteobacteria. In the shared bacterial genera group in raw wastewater, most of the identified genera, except *Comamonas*, were found in tropical WSPs (Belila et al., 2013) and activated sludge (Xia et al., 2010; Ding et al., 2012; Zhang et al., 2012; Zhang et al., 2014; Zhang and Ju, 2015; Ibarbalz et al., 2013). *Comamonas*, which belongs to the denitrifier genera group, was found to be more abundant in mainland China's activated sludge in comparison with other activated sludge samples collected in Hong Kong, Singapore, the United States, and Canada (Zhang et al., 2012). When comparing the core bacterial

groups found in the current study with those of the other studies mentioned above, bacterial community compositions were highly similar at the phylum level, suggesting that similarities in the distribution of bacterial phyla among different municipal wastewater treatment systems located at various geographical locations under different operations and different climates reflect the coherence of microbial ecology at high taxonomic levels. However, when the taxonomic division is investigated at a deeper level, there was less commonality between the bacterial genera detected in the present study than in the other studies.

It is possible that the wastewater in the inflow and outflow ponds, which consisted of domestic wastewater with no influence from agricultural activities, led to differences in bacterial community structures compared to those found in other wastewater studies. Also, the technical limitations and differences in sequencing methods, primers and depth, and PCR amplification bias between this study and other studies may have affected the comparison of bacterial communities, making biologically meaningful comparisons difficult.

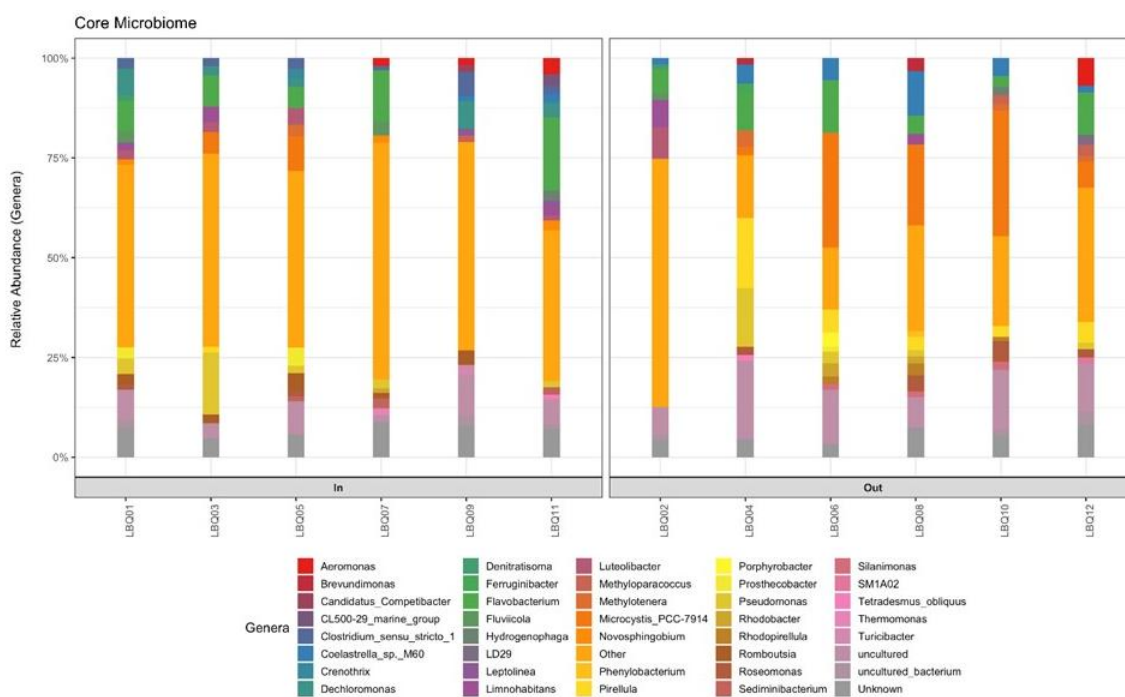


Figure 3.7: Relative abundance of core microbiome (genus level) between the maturation (In: inflow; Out: outflow) pond samples. 1 to 6: inflow and outflow pond samples.

3.3.4. Eukaryotic diversity and abundance

A total of 878 eukaryotic ASVs (> 99% similarity cut-off), ranging from 21 to 183 ASVs per sample, were found using identical sequencing depth in all samples. Of the total number of ASVs, 1 836 (representing 16.09% of the total number of sequences) were unique to inflow samples, 253 (1.09%) were unique to outflow samples, and 351 (82.82%) were shared between the locations.

The eukaryotic diversity between the inflow and outflow ponds, measured in terms of the number of observed ASVs per sample (richness), Simpson index (evenness), and Shannon index, showed a significant difference in alpha diversity (Figure 3.8). Higher eukaryotic species richness was observed in the inflow pond compared to the outflow pond (Chao 1). There was also very high eukaryotic diversity in the inflow pond (Shannon index, $H > 3.50$), while moderate (median $H = 2.50$) diversity was observed in the outflow pond. However, eukaryotic species evenness (Simpson index, D) was slightly lower in the outflow pond than in the inflow pond. Higher Shannon and Simpson diversity indices in the inflow pond suggested a highly diverse eukaryotic community with relatively evenly eukaryotic species distribution. Moreover, moderate Shannon and low Simpson indices in the outflow pond indicated moderate eukaryotic diversity and dominance by a few species.

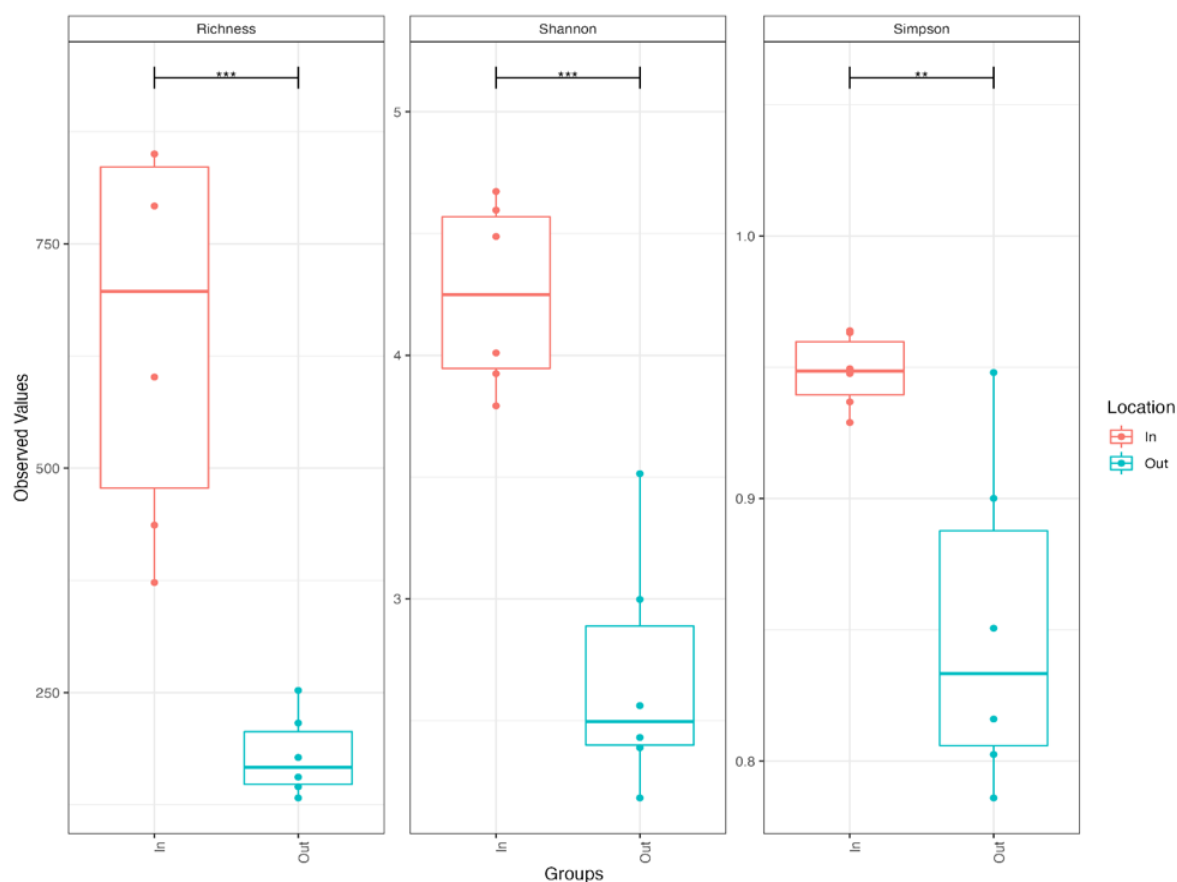


Figure 3.8: Eukaryotic alpha diversity measures (observed number of ASVs richness for Chao1, Shannon diversity, and Simpson index) of inflow (In) and outflow (Out) ponds. The line inside each box represents the median and whiskers represent the lowest and highest levels within the 1.5 interquartile range (IQR). ** ($p < 0.01$) and *** ($p < 0.001$).

The taxonomically assigned ASVs revealed that members of the WSP eukaryotic communities were mainly assigned to Ascomycota, Chytridiomycota, Rozellomycota, Basidiomycota, and Monoblepharomycota. The unidentified phyla have no reference sequences available in the UNITE database (Heeger et al., 2019). The relative abundance of eukaryotes at the phylum level (>1%) showed the predominance of unidentified organisms (68.85% of ASVs), followed by Ascomycota (13.14% of ASVs) across the inflow and outflow samples (Figure 3.9). The high proportion of unidentified eukaryotes highlight a gap in current databases and limits the ecological interpretation of the eukaryotic role. These findings are consistent with multiple environmental sequencing studies, which report that eukaryotic diversity in complex environmental samples frequently exceeds the taxonomic resolution currently

achievable using available reference databases. The underrepresentation of many environmental eukaryotes in curated repositories often constrains accurate classification (De Vargas et al., 2015; Pawlowski et al., 2012). Also, the interpretation of diversity based on ASVs should consider its multifaceted nature. Amplicon Sequence Variants (ASVs) do not correspond to species or even lower taxonomic levels, rather they represent different oligotypes of these same or different taxonomic levels, and even individuals (Salmaso et al., 2020). Therefore, while ASVs provide valuable insights into fine-scale genetic diversity, diversity estimations and ecological interpretations should be made cautiously.

There was a shift in the eukaryotic population in the WSP system as the Rozellomycota population in the inflow pond (9.66% of ASVs) was drastically reduced in the outflow pond (0.27% of ASVs), just like Basidiomycota (inflow: 0.69% and outflow: 0.07% of ASVs). However, the Chytridiomycota population increased in the outflow pond (inflow: 2.07% and outflow: 4.55% of ASVs) (Niu et al., 2017; Zhang et al., 2020). Most of the core eukaryotic phyla, including Ascomycota, Basidiomycota, Chytridiomycota, and Rozellomycota that were identified in this study, have frequently been reported in WWTP environments. Among these, Ascomycota and Basidiomycota are typically the most prevalent due to their metabolic versatility and capacity to adapt to diverse environmental conditions (Manici et al., 2024). Chytridiomycota and Rozellomycota are generally less abundant, are consistently detected, and may fulfil specialised ecological functions (Zhang et al., 2021). Notably, Rozellomycota has been associated with high organic load environments in WWTPs, suggesting a potential role in organic matter degradation. Furthermore, Rozellomycota is especially prevalent in aquatic habitats where it reportedly thrives under nutrient-rich conditions. Importantly, Chytridiomycota and Rozellomycota are part of a group of largely uncharacterised eukaryotic lineages, often referred to as 'dark matter fungi' (Grossart et al., 2016). These basal eukaryotic phyla remain poorly understood, yet they represent a significant portion of eukaryotic diversity in aquatic and engineered ecosystems. Previous studies have corroborated the presence of these phyla in WWTPs, with Niu et al. (2017) detecting Chytridiomycota, Ascomycota, and Basidiomycota while Grossart et al. (2016) identified Chytridiomycota and Rozellomycota within such systems. The inlet

samples exhibited relatively greater variability in the relative abundance of eukaryotic phyla, which may reflect the heterogeneous nature of influent wastewater. The influent typically contains diverse organic substrates, suspended solids, and microbial populations originating from domestic sources. These conditions provide a wide range of ecological niches that support diverse heterotrophic eukaryotes, particularly saprotrophic fungi such as members of the Ascomycota, which play important roles in the decomposition of complex organic matter (Grossart et al., 2016). The presence of Rozellomycota and Chytridiomycota may also be linked to their roles as parasites or decomposers associated with microbial hosts such as algae and other microorganisms commonly present in nutrient-rich wastewater environments (Kagami et al., 2007).

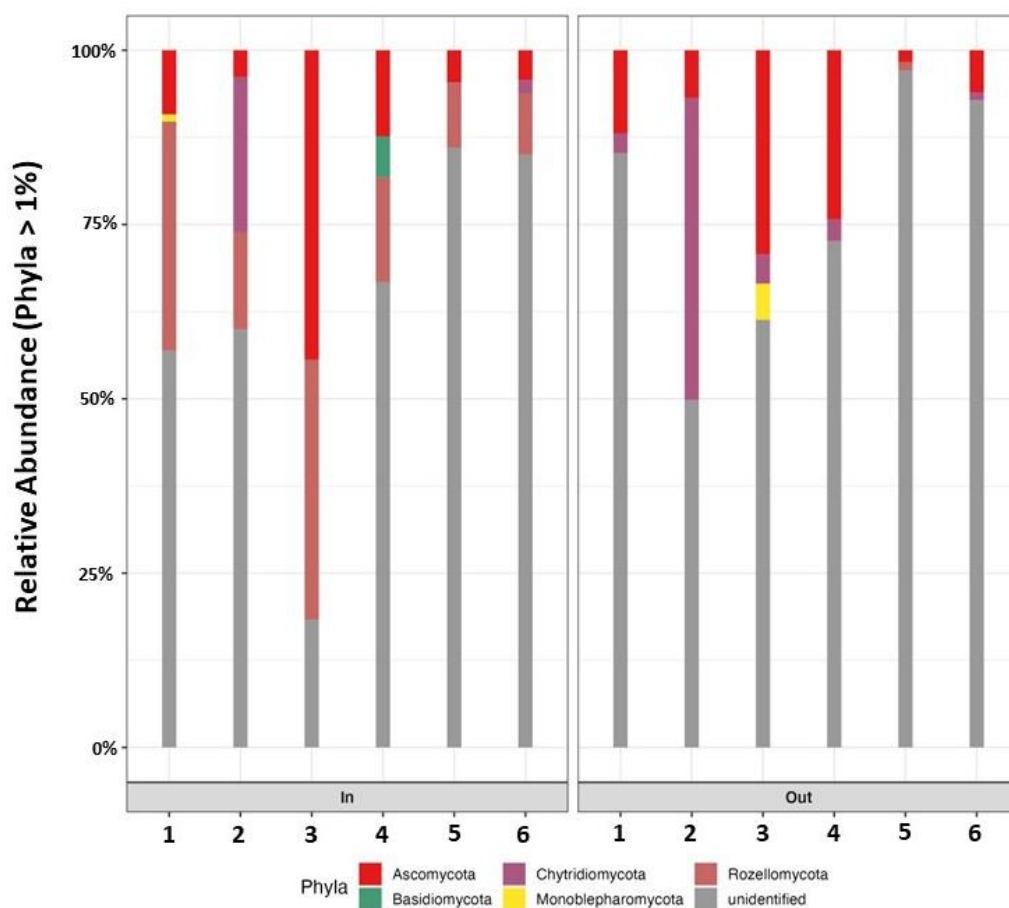


Figure 3.9: Relative abundance of eukaryotic taxa (phylum level >1%) between the maturation (In: inflow; Out: outflow) pond samples. 1 to 6: inflow and outflow pond samples.

Most eukaryotic ASVs remained taxonomically unclassified at the genus level, with unidentified organisms accounting for 85.88% of the total ASVs (Figure 3.10). Among the classified taxa, sequences assigned to the phylum Ciliophora constituted 10.86% of ASVs. While *Ciliophora* is a well-established phylum within the domain Eukaryota—variously classified under Protista, Protozoa, or Chromista—the sequences detected in this study may represent a novel eukaryotic genus closely affiliated with Ciliophora, but not yet represented in current reference databases. The detection of Ciliophora further emphasises the broad eukaryotic coverage of the ITS marker beyond fungi. There was a slight shift in the eukaryotic population. The *Betamyces* and *Metschnikowia* populations were higher in the outflow (0.36% and 0.57%) than in the inflow pond (0.14% and 0.06%). However, *Rhodotorula* sp and *Saccharomyces* sp. populations were higher in the inflow (0.40% and 0.19%) than in the outflow pond (0.2% and 0.00%). *Rhodotorula* sp. was reported to degrade phenolic compounds (Jarboui et al., 2012), while *Saccharomyces* sp. removed cadmium ion (Cd^{2+}) from wastewater (Ibrahim et al., 2022).

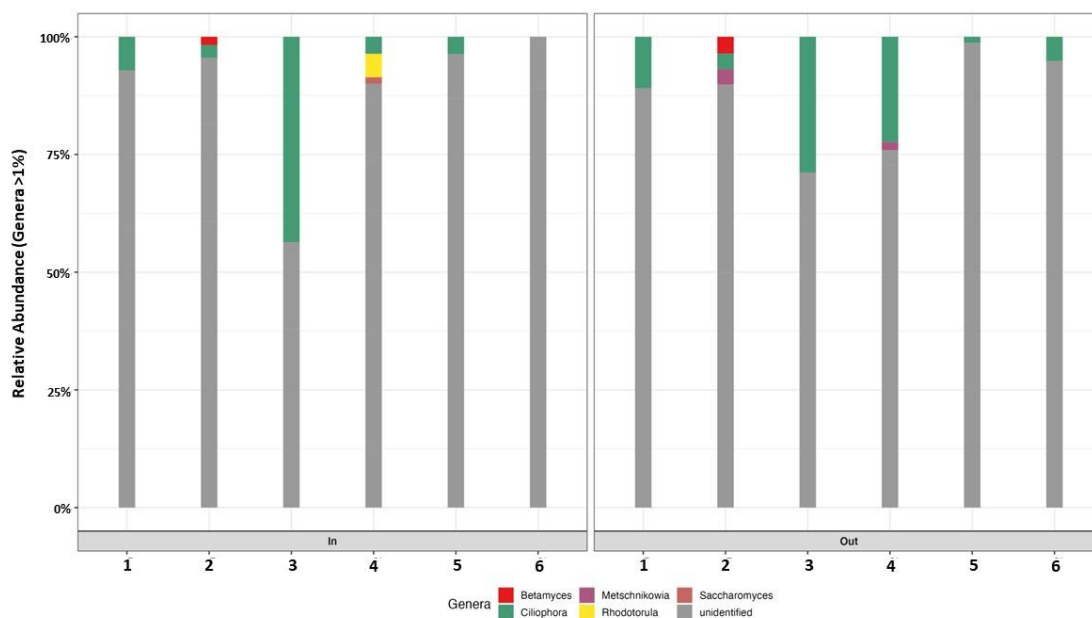


Figure 3.10: Relative abundance of eukaryotic taxa (genus level >1%) between the maturation (In: inflow; Out: outflow) pond samples. 1 to 6: inflow and outflow pond samples.

3.3.5. Relationship between the microbial community, environmental parameters, and the concentration of EDCs

Eukaryotic principal coordinate analysis (PCoA) was used to visualise and compare the dissimilarity matrix of the relative abundance of ASVs among the different samples obtained from the two ponds. The Eukaryotic PCoA showed that the WSP samples clustered according to their location (In and Out). (Figure 3.11 A). Distinct eukaryotic communities were detected between the inflow and outflow ponds (PERMANOVA $F_{1,11} = 2.78$, $R^2 = 22\%$, and $p = 0.001$) indicating that location had a significant effect on eukaryotic diversity. Environmental factors also appear to shape the microbial communities with pH (PERMANOVA $F_{1,11} = 2.48$, $R^2 = 20\%$ and $p = 0.001$), EC (PERMANOVA $F_{1,11} = 1.75$, $R^2 = 15\%$ and $p = 0.028$) and TDS (PERMANOVA $F_{1,11} = 1.87$, $R^2 = 16\%$ and $p = 0.009$) also having a significant effect and along with location explains some of the variance.

The bacterial PCoA showed that the WSP samples formed distinct clusters based on their location (In and Out). (Figure 3.11 B). Distinct bacterial communities were detected between the inflow and outflow ponds (PERMANOVA $F_{1,11} = 3.12$, $R^2 = 24\%$, and $p = 0.002$) indicating that location had a significant effect on bacterial diversity. Environmental factors also appear to shape the microbial communities with pH (PERMANOVA $F_{1,11} = 3.05$, $R^2 = 23\%$ and $p = 0.001$) also having a significant effect and along with location explains some of the variance. Variations in pH are well recognised as a major environmental driver of microbial community composition because pH influences enzyme activity, membrane transport processes, and nutrient solubility, thereby selectively favouring taxa adapted to specific pH conditions (Lauber et al., 2009). Shifts in pH between influent and effluent ponds can therefore promote the proliferation of neutrophilic or alkali-tolerant microorganisms while constraining the growth of pH-sensitive taxa. Similarly, EC and TDS reflect the ionic strength and concentration of dissolved solutes in the water, which can influence microbial community structure through osmotic stress, substrate availability, and the physicochemical conditions governing microbial metabolism (Logue et al., 2016). Other environmental gradients such as pH, salinity, and nutrient availability have been shown to be key determinants shaping

microbial assemblages in wastewater treatment systems and other aquatic environments (Wu et al., 2019). Collectively, these physicochemical factors likely contributed to the clustering patterns observed between inlet and effluent microbial communities in the present study.

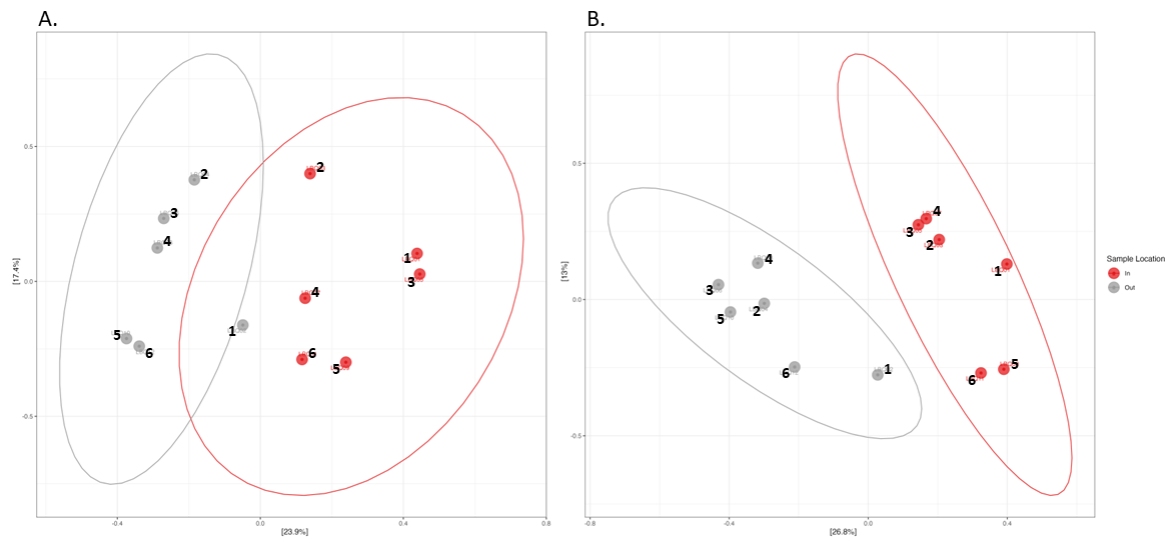


Figure 3.11: Principal coordinate analysis (PCoA) plot of beta diversity based on Bray-Curtis dissimilarities at the ASVs level of the WSP. **(A)** Eukaryotic communities PCoA data revealed that the first two PCoA components explained 23.9 and 17.4% of the variation, respectively. **(B)** Bacteria communities PCoA data revealed that the first two PCoA components explained 26.8% and 13% of the variation, respectively. Red dots: all the samples collected from the inflow pond; grey dots: all the samples collected from the outflow pond; and 1 to 6 depict the inflow and outflow pond samples.

Distance-based redundancy analysis (db-RDA) was used to investigate the association between microbial community structure and environmental (physicochemical) variables across the inflow and outflow samples of the maturation ponds. For the eukaryotic community, the first two constrained axes (CAP1 and CAP2) together explained approximately 77% of the variation in community composition (Figure 3.12 A). However, the overall db-RDA model was not statistically significant (adjusted $R^2 = 0.37$, $p = 0.33$), indicating limited explanatory power of the environmental variables. Despite the lack of overall significance, total dissolved solids (TDS) showed a marginally significant relationship with community

composition ($p < 0.1$), suggesting a potential influence. The ordination plot indicates that sample 2 was aligned along the direction of the TDS and EC vectors, suggesting a possible association. Most inflow samples appeared more tightly clustered and were more influenced by BPA, ATZ, ORP, and temperature. Outflow samples showed greater spread, with samples 3 and 4 showing alignment with DO and pH. These patterns suggest that, while overall environmental influence was weak, specific factors, such as TDS, may contribute to structuring eukaryotic communities under localised conditions.

In terms of the bacterial community, the constrained axes (CAP1 and CAP2) together accounted for a large proportion of the explained variation, but the overall model was not statistically significant (adjusted $R^2 = 0.28$, $p = 0.44$), suggesting limited predictive power of the environmental variables in explaining community composition (Figure 3.12 B). Nevertheless, individual variables showed differing degrees of association with community patterns. Among these, pH was the only statistically significant factor ($p < 0.05$), indicating its likely role as a key driver of bacterial community structure. The ordination also shows that sample 1 (Out) aligned closely with ATZ and ORP, while sample 4 (Out) was positively associated with DO and pH. Several inflow samples clustered together and appeared to be moderately influenced by EE2, BPA, and temperature. These results suggest that while environmental variables collectively explain some variation in community structure, pH may be the most influential individual factor in shaping microbial composition in maturation ponds.

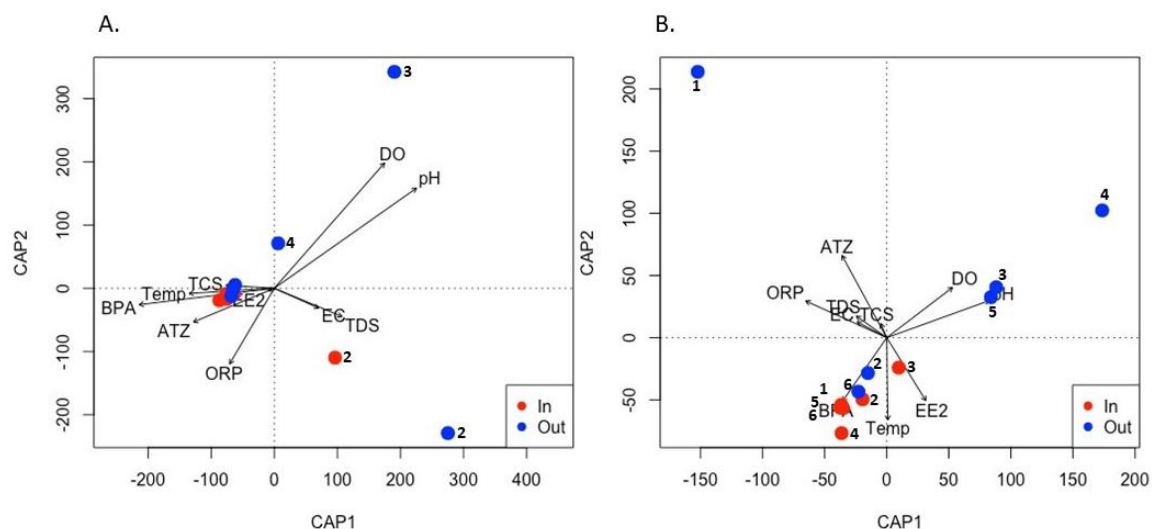


Figure 3.12: Distance-based redundancy analysis (db-RDA) biplot of WSP for microbial communities. **(A)** For eukaryotic communities, the first two constrained axes accounted for 47% and 30% of the explained variance, respectively. **(B)** For bacterial communities, the first two constrained axes accounted for 51% and 29% of the explained variance, respectively. Red dots: the inflow pond; blue dots: the outflow pond, 1 to 6: inflow and outflow pond samples. The arrows depict the physicochemical parameters (ORP, DO, TDS, pH, EC, temp, TCS, BPA, EE2, and ATZ) of the inflow and outflow ponds.

3.3.6. Functional analysis

PICRUSt2 was used to predict the presence of functional pathways related to the maturation ponds in the WSP system under study. A total of 93 996 Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs were obtained from the PICRUSt2 analysis. Among them, 18 877 orthologs had zero KO hits in all samples. Furthermore, 6 573 KEGG orthologs had KO hits of 500 or more in at least one sample out of the 12 WSP samples. The orthologs were identified and grouped according to KEGG functional pathways and Gene Ontology affiliation, which included xenobiotics, carbohydrates, and energy metabolism. The energy metabolism was predicted to be significantly higher in the bacterial community of the inflow pond ($p < 0.05$) than in the outflow pond (Figure 3.13).

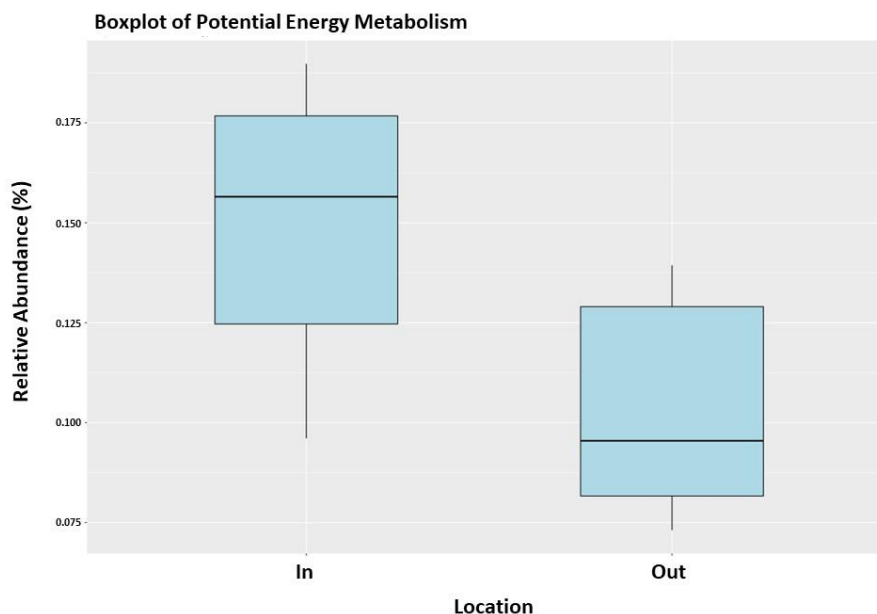


Figure 3.13: Boxplot of PICRUSt2 energy metabolism associated KEGG pathways based on the mean proportions ($p < 0.05$) and identities of 16S rRNA genes in WSP system during the spring and summer seasons between the inflow (In) and outflow (Out) ponds.

The KEGG pathway database was used to analyse the biodegradation pathways of contaminants. Using PICRUSt2, 18 KEGG-specific metabolic sub-pathways involved in xenobiotic degradation were present in the WSP system (i.e., the inflow and outflow ponds) (Figure 3.14). Of these xenobiotic degradation pathways, the atrazine degradation pathway was present and three genes (*ureA*, *B* and *C*; Urease subunits [EC: 3.5.1.5]; *atzF*; allophanate hydrolase [EC:3.5.1.54] and urea carboxylase [EC:6.3.4.6]) were more abundant than *atzD* cyanuric acid amidohydrolase (EC:3.5.2.15) between the ponds. The gene combination of *trzN*/*atzA* - *atzB*, *C* led to the transformation of atrazine to its intermediate cyanuric acid. The literature reports that the presence of the *trzN*, *atzC*, and *trzD* genes indicates atrazine degradation via the hydroxy-atrazine pathway (*trzN*) to cyanuric (*atzC*), followed by biuret, NH_3 , and CO_2 (*trzD*) (Espín et al., 2020). However, the presence of genes does not imply that genes are functional, and this could have been why atrazine accumulated in the outflow pond.

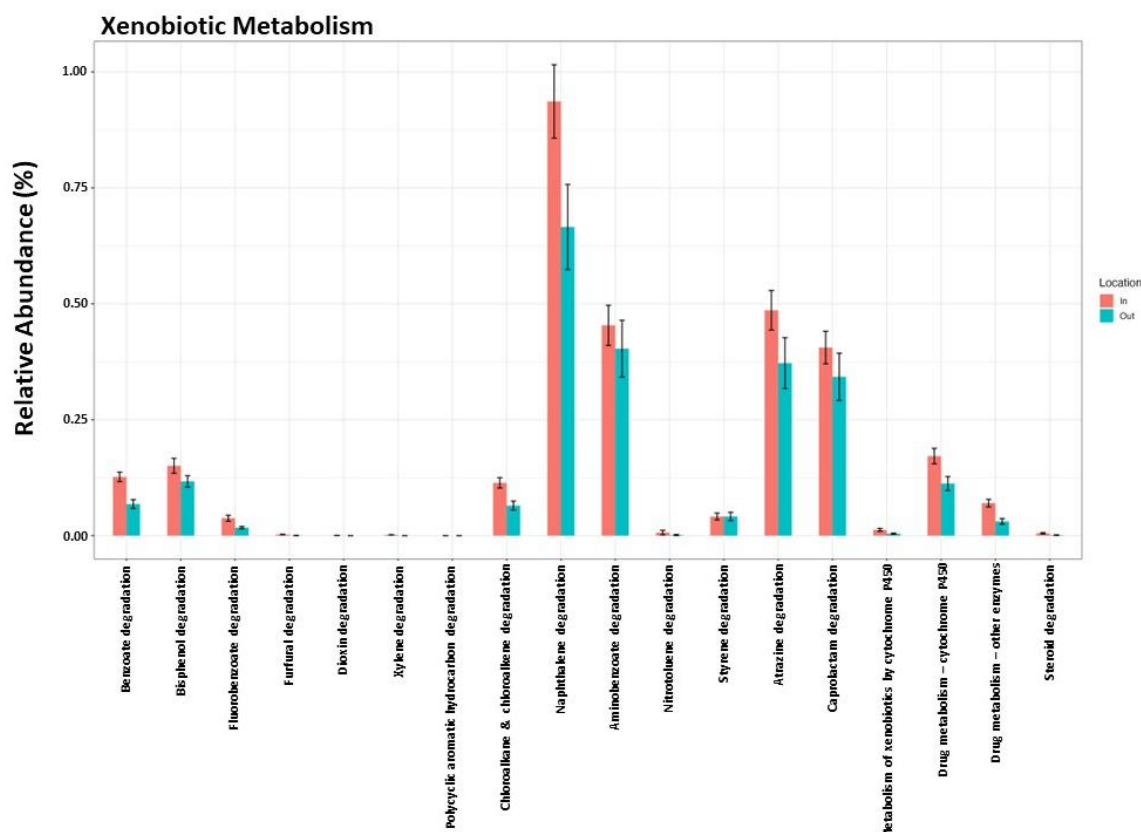


Figure 3.14: Predicted functional xenobiotic metabolism pathways of the wastewater maturation ponds inferred in the KEGG pathways database from the 16S rRNA gene sequences using PICRUST2. Inflow pond = red and outflow pond = blue.

The naphthalene degradation pathway was prominent in the predicted functional xenobiotic metabolism pathways (Figure 3.14), indicating the possible involvement of the bacterial community in naphthalene degradation. In addition, pathways for aminobenzoate, bisphenol, atrazine, and caprolactam degradation were also present.

According to Li et al. (2023), the BPA degradation pathway involves metabolic pathways for various types of compounds, including benzoates, chlorocyclohexane, chlorobenzene, polycyclic aromatic hydrocarbons, xylene, and drug metabolism, specifically cytochrome P450 degradation. In the current study, three genes present in the BPA biodegradation pathway included E1.13.11.41; 2,4'-dihydroxyacetophenone dioxygenase (EC:1.13.11.41), *hapE*; 4-hydroxyacetophenone monooxygenase (EC:1.14.13.84) and *PON*; arylesterase/paraoxonase (EC:3.1.1.2, and EC:3.1.8.1). Yu et al. (2019)

reported that the predicted degradation pathway was due to the known paired relationship between genes, substrates, and products in the KEGG database.

Although the degradation pathway of EE2 is not documented in the KEGG database, Haiyan et al. (2007) argue that EE2 can be degraded to estrone (E1), a steroid hormone. In the current study, nine genes associated with the steroid degradation pathway were present, including *choD* (cholesterol oxidase) (EC:1.1.3.60). Triclosan (TCS) biodegradation pathways remain incompletely elucidated and have not yet been incorporated into the KEGG database. Nevertheless, research has indicated that triclosan degradation involves diverse metabolic processes and enzymatic activities similar to those observed for other xenobiotics. Notably, several studies have identified TcsA, an osmolarity two-component system sensor histidine kinase (EC:2.7.13.3), in *Sphingomonas* sp., which is associated with triclosan degradation (Kagle et al., 2015; Pan et al., 2024). This highlights the need for further investigation into the genetic and enzymatic mechanisms underlying triclosan degradation to facilitate its inclusion in metabolic pathway databases. The microbial community harbours genes associated with some EDC degradation pathways, indicating potential but not confirmed activity. These results are predictive of possible degradation capacity rather than direct evidence of active EDC degradation.

3.4. Conclusions

These findings emphasise the crucial importance of monitoring emerging pollutants (EPs) and microbial communities in wastewater maturation pond systems, particularly in regions where these systems are still in use. Among the four targeted compounds, namely bisphenol A (BPA), atrazine (ATZ), triclosan (TCS), and 17 α -ethinylestradiol (EE2), ATZ and EE2 showed notable persistence in the WSP system under investigation. The selected wastewater treatment plant (WWTP) is the only facility in Bloemfontein that still operate a series of wastewater stabilization or maturation ponds as part of its treatment system. Despite biological treatment, the persistence of endocrine-disrupting chemicals (EDCs) underscores the limitations of current wastewater management practices and signals the urgent need for more effective,

context-specific remediation strategies. Using 16S rRNA and ITS sequencing, the study profiled bacterial and eukaryotic communities across wastewater influent and effluent ponds. Notably, Proteobacteria dominated bacterial communities throughout the system, with increased representation of *Flavobacterium*, *Microcystis*, *Dechloromonas*, and *Pseudomonas* in the effluent sites. These genera were previously associated with EPs degradation, including atrazine and bisphenol A. Eukaryotic communities were more variable with the phyla Ascomycota, Chytridiomycota, Rozellomycota, Basidiomycota, and Monoblepharomycota. A striking observation was the high relative abundance (~30%) of unidentified eukaryotic ASVs, indicating a potentially underexplored reservoir of eukaryotic biodegraders in this WSP system. Multivariate analyses, including db-RDA, revealed that pH and TDS were the most significant environmental variables shaping microbial compositions. Clustering of the wastewater samples into distinct positions, as revealed by PCoA, showed microbial communities were grouped according to their WSP sample location (In and Out). Bacterial functional prediction using PICRUST2 and KEGG pathway analysis suggested an enrichment of genes associated with xenobiotic degradation pathways, including those involved in the metabolism of naphthalene, atrazine, and caprolactam. These predicted functions were predominantly observed in microbial communities from the influent ponds, aligning with the elevated relative abundance of known xenobiotic degraders such as *Pseudomonas* and *Acinetobacter* sp. This suggests that these taxa may play an active role in the initial transformation of EPs within a wastewater system. Although the identification of key EDCs degradation genes highlights potential metabolic capabilities, experimental validation would be necessary to confirm active degradation. These findings suggest that low-energy systems, such as wastewater maturation ponds, support diverse and functionally relevant microbial communities with a limited yet measurable capacity for the attenuation of EPs. The detection of EDC-degrading genes within the studied microbial communities, highlights the opportunities for leveraging these taxa in targeted bioremediation strategies. The persistence of EPs, alongside the high abundance of unidentified eukaryotic taxa, underscores the need for targeted interventions such as bioaugmentation or biostimulation processes to enhance microbial degradation potential. Maturation

ponds provide a cost-effective and low-energy approach for tertiary wastewater treatment, yet their performance in removing EPs such as EDCs can vary with environmental and microbial factors. The findings suggest that indigenous microbial communities possess the genetic potential for EDC degradation, which could be harnessed to improve system performance. To translate these insights into management practices, several strategies can be considered: (i) adjusting hydraulic retention times and aeration regimes to maintain conditions favourable for EDC-degrading taxa; (ii) bioaugmentation with microbial strains carrying key functional genes (e.g., monooxygenase, laccase, or dioxygenase) identified in this study; (iii) incorporating constructed wetlands or algal-bacterial systems downstream of maturation ponds to enhance final polishing; and (iv) establishing molecular monitoring frameworks (qPCR, meta-transcriptomic screening) for early detection of EDC accumulation or functional gene loss. Together, these actions could strengthen the resilience and pollutant removal efficiency of maturation pond systems, aligning with sustainable wastewater management goals.

3.5. References

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Chapter 4

Microbial consortium tolerance to selected endocrine-disrupting chemicals (EDCs)

4.1. Introduction

Endocrine-disrupting chemicals (EDCs) are a group of chemicals that are also known as emerging pollutants (EPs) that cause serious environmental pollution and cause wildlife and public health concerns due to their hormone-like behaviour. EDCs can mimic/inhibit normal functioning hormones and are biologically active even at low concentrations (Gou et al., 2016; Werkneh et al., 2022). Currently, an ever-growing list of diverse chemical substances, such as some natural and synthetic hormones, pesticides, surfactants, plasticizers, pharmaceuticals, and personal care products, are classified as EDCs (Esplugas et al., 2007). Among the various EDCs from the environment, natural estrogens (estrone [E1], 17 β -estradiol [E2], estriol [E3]), synthetic estrogen, 17 α -ethinylestradiol (EE2), phenolic xenoestrogens (4-nonyl phenols [4-NP] and bisphenol-A [BPA]), pesticides, atrazine (ATZ), and antimicrobial agents like triclosan (TCS) have received great attention due to their toxicity and persistence in the environment over the years (Gao et al., 2020; Werkneh et al., 2022).

WWTPs are considered hot spots for chemicals such as EDCs. The failure of conventional wastewater treatment technologies to completely remove a wide range of these compounds has caused human, animal, and environmental health concerns as their concentrations can increase from a few nanograms per litre (ng/L) to μ g/L, which are levels that are high enough to generate acute and chronic toxicity (Li et al., 2014; Verlicchi & Zambello, 2015). WWTPs in temperate and tropical climates use wastewater stabilisation ponds (WSPs) with maturation ponds as secondary or tertiary treatment units in a treatment plant. They are used individually or in a coupling manner. WWTPs are generally cost-effective, reliable, and easily operated, and are commonly constructed in areas where land is readily available. They are

used for the initial treatment of strong industrial wastewater or for the tertiary treatment of domestic wastewater (Mahapatra et al., 2022). Wastewater stabilisation ponds (WSPs) consist of three types: anaerobic, facultative, and maturation ponds. Maturation ponds are mostly used as a tertiary treatment process for improving the effluent quality of wastewater derived from secondary biological processes (Achag et al., 2023; El Hamouri, 2009). These ponds operate through the interaction of sunlight with algae and bacteria.

Microbes can remove EDCs from the environment using different mechanisms (Chaturvedi et al., 2023). Microbial communities have been found to play a significant role in EDC transformation in environments such as WWTPs (Johnson et al., 2015) and river sediment (Peng et al., 2015). A few studies have demonstrated that a mixture of degraders could enhance the degradation rate of individual EDCs compared with pure isolates in culture medium (Chang et al., 2009; Saiyood et al., 2010; Sakai et al., 2007). Bacterial degradation is a particularly easy and prominent way to remove various EDCs that occur in the environment and particularly in wastewater (Husain & Qayyum, 2013). *Ensifer adhaerens*, *Pseudomonas putida*, *Pseudomonas stutzeri*, and *Sinorhizobium fredii* that are isolated from river sediment can degrade BPA (Chang et al., 2011). Six strains belonging to four genera, *Acinetobacter* sp., *Phyllobacterium myrsinacearum*, *Pseudomonas* sp., and *Ralstonia pickettii* were isolated from compost and observed to completely co-metabolise EE2 in the presence of E1, E2, and E3 (Pauwels et al., 2008). Most bacteria belong to the phyla Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. Microbes capable of degrading EDCs possess specialised genes associated with the metabolism of EDCs, such as cytochrome P450 genes (Narayanan et al., 2022).

Despite their demonstrated capacity to degrade EDCs, bacterial communities may experience reduced degradation rates at high EDC concentrations due to toxic effects on cellular integrity and metabolic processes. The inhibitory concentration of EDCs for bacteria is highly species-specific, and therefore it cannot be concluded that all bacteria are sensitive to environmental EDC concentrations (Johnson et al., 2015; Zhang et al., 2016). Triclosan concentrations ranging between 0.0289 – 0.086 mg/L are reportedly

toxic to aquatic bacteria (Clarke et al., 2019; DeLorenzo et al., 2008). De Santana et al. (2019) reported that *Shewanella haliotis* could tolerate up to 150 mg/L of BPA. Concentrations of diethyl phthalate (DEP) above 300 mg/L are considered to have a toxic effect on *Ralstonia pickettii* (Perpetuo et al., 2020), and therefore the determination of the minimum inhibitory concentration (MIC) of EDCs on WSPs' microbial community is vital. Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent (such as a drug or chemical) that inhibits the visible growth of a microorganism after a defined incubation period (Kowalska-Krochmal & Dudek-Wicher, 2021). The investigations reported in this chapter aimed to evaluate the tolerance of indigenous microbial consortia in WSPs to varying concentrations of selected EDCs—specifically bisphenol A (BPA), atrazine (ATZ), 17 α -ethinylestradiol (EE2), and triclosan (TCS)—and to assess potential microbial adaptability.

4.2. Materials and Methods

4.2.1. Chemicals and growth media

Bisphenol A (BPA), atrazine, 17 α -ethinylestradiol (EE2), and triclosan were purchased from Sigma-Aldrich (St. Louis, MO, USA). A highly concentrated stock solution of each EDC (1 000 mg/L) was prepared in methanol and stored in the dark at 4°C. The 10X (strength) mineral salt medium (MSM) of a final volume of 100 mL deionised distilled water and pH 7.0 $\pm$ 0.2 (Ribeiro et al., 2010) composing of 2.67 g Na₂HPO₄·H₂O, 1.4 g KH₂PO₄, 0.20 g MgSO₄·7H₂O, 0.5 g 1.50 g (NH₄)₂SO₄ was autoclaved. In addition, a trace elements (TE) solution (10X strength) containing 2.0 g NaOH, 12.0 g NaEDTA·2H₂O, 2.0 g FeSO₄·7H₂O, 1.0 g CaCl₂, 10.0 g Na₂SO₄, 0.4 g ZnSO₄, 0.4 g MnSO₄·4H₂O, 0.1 g CuSO₄·5H₂O, 0.1 g Na₂MoO₄·2H₂O was dissolved in 100 mL of deionised distilled water and autoclaved (Ribeiro et al., 2010).

4.2.2. Sampling

The sampling site and sample collection were described in section 3.2.1. Samples were stored in an ice cooler box (4°C) and transported back to the laboratory for analysis. The water sample was filtered using

MN GF-1 filters with a pore size of 0.7 μm from Macherey–Nagel (Düren, Germany) to remove any suspended solids.

The initial experiments were conducted on wastewater samples in 5 mL Eppendorf tubes with final working volumes of 3.5 mL. Each water sample (3400 μL) was transferred into Eppendorf tubes containing sterile 90 μL 10X MSM and 10 μL 10X TE and the concentrations of each EDCs supplemented ranged between 0.1, 1, 10, and 100 mg/L (Table 4.1). The test samples were supplemented with EDCs (ATZ, BPA, EE2, and TCS) as the only carbon sources.

Table 4.1: Description of EDCs MIC experimental design

Treatment	Treatment compositions
1. Abiotic control	Autoclaved WSP water (3400 μL) + 10X MSM (90 μL) + 10X TE (10 μL)
2. Biotic control	WSP water (3400 μL) + 10X MSM (90 μL) + 10X TE (10 μL)
3. Test samples	WSP water (3400 μL) + 10X MSM (90 μL) + 10X TE (10 μL) amended with 0.1 – 100 mg/L EDCs*
4. EE2 test samples	WSP water (3400 μL) + 10X MSM (90 μL) + 10X TE (10 μL) amended with 0.1 – 100 mg/L EE2*
5. BPA test samples	WSP water (3400 μL) + 10X MSM (90 μL) + 10X TE (10 μL) amended with 0.1 – 100 mg/L BPA*
6. ATZ test samples	WSP water (3400 μL) + 10X MSM (90 μL) + 10X TE (10 μL) amended with 0.1 – 100 mg/L ATZ*
7. TCS test samples	WSP water (3400 μL) + 10X MSM (90 μL) + 10X TE (10 μL) amended with 0.1 – 100 mg/L TCS*
8. Media control	Water (3400 μL) + 10X MSM (90 μL) + 10X TE (10 μL)
9. Sterile deionise water	Autoclaved deionised water (200 μL)

MSM: Minimum salt medium; TE: Trace elements; WSP: Wastewater stabilisation pond; EDCs: Endocrine-disrupting chemicals; EE2: 17, BPA: bisphenol A, ATZ: atrazine, TCS: triclosan, * final concentration

4.2.3. Determination of the minimum inhibitory concentration (MIC) of EDCs

The optimised MIC protocol for the experiments involved pipetting 200 μL of each test experiment from an Eppendorf tube® into 96-well microplates (flat bottom sterile polystyrene, 0.415 mL capacity, Greiner Bio-One). The 96-well plates were covered with a lid and incubated for 96 hours at 26°C and allowed to shake during exposure period to ensure aerobic conditions in the medium. Optical density (OD) at 600 nm was measured every hour using a SpectraMax M2^e microplate reader with Softmax Pro 4.0 software (Molecular Devices, Sunnyvale, CA, USA) to monitor growth. Negative controls, MSM & TE and autoclaved WSP water supplemented with different EDC concentrations ranging from 0.1 to 100 mg/L were also determined (Figure 4.1).

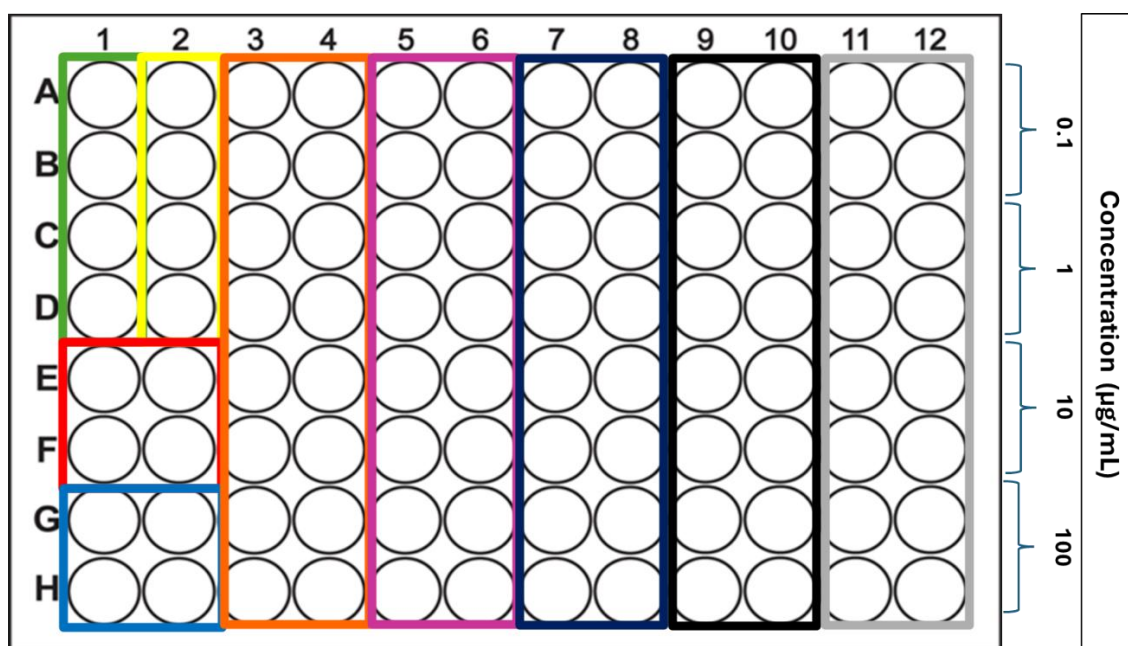


Figure 4.1: The 96-well plate layout used for the MIC assay.

Controls: A1–D1 (green) = Abiotic control (negative); A2–D2 (yellow) = Biotic control (positive); E1,2–F1, 2 (red) = Sterile deionised water; G1, 2–H1, 2 (blue) = Media control (10x MSM and 10x TE with sterile deionised water).

Combined EDC treatment (all four compounds - orange): A3–B4 = 0.1 $\mu\text{g/mL}$, C3–D4 = 1 $\mu\text{g/mL}$, E3–F4 = 10 $\mu\text{g/mL}$, G3–H4 = 100 $\mu\text{g/mL}$.

Individual compound treatments: EE2 (pink): A5–B6 = 0.1 $\mu\text{g/mL}$, C5–D6 = 1 $\mu\text{g/mL}$, E5–F6 = 10 $\mu\text{g/mL}$, G5–H6 = 100 $\mu\text{g/mL}$. BPA (dark blue): A7–B8 = 0.1 $\mu\text{g/mL}$, C7–D8 = 1 $\mu\text{g/mL}$, E7–F8 = 10 $\mu\text{g/mL}$, G7–H8 = 100 $\mu\text{g/mL}$. ATZ (black): A9–B10 = 0.1 $\mu\text{g/mL}$, C9–D10 = 1 $\mu\text{g/mL}$, E9–F10 = 10 $\mu\text{g/mL}$, G9–H10 = 100 $\mu\text{g/mL}$. TCS (grey): A11–B12 = 0.1 $\mu\text{g/mL}$, C11–D12 = 1 $\mu\text{g/mL}$, E11–F12 = 10 $\mu\text{g/mL}$, G11–H12 = 100 $\mu\text{g/mL}$.

4.2.4. Data processing

Data generated from the 96-well microplate reader were obtained as optical density (OD₆₀₀) values for each treatment, with four technical replicates (quadruplets) per sample to ensure reliability. Raw OD values were first exported into spreadsheet format. To correct for background noise, all readings were blank standardised using the media control (10X MSM and 10X TE with sterile deionised water). The average OD of the media-only wells (no inoculum, no test compound) was subtracted from the OD values of each treatment well at the corresponding time point. This ensured that any increase in absorbance reflected microbial growth rather than medium turbidity, instrument noise, or abiotic compound effects. For each sample and concentration, the mean OD₆₀₀ and standard deviation were calculated across the four replicates after blank correction. These values were then used to generate growth curves. This approach provided a robust dataset that accounted for experimental variability, eliminated baseline interference from the growth medium, and allowed accurate comparison of microbial responses to different concentrations of the test compounds.

4.3. Results and Discussion

The MIC assay tested the EDCs' inhibitory effects of 17 α -ethinylestradiol (EE2), bisphenol A (BPA), atrazine (ATZ), and triclosan (TCS) on the microbial growth of wastewater sampled from two separate (i.e., inflow and outflow) maturation ponds. Optical density (OD) at 600_{nm} was used to determine microbial inhibition by turbidity measurements across 96-well plate format. Endocrine-disrupting chemicals (EDCs) were the substances requiring the highest concentration to inhibit growth after 96 hours of incubation, with concentrations of 0.1, 1, 10, and 100 μ g/mL in quadruplicates. These test concentrations were selected to provide a broad gradient that captured both environmentally relevant levels of EDCs and the higher ranges commonly used in laboratory biodegradation and toxicity studies. The controls were included as follows to validate the assays integrity: abiotic controls (A1–D1) to assess background signal; biotic controls (A2–D2) as growth references; sterile deionized water (E1 – F2); and media-only wells (G1–H2) to confirm sterility and media impact. Additional treatment combinations were used to determine

the dose-dependent inhibitory effects of each compound on microbial growth, with each compound tested at different concentrations. Hence, each compound was tested separately at concentrations of 0.1, 1, 10, and 100 $\mu\text{g/mL}$. To guarantee reproducibility between treatments, each concentration was tested in quadruplicate (two columns x two rows per condition).

The performed MIC assay with the combined treatment of all EDCs demonstrated a concentration dependent inhibition of microbial growth over time as determined by $\text{Log OD}_{600\text{nm}}$ (Figure 4.2). At a concentration of 100 $\mu\text{g/mL}$, the mean Log OD values ~ 0.148 ($\text{SD} \pm 0.0382$) remained low and were comparable to the abiotic control mean Log OD values of 0.001 (± 0.0002) throughout the 96-hour duration of the experiment. At concentrations of 0.1 and 1 $\mu\text{g/mL}$, the mean Log OD values observed were 0.212 (± 0.023) and 0.197 (± 0.051), respectively. Abiotic controls, which contained only autoclave water with 10X MSM and 10X trace elements, saw an unexpected increase in Log OD values after just 34 hours of incubation. As there were no compounds present, this effect was likely due to physicochemical changes in the medium or instrumental noise, rather than biological growth. Possible explanations include precipitation of medium components or evaporation-induced concentration effects. The microbial growth in these experiments were comparable to those of a biotic control with mean Log OD values of 0.184 (± 0.019). At these concentrations, the EDCs exerted minimal inhibitory pressure on the WSPs' microbial community. More pronounced inhibition was observed at the concentration of 10 $\mu\text{g/mL}$ with mean Log OD values of 0.086 (± 0.035). As proposed by Gonzalez and Aranda (2023), microbial growth efficiency depended on their resistance to growing in the presence of EDCs, their cell permeability to EDCs, and their enzymatic profile involved in the metabolism of the EDCs. This result highlights the need for more comprehensive methods in research on EDCs. A gap was noted in current research, namely the limited number of studies that assessed certain EDCs within a single compound, even though environmental exposure typically involves a mixture of these compounds. According to the observed data, microbial growth efficiency seemed to decrease at higher concentrations of EDCs.

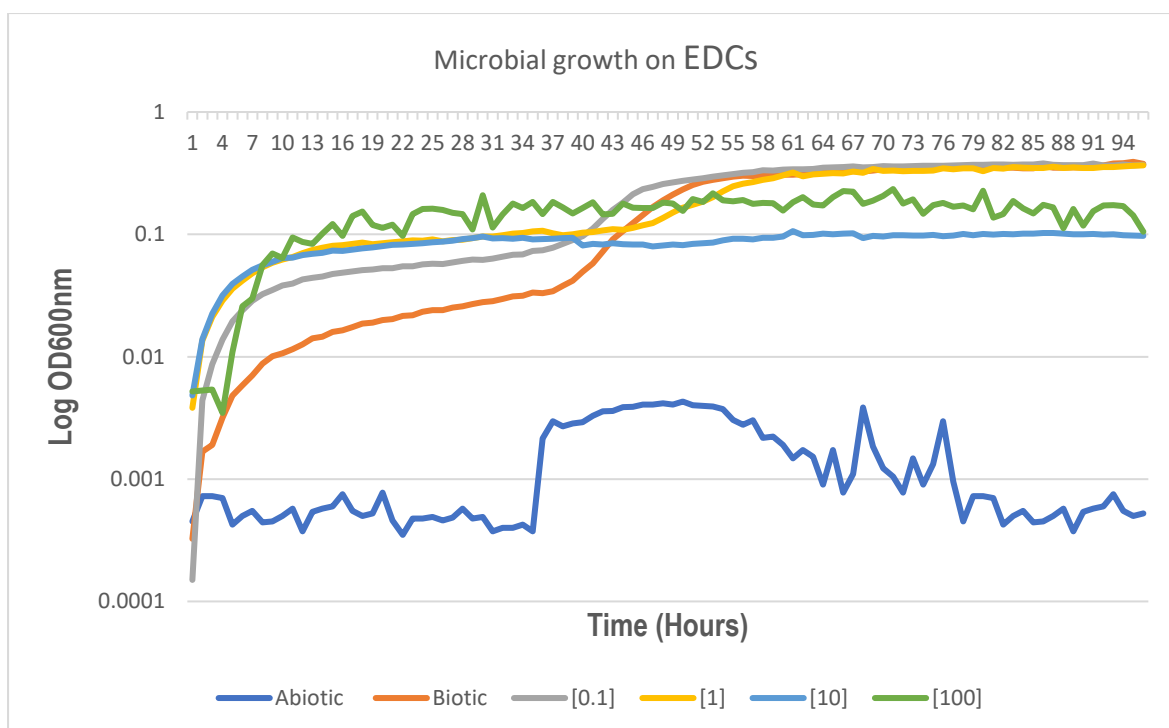


Figure 4.2: Microbial growth in MSM in the WSP system containing a mixture of EDCs at concentrations ranging from 0.1 to 100 mg/L incubated at 26°C.

At the maximum tested concentration of 100 µg/mL, all the EDCs displayed varying degrees of microbial growth inhibition. The concentrations of triclosan (TCS) and atrazine (ATZ) were nearly identical to those of the abiotic control. The highest inhibition activity of microbial growth occurred with mean Log OD_{600nm} values of 0.011 (± 0.001) and 0.013 (± 0.011), respectively. 17α-ethinylestradiol (EE2) mean Log OD_{600nm} values of 0.102 (± 0.034) and bisphenol A (BPA) mean Log OD_{600nm} values of 0.062 (± 0.029) at 100 µg/mL showed moderate inhibition, although residual microbial activity was still observed. At the lowest tested concentrations of 0.1 and 1 µg/mL, the EDCs showed mild to negligible inhibitory effects on microbial growth. At a concentration of 0.1 µg/mL, TCS consortium had mean Log OD values of 0.245 (± 0.019), which were higher than those of the biotic control (Figure 4.3). In a similar study in which EE2 was the only source of carbon and energy, a concentration of 0.6 µg/mL led to better microbial growth than a concentration of 1 µg/mL EE2 (Pauwels et al., 2008). This serves as a clear indication that these compounds may have an inhibitory effect at higher concentrations. The highest concentration that was tested (100 µg/mL) had the most pronounced microbial growth inhibition compared to that of the lower

concentrations. Similar results were obtained when *Sphingomonas* sp. PH-07, *S. wittichii* RW1, and *Burkholderia xenovorans* LB400 were grown in the presence of triclosan at different concentrations (Kim et al., 2011). Triclosan (TCS) and BPA may affect the microbial population similarly, as these compounds are chlorinated diphenols that inhibit the synthesis of cell wall fatty acids and both are widely distributed in WWTPs (Noszczyńska & Piotrowska-Seget, 2018). According to these findings, the EDCs displayed compound-specific inhibitory effects. At the highest concentration, triclosan demonstrated the highest level of antimicrobial activity when assessed individually, followed by atrazine. The determination of MICs revealed compound-specific variability in microbial tolerance, with atrazine (ATZ) showing minimal toxicity at 100 mg/L, while bisphenol A (BPA) was inhibited at approximately 100 mg/L, 17 α -ethinylestradiol (EE2) caused significant inhibition between 10 and 100 mg/L, and triclosan (TCS) exerted the strongest inhibition at 1–10 mg/L. For ease of analysis and experimentation, the concentrations applied in this study were several orders of magnitude higher than those typically reported in wastewater and surface waters. This approach was consistent with other studies that had employed elevated concentrations of EDCs to quantify biodegradation and adsorption during biological removal, where concentrations ranged between 1 and 100 mg/L (Larcher & Yargeau, 2013). While the concentrations of ATZ, BPA, EE2, and TCS used in this study did not directly reflect environmentally observed levels, they nonetheless provided valuable insights into the tolerance thresholds of microbial consortia, the relative toxicity of each compound, and the biodegradation potential of microbial consortia both individually and in mixtures. The choice of 10 mg/L for further analysis was a practical compromise, as this concentration was sufficient to elicit inhibitory responses in the more toxic compounds (EE2 and TCS) while still permitting measurable microbial activity in the case of ATZ and BPA. Lower concentrations produced negligible inhibition and thus limited interpretive value, whereas 100 mg/L caused near-complete suppression of microbial growth, constraining comparative assessment. Thus, the selected working concentration of 10 mg/L provided an informative benchmark for evaluating microbial responses across the four EDCs under study.

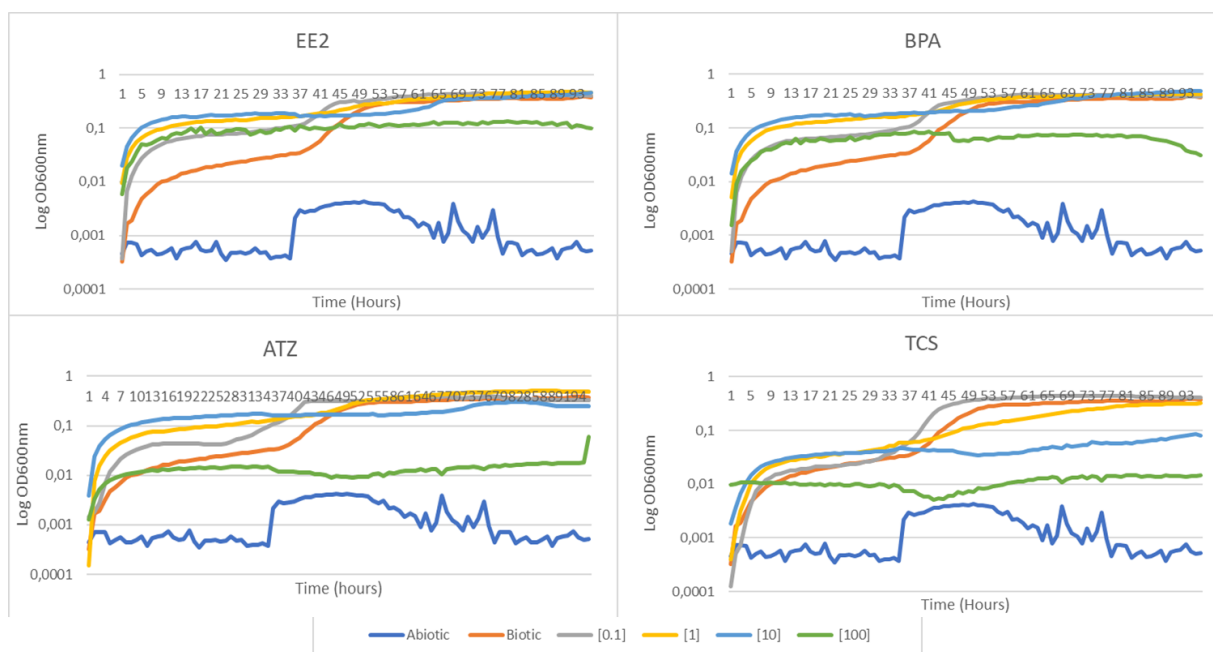


Figure 4.3: Dose-response analyses of individual EDCs on WSP microbial growth. Each compound—triclosan (TCS), bisphenol A (BPA), atrazine (ATZ), and 17 α -ethinylestradiol (EE2)—was tested separately in minimal salts medium (MSM) across a concentration range of 0.1 to 100 mg/L to assess microbial growth inhibition.

4.4. Conclusions

The investigations discussed in this chapter aimed to evaluate the tolerance of microbial consortia from the selected WSP system to selected endocrine-disrupting chemicals (EDCs), namely triclosan, bisphenol A (BPA), atrazine, and 17 α -ethinylestradiol (EE2). This was done to address the problem of limited knowledge about the threshold concentrations at which the selected pollutants would inhibit microbial growth. By determining the minimum inhibitory concentrations (MICs) of EE2, BPA, ATZ, and TCS, the findings provided valuable insights into the microbial tolerance under EDCs stress. The results revealed a clear difference in microbial tolerance across the selected compounds. Collectively, these compounds had little to no effect on microbial growth in terms of inhibition at lower concentrations (0.1 – 1 μ g/mL), as growth patterns remained comparable to those observed in the biotic control. However, inhibition was observed at higher concentrations. Individually, EE2 and BPA were the least toxic, with

growth sustained even at the highest concentration tested (100 µg/mL). Moderate inhibition was observed for ATZ, with more evident inhibition at 100 µg/mL, suggesting that microbial growth could partially adapt or metabolise ATZ even at lower concentrations. The implications of these findings are profound for wastewater treatment as they underline the potential of microbial communities in WSPs to naturally attenuate certain EDCs, such as ATZ and BPA, and pollutants like EE2 and particularly TCS. The latter poses a significant challenge that could impair microbial function even at relatively low concentrations. This highlights the need to fully understand the microbial community capabilities of WSP in removing EDCs.

The researcher acknowledges that the study was hindered by certain limitations. The first is that the assays were conducted under controlled laboratory conditions, which might not have fully replicated the complex interactions that occur in WSP environments. In real WSP environments, these compounds often coexist and may interact synergistically or antagonistically. However, this study sought to examine microbial response to both single compound exposures and mixtures of selected compounds. Also, the work was limited to assessing microbial growth in the presence of these compounds without investigating whether actual degradation occurred or identifying the specific microbial taxa involved. Future research directions should focus on investigating microbial consortia over longer periods, as this will provide valuable insights into the resilience of microbial communities. In conclusion, this chapter demonstrated that microbial tolerance to EDCs in WSPs is compound-specific and concentration-dependent. The minimum inhibitory concentration (MIC) ranges defined for BPA, EE2, TCS, and ATZ could provide critical data for predicting the fate of these compounds. Understanding microbial resilience is not only critical for the bioremediation of WSPs, but also for safeguarding aquatic ecosystems against the risks posed by the persistence of these EDCs.

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Chapter 5

Removal of endocrine-disrupting chemicals by the enriched indigenous microbial consortia on a laboratory scale

5.1. Introduction

Endocrine-disrupting chemicals (EDCs) are anthropogenic and natural chemicals that can mimic or block normal hormonal actions, thereby either triggering or preventing cellular responses (Figure 5.1). This interference can disrupt critical endocrine functions such as reproduction, growth, and development (Flint et al., 2012).

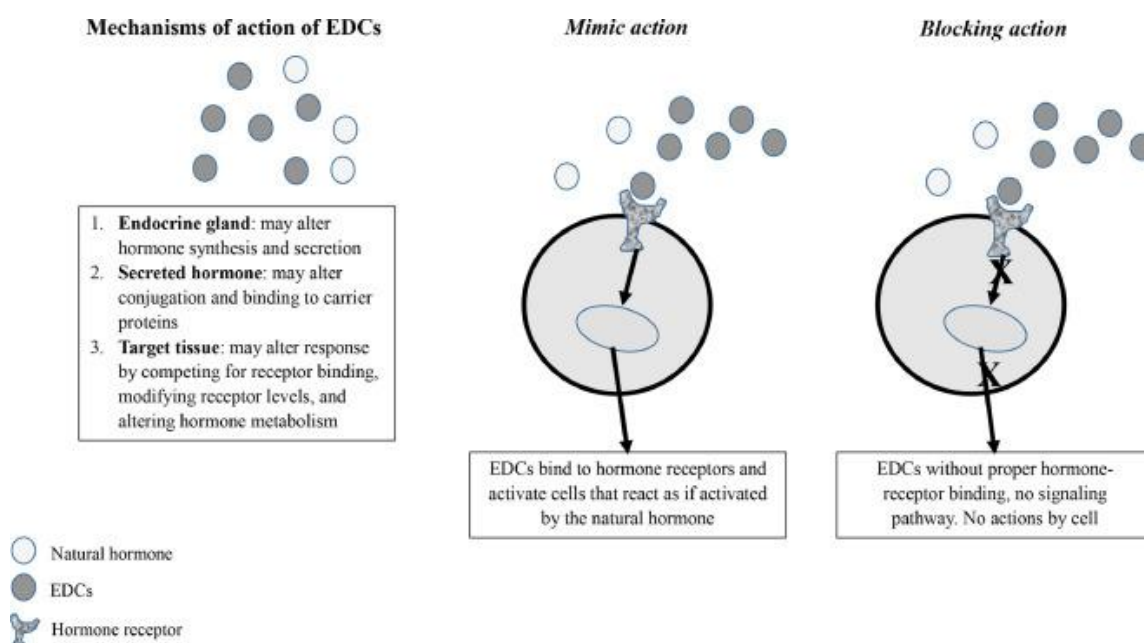


Figure 5.1: EDCs mechanism of action. Adapter from Predieri et al., 2022.

Many other chemicals known to be EDCs are not acutely toxic at environmental concentrations, and their effects on wildlife and humans often do not become apparent until early adulthood when they primarily affect the reproductive system (Colborn et al., 1994; Flint et al., 2012). Slight changes in concentrations of these chemicals can elicit significant physiological responses, potentially leading to permanent changes in mature organisms and their offspring due to the specific interaction between hormones and

their receptors (Colborn et al., 1994; Diamanti-Kandarakis et al., 2009). The occurrence of EDCs in the environment is primarily associated with various products such as pesticides, fuels, surfactants, heavy metals, industrial chemicals, plastics and plasticizers, food additives, food and drink packaging, personal care products, and pharmaceuticals. The presence of EDCs in municipal and industrial effluents has become a significant concern for marine and coastal environments near urban areas (Yamazaki et al., 2015). Many wastewater treatment plants (WWTPs) are not entirely effective in removing EDCs (Kim et al., 2007), and the physical properties of EDCs, such as solubility and sorption, can influence their biodegradation during treatment (Ying et al., 2003). Aquatic ecosystems found in contaminated water sources with high levels of specific EDCs are particularly affected (Lin & Tsai, 2009). Globally, and particularly in South Africa, EDCs have been detected in wastewater effluents, surface waters, and sediments in rivers and marine environments, with particular prevalence rates in KwaZulu-Natal (Manickum & John, 2014), Limpopo (Aneck-Hahn et al., 2009), and Gauteng (Archer et al., 2017).

Various studies have been conducted on the degradation of EDCs in river water, sediments, and sludge from WWTPs (Ike et al., 2000; Kang & Kondo, 2002; Mohapatra et al., 2010; Suzuki et al., 2004; Ying & Kookana, 2003; Zhao et al., 2008). With advancements in the chemical analysis of EDCs and the relentless efforts of environmental researchers, studies on the occurrence and fate of targeted EDCs in WWTPs have significantly increased. As a result, an increasing number of these compounds have been detected and are being monitored (Im & Löffler, 2016). However, despite identifying multiple metabolic pathways, the structure, effects, and fate of the intermediates and metabolites remain unclear (Metcalf et al., 2022; Schmidt & Mašič, 2012). It has been observed that EDC nonylphenol (NP) is more resistant to degradation than its ethoxylate counterparts. Nonylphenol ethoxylates (NPEs) can break down into NP, which is more persistent in the environment (Metcalf et al., 2022; Priac et al., 2017). Moreover, several estrogen-degrading bacteria have been isolated from wastewater, but there is little information about estrogen degradation in other environments. Additionally, the synthetic hormone EE2 has been found to be more resistant to biodegradation than natural estrogens (Cajthaml et al., 2009).

Understanding the biodegradation kinetics and estrogenic potential of estrogen metabolites is crucial for determining their fate in the environment (C. P. Yu et al., 2013).

Isolated pure cultures have been the primary focus of most previous studies on biodegradation. Numerous aerobic and anaerobic bacterial strains have been identified as efficient EDC degraders, including *Achromobacter* sp., *Alcaligenes* sp., *Burkholderia* sp., *Comamonas* sp., *Dehalococcoides* sp., *Pseudomonas* sp., *Ralstonia* sp., *Rhodococcus* sp., and *Sphingomonas* sp. (Antony et al., 2022; Brzeszcz & Kaszycki, 2018). Pesticides and plasticisers that can hydrolyze bacteria such as *Streptomyces* sp., *Fusarium solani*, *Rhodococcus rhodochrous*, *Aspergillus niger*, and *Bacillus pallidus*, and *Pseudomonas fluorescens* can produce nitrilase enzymes to degrade cyanide into formate and ammonia (Mousavi et al., 2021). The only genera presenting five or more estrogen-degrading bacteria are *Acinetobacter*, *Bacillus*, *Leptothrix*, *Phyllobacterium*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Shewanella*, and *Sphingomonas* (Budeli et al., 2021). However, these isolates might not be the dominant degraders in contaminated environments (Jiao et al., 2016), and individual microorganisms may lack all the enzymes required for the complete mineralisation of xenobiotics.

Microbial consortia with broad enzymatic capacities are more advantageous than individual microorganisms due to synergistic interactions within microbial communities, leading to more effective and robust complete degradation of contaminants (Cao et al., 2022). The biodegradation of contaminants, especially mixed xenobiotics, relies on the metabolic cooperation of microbes in the population (Morris et al., 2013; Mukherjee et al., 2022), as metabolic intermediates produced by certain microbes may serve as growth substrates for others in the community (Johnson et al., 2012). The recent development of high-throughput sequencing technologies has significantly advanced the investigation into microbial ecology in xenobiotic contamination.

The recent trajectory of microbial ecology in biodegradation has mainly focused on the microbial community composition and diversity in contaminated sites (Pannekens et al., 2019; Shahi et al., 2016)

and, in recent years, characterizing enriched consortia capable of degrading various contaminants has been of great interest (Spini et al., 2018; Vaidya et al., 2018). The clean-up of contaminated environments begins with an understanding of the ecology and phylogeny of the cultivatable indigenous microbial consortia from diverse contaminated sites. Despite this, detailed information about the clean-up of mixed contaminants using enriched consortia is still in its early phase of exploration. For instance, researchers still have limited understanding of the cultivatable aerobic xenobiotic degraders from diverse microbial sources, the differences in major degraders when growing on different xenobiotics, and the variations in major degraders and degradation efficiency across diverse initial microbial sources. It was against this background that four representative EDCs were chosen for this study, namely the widely used herbicide atrazine (ATZ), the industrial chemical bisphenol A (BPA), the antibacterial agent triclosan (TCS), and the synthetic steroid 17 α -ethynylestradiol (EE2). These four chemicals have been detected in WSPs at concentrations ranging from ng/L to μ g/L, as outlined in Chapter 3. Thus, the investigation reported in the current chapter aimed to stimulate indigenous microbial consortia for the removal of the mixed EDCs in wastewater effluent from the selected waste stabilisation pond system (WSPs) as inoculum.

5.2. Materials and Methods

5.2.1. Sample collection and physicochemical parameter determination

The dataset presented in the current chapter, is separate from the sampling campaign conducted at an earlier stage of the study, Chapter 3. These samples were collected specifically to inform the design and optimisation of the enrichment experiments, rather than to characterise in situ microbial diversity and physicochemical conditions as presented in Chapter 3. Water samples were collected from the inflow pond of the waste stabilisation maturation pond system as described in Section 3.2.1. Additional analyses of biochemical oxygen demand (BOD), chemical oxygen demand (COD), phosphate (P), sulfate (SO_4^{2-}), and ammonium (NH_4^+) were also conducted. The samples were analysed within hours after sampling. The water sample was filtered using MN GF-1 filters with a pore size of 0.7 μm obtained from Macherey–Nagel (Düren, Germany) to remove any suspended solids.

5.2.2. Chemicals

High purity (>98%) chemical standards for bisphenol A (BPA), atrazine (ATZ), 17 α -ethinylestradiol (EE2), and triclosan (TCS) were purchased from Sigma Aldrich (St. Luis, MO, USA), while internal standards (positive mode: atrazine-d5 and negative mode: bisphenol A-d16) were provided by the Facility for Genomics and Proteomics, Microbial, Biochemical and Food, University of Free State. The stock solution for each standard was prepared in methanol at a concentration of 1 000 mg/L. High-performance liquid chromatography (HPLC), graded methanol (MeOH), formic acid, and ammonium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO). Ultra-pure water (18 Ω) that was prepared using a Milli-Q purification device (Millipore, Billerica, MA, USA) was used in all the experiments.

5.2.3. Enrichment media and EDC removal studies

5.2.3.1. Culture media preparations

A modified mineral salt medium (MSM) culture was prepared as follows (amount per litre of deionised distilled water): Na₂HPO₄·H₂O (2.67 g), KH₂PO₄ (1.4 g), MgSO₄·7H₂O (0.20 g), (NH₄)₂SO₄ (0.5 g), and a trace elements solution. The trace-elements solution contained (amount per litre of deionised distilled water): NaOH (2.0 g), NaEDTA· 2H₂O (12.0 g), FeSO₄·7H₂O (2.0 g), CaCl₂ (1.0 g), Na₂SO₄ (10.0 g), ZnSO₄ (0.4 g), MnSO₄·4H₂O (0.4 g), CuSO₄·5H₂O (0.1 g), Na₂MoO₄·2H₂O (0.1 g), and the pH was adjusted to 7.0 using 1 M NaOH (Ribeiro et al., 2010).

5.2.3.2. Experimental setup

To enrich microbial consortia, 340 mL of WSP water used as the inoculum was added to 1 L Erlenmeyer flasks containing 10 mL of 10X MSM (w/v) to prepare a working volume of 350 mL (Figure 5.2). The test experiment contained enriched culture and each flask was supplemented with a mixture of ATZ, BPA, EE2, and TCS, each at a concentration of 10 mg/L (based on the MIC results in Chapter 4) as the sole carbon and energy source. Experiments were performed in duplicate under sterile conditions.

Autoclaved WSP water samples (killed inoculum 350 mL) were used as the controls for the test experiment (control experiment). Flasks were incubated in an orbital shaker at 26°C at 150 rpm. An aliquot of 3.5 mL (1% of the final volume) from week 1 was transferred to fresh MSM containing ATZ, BPA, EE2, and TCS (10 mg/L each). The flasks were incubated on an orbital shaker at 26°C and 150 rpm for a total experimental duration of 35 days, with samples collected at 7-day intervals. Sampling was done every 24 h for the first 7 days, and thereafter every 7 days until Day 35. Samples were collected for total plates count, growth studies, microbial diversity studies, and LC-MS/MS.

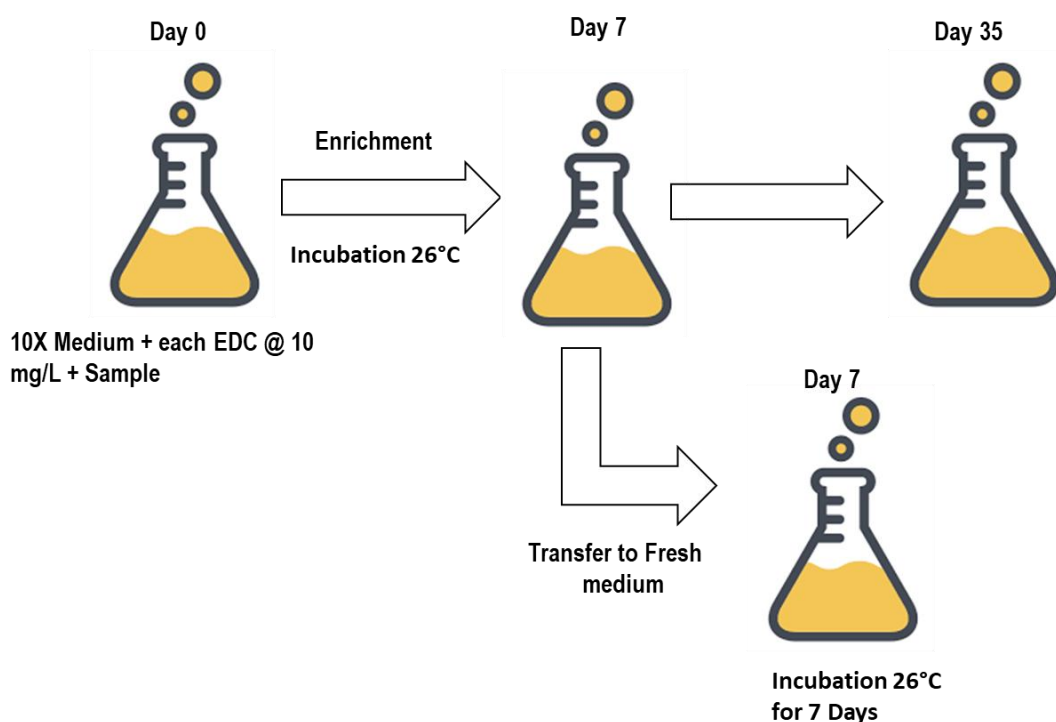


Figure 5.2: Experimental setup of flask culturing.

5.2.4. Total plate count and growth studies

To enumerate the number of viable microorganisms, 0.1 mL of culture was spread onto nutrient agar (NA) and allowed to dry. The plate was then incubated at 37°C for 24 hours, after which colony counting and calculation were performed using the automated colony counter Interscience Scan 1200 (Interscience, St. Nom, France). The growth of the culture was monitored by measuring the optical density

using a spectrophotometer (Helios Epsilon Thermo Spectronic, USA) at a wavelength of 600 nm (OD_{600nm}).

5.2.5. DNA extraction and 16S rRNA gene high-throughput sequencing

To gain an overview of the enrichment culture microbial community, the genomic DNA of the microbial community was first isolated from all the samples, including after successive serial transfers into fresh medium. The microbial biomass was harvested from 8 mL of the enrichment withdrawn from the flasks and then centrifuged at 8 500 rpm for 10 minutes at 4°C using a Neofuge 15R centrifuge (Heal Force, Shanghai, China). Total community DNA was extracted from the pellets using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The genomic DNA extracts were sent to Inqaba Biotech™, Pretoria, South Africa, for high-throughput sequencing. Briefly, each DNA extract was quantified using the Qubit™ fluorometer with a Qubit dsDNA BR assay kit (ThermoFisher Scientific, South Africa). The universal bacterial primer set 27F (AGRGTTYGATYMTGGCTCAG) and 1492R (RGYTACCTTGTTACGACTT) was used to amplify the full-length 16S rRNA gene from genomic DNA. The forward and reverse 16S rRNA primers were tailed with sample-specific PacBio barcode sequences (PacBio M13 barcodes) for multiplexed sequencing. Negative controls were included.

The KAPA HiFi Hot Start DNA Polymerase (KAPA Biosystems) was used to perform PCR amplification, with initial denaturing at 95°C for 3 min followed by 25 amplification cycles consisting of denaturing at 98°C for 20 s, annealing at 57°C for 15 s, and extension at 72°C for 60 s, followed by a final extension step at 72°C for 60 s. Post-amplification quality control was performed. DNA amplified from each sample was pooled in equimolar concentration before library preparation, and the purification step was then performed using SMRTbell clean-up bead based (PacBio, USA). Following the manufacturer's procedure, the amplified DNA pool was processed using the SMRTbell Prep Kit 3.0 (PacBio, USA).

The fluorescence concentration and average size of the library pool obtained were evaluated using the Qubit 1X dsDNA HS assay kit (Qubit™ Fluorometer, Invitrogen, USA) and 5400 Fragment Analyzer (Agilent Technologies, USA). Sequencing primer annealing and polymerase binding steps were carried

out using the Sequel II Binding Kit 2.1 and the Sequel II DNA Internal Control Complex 1.0 (PacBio, USA) before loading the pool into the sequencer. Sequencing was performed using the Sequel II Sequencing Kit 2.0 (PacBio, USA) on the Sequel II PacBio system at Inqaba Biotec, Pretoria, South Africa.

5.2.6. Data processing and statistical evaluation

The PacBio data were processed using the DADA2 R Statistics package (v1.20.0) (Callahan et al., 2016; R Core Team, 2021). The primer sequences were first removed, and the reads were trimmed by length. Reads exceeding 5 expected errors were removed, sequencing errors were dereplicated, and the error rate was estimated using loessErrfun. True sequence variants were inferred, and the forward and reverse reads were merged to obtain the full denoised sequences with a minimum overlapping region of 15 bp. ASVs identified as chimeric were removed, and the non-chimeric reads were annotated to SILVA v.138.1 database (Quast et al., 2013; Yilmaz et al., 2014).

The data were demultiplexed and a quality check was done following the DADA2 R Statistics package (v1.20.0) (Callahan et al., 2016; R Core Team, 2021) to briefly reorient the sequences, remove forward and reverse primers, filter and trim the sequences by length and average quality, dereplicate and estimate sequencing errors using the PacBio error model, and infer the compositions of the samples. ASVs identified as chimeric were removed, and the remaining ones were annotated (Callahan et al., 2016; Quast et al., 2013; Yilmaz et al., 2014).

5.2.7. Analysis of EDCs using Liquid-Chromatography Tandem Mass Spectrometry (LC-MS/MS)

Samples were analysed using a Sciex QTRAP4500 hybrid triple quadrupole ion trap mass spectrometer with a Shimadzu HPLC stack as a front end. All data acquisition and processing were performed using SciexOS software.

Twenty (20) µL of each extracted sample was separated on a C18 column (Triclosan: Kinetix Biphenyl 150mm x 4.6mm, Phenomenex and EE2, BPA and atrazine: Gemini NX, 150mm x 4.6mm, Phenomenex) at a flow rate of 300 µL/min using a 4 min gradient from 5% solvent A (triclosan and atrazine: H₂O/0.1%

formic acid and EE2, BPA: H₂O/0.1% NH₄OH) to 95% solvent B (triclosan and atrazine: Acetonitrile/0.1% formic acid and EE2, BPA: H₂O/0.1% NH₄OH). The total run time was 12 min to allow for column re-equilibration after the gradient. Eluting analytes were analysed in negative mode and ionised by electrospray in the TurboV ion source at 500°C heater temperature to evaporate excess solvent, 35 psi nebuliser gas, 50 psi heater gas, and 50 psi curtain gas. The ion spray voltage was set at 4 500V for negative mode and 5 500V for positive mode.

Targeted analyses of EE2, BPA, TCS, and ATZ were performed using 2 multiple reaction monitoring (MRM) transitions. The peak area on the chromatogram generated from the first and most sensitive transition was used as the quantifier, while the second transition was used as a qualifier. The qualifiers served as an additional level of confirmation for the presence of the analyte, as the retention time for these two transitions needed to be the same.

Table 5.1: The MRM transitions

Analyte	Precursor (DA)	Fragments (DA)
BPA	227.1	133 (quantifier) 93
EE2	287	35 (quantifier) 88
TCS	286	35 (quantifier) 241.2
ATZ	216	104 (quantifier) 174

LC-MS/MS analysis was used to monitor the removal of TCS, EE2, BPA, and ATZ. As no calibration curves were prepared for absolute quantification, the data are presented as raw peak areas under curve (AUC), which are assumed to be proportional to analyte concentration (Athamneh et al., 2022; Do et al., 2020; Pena et al., 2016). While this approach did not provide absolute concentrations, it allowed for comparative assessment of relative changes in the levels of EDCs across the enrichment culture flasks over time.

5.3. Results and Discussion

5.3.1. Physicochemical parameters

The physicochemical characteristics of the samples that were collected at different times are summarised in Figure 5.3. For this reason, the dataset presented is distinct, and not directly comparable to the datasets reported in Chapter 3. The results revealed a range of variations across key indicators of wastewater quality. Dissolved oxygen (DO) ranged from 1.91 to 5.19 mg/L, with a mean of 3.83 (± 0.83) mg/L, suggesting moderate to poor oxygenation. This could have been due to the decomposition of organic matter or possible microbial activities. The pH values were generally alkaline, ranging from 8.96 to 9.54, with a mean of 9.10. This was probably impacted by biological processes, including carbonate-bicarbonate buffer systems and photosynthesis. Conversely, two previous samplings revealed DO and pH values of 1.7 (± 0.9) and 7.7 (± 0.1) mg/L for the first visit, and 1.7 (± 0.5) and 7.8 (± 0.5) mg/L for the second visit, respectively.

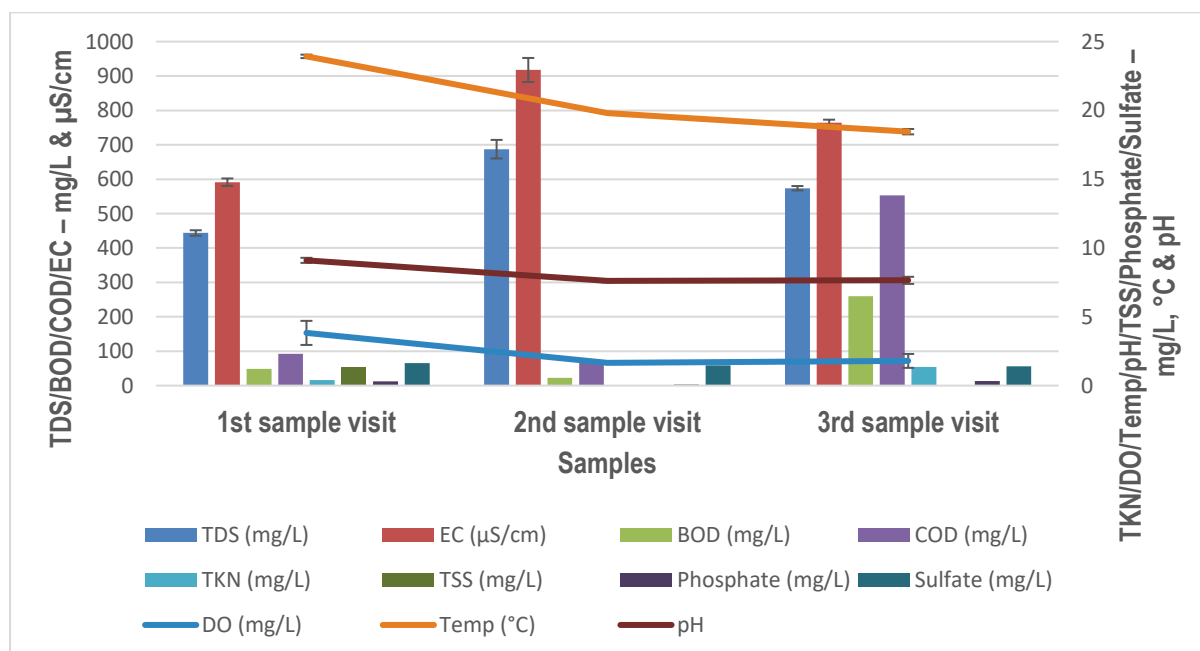


Figure 5.3: Comparison of physicochemical parameters across three sampling visits. Primary (left) axis shows clustered columns for TDS, EC, BOD, TKN TSS, Phosphate, Sulfate and COD. Secondary (right) axis shows lines for, DO, Temp, and pH. Error bars: shown only for TDS, EC, DO, Temp, and pH using per-sample standard deviations as error bars. Other parameters have no error bars.

Temperature readings with a mean value of $\sim 24^{\circ}\text{C}$ showed normal ambient temperatures. Electrical conductivity (EC) measurements ranged from 582 to 616 $\mu\text{S}/\text{cm}$, with a mean value of 591.4 $\mu\text{S}/\text{cm}$, indicating moderate ions, including metal(loids) such as iron (Fe) and arsenic (As) in the influent of the maturation pond in the WSP system, which is similar to the finding of Valles-Aragón et al. (2014). Also, the mean values of the total dissolved solids (TDS) of 407.35 mg/L were within acceptable limits ($< 2\,000$ mg/L) for effluents discharged into surface water, according to WHO standards (Akan et al., 2008) and the standards for South African water systems that apply for domestic use (0 to 450 mg/L) (Odume et al., 2024). Chemical parameter measurements were only done once and the results showed that the concentration of biochemical oxygen demand (BOD) was 49 mg/L, the chemical oxygen demand (COD) was 92 mg/L, the total Kjeldahl nitrogen (TKN) was 15.9 mg/L, orthophosphate (P) was 12.3 mg/L, sulfate (SO_4^{2-}) was 65.4 mg/L, and total suspended solids (TSS) were 54 mg/L. Compared to the results obtained for a sampling phase conducted earlier in the 3rd sampling visit, elevated concentrations were found for BOD at 260 mg/L, COD at 553 mg/L, TKN at 53.9 mg/L, and P at 13.1 mg/L, while lower readings were observed in 2nd sampling visit, namely 23.0 mg/L (BOD), 66.0 mg/L (COD), < 0 mg/L (TKN), and 2.75 mg/L (P). The significant differences in the recorded physiochemical property concentrations were likely due to seasonal variation as changes in these parameters could have impacted water quality. Both observed concentrations of BOD and TSS were higher than the stipulated limits of 30 mg/L, while COD was lower than the 125 mg/L limit (Department of Water and Sanitation [DWS], 2018). The relative oxygen depletion effect of wastewater contamination is measured by both BOD and COD, and both have been widely used to measure the pollution effect. BOD measures the oxygen demand of biodegradable pollutants, while COD measures the oxygen demand of oxidisable pollutants. A high BOD concentration (< 30 mg/L) indicates low water quality. COD concentrations (< 125 mg/L) are typically higher than those of BOD because COD measures the total organic material in water samples, while BOD measures only the prevalence of biodegradable organic material (Maddah, 2022; Sharma & Gupta, 2014).

5.3.2. Enumeration of viable microorganisms and microbial growth monitoring

Microbial growth in the enrichment cultures was monitored over a 35-day incubation period using both optical density (OD_{600nm}) and colony-forming unit (CFU/mL) counts (Figure 5.4). Both measures indicated that some microorganisms in the water inoculum from the maturation ponds were viable and had adapted to the conditions in the enrichment culture supplemented with EDCs at $10 \mu\text{g/mL}$, as referred to in terms of the MIC discussed in Chapter 4. The initial values of OD increased from $\text{Log OD } 0.0645 \pm 0.0015$ at Day 0 to $\text{Log OD } 0.3865 \pm 0.0865$ by day 14, indicating active cell growth. Thereafter, OD values remained within the range of $\text{Log OD } 0.36$ to 0.41 , suggesting that the culture had reached a stationary phase. Similarly, CFU counts increased rapidly during the first week, from $5.8 \times 10^5 \pm 1.6 \times 10^5$ to $8.6 \times 10^6 \pm 1.5 \times 10^6$ CFU/mL at Day 7. The counts reached a maximum of $1.23 \times 10^7 \pm 3.05 \times 10^6$ CFU/mL on Day 35.

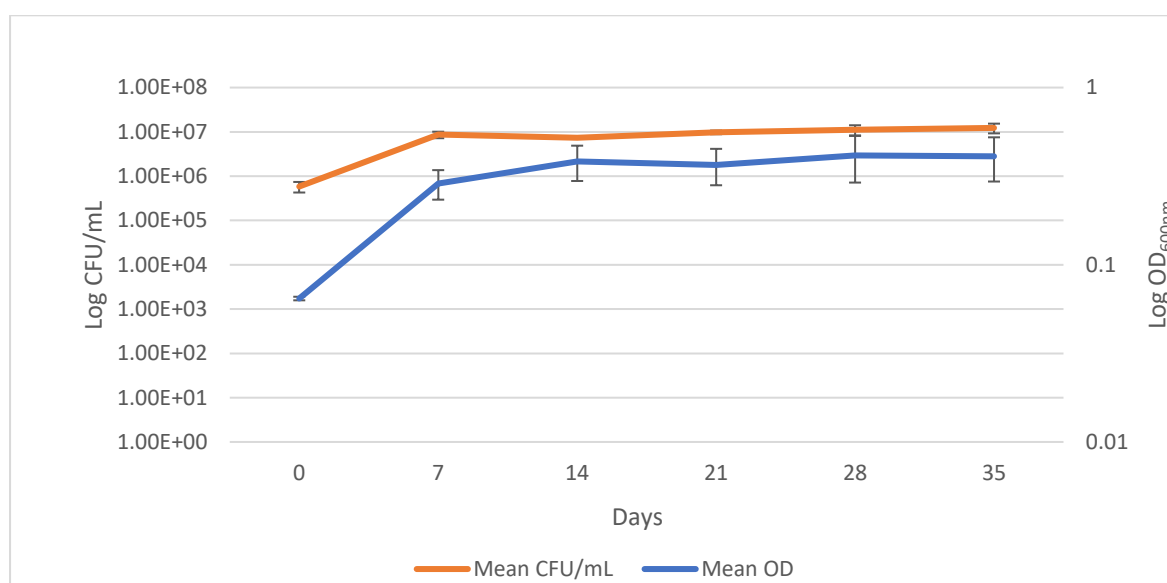


Figure 5.4: Microbial growth of enriched cultures contained in the water sample collected from the inflow waste maturation pond as inoculum and supplemented with modified mineral salt medium (MSM) and a mix of EDCs (EE2, BPA, TCS, ATZ) at $10 \mu\text{g/mL}$ each. Growth is presented as optical density at 600 nm and total viable counts as CFU/mL. Mean values were plotted with standard deviations indicated as error bars.

5.3.3. Microbial diversity studies

PacBio-based PCR amplification targeting the 16S rRNA gene was employed to characterise bacterial communities within the enrichment cultures. Initial amplification attempts were unsuccessful for a subset of samples, necessitating a series of optimisation steps, including DNA dilution to minimise inhibitor effects, adjustment of template input concentrations, and modification of PCR cycling conditions. Despite these efforts, amplification could not be consistently achieved for all samples. Similar challenges have been reported for environmental and enrichment-derived DNA, where co-extracted inhibitors and DNA degradation compromise PCR efficiency even after optimisation (Radström et al., 2004; Schrader et al., 2012). Consequently, only samples that yielded reproducible and high-quality amplicons were included in downstream sequencing analyses. While this resulted in a reduced dataset, the retained samples provided sufficient resolution to assess dominant bacterial taxa within the enrichment cultures. A marked shift in microbial community structure was observed between the initial inoculum (Day 0) and the transfer flask sampled after 7 days of enrichment. At Day 0, the community was diverse and dominated by *Pseudomonadota* (34%), *Actinomycota* (27%), *Planctomycota* (21%), and *Bacteriodota* (14%) at the phylum level. Genera such as *QWPN01* (19%), *Polynucleobacter* (16%), *Cloacibacterium* (12%), and *Limnohabitans* A (10%) were most abundant, while at the species level (Figure 5.5) *QWPN01* sp014192425 (16%) and a large proportion of unassigned taxa (15%) were detected. This composition reflected a typical heterogeneous microbial community in the wastewater-impacted aquatic environment under study, with both characterised and uncharacterised lineages potentially contributing to removal capacity.

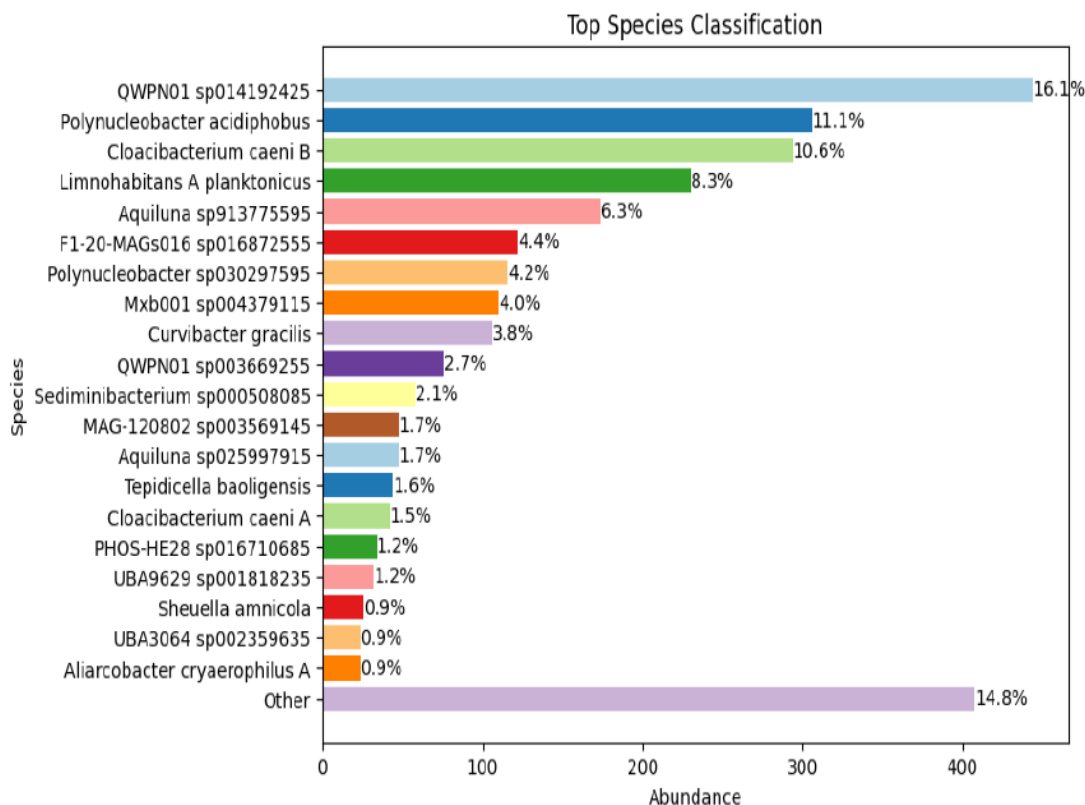


Figure 5.5: Relative abundance (RA >1%) of bacterial taxa at the species level observed on Day 0 enrichment flask.

In contrast, after transfer to fresh MSM supplemented with EDCs, the microbial profile shifted dramatically. The community became highly specialised and was dominated by a few taxa, namely *Achromobacter* (65%), *Stenotrophomonas* (20%), and *Pseudomonas* (11%) at the genus level (Figure 5.6). At the species level, enrichment was strongly represented by *Achromobacter aegrifaciens* (25%), *Stenotrophomonas muris* (17%), *Achromobacter* sp. 002902905 (17%), and *Achromobacter insuavis* (14%).

These findings suggested that the enrichment conditions imposed by MSM and the presence of high concentrations of EDCs created a strong selective pressure favouring specialised degraders. Both *Achromobacter* and *Pseudomonas* are well-documented for their ability to degrade aromatic compounds,

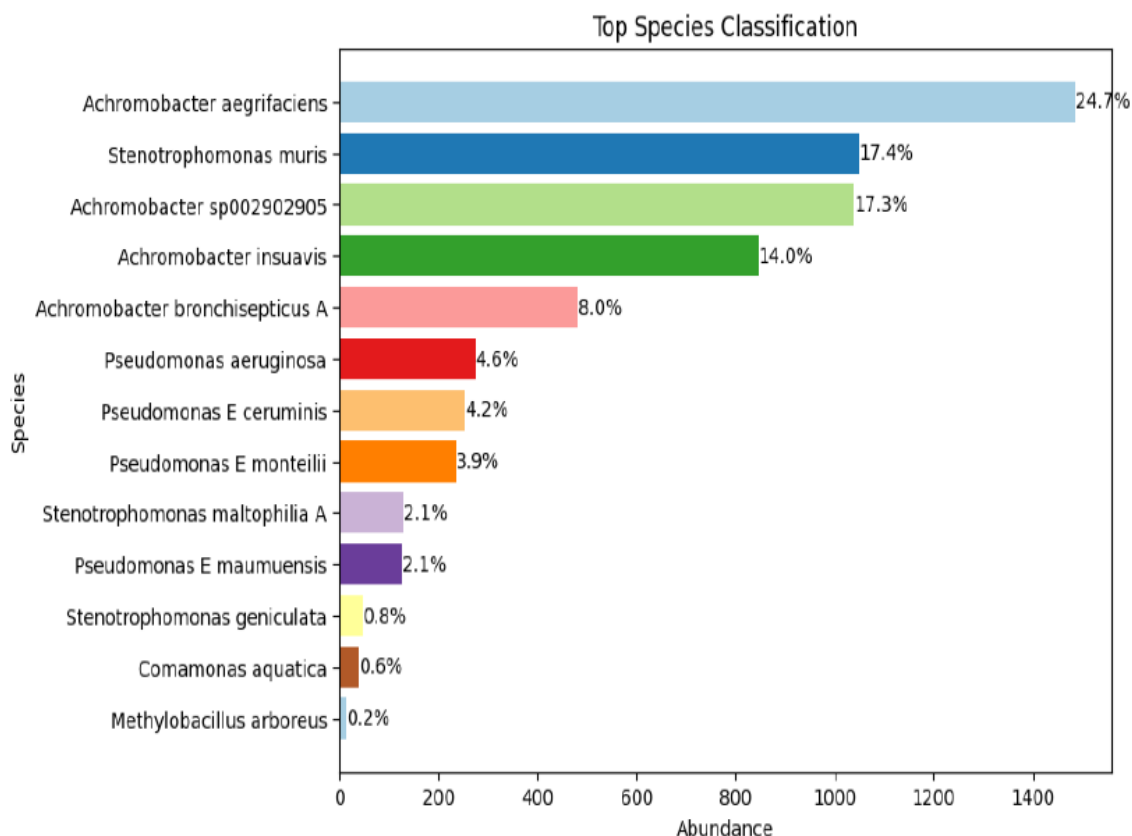


Figure 5.6: Relative abundance (RA >1%) bacterial taxa at the species level observed from transfer enrichment flask after 7 days.

Pesticides, and xenobiotics, including EDCs (Guo et al., 2019; Li et al., 2021). Similarly, *Stenotrophomonas* species are often reported in contaminated environments and are associated with versatile metabolic pathways that confer resistance to chemical stressors (Ryan et al., 2009). These results indicated that, while the inoculum harboured a broad and taxonomically diverse community, the enrichment process led to the emergence of a narrow but highly specialised consortium. This microbial community restructuring highlights the adaptive abilities of microbial populations under selective environmental pressure and underscores the ecological importance of specific genera, particularly *Achromobacter* and *Pseudomonas*, in the removal of xenobiotic pollutants.

5.3.4. Removal of EDCs in an enrichment culture

The removal of EDCs by consortium of the maturation pond enrichment culture was assessed by monitoring the concentrations of EDCs (ATZ, EE2, BPA, and TCS) at time intervals using LC-MS/MS. The removal profiles were measured using LC-MS peak area (AUC) values for the MRM transition of each EDC, which served as semi-quantitative indicators of concentration. This approach is widely applied in degradation studies when an absolute calibration curve is not feasible (Athamneh et al., 2022; Do et al., 2020; Pena et al., 2016).

Using the maturation pond water samples as inoculum, enrichment flasks were set up in duplicate, with all flasks supplemented with ATZ, EE2, BPA, and TCS at an initial concentration of 10 mg/L each, serving as the source of carbon and energy. Abiotic control flasks contained autoclaved maturation pond water. After the first week, the contents of the enrichment flasks were transferred into fresh medium with the same four compounds at 10 mg/L and incubated for 35 days. Weekly sampling and analyses were conducted using LC-MS/MS to monitor the degradation of each compound.

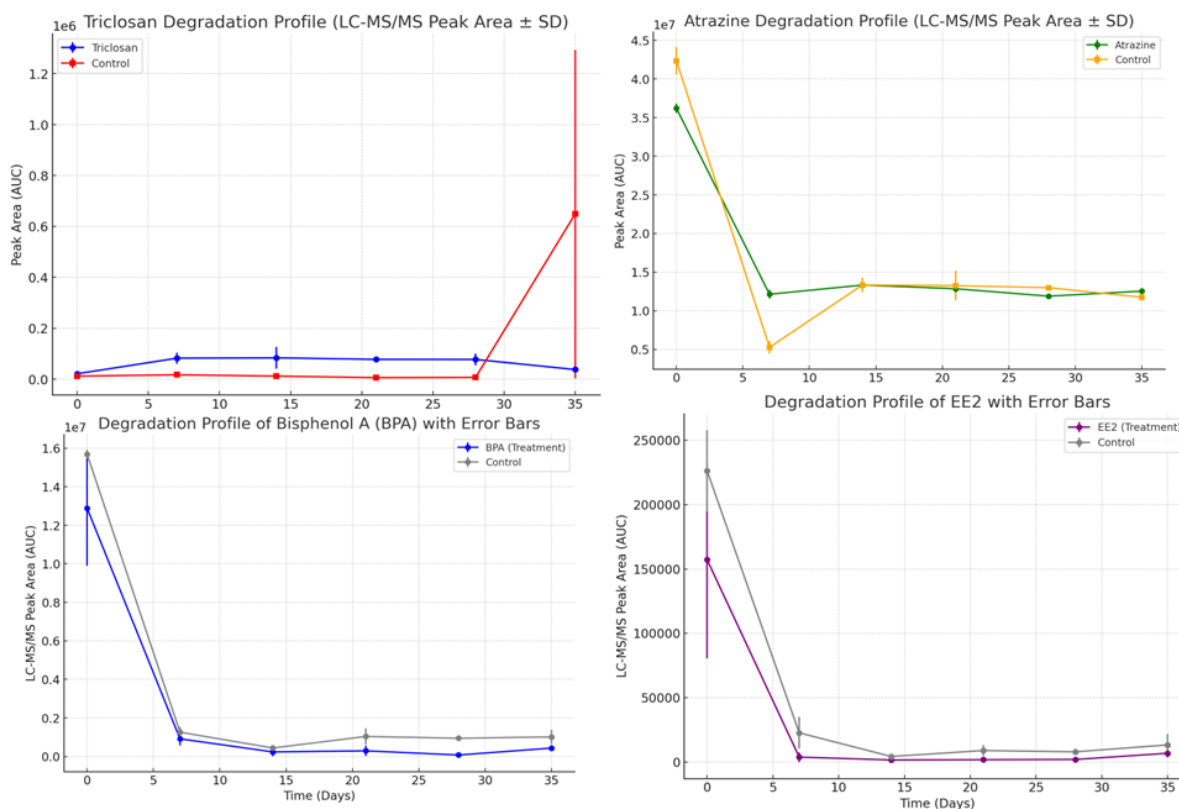


Figure 5.7: Removal profiles of EDCs in the enrichment medium containing EE2, ATZ, BPA, and TCS. The EDCs' areas under curve (AUC) were measured by LC-MS analysis over a period of 35 days of enrichment. Data are presented as average and standard deviation as error bars.

For EE2, a rapid decrease in chromatographic peak area (approximately 98%) was observed within the first 7 days in the enrichment cultures, with near complete removal by Day 14 (Figure 5.7). However, a similar decline was also recorded in the abiotic control, followed by minor fluctuations at trace levels in both systems. Comparable behaviour was observed for BPA, where peak area reductions exceeding 90% in the enrichment cultures and approximately 97% in the abiotic control were achieved within the first 14 days, with consistently low levels thereafter. The close correspondence between test and control profiles suggests that sorption-driven partitioning, rather than enzymatic transformation, dominated the apparent removal of these compounds.

ATZ exhibited slower and partial removal. In the enrichment cultures, an initial decrease of approximately 66% was observed by Day 7, after which no substantial further reduction occurred. A similar pattern was

observed in the abiotic control, where an overall reduction of approximately 87% was recorded, accompanied by a transient increase in ATZ peak area between Day 7 and Day 14. Since an autoclaved water sample collected from the site serves as the abiotic control, a similar pattern observed in the abiotic control may be due to ATZ sorption by microbial dead cells. This increase is consistent with desorption or re-equilibration processes commonly reported for moderately hydrophobic herbicides in closed laboratory systems (USEPA, 1990; Phan et al., 2022). Although ATZ biodegradation is known to require specialised microbial populations possessing s-triazine-degrading genes (Mandelbaum et al., 1995; Strong et al., 2002; Udiković-Kolić et al., 2012), the absence of divergence between enrichment and control flasks in this study precludes confirmation of a passive, non-metabolic process.

Following transfer into fresh medium, reduced removal was observed for EE2 (74%) and BPA (58%), while ATZ and TCS showed increases in peak area. These shifts further support the interpretation that removal could be governed by reversible sorption–desorption rather than sustained microbial metabolism, as changes in medium composition and equilibrium conditions can promote desorption of previously partitioned compounds.

The comparable removal of EE2, BPA, and ATZ observed in both the enrichment cultures and the abiotic control experiments indicates that non-biological processes played a role under the experimental conditions employed. Given the hydrophobic nature of these compounds, the apparent observed reduction in aqueous concentration could mostly be attributed to sorption onto flask surfaces, suspended particulates, inorganic salts in the medium, and microbial biomass, irrespective of metabolic activity. Similar sorption-driven losses have been widely reported for steroid hormones (e.g., estrone and 17 β -estradiol) and phenolic compounds such as bisphenol A, nonylphenol, and triclosan in laboratory systems, where adsorption to biomass, reactor surfaces, and solids had obscure or confound the detection of biological degradation (Ternes et al., 1999; Joss et al., 2004; Singer et al., 2002; Urase & Kikuta, 2005). The observed reduction in chromatographic area peak of analytes in both enrichment cultures and abiotic control flasks therefore suggests sorption, rather than biodegradation, as the primary

removal mechanism. As a result, based solely on changes in area peak, it was not possible to distinguish biological transformation from physicochemical partitioning, underscoring the need for complementary approaches, such as metabolite identification, mass balance analysis, or isotope-based techniques, to confirm biodegradation (USEPA, 1990; Gonzalez et al., 2023). A high capacity of *Streptomyces roseorubens* in removing nickel ions was reported when Long et al. (2018) investigated the non-living strain of *Streptomyces roseorubens* for its ability to adsorb nickel ions from an aqueous solution. Syed et al. (2015) also reported the biosorption of heavy metals, such as lead, chromium, and copper, from heavy metal solutions by *Bacillus* sp. Furthermore, Shalaby et al. (2023) reported the removal of metallic nanoparticles from aqueous solutions via biosorption by *Aspergillus* sp. However, data on the removal of EDC by sorption (biosorption or adsorption) using live or dead microbial cells are limited.

Triclosan (TCS) exhibited a distinct analytical behaviour compared to the other compounds. In both enrichment and control flasks, an apparent increase in TCS peak area was observed over the 35-day incubation period. Given the chlorinated nature and high hydrophobicity of TCS, this behaviour is consistent with known matrix-induced analytical artefacts, including ion suppression or enhancement in electrospray ionisation caused by inorganic salts present in the MSM (Trufelli et al., 2011; Petrie et al., 2015). The emergence of chlorinated transformation products with overlapping mass spectral responses may also contribute to apparent signal increases (Balakrishnan & Mohan, 2021; Chen et al., 2011; Lee et al., 2014). Importantly, the potential for medium-induced interference with TCS analysis is well recognised, and alternative strategies such as modifying medium composition, employing complementary extraction solvents, or using an alternative nutrient matrix represent viable approaches to mitigate these effects. However, the present study prioritised maintaining consistent enrichment conditions across all target EDCs to allow comparative assessment of compound behaviour within a single experimental framework. As a result, no definitive conclusions regarding TCS biodegradation could be drawn, and the observed trends are best interpreted as reflecting analytical and physicochemical influences rather than biological transformation.

5.4. Conclusions

The investigations discussed in this chapter evaluated the biodegradation of four priority endocrine-disrupting compounds (EDCs), namely atrazine (ATZ), bisphenol A (BPA), 17 α -ethinylestradiol (EE2), and triclosan (TCS) in enrichment cultures using mineral salts medium (MSM) inoculated with maturation pond water. The problem addressed was the persistence of these pollutants in such aquatic environments where they can pose risks to the ecosystems and human health. It was important to better understand their fate under biologically active conditions. The results demonstrated that BPA and EE2 were rapidly removed, with significant reductions observed within the first 7 to 14 days, indicating effective removal by biosorption or adsorption supported by the decrease in the concentrations of these compounds in the abiotic control experiments. The removal of ATZ was slower than that of BPA and EE2, but the concentration steadily decreased relative to the control. In contrast, there was an increase in TCS concentration, but the result was not conclusive, suggesting substantial interference of the medium with its ionization in LC-MS/MS analysis. This was a key limitation of the study, as TCS removal could not be directly evaluated under enrichment conditions. There is a dearth of research on the sorption of EDCs by microbial cells; hence, the findings of this study highlight the potential of wastewater-derived microbial consortia in removing EE2, BPA, and ATZ from EDC-contaminated water through the sorption of contaminants containing both live and dead microbial cells. Furthermore, the findings also emphasise the challenges of analytical interference and the compound-specific variability in degradation behaviour. Future work should focus on optimising extraction and detection methods for TCS in complex matrices, extending the monitoring of degradation metabolites and applying molecular tools (e.g., 16S rRNA sequencing and functional gene analysis) to link EDC removal trends with the shifts in microbial community.

In summary, while the study confirmed the biodegradability of EE2 and BPA, the partial transformation of ATZ and the analytical challenges associated with TCS underscore the importance of integrating chemical and microbiological analyses. Addressing these challenges will be essential to better

understand the environmental fate of EDCs and microbial communities, and to inform the development of bioremediation strategies for the wastewater maturation ponds under study, and possibly for those operating under similar conditions.

5.5. References

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Chapter 6

General concluding remarks and recommendations for future studies

1.6 General Concluding Remarks

There is a notable lack of research on and comprehensive documentation of the design, efficiency, and performance of wastewater treatment plants (WWTPs), particularly concerning the removal of emerging pollutants (EPs) such as endocrine-disrupting compounds (EDCs), pharmaceuticals, and other micropollutants. In general, the microbial communities in WSPs, particularly in the South African environment, are understudied. This work provided important insights into the composition of microbial communities in WSPs, particularly in inflow and outflow maturation ponds, through 16S rRNA and ITS gene amplicon analysis using the PacBio sequencing platform. In this study, the microbial composition and structure of wastewater maturation ponds (i.e., an inflow and an outflow pond) were evaluated while their potential to remove EDCs was also assessed. This research provides important insights into the prevalence and persistence of EDCs, particularly bisphenol A (BPA), atrazine (ATZ), triclosan (TCS), and 17 α -ethinylestradiol (EE2) in the selected wastewater stabilisation system's maturation ponds under study. The 16S rRNA and ITS sequencing revealed that Proteobacteria, Ascomycota, Chytridiomycota, Rozellomycota, Basidiomycota, and Monoblepharomycota were the dominant bacterial and fungal phyla. Both inflow and outflow ponds contained elevated levels of *Pseudomonas* sp. and *Acinetobacter* sp. that are known xenobiotic degraders. A high relative abundance (~68%) of ASVs was associated with unidentified eukaryotes, which could be attributed to the lack of reference sequences for these unidentified phyla in the UNITE database. The distance-based RDA revealed that pH and TDS were the environmental variables shaping the microbial communities. PICRUSt2 and KEGG pathway analyses suggested that functional genes could have been involved in naphthalene, atrazine, and caprolactam metabolism.

Tolerance assay was used to determine microbial sensitivity to EDCs by minimum inhibitory concentrations (MICs) at different concentrations; i.e., 0.1, 1.10, and 100 mg/L. The results indicated that the combination of ATZ, BPA, EE2, and TCS at lower concentrations (0.1 to 1 mg/L) did not affect microbial growth as their growth was similar to that of the biotic control. However, at higher concentrations, growth was restricted, but not completely restricted. The influence of each compound was also evaluated, and the results suggested that EE2 and BPA, even at the highest concentration (100 mg/L), still allowed microbial growth to occur. While ATZ showed moderate inhibition, TCS showed significant inhibition at 10 and 100 mg/L. The results suggest that EDCs at lower concentrations can be utilised by microbial communities in WSP systems, yet they pose a challenge at higher concentrations.

The enumeration of viable microorganisms during the assessment of EDC removal (at 10 mg/L of ATZ, BPA, EE2, and TCS each) by the enriched microbial consortia revealed that the colony-forming units (CFU/mL) reached a maximum of 1.23×10^7 CFU/mL by Day 35, while the optical density for the growth study was measured at 0.41 (Log OD_{600nm}). The 16S rRNA gene amplicon analysis using the PacBio sequencing platform showed that QWPN01 sp014192425 (16%) and unassigned taxa (15%) dominated the enrichment culture at species level, resulting in a microbial shift.

The potential removal of EDCs by the enriched indigenous consortia, as tracked by LC-MS/MS and measured by peak area (AUC values) for the MRM transition of each EDC, served as semi-quantitative indicators of concentration. This revealed a rapid removal of BPA and EE2 within the first 7 to 14 days. In comparison, there was a steady, slower and partial removal of ATZ, while each abiotic control showed corresponding similar removal trends. This finding indicates that BPA, EE2, and ATZ were likely removed by sorption into the microbial cells (biosorption) or onto the surface of the microbial cells (adsorption) rather than degraded. The detection of TCS presented challenges in the measurement as the concentration increased. This suggested that medium interference could have occurred due to ionization in the LC-MS/MS analysis process.

This study provides valuable information about the presence of EDCs in WSPs as it illuminates their fate in the presence of the diverse microbial communities under study and highlights the possible mechanisms by which in-situ microorganisms may remove EDC contaminants.

6.1. Future studies

The following comments and recommendations emerged from the findings of this study:

- 1) The degradation pathways, transformation rates, and overall fate of EDCs that occur in the environment in relevant concentration levels remain insufficiently understood. To better assess the risks, they pose to the environment and human health, it is crucial to conduct comprehensive studies that investigate the behaviour, persistence, and breakdown at the low concentrations at which they are detected in the environment. Such investigations will help improve the accuracy of environmental risk assessments and inform the development of more effective mitigation strategies.
- 2) Investigating EDC removal under anaerobic conditions is crucial, as these conditions are prevalent in various natural and engineered environments, including sediments, wetlands, landfills, and specific zones of wastewater treatment systems. Anaerobic biodegradation pathways differ from aerobic pathways due to differences in microbial compositions and their enzymes. Therefore, studying the fate of EDCs in oxygen-depleted environments is essential in gaining a comprehensive understanding of their persistence, potential for accumulation, and means of removal, as well as their long-term ecological impacts.
- 3) The formation of highly persistent metabolites in the aerobic sludge systems under study raises significant concerns regarding their potential long-term impact on both engineered and natural environments. Given their stability and resistance to degradation, these metabolites may accumulate and pose risks to ecological and human health. Therefore, it is essential to investigate the concentrations, behaviour, and fate of this metabolite within WSP systems, as well as in surrounding environmental compartments such as surface water, groundwater, and

sediments. Understanding its persistence, transport, and potential for bioaccumulation will provide critical insights that will inform environmental risk assessment strategies and the development of effective mitigation measures.

- 4) Comprehensive toxicological studies of all EDCs present in waste stabilisation ponds (WSPs), particularly in their maturation ponds, should be systematically conducted to better understand the potential risks they pose to human health and aquatic ecosystems. Such studies are essential in identifying acute and chronic toxicity effects, endocrine interference mechanisms, and potential synergistic interactions among multiple EDCs under environmentally realistic conditions. Furthermore, there is a pressing need to expand the existing catalogue of identified EDCs in WSP systems by employing advanced analytical techniques such as high-resolution mass spectrometry and non-targeted screening. Such investigations will enable the detection of previously unrecognised or emerging contaminants, contributing to a more complete assessment of chemical risks and supporting the development of better-informed regulatory frameworks and treatment strategies.
- 5) The isolation and characterisation of pure culture bacterial strains capable of removing EDCs are critical steps towards understanding the microbial mechanisms involved in EDC biotransformation. By cultivating and studying specific microbial degraders under controlled conditions, we can elucidate the metabolic pathways, enzymatic systems, and genetic determinants responsible for the breakdown of EDCs. Such isolates not only provide insight into the natural attenuation potential of microbial communities, but also offer promising candidates for bioaugmentation strategies in wastewater treatment systems. Detailed characterisation, including phylogenetic identification, degradation kinetics, and the identification of intermediate metabolites, will enhance our ability to design effective bioremediation strategies for mitigating EDC contamination in various environmental settings.