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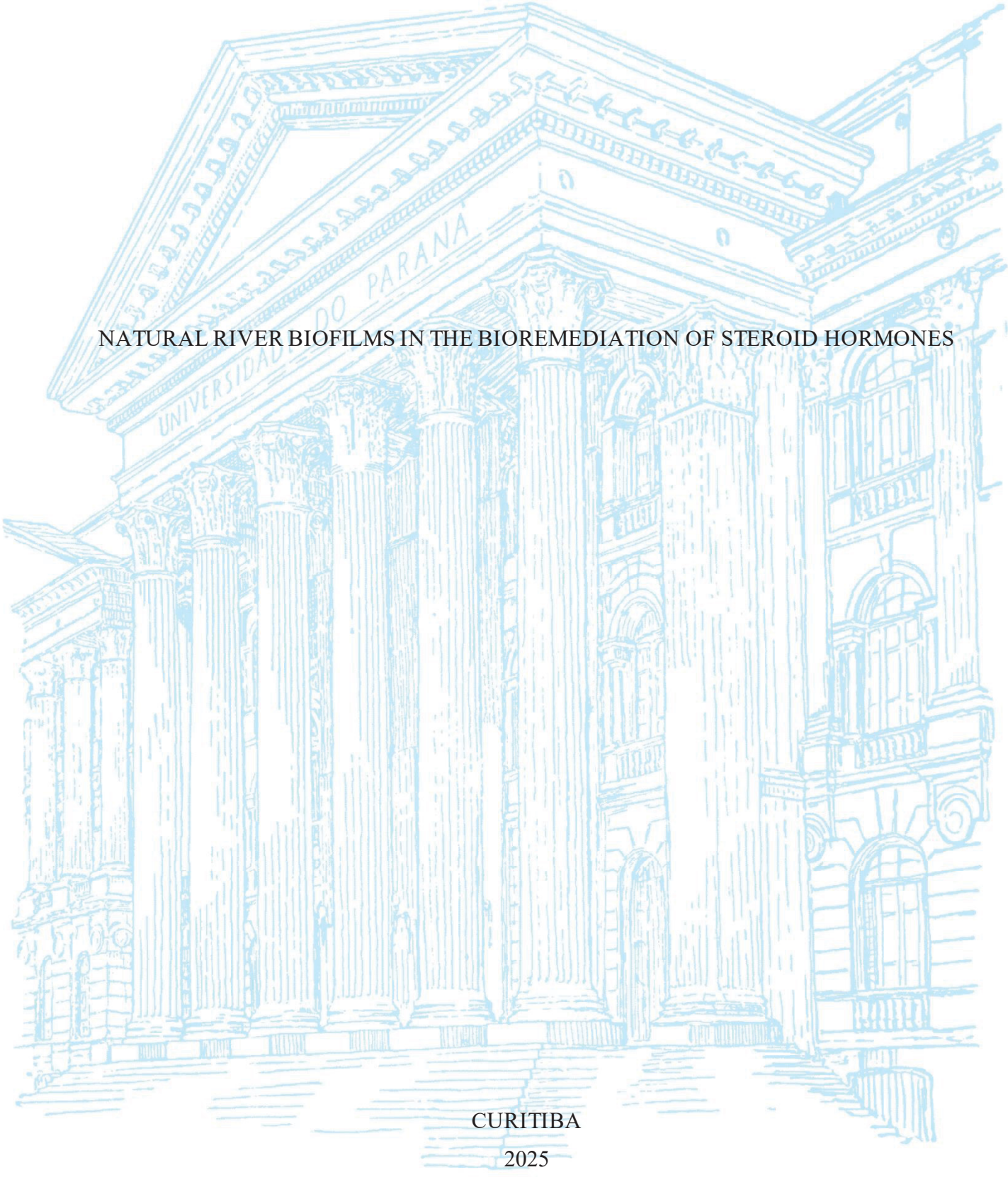

NATURAL RIVER BIOFILMS IN THE BIOREMEDIATION OF STEROID HORMONES

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2025

TATIANI ANDRESSA MODKOVSKI

NATURAL RIVER BIOFILMS IN THE BIOREMEDIATION OF STEROID HORMONES

Tese submetida como requisito para obtenção do título de Doutora, no Programa de Pós-Graduação em Engenharia de Recursos Hídricos e Ambiental (PPGERHA), Setor de Tecnologia, Universidade Federal do Paraná.

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A outorga do título de doutora está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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"A vida não é só trabalhar. É preciso deixar um bom capítulo para a loucura que cada um tem. Você é livre quando gasta o tempo da sua vida em coisas que te motivam, que você gosta. Para uma pessoa pode ser jogar futebol, para outra pescar, para outra investigar uma molécula, para outra a arte, sei lá. É que, somos diferentes."
Pepe Mujica

"Life is not only about working. It is necessary to leave a good chapter for the madness that each one has. You are free when you spend the time of your life on things that motivate you, that you like. For one person, it can be playing soccer, for another fishing, for another investigating a molecule, for another art, I don't know. It's just that we are different."
Pepe Mujica

RESUMO

Hormônios esteroides são compostos orgânicos, que podem ser naturais ou sintéticos, utilizados na medicina humana e veterinária. No entanto, eles também são considerados desreguladores endócrinos devido à sua atividade hormonal, o que é uma preocupação quando esses compostos aparecem nos ambientes naturais. Uma técnica para eliminar esses contaminantes em ambientes aquáticos é a biorremediação, que utiliza microrganismos para remover substâncias indesejadas. Em águas naturais, por exemplo, comunidades microbianas complexas formam biofilmes, os quais podem adsorver, bioacumular, biodegradar e transformar esses contaminantes presentes no ambiente. Esta pesquisa teve como objetivo avaliar se biofilmes naturais de rios tem capacidade de remoção dos hormônios esteroides Estrona (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2) e Progesterona (PRO) de ambientes aquáticos. A pesquisa envolveu amostras de biofilme obtidas do Rio Alb em Karlsruhe, Alemanha, e do Rio Barigui em Curitiba, Brasil. Os biofilmes foram mantidos, tanto em ambientes fluviais naturais quanto em microcosmos controlados de laboratório, para conduzir testes de remoção dos compostos alvo. Os resultados experimentais demonstraram a remoção completa de E1, E2 e EE2 sob condições específicas, apresentando como principais mecanismos envolvidos na remoção a biodegradação e/ou biotransformação. A análise microbiológica mostrou que *Proteobacteria, Bacteroidota, Actinobacteria, Firmicutes* e *Deinococcota*, representaram os principais filos responsáveis pelo processo, mas as duas regiões de estudo apresentaram gêneros dominantes diferentes. Os micropoluentes presentes no ambiente afetam a comunidade de biofilmes, o que leva à sensibilidade entre certas espécies, enquanto promove resiliência e possível adaptação entre outras. Portanto, esta tese revelou o potencial dos biofilmes fluviais para a remoção simultânea de hormônios esteroides, fornecendo assim um método capaz de ser usado em aplicações de tratamento de águas residuais. Além disso, o estudo fornece insights valiosos sobre os mecanismos de remoção de hormônios esteroides em água superficial de rios urbanos.

Palavras-chave: Perifíton; Hormônios esteroides; Contaminantes emergentes; Tratamento de água; Biofilmes; Amostrador passivo.

ABSTRACT

Steroid hormones are organic compounds that can be either natural or synthetic, and are used in both human and veterinary medicine. However, they are also considered endocrine disruptors due to their hormonal activity, which is a concern when these compounds are released into the environment. One technique for eliminating these contaminants in aquatic environments is bioremediation, which uses microorganisms to remove unwanted substances. In natural waters, for example, complex microbial communities form biofilms, which can adsorb, bioaccumulate, biodegrade, and transform these contaminants present in the environment. This study aimed to evaluate the ability of natural river biofilms to remove the steroid hormones Estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2), and Progesterone (PRO) from aquatic environments. The research involved biofilm samples obtained from the Alb River in Karlsruhe, Germany, and the Barigui River in Curitiba, Brazil. The biofilms were maintained both in natural river environments and controlled laboratory microcosms to conduct removal tests of target compounds. The experimental results demonstrated the complete removal of E1, E2, and EE2 under specific conditions, with biodegradation and/or biotransformation being the main mechanisms involved in the removal. The microbiological analysis showed that *Proteobacteria, Bacteroidota, Actinobacteria, Firmicutes*, and *Deinococcota*, represented the main phyla responsible for the process, but the two study regions showed different dominant genera. Micropollutants present in the environment affect the biofilm community, leading to sensitivity among certain species while promoting resilience and possible adaptation among others. Therefore, this thesis revealed the potential of river biofilms for the simultaneous removal of steroid hormones, thus providing a method capable of being used in wastewater treatment applications. Furthermore, the study provides valuable insights into the mechanisms of steroid hormone removal in the surface water of urban rivers.

Keywords: Periphyton; Steroid hormones; Emerging contaminants; Bioremediation; Water treatment; Biofilms; Passive sampler.

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ABREVIATIONS

ANA	Agência Nacional das Águas (National Water Agency)
AOPs	Advanced Oxidation Processes
AZI	Azithromycin
BA1	Site 1 at River Barigui
BA2	Site 2 at River Barigui
BOD	Biochemical Oxygen Demand
BPA	Bisphenol A
CEC	Cation Exchange Capacity
CMR	Curitiba Metropolitan Region
COD	Chemical Oxygen Demand
CONAMA	Conselho Nacional do Meio Ambiente (National Environmental Council)
CSO	Combined Sewer Overflow
CSS	Combined Sewer Systems
CV	Cristal Violet
CWA	Clean Water Act (U.S.)
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
DOX	Antihistamine Doxylamine Succinate
DWF	Dry Weather Flow
DWS	Downstream Site
E1	Estrone
E2	17β-Estradiol
E3	Estriol
ECs	Emerging Contaminants
EDC	Endocrine-Disrupting Compound
EE2	17α-Ethinylestradiol
EPA	Environmental Protection Agency (United States)
EPM	Extracellular Polymeric Matrix
EPS	Extracellular Polymeric Substances
ERY	Erythromycin

EU	European Union
FPBR	Fiber Periphyton Bioreactor
FTB	Floating Treatment Bed
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS/MS	Gas Chromatography Coupled with Tandem Mass Spectrometry
GPC	Gel Permeation Chromatography
HRT	Hydraulic Retention Time
IUCN	International Union for Conservation of Nature
IAT	Instituto Água e Terra (Water and Land Institute)
IOP	Iopromide
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LV	Levonorgestrel
NbS	Nature-based Solutions
NLCD	National Land Cover Database
NOR	Norfloxacin
NR	Norethisterone
OTUs	Operational Taxonomic Units
PAH	Polycyclic Aromatic Hydrocarbons
PE	Polyethylene
PET	Polyethylene Terephthalate
PFAS	Poly-Fluoroalkyl Substances
PFOA	Perfluorooctanoic Acid
PFOS	Perfluorooctane Sulfonate
PP	Polypropylene
PRO	Progesterone
PVC	Polyvinyl Chloride
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RNA-seq	Transcriptomics Sequencing
ROX	Roxithromycin
SDGs	Sustainable Development Goals
SDWA	Safe Drinking Water Act (U.S.)
SEM	Scanning Electron Microscopy

SEMA	Secretaria do Meio Ambiente E Infraestrutura (Secretariat of Environment and Infrastructure)
SMX	Antibiotic Sulfamethoxazole
SNIRH	Sistema Nacional de Informações Sobre Recursos Hídricos (National Water Resources Information System)
SNIS	Sistema Nacional de Informações Sobre Saneamento (National Sanitation Information System)
SPE	Solid Phase Extraction
TDS	Total Dissolved Solids.
TPs	Transformation Products
UASB	Anaerobic Upflow Sludge Blanket
UHPLC	Ultra-High Performance Liquid Chromatography
UPS	Upstream site
USEPA	United States Environmental Protection Agency
WGS	Whole Genome Sequencing
WHO	World Health Organization
WTP	Water Treatment Plants
WWTP	Wastewater Treatment Plant

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PREFACE

This doctoral thesis is organized into seven chapters, each contributing to an understanding of the function of natural river biofilms in the bioremediation of emerging contaminants, particularly steroid hormones.

Chapter 1 introduces the research by presenting the central hypotheses, the scientific justification for the study, and its objectives. This section shows the ideas that guided the development of the thesis

Chapter 2 offers a literature review, which serves as the theoretical and conceptual foundation for the research. It contextualizes the relevance of microbial communities in aquatic ecosystems and their potential in environmental remediation strategies.

Chapter 3 comprises a review article focused on the bioremediation of emerging contaminants by river biofilms. This manuscript has been submitted to the *Environmental Management* journal and is currently under review.

Chapter 4 presents a description of the study areas, located in Brazil and Germany, that were selected for experimental investigation. This contextual framework supports the subsequent chapters, which explore the bioremediation potential of natural river biofilms in distinct environmental conditions.

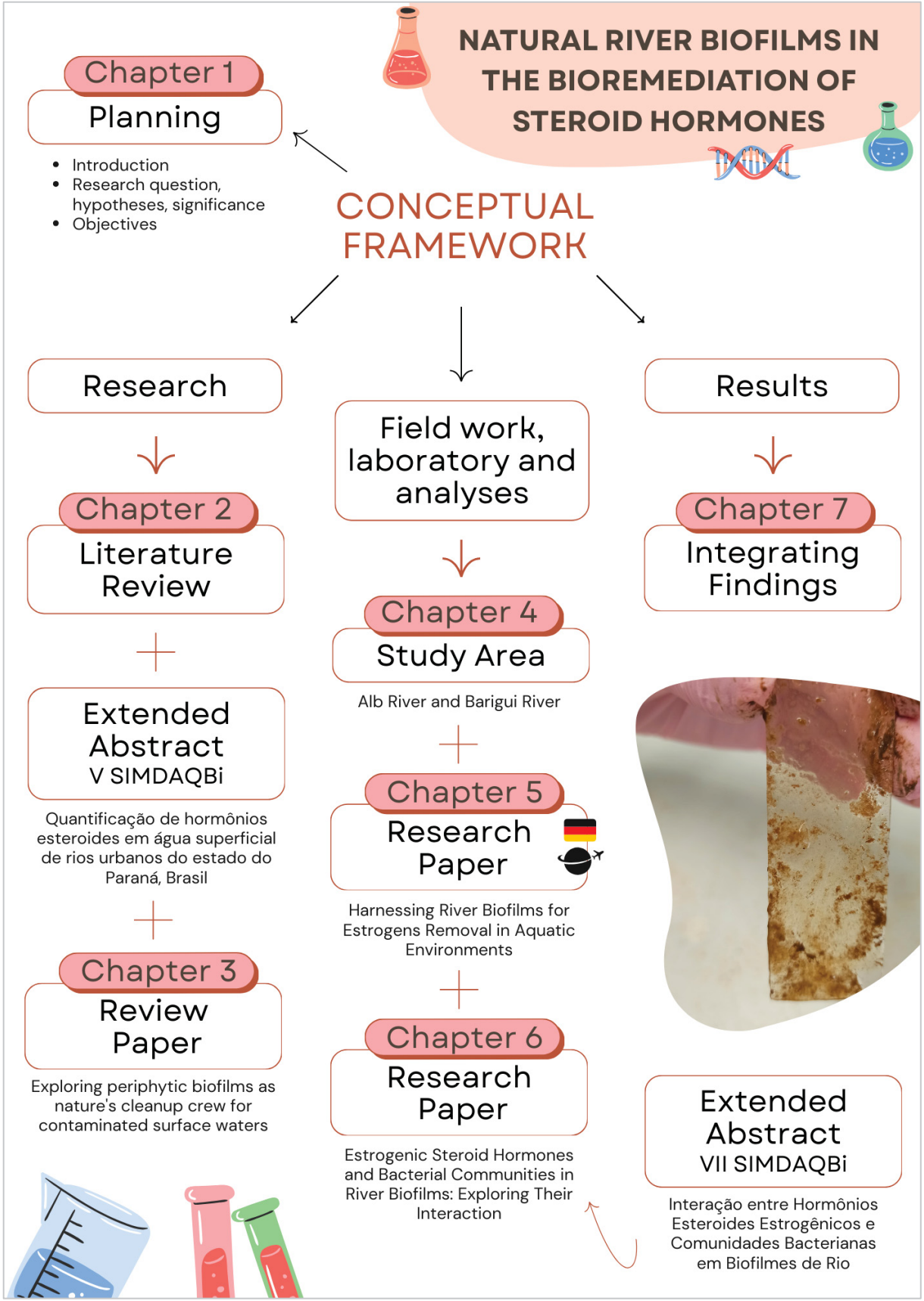
Chapter 5 includes an article based on data obtained through experimental work carried out in collaboration with the Karlsruhe Institute of Technology (KIT), as part of an international doctoral exchange program. This manuscript has been submitted to the *Journal of Environmental Chemical Engineering* and is currently under review.

Chapter 6 presents the third research article, which is based on field and laboratory investigations conducted using samples from the Barigui River in Curitiba, Paraná. It continues the investigation of how biofilms contribute to the removal of steroid hormones in an urban tropical setting.

Finally, **Chapter 7** synthesizes the major findings of the research, discussing their implications, limitations, and contributions to the field of environmental engineering and water quality management.

To support the reader's understanding of the thesis structure, a conceptual framework diagram (Figure 1) is included. It illustrates the logical progression of the chapters and how each stage, from planning to research dissemination, contributes to addressing the central research question. The figure also shows the extended abstracts presented at conferences, highlighting the dissemination of partial results during the PhD.

FIGURE 1 – THE CONCEPTUAL FRAMEWORK OF THE THESIS.



SOURCE: The author (2025).

Chapter 1

"Estou entre aqueles que acham que a ciência tem uma grande beleza."
Marie Curie

"I am among those who believe that science has great beauty."
Marie Curie

1 INTRODUCTION

Water is a natural resource, essential for the development and maintenance of life on Earth, and problems related to its quantity and quality are not just a concern today. With rapid industrialization and urbanization, unregulated chemical and biological substances have been discharged into the environment on a significant scale, and these substances are known as emerging contaminants (ECs) (Morin-Crini *et al.*, 2022). Found in concentrations ranging from nanograms to micrograms per liter, they are continuously released into the environment through various pathways, including domestic, industrial, and hospital effluents, stormwater, and agricultural runoff (Parida *et al.*, 2021).

To control and prevent risks to the environment and living organisms, institutions establish environmental laws and watch lists to regulate the presence of microcontaminants in environmental matrices. In April 2024, the European Parliament adopted an agreement with the Council on revising the European Union's water management and urban wastewater treatment standards to better protect public health and the environment. The amendment requires an additional quaternary treatment to remove at least 80% of a broad spectrum of micropollutants, which will be mandatory for all treatment plants with a capacity above 150,000 population equivalents (and for plants above 10,000 population equivalents, based on a risk assessment) by 2045 (EU, 2024). In Brazil, there are no official regulations, environmental laws, or watch lists regarding ECs in environmental matrices. Most regulations apply to drinking water and include nutrients, heavy metals, and some pesticides (CONAMA, 2011), but pharmaceuticals, plasticizers, hormones, personal care products, and other compounds are generally not regulated (Reichert *et al.*, 2019).

Pharmaceuticals are among the most important examples of ECs (Rodrigues *et al.*, 2025). Antibiotics, anti-inflammatories, antidepressants, analgesics, and endocrine-disrupting

compounds (EDCs) are among the most commonly prescribed medications worldwide (Fernandes *et al.*, 2020). Estrogens, a class of EDCs, can be either naturally produced by humans and animals or synthetically manufactured, with primary applications in contraceptives and hormone therapies (Du *et al.*, 2020). Another relevant EDC is progesterone, which also exists in both natural and synthetic forms and is used in human and veterinary treatments (Shen *et al.*, 2018).

Following pharmaceutical ingestion, a considerable fraction of these compounds is excreted via urine and feces, either as the parent compound or as biologically active metabolites (Torres *et al.*, 2015). Conventional Wastewater Treatment Plants (WWTPs) were designed primarily to remove suspended solids, biodegradable organic matter, nutrients, and pathogenic microorganisms, and therefore are not focused on removing micropollutants present at trace levels (Tran; Reinhard; Gin, 2018). The removal efficiency of ECs in WWTPs is dependent on the physicochemical properties of the compounds, including hydrophobicity, molecular structure, functional groups, ionization state, molecular weight, and morphology. WWTPs typically comprise sequential treatment stages: primary, which involves mechanical separation; secondary, based on biological degradation; and in some cases, tertiary treatment, which includes advanced physicochemical processes such as sorption, oxidation, and membrane filtration (Shahid *et al.*, 2021).

A study showed that removal efficiencies for steroid hormones in WWTPs vary, with average rates of 37.8% for Estrone, 75.9% for 17β-estradiol, and 74.8% for Estriol (Liu *et al.*, 2015), and can be even lower depending on the type of treatment and operational conditions (Paterakis *et al.*, 2012; Rodriguez-Narvaez *et al.*, 2017). Global reviews indicate that this variability is influenced by factors such as human and animal metabolism and excretion rates of EDCs, wastewater composition, WWTPs size, and the specific treatment processes employed (Du *et al.*, 2020; Shabbir *et al.*, 2022).

The presence of these compounds in aquatic environments is of concern due to their adverse effects on ecosystems (Bai *et al.*, 2022). Even at trace concentrations, prolonged exposure to EDCs can impact both human and environmental health. EDCs are known to interfere with hormonal functions, leading to various health issues in humans and aquatic organisms. In humans, they have been associated with reproductive disorders, thyroid dysfunction, Alzheimer's disease, cancer, and obesity. In aquatic organisms, EDCs can disrupt reproductive processes, affect hatchability rates, and alter vitellogenin levels (Kasonga *et al.*, 2021; Vilela; Bassin; Peixoto, 2018).

As a result, there is increasing scientific interest in understanding the environmental occurrence, transformation pathways, and removal mechanisms of ECs (Montagner *et al.*, 2019). One promising technique for treating water contaminated with hormones is bioremediation. This technique offers several advantages over conventional treatment methods, including reduced use of hazardous chemicals, minimal environmental disruption, and cost-effectiveness in degrading and detoxifying complex pollutants (Yadav; Chandra, 2020).

Biodegradation refers to the partial or total decomposition of chemical pollutants through the metabolic activities of microorganisms (Hatzinger; Kelsey, 2005). The microorganisms used in bioremediation, fungi and bacteria, can be easily cultivated, and biodegradation is considered one of the most effective processes for the removal of ECs (Bilal *et al.*, 2019). These microorganisms degrade pollutants via specific metabolic pathways, in which enzymes play a central role by converting complex compounds into less toxic substances, such as carbon dioxide and water (Vijayalakshmidevi; Muthukumar, 2015).

Autochthonous microorganisms exhibit the capacity for the bioremediation of ECs in surface water systems. This strategy can be observable on native microbial communities already adapted to the impacted environment to efficiently target and degrade specific pollutants (Fernandes *et al.*, 2020). River biofilms (periphyton) consist mainly of bacteria and algae embedded in an organic polymer matrix, which retains nutrients present in the water and provides protection for microorganisms within the biofilms (Wu, 2017). Biofilm microbial communities are diverse and play a critical function in biogeochemical cycling, acting as good indicators of water contamination, and harboring the ability to biodegrade pollutants (Argudo *et al.*, 2020; Valdés *et al.*, 2021).

Natural river biofilms support bioremediation by hosting microorganisms with high adaptability and survival capacity under adverse conditions, making them a possible treatment strategy (Sharma, A. *et al.*, 2020). Biofilms support synergistic interactions within microbial communities, including potential genetic exchange, which enhances the biodegradation process (Yadav; Chandra, 2020). The use of natural biofilms in the bioremediation of various contaminants has attracted growing attention in research and development (Bursztyn Fuentes; Montes; Rodríguez, 2024; Carles *et al.*, 2019; Liang *et al.*, 2024; Shabbir *et al.*, 2022; Yan *et al.*, 2023).

Biodegradation of steroid hormones by natural river biofilms can be considered a viable and environmentally friendly technique for the treatment of waters contaminated with these compounds. As previously mentioned, conventional WWTPs are not fully effective in removing these hormones, which, even at trace concentrations, can have adverse effects on

aquatic ecosystems and human health. Although river biofilms have been studied in the bioremediation of several pollutants, the application of river biofilms in the removal of steroid hormones is rarely reported. Biofilm-mediated biodegradation stands out as an effective and low-cost strategy for the remediation of contaminated surface waters. In addition, microbial bioremediation is a sustainable technique, can be performed *in situ,* and is capable of eliminating pollutants without generating negative environmental impacts.

In this context, this thesis aimed to investigate the capacity of natural river biofilms to bioremediate waters contaminated with the steroid hormones Estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2), and Progesterone (PRO). The microcosm experiments were conducted using biofilms collected from the Alb River in Karlsruhe, Germany, and the Barigui River in Curitiba, Brazil, to compare the bioremediation potential of microbial communities from different geographic regions. Furthermore, the removal mechanisms of these hormones were studied. Therefore, this thesis highlighted the potential of natural biofilms from urban rivers to mitigate emerging contaminants from contaminated water, while reporting the self-cleaning processes of these ecosystems.

1.1 RESEARCH QUESTION, HYPOTHESES, AND SIGNIFICANCE OF THE THESIS

The research question that guided this thesis is:

Can microorganisms present in natural river biofilms be used for the bioremediation of emerging contaminants in these ecosystems?

Given that natural river biofilms are capable of bioaccumulating emerging contaminants and are recognized as bioindicators of environmental pollution, it is assumed that they can also biodegrade these compounds. Therefore, they can be used as a tool in bioremediation processes. Based on this premise, the hypotheses formulated for this study are:

- Natural river biofilms can remove the emerging contaminants E1, E2, EE2, and PRO from aquatic environments;
- The predominant mechanism involved in the removal of these compounds is biodegradation.

Population growth has caused a number of environmental problems, raising concerns for both government authorities and society. Contributing to this is the continued release of chemicals and pharmaceuticals into aquatic environments, causing adverse effects on ecosystems and public health.

Considering the urgent need to improve the quality of surface waters, this study aimed to evaluate the effectiveness of bioremediation of steroid hormones through the biodegradation of these compounds by microorganisms. The research focused on the biodegradation of steroid hormones by autochthonous microorganisms embedded in natural river biofilms.

Although some studies have investigated the use of biofilms for the degradation of various pollutants in rivers and lakes, the application of natural biofilms in the bioremediation of pharmaceutical compounds remains an emerging field. The simultaneous removal of steroid hormones such as E1, E2, EE2, and PRO by natural river biofilms has not yet been thoroughly explored.

The efficiency of steroid hormone biodegradation by biofilms may represent an effective and low-cost process for removing contaminants from riverine environments. Moreover, microbial bioremediation is an *in situ* technique that does not cause environmental harm or generate toxic by-products. This approach also highlights rivers' natural self-purification capacity.

Therefore, this study is justified by the scarcity of research on the use of bioremediation with river biofilms for the removal of steroid hormones. Evaluating this potential is interesting, especially when considering the presence of these compounds in surface waters that are used for drinking water supply. Finally, exploring innovative technologies based on natural processes already existing in the environment is a promising strategy to address environmental challenges, integrating environmental engineering with a multidisciplinary perspective.

1.2 OBJECTIVES

1.2.1 General objective

The general objective of this thesis is to propose a strategy to evaluate the capacity of natural river biofilms to biodegrade steroid hormones, aiming at the bioremediation of water contaminated with these compounds.

1.2.2 Specific objectives

To achieve this main objective, the following specific objectives were established:

- To verify the ability of river biofilms to promote the removal of the steroid hormones E1, E2, EE2, and PRO in microcosm systems;
- To determine the removal percentages of the steroid hormones based on chromatographic analyses using Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS);
- To identify the microorganisms present in the biofilm microbiome associated with the steroid hormone exposure and removal;
- To identify the main mechanisms involved in the bioremediation of steroid hormones by natural biofilms.

Chapter 2

"Se assumirmos que chegamos à verdade absoluta, nós paramos de procurar, nós paramos de desenvolver."
Jocelyn Bell Burnell

"If we assume we've arrived at the absolute truth, we stop searching, we stop developing."
Jocelyn Bell Burnell

2 LITERATURE REVIEW

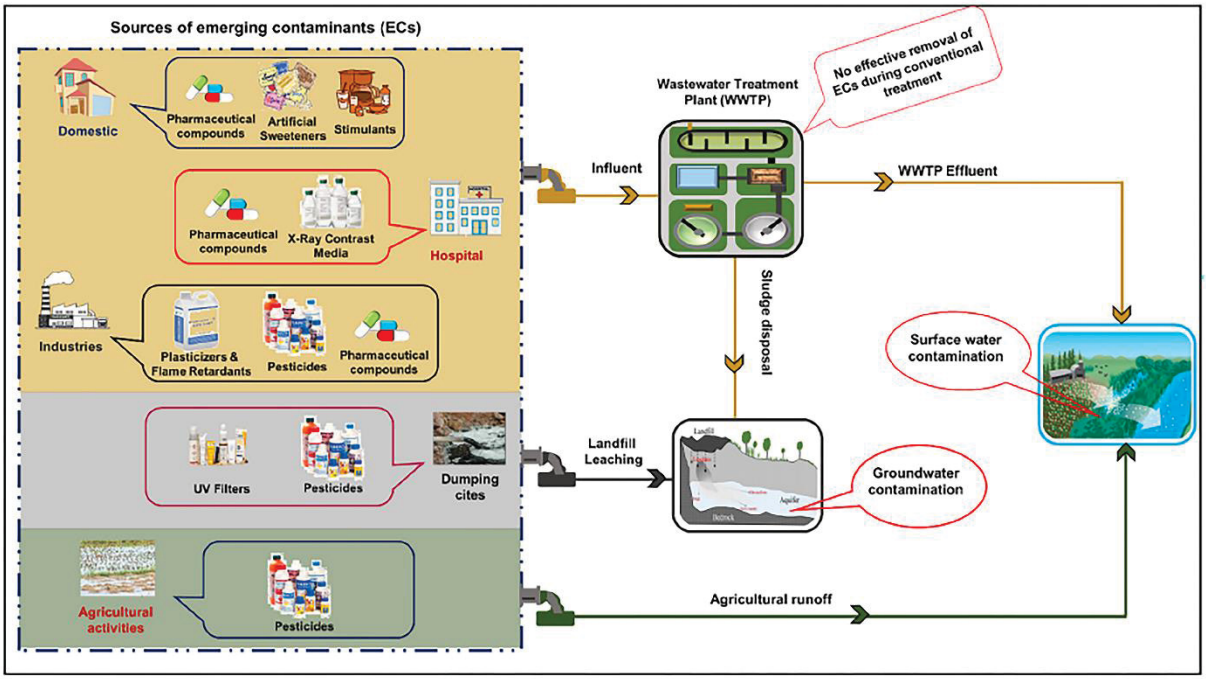
2.1 EMERGING CONTAMINANTS

Since the end of the last century, micropollutants have been released into aquatic environments, resulting in environmental problems and public health concerns (Ku, 2011). Among these micropollutants are the ECs, which are typically found in the environment at low concentrations, usually in the range of nanograms per liter or micrograms per liter (Méndez *et al.*, 2016). The detection of these compounds in aquatic environments is relatively recent, which is why they are referred to as "emerging."

According to the literature, a wide variety of ECs have been detected in the environment, including antibiotics, anti-inflammatory drugs, hormones, lipid-regulating medications, antidepressants, anticonvulsants, X-ray contrast media, nanomaterials, microplastics, plasticizers, polyfluoroalkyl substances, UV filters, pesticides, personal care products, stimulants, insect repellents, fire retardants, artificial sweeteners, among others (Bilal *et al.*, 2019; Biswas *et al.*, 2024; Li *et al.*, 2024; Parida *et al.*, 2021).

ECs reach water bodies through mainly by anthropogenic pathways. Point sources of pollution include hospital effluents, industrial discharges, and WWTPs' effluents. In contrast, non-point sources involve catchment runoff, atmospheric deposition, waste dumping sites, and septic tanks. Pharmaceutical compounds, for instance, undergo metabolic processes after ingestion. However, in many cases, these substances are not fully metabolized. The unmetabolized fraction is excreted through urine and feces, eventually entering wastewater streams (Parida *et al.*, 2021). Figure 2.1 illustrates the main sources and pathways through which ECs are introduced into aquatic environments.

FIGURE 2.1 – SOURCES AND PATHWAYS OF DIFFERENT ECS INTO THE AQUATIC ECOSYSTEM.



SOURCE: Parida *et al.* (2021).

How pollution by emerging contaminants occurs varies from country to country. Factors such as patterns of pharmaceutical use, product marketing and advertising, regional epidemiology, and medical prescription regulations can influence the concentrations of each compound found in aquatic systems. Additionally, other variables play a key role in the study of these compounds, including the presence of industrial activities, demographic, economic, and social data, climatic factors, effluent treatment methods, disposal practices, and the dilution capacity of water bodies (Boxall *et al.*, 2012).

Several factors can influence the concentrations of emerging contaminants found in aquatic environments. Examples include patterns of pharmaceutical use, product marketing and advertising, epidemiology, and regional prescription regulations (Mizukawa *et al.*, 2019). Therefore, the fate, monitoring, behavior, and removal of emerging contaminants in the environment require further studies.

Current regulations do not establish maximum allowable limits for these compounds in water. In Brazil, existing legislation addresses minimum concentration levels for various substances in water bodies, but there are no specific regulations concerning ECs. Most regulations focus on nutrients, heavy metals, and some pesticides in drinking water (CONAMA, 2011), while pharmaceutical and personal care products, plasticizers, and other emerging contaminants are generally unregulated (Reichert *et al.*, 2019).

At the international level, certain regulatory frameworks identify substances that should be monitored in aquatic environments; however, they often do not establish maximum concentration limits. An example is the “Watch List” mechanism developed by the European Union, which includes several pharmaceutical compounds of concern, such as the anti-inflammatory drug ibuprofen, the antibiotics azithromycin and erythromycin, and the hormone estrone (Reichert *et al.*, 2019). The first Watch List was introduced in 2015 and has since been updated in 2018, 2020, 2022, and most recently in 2025 (EU, 2015, 2018, 2020, 2022, 2025). Furthermore, in April 2024, the European Parliament approved an agreement with the Council to revise the EU’s water management and urban wastewater treatment standards, aiming to enhance public health and environmental protection. As part of these revisions, a mandatory quaternary treatment stage was introduced, requiring the removal of at least 80% of a broad range of micropollutants. This requirement will apply to all treatment plants with a capacity exceeding 150,000 population equivalents, and to those above 10,000, subject to risk assessment, by the year 2045 (EU, 2024).

In the United States, the Environmental Protection Agency (EPA) is responsible for regulating water contaminants through two key laws: the Safe Drinking Water Act (SDWA) and the Clean Water Act (CWA). The SDWA focuses on public health by establishing maximum contaminant levels for drinking water, while the CWA addresses water quality by controlling pollutant discharges and setting standards for surface waters. To date, the EPA has prioritized 126 substances for regulation, with defined concentration limits (Li *et al.*, 2024).

In China, the "Action Plan for the Prevention and Control of New Pollutants," released by the State Council in May 2022, introduced preliminary measures to manage priority emerging pollutants throughout their lifecycle, including production, processing, use, import, export, and emission control. Substances such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) were specifically highlighted. Additionally, China’s 14th Five-Year Plan (2021–2025) includes actions to assess, monitor, and identify high-risk chemical pollutants (Xuan *et al.*, 2024).

There is increasing concern about the quality of water used for human consumption, and most WWTPs are not designed to remove these types of micropollutants completely (Joanna *et al.*, 2018; Rout *et al.*, 2021). Conventional wastewater treatment relies on a mix of physical, chemical, and biological processes to eliminate solids, organic matter, and sometimes nutrients, typically classified into preliminary, primary, secondary, tertiary, or advanced stages (Kasonga *et al.*, 2021).

ECs are persistent in water, and to improve removal efficiency, integrated approaches combining biological processes with advanced oxidation techniques, physical adsorption-based treatment methods, and membrane-based separations have been developed (Li *et al.*, 2024). While these methods show promising results, their high energy demands and costs present challenges for large-scale applications (Kasonga *et al.*, 2021; Parida *et al.*, 2021).

As an alternative technique, the use of microorganisms such as bacteria, fungi, algae, and protozoa for the biodegradation of ECs has gained attention. This process, known as bioremediation, represents a more sustainable and cost-effective strategy for the treatment of these pollutants (Li *et al.*, 2024). The principles, mechanisms, and applications of bioremediation will be discussed in Section 2.3.

2.2 STEROID HORMONES

Pharmaceuticals, among ECs, are detected in various ecosystems. These compounds comprise more than 4,000 molecules with diverse physicochemical and biological properties, as well as distinct mechanisms of action (Caracciolo *et al.*, 2012). Their use is constant in human life, and they are often not fully metabolized inside the human body. Consequently, they are excreted and released into the environment either in their original form or as metabolites (Anawar; Ahmed, 2019).

Steroid hormones are among the most widely used pharmaceuticals. Based on their structural characteristics, steroids can be subdivided into five classes: androgens, estrogens, glucocorticoids, mineralocorticoids, and progestogens. Estrogens and progestogens, in particular, are commonly used in their synthetic forms, primarily in contraceptives. They are also naturally produced and excreted by humans and animals (Ojoghoro; Scrimshaw; Sumpter, 2021).

Estrogens are known as female sex hormones and are essential compounds for the female reproductive system, although they are also present in men at lower levels. Naturally occurring estrogens include Estrone (E1), Estradiol (E2), and Estriol (E3), while the main synthetic estrogen is Ethinylestradiol (EE2) (Ojoghoro; Scrimshaw; Sumpter, 2021). Synthetic estrogens are used in contraceptives, menopause management, and treatments for breast and prostate cancer, among other conditions (Torres *et al.*, 2021).

Progestogens are steroid hormones that play an important role in the estrous and menstrual cycles, as well as during pregnancy (Ojoghoro; Scrimshaw; Sumpter, 2021). These hormones are produced in natural form as Progesterone (PRO) and in synthetic forms such as

Norethisterone (NR) and Levonorgestrel (LV). LV is typically combined with EE2 in oral contraceptives. NR is used in hormonal regulation, and treatment of endometriosis, and pelvic pain (Ilyas; Van Hullebusch, 2020).

Steroid hormones are excreted in urine and feces after being ingested, either in their active forms or as metabolites, such as glucuronide or sulfate conjugates. Their entry into water bodies occurs through multiple pathways, including both treated and untreated domestic wastewater, as well as industrial discharges from pharmaceutical production, runoff and leaching from livestock farming and aquaculture activities, and landfill leachates where pharmaceuticals are frequently disposed of inappropriately (Ilyas; Van Hullebusch, 2020; Torres *et al.*, 2021).

Even at low concentrations, the presence of these compounds in water can induce adverse effects on the hormonal functions of humans and other organisms. Pharmaceuticals tend to be persistent and may cause considerable toxicological effects, including endocrine disruption, reduced nutrient uptake by plants, biodiversity loss, contamination of the food chain, human infertility, and cancer (Caracciolo *et al.*, 2012). Studies indicate that estrogens can affect aquatic organisms by causing male feminization, inhibition of sexual organs, and even sex reversal (Ilyas; Van Hullebusch, 2020; Torres *et al.*, 2021). In humans, they may lead to issues such as infertility or reduced fertility, as well as disruption of the endocrine system (Torres *et al.*, 2021).

The presence of hormones in surface waters has been detected in several countries, including Belgian (Mirmont *et al.*, 2021), Luxembourg (Pailler *et al.*, 2009), Serbia (Jauković *et al.*, 2017), Canada (Goeury *et al.*, 2022), Brazil (Torres *et al.*, 2015). In Brazil, Ide *et al.* (2017) detected the presence of the hormones E1, E2, and EE2 in water samples from the Iguaçu River (located in the state of Paraná, in Southern Brazil), at concentrations of 0.94 μg L^{-1}, 14.42 μg L^{-1}, and 1.48 μg L^{-1}, respectively. Another study conducted in Brazil by Liz *et al.* (2017) also reported the presence of E2, E3, and EE2 in wastewater samples from treatment plants located in the city of Curitiba, Paraná, confirming the frequent occurrence of these contaminants in water.

Several studies have reported the presence of steroid hormones in surface waters of urban rivers in the state of Paraná. Chart 2.1 presents a summary of steroid hormone concentrations found in rivers in Paraná. The most frequently reported compounds were the estrogens E1, E2, and EE2. These substances are prone to bioaccumulation and biomagnification in various matrices and organisms (Kumar *et al.*, 2022; Świacka *et al.*, 2022).

An important factor that has facilitated the detection of these contaminants in the environment is the development of analytical methods sensitive to the low concentrations of these hormones. As a result, researchers have been investigating extraction methodologies and pre-concentration steps for determining the concentration of these compounds through chromatographic analyses (Merlo *et al.*, 2019).

Another important factor that has been evaluated regarding the fate of steroid hormones in water resources is the analysis of different environmental matrices. When the presence of hormones is studied in surface water, groundwater, sediment, and biofilm samples, it allows for the evaluation of both the dissolved and particulate phases of these hormones, providing more comprehensive information on contamination (Goeury *et al.*, 2022; Yarahmadi *et al.*, 2018).

CHART 2.1 – MAXIMUM CONCENTRATIONS OF STEROID HORMONES DETECTED IN RIVERS OF THE STATE OF PARANÁ, BRAZIL.

	Concentration (µg L^{-1})	**Location**	**Reference**
E1	0,92	Atuba River	(Machado *et al.*, 2014)
E2	13,45	Atuba River	
EE2	4,53	Atuba River	
PRO	0,45	Atuba River	
E1	0,95	Iguaçu River	(Ide, 2014)
E2	5,88	Belém River	
EE2	1,59	Palmital River	
E1	1,09	Belém River	(Mizukawa, 2016)
E2	8,46	Belém River	
EE2	2,81	Belém River	
E1	0,94	Iguaçu River	(Ide *et al.*, 2017)
E2	1,42	Iguaçu River	
EE2	1,48	Iguaçu River	
E1	0,09	Rivers of CMR	(Baudisch, 2017)
E2	0,87	Rivers of CMR	
EE2	0,24	Rivers of CMR	
EE2	1,65	Tibagi River	(Reichert, 2017)
E2	0,53	Tibagi River	
PRO	0,79	Tibagi River	
E1	0,83	Iguaçu River	(Scipioni, 2018)
E2	2,30	Iguaçu River	
EE2	3,00	Iguaçu River	
E1	0,32	Palmital River	(Filippe, 2018)
E2	1,71	Palmital River	
EE2	0,66	Palmital River	
PRO	0,52	Palmital River	
E1	2,40	Belém River	(Barcellos, 2014)
E2	210,00	Belém River	
EE2	5,80	Belém River	

CMR = Curitiba Metropolitan region, E1 = Estrone, E2 = Estradiol, EE2 = Ethinylestradiol and PRO = Progesterone.

SOURCE: Modkovski *et al.* (2022).

Conventional WWTPs can't easily remove steroid hormones and some other emerging contaminants (Shabbir *et al.*, 2022). Du *et al.* (2020) conducted a five-year global review on the presence of steroid hormones in natural water bodies, drinking water, and WWTPs' effluents. According to their findings, the concentrations of these compounds in treated effluents vary, mainly due to factors such as human and animal metabolism and excretion rates, fluctuations in water consumption and its consequences, wastewater composition, size, and treatment settings of WWTPs, and operating conditions. Furthermore, Liu *et al.* (2015) compiled data from 14 countries between 1999 and 2012, reporting average removal efficiencies of 37.8%, 75.9%, and 74.8% for E1, E2, and E3, respectively.

A study conducted in a WWTP in the United Kingdom reported removal rates of 79% for E1, 0% for E2, 45% for E3, and 34% for EE2 using activated sludge. Furthermore, the total removal of steroid estrogens (sum of endocrine steroid estrogens) in primary sludge digestion reached 53% and 51% under mesophilic and thermophilic conditions, respectively (Paterakis *et al.*, 2012). Similarly, Rodriguez-Narvaez *et al.* (2017) reported removal efficiencies for activated sludge systems in WWTPs ranging from 0–36% for E1, 0–8% for E2, 0–1.67% for E3, and 0–4.2% for EE2, further highlighting the limited and variable performance of conventional biological processes in eliminating these micropollutants.

Secondary treatment processes, such as activated sludge systems and membrane bioreactors, are typically the primary methods for removing contaminants in conventional WWTPs. However, incorporating additional treatment stages (tertiary or advanced treatments) can improve the removal efficiency of various pollutants. As scientific knowledge advances, existing technologies for steroid hormone treatment are being improved to create more efficient, sustainable, and environmentally friendly solutions. In particular, the persistence of synthetic hormones such as EE2 has encouraged research into advanced treatment methods capable of achieving near-complete removal even at very low concentrations (Bayode *et al.*, 2024).

Techniques for removing these contaminants have been investigated, such as adsorption and biodegradation. Due to their hydrophobicity and nonpolar nature, steroid hormones can be easily adsorbed onto biofilms and sediments, the matrices commonly found in aquatic environments, and are often considered bioindicators of environmental contamination (Yarahmadi *et al.*, 2018; Ying; Kookana; Dillon, 2003). Therefore, studies evaluating the biodegradation process carried out by the microorganisms present, particularly in the biofilm, are of great interest, as they may be capable of degrading both the hormones that remain dissolved in water and those adsorbed onto this matrix.

2.3 BIOREMEDIATION

According to Boopathy (2000), bioremediation is the use of microorganisms to destroy or reduce the concentration of hazardous wastes at a contaminated site. This technology relies on the metabolic capacity of microorganisms to degrade different pollutants and presents advantages over conventional physicochemical methods, such as lower environmental impact and reduced operational costs (Li *et al.*, 2024). However, bioremediation also faces certain limitations, as not all compounds are biodegradable, and in some instances, microbial activity may lead to the formation of intermediate metabolites that are even more toxic than the original substances (Boopathy, 2000).

Microorganisms and their metabolic pathways are considered the main agents responsible for large-scale transformations in the biosphere. They are capable of degrading contaminants through complete mineralization or cometabolism, under both aerobic and anaerobic conditions. Furthermore, microorganisms exhibit short replication times, rapid evolution, and adaptability to new environmental conditions, making them highly suitable for bioremediation applications (Dvořák *et al.*, 2017).

Bioremediation can be performed either *in situ* or *ex situ*. *In situ* bioremediation refers to the treatment of contaminated material directly at the site, avoiding the costs and environmental impacts associated with excavation and transportation (Anawar; Chowdhury, 2020). In contrast, *ex situ* bioremediation involves the removal of contaminated material for treatment in a different location (Singh *et al.*, 2020).

In situ bioremediation can be carried out through intrinsic bioremediation, biostimulation, or bioaugmentation. In intrinsic bioremediation, natural microbial processes are responsible for contaminant degradation. Biostimulation involves enhancing the activity of indigenous microorganisms through the addition of nutrients, such as nitrogen and phosphorus, and surfactants. Bioaugmentation consists of the inoculation of selected non-indigenous (allochthonous) microorganisms capable of degrading specific pollutants (Lacerda; Navoni; Amaral, 2020).

Several factors must be considered when implementing bioremediation processes. These include achieving a favorable cost-benefit ratio, ensuring that the end products are non-toxic or less toxic than the original compounds, and maintaining environmental conditions that support microbial activity. Thus, it is common to optimize physical and chemical parameters such as pH, aeration, agitation, temperature, and nutrient supplementation to promote microbial growth (Yadav; Chandra, 2020). As highlighted by Boopathy (2000), the success of

bioremediation depends on the presence of suitable microorganisms in a conducive environment that enables effective pollutant degradation.

One strategy in bioremediation using fungi and bacteria involves the use of autochthonous microorganisms, for example, native strains isolated from the environment being treated. Using microorganisms adapted to the contaminated site offers advantages, such as improved survival, acclimatization, and effectiveness under site-specific conditions (Sarkar *et al.*, 2020). Several studies have reported the isolation of microorganisms for the removal of contaminants such as the pharmaceuticals paroxetine and bezafibrate (Fernandes *et al.*, 2020), doxycycline (Wen *et al.*, 2018), polycyclic aromatic hydrocarbons (Quintella; Mata; Lima, 2019), metals (Sharma, R. *et al.*, 2020), and combinations of metals and dyes (Mishra; Malik, 2014).

The bioremediation of steroid hormones by microorganisms may occur through two main biodegradation mechanisms: metabolism and cometabolism. In metabolism, the microorganisms use the compound as a source of carbon and/or energy for growth. In cometabolism, enzymes produced by the microorganisms degrade the compounds incidentally, without providing any direct energetic benefit. Cometabolism refers to the breakdown of a substance that does not supply carbon, energy, or essential nutrients, and this process can only occur in the presence of a primary substrate that induces the required enzymes (Boopathy, 2000).

Two groups of enzymes stand out as particularly promising for this application: peroxidases and laccases. These enzymes act as efficient biocatalysts in the transformation of organic pollutants into non-toxic compounds. Peroxidases and laccases operate under mild environmental conditions (ambient temperature and neutral pH), require less aggressive oxidants (oxygen for laccases and hydrogen peroxide for peroxidases), do not generate sludge, and offer simple application and control (Brugnari *et al.*, 2021; Méndez *et al.*, 2016).

Autochthonous fungi and bacteria have the potential to naturally eliminate environmental contaminants. However, limitations such as insufficient microbial populations, limited access to the contaminated zone, and lack of nutrients can hinder the natural attenuation process. In contrast, mobile, biofilm-forming microorganisms demonstrate greater versatility in contaminant biodegradation (Edwards, 2014). As a result, the application of biofilms in the bioremediation of emerging contaminants has been reported as a promising strategy for the treatment of contaminated water.

2.4 RIVER BIOFILMS

River biofilms are considered essential components of aquatic biota. They are composed of fungi, bacteria, algae, and protozoa embedded in a matrix of extracellular polysaccharides, lipids, and proteins that facilitate their attachment to inert surfaces. These biofilms are essential for ecosystems, participating in carbon and nutrient cycling, river self-purification, and are also applied in various biotechnological processes (Sharma, A. *et al.*, 2020; Valdés *et al.*, 2021).

The term periphyton is also used to describe natural biofilms that colonize aquatic environments. Some authors classify periphyton based on the type of natural substrate they colonize. According to Allan and Castillo (2007), *epilithon* refers to biofilms growing on hard substrates like rocks; *epipelon* develops on organic (muddy) sediments; *epipsammon* is associated with sand grains; and *epixylon* colonizes wood surfaces. Biggs; Hickey (1994) added two more categories: *epizoic*, which colonizes the surfaces of animal shells or larvae, and *metaphyton*, which is found in sediments associated with free-floating algae, not attached to any substrate.

Biofilms can be perceived as either beneficial or detrimental, depending on the context. Beneficial biofilms naturally form in environments such as rivers, lakes, and oceans and may associate with plant roots, aiding in nutrient supply. Another example of beneficial biofilms includes those applied in environmental biotechnology for wastewater treatment, where they are responsible for removing both organic and inorganic pollutants from contaminated water. Studies have documented the contribution of bacterial biofilms in the bioremediation of metals (Ma *et al*., 2018; Yang *et al*., 2016), dyes (Shabbir *et al*., 2017; Shabbir; Faheem; Wu, 2018), pharmaceuticals (Liang *et al*., 2024), pesticides (Chen *et al*., 2015; Dash; Osborne, 2020; Tien *et al.,* 2013; Tien; Huang; Chen, 2017), and other pollutants (Santos *et al*., 2019).

In natural environments, biofilms exhibit a tolerance to environmental stress due to their composition of diverse microbial species. Their rapid physiological responses to toxic substances, combined with their high biomass and contaminant adsorption capacity, make them effective bioindicators for environmental pollution monitoring (Sharma, A. *et al.*, 2020; Valdés *et al.*, 2021). Moreover, biofilms can transfer emerging contaminants to higher trophic levels in the food chain due to their sorptive properties and their role as a food source for various organisms (Valdés *et al.*, 2021).

Due to synergistic interactions and the potential for genetic exchange within biofilm communities, these systems are capable of degrading a wide variety of compounds. The use of

microbial consortia instead of individual microorganisms for pollutant degradation has been reported as a good strategy. Cooperative interactions among different bacterial or fungal strains can produce synergistic effects, enhancing the degradation of contaminants (Fernandes *et al.*, 2020).

Given their microbial complexity, natural river biofilms are used in wastewater treatment for the removal of contaminants, nutrients, and organic matter. Several types of bioreactors have been developed for this purpose (Ma *et al.*, 2018). Employing natural biofilms in biodegradation processes can minimize the adaptation challenges often faced when microorganisms are introduced to new contaminated environments, since these biofilms originate from areas already exposed to such pollutants (Kowalczyk *et al.*, 2016).

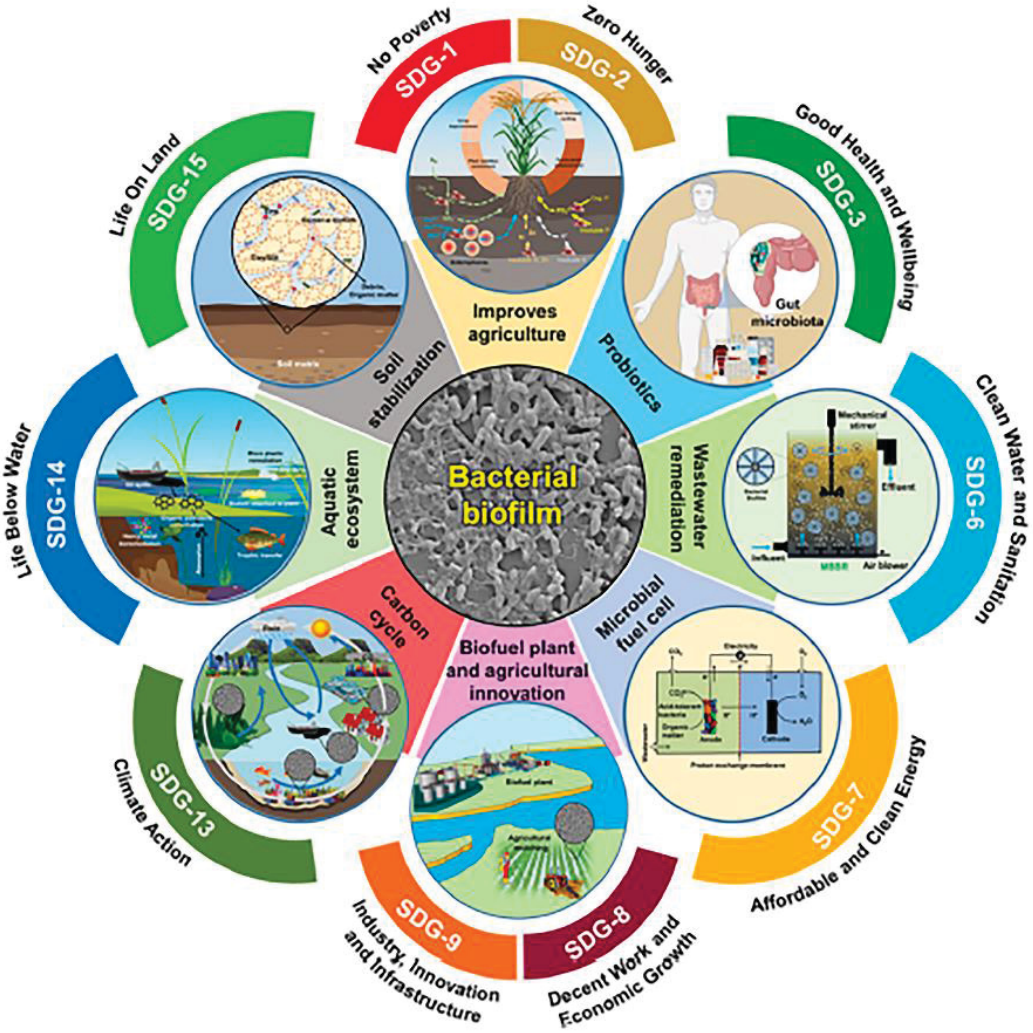
Biofilms also make bioremediation bacteria more resistant, protecting them from environmental toxins. Structured within a matrix of extracellular polymeric substances (EPS), microorganisms are shielded from harmful compounds. In addition, the exopolysaccharides within the EPS matrix act as biosurfactants, aiding in the solubilization of hydrophobic pollutants and increasing their bioavailability for bacterial degradation (Gutierrez *et al.*, 2013).

Biofilm-based remediation has been recognized as a promising approach to address complex global challenges and contribute to the achievement of the Sustainable Development Goals (SDGs) (Das *et al.*, 2024). Established by the United Nations, the SDGs provide a comprehensive framework for tackling interconnected global issues such as economic prosperity, social equity, and sustainable environmental management (Saxena *et al.*, 2021). Due to their diverse metabolic functions and ecological adaptability, bacterial biofilms contribute to environmental protection, social resilience, and economic development (Figure 2.2).

In agriculture, biofilms enhance soil fertility, promote plant growth, and suppress diseases, thereby supporting food security (SDG 2) and poverty reduction (SDG 1). Their antimicrobial properties and role in probiotic function also contribute to disease prevention and public health (SDG 3). In the water sector, biofilms are effective in treating contaminated wastewater, helping to ensure clean water and sanitation (SDG 6). Moreover, bacterial biofilms play a pivotal role in sustainable energy production, particularly in biofuel generation using waste materials, contributing to affordable and clean energy (SDG 7). In economic and industrial contexts, biofilms support innovation in bioprocesses and biotechnology, aligning with goals for decent work and economic growth (SDG 8) and industry, innovation, and infrastructure (SDG 9). From an environmental standpoint, biofilms aid in carbon sequestration, reduce greenhouse gas emissions, and improve ecosystem resilience, directly supporting climate action (SDG 13). They also promote the health of aquatic systems (SDG 14) through

nutrient cycling and habitat formation, and contribute to land conservation and biodiversity protection in terrestrial environments (SDG 15) (Das *et al.*, 2024).

FIGURE 2.2 – CONTRIBUTION OF BACTERIAL BIOFILM TO ENVIRONMENTAL REMEDIATION. IT ALIGNS WITH SEVERAL SUSTAINABLE DEVELOPMENT GOALS (SDGS), INCLUDING POVERTY ERADICATION (SDG 1), ZERO HUNGER (SDG 2), GOOD HEALTH AND WELL-BEING (SDG 3), CLEAN WATER AND SANITATION (SDG 6), AFFORDABLE AND CLEAN ENERGY (SDG 7), DECENT WORK AND ECONOMIC GROWTH (SDG 8), INDUSTRY, INNOVATION, AND INFRASTRUCTURE (SDG 9), CLIMATE ACTION (SDG 13), LIFE BELOW WATER (SDG 14), AND LIFE ON LAND (SDG 15).



SOURCE: Das *et al.* (2024).

A deeper understanding of the interactions between biofilms and ECs as steroid hormones, may contribute to the development of more effective and sustainable water treatment strategies. In this context, some of the following chapters of this thesis are organized as scientific articles that explore different aspects of this topic.

Chapter 3

"Sem água, sem vida.
Sem azul, sem verde."
Sylvia Earle

"No water, no life.
No blue, no green."
Sylvia Earle

3 EXPLORING PERIPHYTIC BIOFILMS AS NATURE´S CLEANUP CREW FOR CONTAMINATED SURFACE WATERS

Abstract

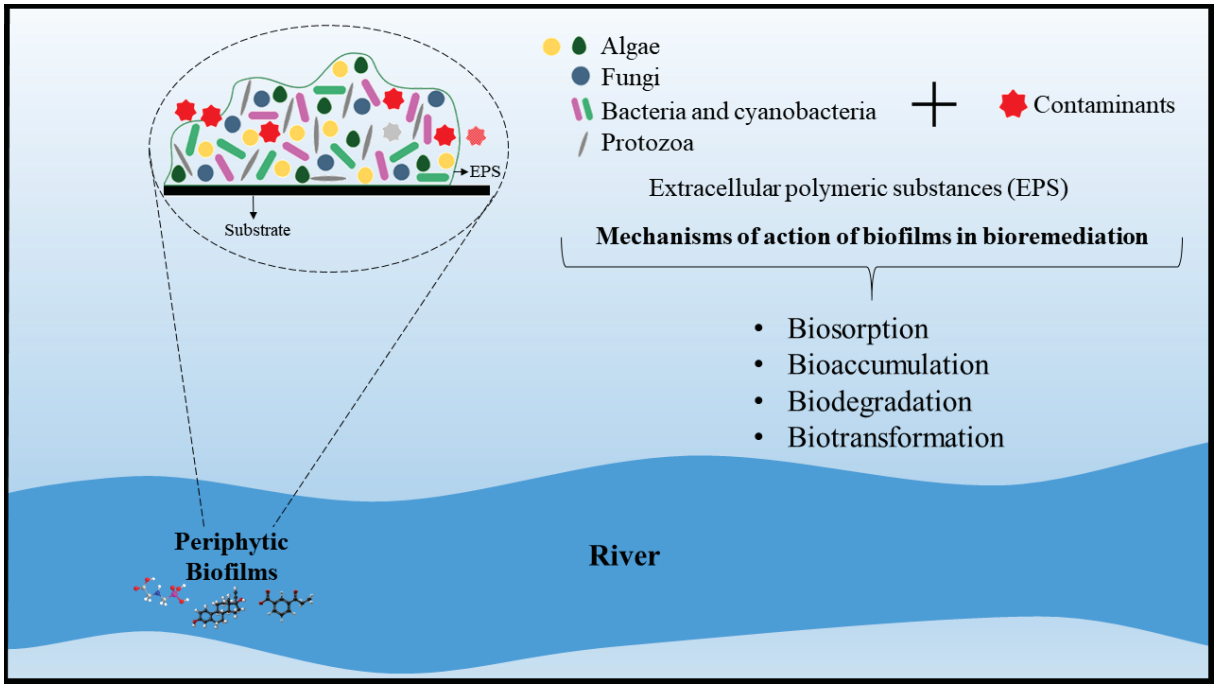
Periphytic biofilms, formed by fungi, bacteria, algae, and protozoa within an extracellular matrix, colonize various surfaces in river water and play a key role in carbon and nutrient cycling and river self-purification. Given their ecological importance, understanding the mechanisms these biofilms employ in contaminant bioremediation is essential for optimizing their application in environmental management. To achieve this, it is crucial to differentiate processes such as sorption, bioaccumulation, biodegradation, and biotransformation, which are key to evaluating bioremediation strategies using biofilms. This review highlights the effectiveness of biofilms in contaminant removal, even at low concentrations, due to their extensive adherence to solid surfaces in river systems. Furthermore, it explores the potential mechanisms of biofilm action in bioremediation. The review also addresses current challenges and prospects for enhancing the self-purification of aquatic ecosystems, alongside applying green bioremediation technologies utilizing periphytic biofilms. Such advancements aim to contribute to the sustainable management of water resources and restore aquatic ecosystem health.

Keywords: Biofilm, biodegradation, bioremediation, river, self-cleaning.

Highlights

- Biofilms are fundamental for self-purification and bioremediation of aquatic ecosystems
- Cells within a biofilm are more resistant to environmental fluctuations
- Biofilm removal efficiency is significant even at low concentrations
- The main mechanisms in the biofilms are sorption, bioaccumulation, biodegradation, and biotransformation
- Molecular analyses can prove the biofilm's mechanisms

FIGURE 3.1 – GRAPHICAL ABSTRACT.



SOURCE: The author (2025).

3.1 INTRODUCTION

The increasingly intense use of pharmaceuticals, personal care products, endocrine disruptors, insecticides, pesticides, metals, and dyes, among numerous other contaminants, underscores the need to investigate their occurrence (Montagner *et al.*, 2019), fate, persistence, impacts, and strategies for their removal (Maia *et al.*, 2023; Mukhopadhyay *et al.*, 2022). These substances, referred to as emerging contaminants (ECs), have been detected globally in surface water, wastewater, and groundwater, where they pose risks to ecosystems, aquatic organisms, and human health (Mishra *et al.*, 2022).

Among the natural processes with potential to mitigate these contaminants, microbial biofilms stand out as the most dominant form of life in streams (Battin *et al.*, 2003). River biofilms are essential components of aquatic ecosystems, composed primarily of algae, bacteria, fungi, microfauna, and EPS that attach to solid surfaces such as gravel, sediments, or aquatic plants (Battin *et al.*, 2016). They can capture particles and fine sediments that accumulate in the water and play a fundamental role in biogeochemical cycling, chemical exchanges between surface water and the riverbed (Gong *et al.*, 2023).

Biofilms exhibited high concentrations of certain contaminants, emphasizing their strong attraction and interaction with these substances (Wang *et al.*, 2019; Writer *et al.*, 2011). Therefore, considering the biofilms' ability to sorb contaminants, they can be regarded as pollution indicators and act as sinks for effectively removing contaminants (Shabbir *et al.*, 2017a). Additionally, due to their diverse microbial communities, river biofilms can significantly contribute to the degradation of ECs in aquatic environments, making them essential components in natural attenuation and in situ bioremediation processes (Lu *et al.*, 2014; Nadeau *et al.*, 2021; Sharma *et al.*, 2020; Singh *et al.*, 2020).

Conventional domestic wastewater treatment plants, such as stabilization ponds and activated sludge systems, were not originally designed to handle recalcitrant organic pollutants, including ECs (Morin-Crini *et al.*, 2022). Physicochemical methods and advanced oxidation processes, such as adsorption, membrane filtration, and ozonation, have demonstrated efficiency in contaminant removal. However, they are often limited by high energy requirements and operating costs, and the potential generation of toxic byproducts (Hu *et al.*, 2024; Das *et al.*, 2023). Biological treatments, on the other hand, are more sustainable and cost-effective, but traditional approaches based on suspended microbial cultures may lack stability and efficiency in degrading persistent compounds. In this context, biofilm-based systems represent a novel and advantageous strategy. Structured microbial communities provide greater

resilience, adaptability, and multifunctionality than planktonic microorganisms, improving contaminant sorption and degradation (Mishra *et al.*, 2022).

Bioremediation is a cost-effective and environment-friendly treatment technology for biodegrading organic and inorganic pollutants in contaminated areas, utilizing microorganisms' metabolic capabilities (Padma *et al.*, 2023; Sharma; Kumar, 2021). Indigenous microorganisms have significant potential to degrade contaminants due to their adaptability, genetic diversity, and functionality, removing pollutants through processes such as bioaccumulation, biosorption, biotransformation, and biomineralization (Padma *et al.*, 2023; Tang *et al.*, 2022).

Natural river biofilms hold significant potential for contaminant removal, however their application in bioremediation remains underexplored. The dynamic and responsive behavior of biofilms in contaminated environments, which allows them to adapt and effectively remove pollutants, warrants further investigation. The novelty of this review lies in synthesizing current knowledge on natural river biofilms as agents for the bioremediation of contaminated water, highlighting the essential mechanisms they use to interact with and eliminate contaminants, and identifying the environmental factors that affect their effectiveness. Thus, this work offers new perspectives and practical considerations on the use of natural river biofilms in sustainable water management.

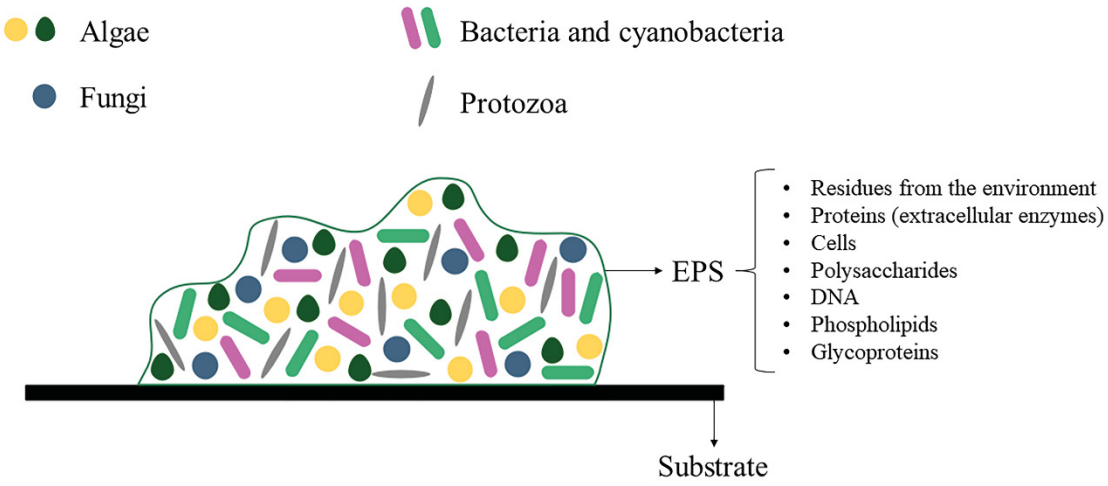
3.2 DIVERSITY AND FUNCTION OF PERIPHYTIC BIOFILMS

Microorganisms are the most prevalent life forms on Earth, and approximately 99.9% of microbial biomass in subsurface environments adheres to surfaces in the form of biofilm (Su *et al.*, 2023). Biofilms are complex and dynamic structures as they are constantly exposed to changes in aquatic ecosystems. Periphyton is a biofilm that grows on submerged surfaces in rivers, lakes, swamps, marshes, beaches, rice fields, and other locations (Tang *et al.*, 2023). As shown in Figure 3.2, they are composed of microbial cells, which can be eukaryotic or prokaryotic, adhered to an inert surface (Fernandes *et al.*, 2020). The interaction of these microorganisms with various interfaces includes connections between solid and liquid phases, liquid and liquid phases, liquid and gas phases, solid and gas phases, and even interactions among the microorganisms themselves in the form of aggregates (Sentenac *et al.*, 2022).

The terminology of biofilms may vary according to the surface on which they are adhered. When adhering to rocks, biofilms are called "*epilithic biofilm*" or "*epilithon*", on wood are "*pixylic biofilm*", on sand grains are "*episammon*", on mud sediments are "*epipelon*", on sediments are "*metaphyton*", and on plant organisms, they are called "*epiphyton*". All these

terminologies are covered by the terms “periphyton” or “periphytic biofilms”, which make up river biofilms. In addition to all these natural substrates, periphytic biofilms also grow by adhering to artificial substrates, such as plastics, glass, and other materials found in water (Sentenac *et al.*, 2022).

FIGURE 3.2 – PHYSICAL STRUCTURE OF PERIPHYTIC BIOFILMS WITH THEIR MAIN COMPONENTS. ABBREVIATIONS: DNA (DEOXYRIBONUCLEIC ACID), EPS (EXTRACELLULAR POLYMERIC SUBSTANCES).



SOURCE: The author (2025).

In addition to microbial cells, periphytic biofilms contain EPS and water. The EPS matrix comes from residues from the environment and the metabolism of these microorganisms. Water is the most significant fraction (97%) of the biofilm matrix (Fernandes *et al.*, 2020). Furthermore, the composition of EPS and water provides a hydrated environment that takes longer to dry than the surrounding environment and protects the biofilm cells from fluctuations (Flemming; Wingender, 2010).

The part composed of EPS acts as a protection for microorganisms and as a structure that promotes the binding of all fractions within the biofilm. This fraction contains proteins (extracellular enzymes), cells, polysaccharides, DNA (Deoxyribonucleic Acid) - genes for horizontal gene transfer, phospholipids, and glycoproteins (Figure 3.2). The EPS of a biofilm varies from one location to another, as it depends a lot on the microorganisms present there, the available nutrients, and other physical parameters, such as the shear forces suffered, pH, and temperature (Fernandes *et al.*, 2020; Flemming; Wingender, 2010).

Certain microorganisms in biofilms can also survive in planktonic communities or move from biofilms to planktonic communities and vice versa. These microorganisms in

biofilms can persist in the environment due to the surrounding EPS matrix. Conversely, when they are in planktonic form, they can disperse and establish themselves in new environments (Sentenac *et al.*, 2022). It is important to emphasize that bacteria dominate the microorganisms present due to their fast reproduction and adaptability to many conditions (Fernandes *et al.*, 2020).

The biodegradation conducted by biofilms represents better real-world degradation scenarios compared to planktonic assays. This is because most environmental biodegradation events occur through biofilm-substrate interactions. Biofilms provide the spatial and temporal aspects of degradation, including how it relates to biofilm attachment and growth, chemical movement, mobility, and characteristics of cells. Additionally, biofilm assays offer insights into how different species interact, which helps identify synergistic or competitive relationships that may either enhance or hinder biodegradation processes (Nadeau *et al.*, 2021).

Biofilms play a significant role in river ecosystems, driving enzymatic activities and contributing to critical processes such as biogeochemical cycling, organic matter cycles, primary production, and ecosystem respiration. They also facilitate the natural purification of surface waters and stabilize sediments, thereby mitigating erosion. These functions highlight the role of biofilms in maintaining the ecological balance and health of riverine environments (Gerbersdorf; Wieprecht, 2015; Dömölki *et al.*, 2022).

In addition, biofilms have essential advantages such as improved horizontal gene transfer capacity, ionic charges on the surface, and binding sites in cells and in the EPS matrix, which make them capable of performing the sorption of different compounds. Therefore, biofilms are considered good bioaccumulators of nutrients and microcontaminants such as pesticides, drugs, metals, microplastics, and others (Fernandes *et al.*, 2020). However, to propose water treatment approaches using these organisms, either *in situ* or *ex situ*, it is required to deepen knowledge and examine and evaluate the removal of contaminants by periphytic biofilms.

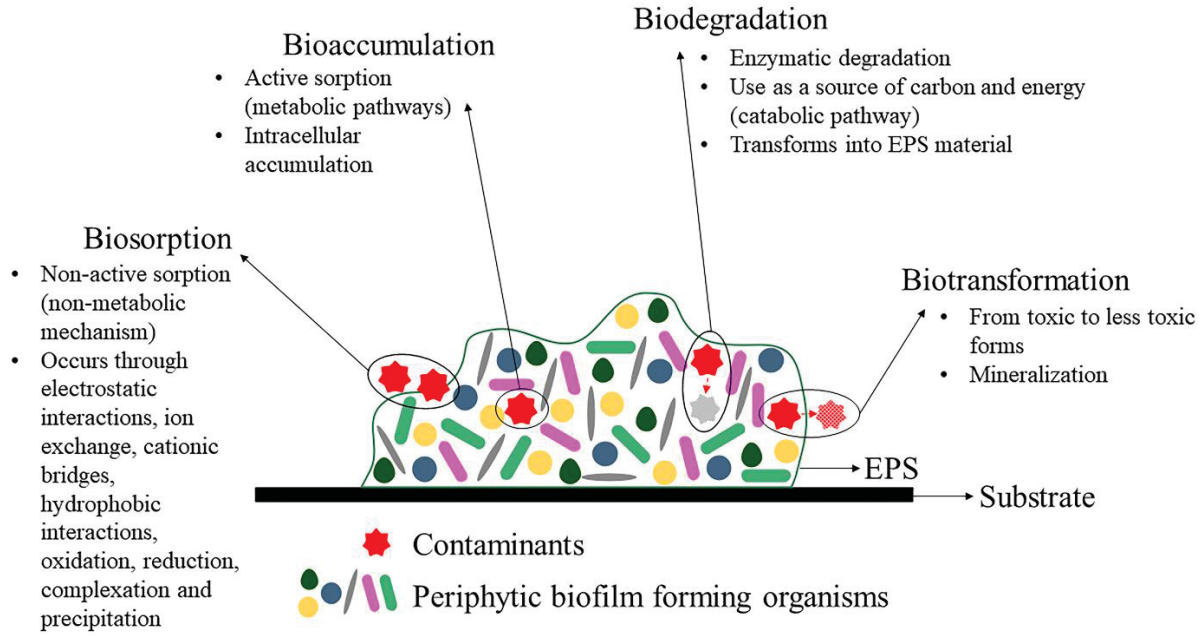
3.3 POSSIBLE MECHANISMS OF ACTION OF BIOFILMS IN BIOREMEDIATION

Biofilm microorganisms in aquatic environments face challenging and selective conditions, leading to structural and metabolic adaptations that enable their survival. This adaptive strategy results in varied responses to contaminants, including degradation and biotransformation (Desiante *et al.*, 2021). In this way, biofilms also play a role in the bioremediation and self-purification of the environments they inhabit.

Biofilm-mediated bioremediation of environmental contaminants utilizes both cells and EPS (Mahto *et al.*, 2022). In the biofilm and matrix-EPS, different exoenzymes degrade various xenobiotics, charged groups trap organic contaminants via biosorption, and bacteria use these toxic contaminants as a carbon and energy source for their growth and development. Hence, natural biofilms can eliminate microcontaminants from aquatic environments, functioning as a natural repository through passive sorption or engaging in various biological processes such as biosorption, bioaccumulation, biodegradation, or biotransformation (Desiante *et al.*, 2021).

The mechanisms of action involved in the bioremediation of contaminants by biofilms are fundamental to understanding this process, but distinguishing between biodegradation, biotransformation, sorption, and bioaccumulation is challenging. Figure 3.3 summarizes the mechanisms employed by periphytic biofilms in the bioremediation of various contaminants. This figure clarifies the metabolic and physical pathways involved in each mechanism, offering a detailed view of the role of each in the pollutant removal process and contributing to a better understanding of the dynamics of bioremediation.

FIGURE 3.3 – SCHEMATIC DIAGRAM DEMONSTRATING THE POSSIBLE MECHANISMS USED BY PERIPHYTIC BIOFILMS IN THE BIOREMEDIATION OF CONTAMINANTS.



SOURCE: The author (2025).

3.3.1 Biosorption and bioaccumulation

Biosorption and bioaccumulation are fundamental mechanisms by which periphytic biofilms contribute to the remediation of contaminants in aquatic environments. Sorption is a process involving both adsorption and absorption, and it can be either active or passive. When sorption is active, it involves metabolic pathways and is referred to as bioaccumulation (Chan *et al.*, 2022). On the other hand, biosorption is a passive, non-metabolic mechanism without energy consumption, which results from the surface sorption of contaminants by microorganisms and EPS in periphyton (Liu *et al.*, 2017). While biosorption can occur in living and dead cells, bioaccumulation is exclusive to living cells that metabolize the absorbed substances for growth and development (Bhunia *et al.*, 2022). As illustrated in Figure 3.3, in biosorption, contaminants are retained on the external surface of the biofilm, whereas in bioaccumulation, contaminants are internalized within the biofilm matrix through active cellular processes.

Periphytic biofilms, due to the complex structure of their EPS matrix, are highly effective at sorbing a wide range of contaminants, including both polar and non-polar compounds. Non-polar contaminants, such as hydrophobic organic pollutants, are primarily adsorbed based on their octanol-water partition coefficient (log Kow), with higher log Kow values indicating greater sorption affinity. Conversely, polar contaminants are sorbed through electrostatic interactions, cation exchange, hydrophobic interactions, and cation bridging. These passive sorption mechanisms are mediated by functional groups located on the surface of EPS, cell membranes, and cell walls (Fernandes *et al.*, 2020). The non-specific nature of biofilm sorption allows for the accumulation of essential nutrients and toxic substances, which is crucial for biofilms' environmental monitoring and remediation.

Regarding metal remediation, bioaccumulation involves the active uptake of metal ions into the microbial cytoplasm, utilizing metabolic processes that convert these ions into less toxic forms. In contrast, passive biosorption of metals occurs through electrostatic interactions between positively charged metal ions and negatively charged functional groups on the biofilm surface. These interactions facilitate complexation, precipitation, and ion exchange, allowing biofilms to immobilize and detoxify metals without direct metabolic involvement (Mahto *et al.*, 2022; Wu, 2017).

In 2024, Ji and Zhao published a review on the relation between biofilms and Perfluoroalkyl and poly-fluoroalkyl substances (PFASs), also known as "forever chemicals." According to the authors, biofilms have rough surfaces that contain complex functional groups

capable of adsorbing and retaining PFASs in water through known and unknown mechanisms. Studies show that long-chain PFASs containing more than seven fluorinated carbons are more likely to accumulate in biofilms than short-chain PFASs. However, further studies are required to investigate the mechanisms involved in the adsorption of PFASs in biofilms, as both long-chain and short-chain PFASs are widely present in the aquatic environment.

Furthermore, biofilms colonizing substrates like microplastics significantly enhance the retention of contaminants. For instance, Bhagwat *et al.* (2021) demonstrated that microplastic fibers coated with biofilms accumulated 75 times more perfluorooctane sulfonate (PFOS) than those without biofilm coverage. Functional groups such as carboxyl and sulfonic acid on biofilm surfaces facilitate this, aiding in the hydrophobic and electrostatic capture of the contaminants (Ji; Zhao, 2024).

Microorganisms within periphytic biofilms can utilize the organic contaminants they adsorb as nutrient sources, transforming them into cellular material like cytoplasm and promoting the production of EPS. This capacity for both sorption and subsequent biodegradation of organic pollutants highlights the dual role of biofilms in contaminant removal (Wu, 2017). For example, Fernandes *et al.* (2019) reported that epilithic biofilms are capable of both adsorbing and biodegrading the herbicide glyphosate, suggesting that the combination of biosorption and biodegradation can effectively eliminate such pollutants from aquatic environments.

3.3.2 Biodegradation and biotransformation

Biodegradation encompasses a range of processes through which microorganisms transform or break down substances in the environment. The substrate may be completely mineralized or only partially transformed (biotransformation), generating metabolites that cannot be further utilized and may accumulate in the medium. In some cases, these metabolites bind to matrix components, such as humic acids, becoming immobilized. Although the original contaminant is removed in all these scenarios, partially degraded products can be more problematic, particularly when the goal is to remediate contaminated environments (Kiel; Engesser, 2015).

Biotransformation is the alteration of compounds by an organism, resulting in changes to its chemical and toxicological properties. It also encompasses the metabolic alterations that occur in organisms due to exposure to xenobiotics (Valentová, 2023). Microorganisms in biofilms have catabolic enzymes that degrade persistent organic pollutants. In addition,

microorganisms biotransform and mineralize different toxic organic pollutants. These processes can occur in the presence or absence of oxygen. For example, anaerobic metabolism uses reductive reactions to degenerate the aromatic ring of hydrocarbons. On the other hand, the aerobic metabolism process uses monooxygenase and dioxygenase enzymes to incorporate oxygen atoms into hydrocarbons, leading to the hydroxylation of the aromatic ring. When bacteria use the catabolism pathway of organic pollutants, metabolic end products are formed that enter the Krebs cycle, leading to the formation of CO_2, water, and energy for the growth and maintenance of microorganism cells (Mahto *et al.*, 2022).

Biodegradation is crucial for removing contaminants and transforming environmental substances (Bose *et al.*, 2021). Carles *et al.* (2019) performed a study on periphytic biofilm, which showed that biofilms can biodegrade glyphosate in conditions similar to the environment. The experiment did not detect any glyphosate and the metabolite AMPA at the end of the study. The presence of phosphorus was also evaluated, and the best results were for the lowest initial phosphorus concentration. This is because microorganisms in the biofilm use glyphosate as a source of phosphorus. The natural biofilm uses catabolism to degrade glyphosate and obtain energy. Also, according to Carles and Artigas (2020), microorganisms that use glyphosate as a source of phosphorus have an enzymatic complex that allows an orthophosphate molecule to be released from glyphosate or AMPA through biodegradation pathways.

Some authors have stated that biofilm microorganisms require extra carbon sources to degrade certain compounds effectively (Shabbir *et al.*, 2020; Wang *et al.*, 2019). For instance, when glucose is added as a carbon source for microorganisms to obtain energy, the biofilm's ability to break down the insecticide methomyl carbamate is improved (Chen *et al.*, 2015). In contrast, in petroleum biodegradation, bacteria use polycyclic aromatic hydrocarbons (PAH) as a carbon source and degrade them through several catabolic pathways. Biofilms use emulsification, solubilization, and biodegradation mechanisms to remove petroleum hydrocarbons. The biofilm's emulsification breaks down large oil droplets into smaller ones, while EPS increases hydrocarbon dispersion and solubility, making biodegradation more efficient (Mahto *et al.*, 2022).

The removal of textile dyes combines adsorption and biodegradation through natural biofilms. According to Shabbir *et al.* (2017a), dyes are first adsorbed, and biodegradation occurs once adsorption sites are saturated. Biofilms enhance this process due to their large surface area, active sites, and transport pathways. Dye biodegradation involves azo bond cleavage, desulfonation, oxidation, reduction, breakdown into open-chain hydrocarbons, and

deamination, with enzymes such as azo reductase, peroxidases, and laccases playing key roles (Shabbir *et al*., 2017a).

Liang *et al*. (2024) identified 15 enzymes highly correlated with the biotransformation of azithromycin, energy supply, and antibiotic resistance processes. Key enzymes mentioned include aryl-alcohol dehydrogenases, hydroxylamine dehydrogenase, and monooxygenases, which are directly involved in azithromycin degradation. The study emphasizes that these enzymes facilitate the breakdown of the antibiotic, allowing the microbial community to adapt and enhance its metabolic pathways for more efficient biodegradation. This enzymatic activity is crucial for the overall health of the periphyton community and its ability to mitigate the impacts of antibiotic contamination in aquatic environments.

Mauch *et al*. (2023) identify key biochemical pathways involved in the biotransformation of iopromide (IOP) by periphyton and microbial communities. It highlights the oxidation of hydroxy groups, cleavage of amide-methylene bonds, and oxidative decarboxylation as primary mechanisms. Co-oxidation, driven by ammonia mono-oxygenase from ammonia-oxidizing bacteria, also plays a significant role in the process, especially under aerobic conditions. These pathways demonstrate the complex interactions between microbial metabolism and environmental factors in the degradation of IOP in aquatic ecosystems.

Biotransformation is related to the enzymatic activities of aquatic microbial communities, and evaluating them is important to improve the degradation of micropollutants in river remediation. To better propose contaminants biotransformation strategies, it is necessary to know the enzymes involved in this process and this is a lack of knowledge in this area. Mass balance calculations and the stoichiometry of compounds help when wanting to prove the biotransformation mechanism. However, to deeply understand and elucidate the biotransformation of contaminants by biofilms, it is necessary to explore the molecular mechanism that occurs (Liang *et al*., 2024).

Summarizing both processes, as described by Birolli *et al*. (2019), biodegradation takes place within a biotransformation process when various reactions in the substrate lead to the formation of small molecules and mineralization into water and CO_2. The boundaries between these phenomena are not well defined, but their main distinction lies in the study's focus and the extent of substrate modification.

3.4 REMOVAL OF CONTAMINANTS BY PERIPHYTIC BIOFILMS

Bioremediation involves detoxifying polluted environments through the action of microorganisms or their byproducts. This process can facilitate the transformation of contaminants into less harmful or inert compounds, such as CO_2 and water, which pose minimal risk to humans and the environment (Hidalgo *et al.*, 2024). Periphytic biofilms can absorb or biodegrade nutrients or contaminants in their matrix, detoxifying the surrounding environment. Several compounds, such as persistent organic pollutants, pesticides, heavy metals, human and veterinary drugs, nanomaterials, and plastics, can be captured and metabolized in natural biofilms (Sentenac *et al.*, 2022).

Table 3.1 summarizes studies conducted between 2014 and 2024 exploring using periphytic biofilms as a bioremediation method for removing contaminants in aquatic environments. The table details the evaluated compounds, their initial concentrations in the studies, and the removal mechanisms employed by the microorganisms present in the biofilms.

TABLE 3.1 – STUDIES REPORTING THE USE OF PERIPHYTIC BIOFILM IN THE BIOREMEDIATION OF CONTAMINANTS FROM 2014 TO 2024.

Compound	**Initial concentration**	**Removal mechanisms**	**Efficiency**	**References**
Cu	0.5 e 2 mg L^{-1}	Adsorption on extracellular polymeric substances, cell surface adsorption, and intracellular uptake	99 e 98 %	(Zhong *et al.*, 2020)
Cu (II)	2, 5, 10 and 20 mg L^{-1}	Biosorption	98, 90, 78 e 78 %	(Liu *et al.*, 2018)
Cu	2 e 10 mg L^{-1}	-	99 %	(Ma *et al.*, 2018)
Inorganic phosphorus	13 mg L^{-1}	Adsorption	90 %	(Lu *et al.*, 2014)
Glyphosate	< 10 µg L^{-1}	Degradation	100 %	(Carles *et al.*, 2019)
Glyphosate	0.132 mM of P-equivalents of glyphosate	Degradation	100 %	(Rossi *et al.*, 2021)
Methomyl	50 mg L^{-1}	Degradation	90.6 %	(Chen *et al.*, 2015)
Carbofuran	50 mg L^{-1}	Degradation	52.5 %	(Tien *et al.*, 2017)
Metazachlor, metribuzin and bentazone	540, 62, 150 µg L^{-1}	Biodegradation	> 94 %	(Bighiu; Goedkoop, 2021)
Lindane and norfloxacin (NOR)	Lindane (0.2 – 2.0 µmol L^{-1}) and NOR (40 – 400 µmol L^{-1})	Adsorption	45.6 % (Lindane) and 98.9 % (NOR)	(Dong *et al.*, 2018)
Ofloxacin	0.1 – 10 mg L^{-1}	Sorption	-	(Zhang *et al.*, 2018)
Phenanthrene and ofloxacin	0.3 – 6.0 and 0.1 – 3.0 µmol L^{-1}	Sorption	-	(Wang *et al.*, 2019)
Azithromycin (AZI)	5, 50, 200, 500, 5000 µg L-1	Biodegradation	88.65, 87.50, 80.62, 54.37 and 44.70 %	(Liang *et al.*, 2024)
Venlafaxine, diuron and triclosan	10 µg L^{-1} for diuron and triclosan, and 50 µg L^{-1} for venlafaxine	Bioaccumulation and biotransformation	15 % (Venlafaxine) 6,7 % (Diuron) 6 % (Triclosan)	(Santos *et al.*, 2019)
Iopromide (IOP)	100 µg L-1	Transformation	93%	(Mauch *et al.*, 2023)
17α- ethinylestradiol (EE2), bisphenol A (BPA) and polypropylene microplastic (PP)	0.2 – 5 mg L^{-1}	Biodegradation	100 % 100 % 5 – 12 %	(Shabbir *et al.*, 2022)
Microplastics: Polypropylene (PP), polyethylene (PE) and polyethylene terephthalate (PET)	Dimensions pellets < 1000 µm	Biodegradation	18 % (PP) 14 % (PE) 19.7 % (PET)	(Shabbir *et al.*, 2020)
Antibiotic sulfamethoxazole (SMX) and antihistamine doxylamine succinate (DOX)	SMX (0, 0.05, 1, 2, and 5 mg L^{-1}) and DOX (0, 0.05, 0.1, 0.2, and 1 mg L^{-1})	Bioadsorption, Bioaccumulation and Biodegradation	78.7, 18.9, 1.7 % (SMX) and 24.3, 15.9, 58.6 % (DOX)	(Yadav *et al.*, 2021)
Erythromycin (ERY) and roxithromycin (ROX)	0.5 µg L^{-1}, 5 µg L^{-1} and 50 µg L^{-1}	Biodegradation and indirect photodegradation	51.63 – 66.87% (ERY) and 41.85 – 48.27% (ROX)	(Yan *et al.*, 2023)
63 target substances	1 mg L^{-1}	Biotransformation and Bioaccumulation	-	(Desiante *et al.*, 2021)
Orange methyl azo dye	25 – 500 mg L^{-1}	Biotransformation and Bioaccumulation	100 %	(Shabbir *et al.*, 2017a)
Crystal violet dye	25 – 1000 mg L^{-1}	Biotransformation and Bioaccumulation	100 %	(Shabbir *et al.*, 2018)

Zhong *et al.* (2020) demonstrated that periphyton efficiently biosorb copper (Cu^{2+}) from wastewater, reducing Cu concentrations across varying exposure levels. The removal efficiency increased significantly within the first 48 hours of treatment, reaching approximately 86% for exposure to 0.5 mg L^{-1} Cu^{2+} and 91% for 2 mg L^{-1}. The efficiency continued to rise over time, with final removal rates of about 99% and 98% after 108 hours. Although exposure to 2 mg L^{-1} Cu^{2+} inhibited total chlorophyll-a content, the metabolic activity of heterotrophic microorganisms and the rates of chemical oxygen demand (COD) removal remained unaffected.

In addition to these findings, Ma *et al.* (2018) also reported promising results when investigating periphytic biofilms under similar conditions. In their study, periphytic biofilms demonstrated efficiency, achieving up to 99 % Cu removal, even at higher concentrations (2 to 10 mg L^{-1}). Additionally, the biofilms adapted to Cu stress by regulating their microbial community composition, which included diverse species such as diatoms and green algae, enhancing their overall capacity for heavy metal removal from wastewater.

Liu *et al.* (2018) tested immobilizing the biofilm to enhance Cu removal. The authors immobilized the biofilm onto fibers to develop a novel bioreactor. The results indicate that periphyton effectively captures Cu at initial concentrations ranging from 2 to 20 mg L^{-1}, primarily due to the overproduction of EPS and the porous structure of the periphyton, with biosorption serving as the primary removal mechanism.

In Table 3.1, the pesticide group is the second largest group of studies evaluating bioremediation in aquatic environments using biofilms. Biofilms contribute to the biodegradation of glyphosate in aquatic ecosystems through their microbial diversity, which enables a range of metabolic pathways for pollutant degradation. They can effectively break down glyphosate, utilizing it as a phosphorus source, which is significant given that glyphosate contains phosphorus (Carles *et al.*, 2019). A study investigated the biodegradation potential of five bacterial strains isolated from glyphosate-contaminated streams, focusing on their ability to metabolize glyphosate and its byproduct, AMPA, as phosphorus sources (Rossi *et al.*, 2021). Both studies demonstrated 100% glyphosate removal under the evaluated conditions.

Periphytic biofilms have also shown potential in the biodegradation of other pesticides, such as methomyl. Bacterial consortia isolated and acclimatized from biofilms could remove up to 91% of methomyl in 7 days. Additionally, exposure to methomyl altered the biofilm community structure, favoring species tolerant to the pesticide, which may enhance the overall degradation efficiency (Chen *et al.*, 2015). Bacteria that are resistant to or capable of degrading methomyl can produce enzymes that break down the pesticide, metabolize it as a carbon source,

or employ mechanisms that prevent it from interfering with their cellular functions. As a result, these bacteria are better equipped to survive and prosper in the presence of methomyl, making them more effective at its degradation (Perpetuini *et al*., 2023).

Tien *et al*. (2017) isolated microbial consortia from natural river biofilms by targeting areas that may have been influenced by agricultural activities, which often involve pesticides like carbofuran. The biofilms were then acclimatized and cultured in a laboratory to study their carbofuran degradation abilities under controlled conditions. They achieved a removal rate of 52.5% after 7 days of exposure, compared to only 4.9% removal in control groups without biofilms. The research identified specific bacterial strains, including *Sphingobacterium multivorum*, as effective carbofuran degraders.

Bighiu and Goedkoop (2021) outline future research directions, including identifying specific microbial communities and the genes involved in the biodegradation process. The authors emphasize the importance of conducting field studies to assess degradation rates in natural environments and suggest testing a broader range of herbicides to evaluate degradation patterns across different chemical classes. Finally, implementing long-term monitoring programs is essential to understanding the dynamics of herbicide runoff and leaching from agricultural soils. These efforts aim to enhance our understanding of the ecological roles of biofilms in managing herbicide pollution in aquatic ecosystems.

Also, according Table 3.1, pharmaceuticals are the most frequently cited ECs in studies on bioremediation of aquatic environments using periphytic biofilms. Natural biofilms exhibit affinity for organic contaminants, including steroidal hormones, lindane, norfloxacin, ofloxacin, phenanthrene, azithromycin, venlafaxine, iopromide, sulfamethoxazole, doxylamine succinate, erythromycin, and roxithromycin (Dong *et al*., 2018; Liang *et al*., 2024; Mauch *et al*., 2023; Santos *et al*., 2019; Wang *et al*., 2019; Yadav *et al*., 2021; Yan *et al*., 2023; Zhang *et al*., 2018).

Although these studies assess the removal capacity of pharmaceuticals by biofilms, the removal efficiency is generally lower compared to other classes of contaminants (see Table 3.1). Dong *et al.* (2018) investigated the sorption of lindane and norfloxacin in biofilms in the presence of heavy metals, such as lead (Pb), cadmium (Cd), chromium (Cr), and arsenic (As). The authors attributed the reduced sorption of lindane in the presence of heavy metals to competitive interactions for sorption sites on biofilm surfaces. In contrast, the presence of metals enhanced the sorption capacity of biofilms for norfloxacin.

Erythromycin and roxithromycin were degraded by periphyton, with removal rates of 51.63–66.87% and 41.85–48.27%, respectively. Side chain and ring cleavage were identified

as the main degradation pathways. Furthermore, while biofilms degrade these antibiotics at high concentrations, the antibiotics also alter river periphyton's structural composition and photosynthetic processes (Yan *et al*., 2023). Azithromycin was removed by river biofilms, with removal rates ranging from 44.70% to 88.65% on the 14th day, through three main processes: biosorption, bioaccumulation, and biodegradation. The authors also observed genetic modifications in the biofilm following antibiotic exposure, indicating that biofilms undergo adaptive changes to be resilient to this environmental stress (Liang *et al*., 2024).

The prevalence and environmental impact of microplastics, which are among the most widely found pollutants in aquatic environments and marine organisms, necessitates the development of effective removal technologies. Shabbir *et al.* (2020) introduce that periphytic biofilm biodegrades microplastics, with degradation rates of 9.52%–18.02% for polypropylene (PP), 5.95%–14.02% for polyethylene (PE), and 13.24%–19.72% for polyethylene terephthalate (PET). The biodegradation has been confirmed by microplastic weight loss, Scanning Electron Microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) analyses. These biofilms are resilient in complex environments and don't require sterile conditions, making them a cost-effective solution for wastewater treatment. The presence of different carbon sources, especially glucose, enhanced biodegradation efficiency, and altered microbial community structure,

Another study demonstrated an innovative approach for the simultaneous removal of microplastics and endocrine-disrupting chemicals using periphytic biofilms, a capability not previously emphasized in studies focused on individual pollutant removal. The study employed four types of periphytic biofilms to remove 17α-ethinylestradiol (EE2), bisphenol A (BPA), and polypropylene (PP). EE2 and BPA (0.2 mg/L each) were completely (~100%) removed within 36 days. Advanced analytical techniques, including Ultra-high performance liquid chromatography (UHPLC), and Gas chromatography coupled with tandem mass spectrometry (GC-MS/MS), were used to evaluate the biodegradation processes and mechanisms. Gel permeation chromatography (GPC) and SEM further validated the biodegradation of PP. Additionally, the study highlights significant changes in microbial community dynamics, particularly an increase in *Proteobacteria* with adding humic acid, which may enhance biodegradation efficiency (Shabbir *et al*., 2022). This study highlights the potential of periphytic biofilms for the simultaneous removal of pollutants with different chemical properties, offering a promising method for wastewater treatment applications.

Shabbir *et al.* (2018) reported that periphyton bioreactors completely removed crystal violet (1000 mg L^{-1}) within 144–168 h, with removal rates directly proportional to biomass

concentration. Immobilized periphyton also showed strong biodegradation capacity, as minimal desorption occurred after complete decolorization. In a previous study, the same group achieved full decolorization of the azo dye amaranth within 20 h under static conditions, while agitation slowed the process, indicating that static environments favor degradation. Overall, immobilized periphyton demonstrated high potential for dye removal, especially under optimized conditions of pH 7 and 30 °C (Shabbir *et al*., 2017b).

Liu *et al.* (2016) tested the effectiveness of using periphyton in a planted floating treatment bed (FTB) to remove contaminants from rivers. The periphyton limited plant root growth and the plants showed shading effects on the periphyton, but the FTB and periphyton combination successfully removed nitrogen and phosphorus. This indicates that the system can be scaled up to remove nutrients and contaminants from polluted rivers.

In addition to all these examples of contaminant removal by biofilms, they can be used to prevent the proliferation of cyanobacteria. In this case, biofilms consume water nutrients (nitrogen and phosphorus), causing competition for nutrients, thus controlling the bloom of cyanobacteria (Ko *et al*., 2019). Harmful algal blooms caused by cyanobacteria are a global issue and pose several dangers to ecosystems and humans. Periphyton, which is a mixture of heterotrophic and photoautotrophic microorganisms, has been found to effectively reduce the proliferation of cyanobacteria. In an outdoor mesocosm environment (1000 L), Van Le *et al.* (2023) investigated the response of bacterial communities to periphyton using high-throughput 16S rRNA gene sequencing data. The study found that periphyton reduced the concentration of planktonic chlorophyll-a by 38.6% and decreased the cell density of Microcystis and Dolichospermum by 70.8% and 94.8%, respectively. The results suggest that periphyton-modified bacterial interactions favor the growth of organic matter-degrading bacteria.

3.5 MASS BALANCE AND STOICHIOMETRY

Stoichiometric and microbial energy reactions occur in biofilm-based bioremediation, where multi-species communities interact. During this process, pollutants are removed from the water and assimilated into the microbial biomass. These chemical reactions are predominantly driven by microorganisms, aiming to capture energy for cellular synthesis and maintain metabolic activity (Wu, 2017). Therefore, studying and describing these processes' stoichiometry and mass balance is important.

The mass balance of compounds in a bioremediation process is crucial for distinguishing bioaccumulation from biodegradation and biotransformation. Desiante *et al.*

(2021) emphasize that a complete mass balance provides insight into the active bioaccumulation of contaminants in biofilms, enabling the assessment of their role in detoxifying surface waters from foreign substances. The authors aimed to study the fate of complex mixtures of micropollutants at environmentally relevant concentrations in biofilms, specifically focusing on differentiating between biotransformation, passive sorption, and active bioaccumulation. Through mass balance, the amount of contaminant present in the liquid phase represents what was not removed by the biofilm. The amount present in the biofilm at the end of the test was bioaccumulated, and these two concentrations, subtracted from the initial concentration added, represent biotransformation. Tests and mass balance calculations were performed with live and dead biofilm biomass to differentiate active bioaccumulation from passive sorption.

Santos *et al.* (2019) evaluated the mass distribution of selected organic microcontaminants between water and biofilm in mesocosms. The concentrations obtained for each microcontaminant after 72 hours of exposure, as well as the volume of water and the mass of biofilm, were used for the mass balance. The authors used the following equations:

$$\text{Contaminant remaining in water (\%)} = \frac{C_w(72\text{h}) \times \text{V}}{C_w(0\text{h}) \times \text{V}} \times 100 \quad (1)$$

$$\text{Bioaccumulation (\%)} = \frac{C_{biofilm}(72\text{h}) \times m_{biofilm}}{C_w(0\text{h}) \times \text{V}} \times 100 \quad (2)$$

$$\text{Biotransformation (\%)} = \frac{\sum C_w \text{ TP}(72\text{h}) \times \sum C_{biofilm} \text{TP}(72\text{h}) \times m_{biofilm}}{C_w(0\text{h}) \times \text{V}} \times 100 \quad (3)$$

Where C_w (µg L^{-1}) is the concentration of the corresponding microcontaminant in water at time 0h or 72h, V (L) corresponds to the volume of water used in the exposure experiment, $C_{biofilm}$ (µg g^{-1}) is the concentration of the corresponding microcontaminant in biofilm, $m_{biofilm}$ (g) corresponds to the mass of biofilm (in dry weight), Σ C_w TP (µg L^{-1}) is the sum of the concentration of all TPs (transformation products) in water at 72h and, Σ $C_{biofilm}$ TP (µg g^{-1}) is the sum of concentration of all TPs in biofilm at 72h.

Biotransformation was assessed by considering the total amount of transformation products (TPs) generated, taking into account their occurrence in both water and biofilm. In this context, all possible types of transformations (e.g., biotransformation, phototransformation,

etc.) that may take place within the mesocosm during the exposure experiments were included (Santos *et al*., 2019).

Liu *et al.* (2023) conducted a study on the degradation of phenol and formaldehyde using the fungus *Aspergillus nomius* SGFA1. The study involved analyzing the biodegradation and biomass accumulation of phenol and formaldehyde based on the dry weight of the biomass. Mass balance and stoichiometric analysis showed that 0.26 g g^{-1} and 0.05 g g^{-1} of carbon from phenol and formaldehyde were converted into biomass. The remaining carbon was converted into H_2O and CO_2. In this particular case, the conversion rate of biomass to phenol was higher than formaldehyde. This demonstrates the ability of the microorganism to effectively remove phenol and formaldehyde simultaneously, providing new information on the use of microorganisms to reduce environmental pollution.

The stoichiometric equations involved in bioremediation when organic matter is removed are as follows (Wu *et al*., 2014):

$$0.0417C_6H_{12}O_6 + 0.25H_2O = 0.25CO_2 + H^+ + e^- \quad (4)$$

$$0.25O_2 + H^+ + e^- = 0.5H_2O \quad (5)$$

$$0.0417C_6H_{12}O_6 + 0.25O_2 = 0.25CO_2 + 0.25H_2O \quad (6)$$

Organic matter is represented by $C_6H_{12}O_6$, which is oxidized to produce CO_2, HCO_3, and H_2O. Molecular oxygen acts as the electron acceptor, and the CO_2 produced is utilized by photoautotrophic microorganisms (cyanobacteria and diatoms) in periphyton communities for their growth. This process promotes the development of the periphytic community by creating a habitat for the attachment of other microorganisms (Wu *et al*., 2014).

3.6 PHYSICAL AND CHEMICAL CONDITIONS ON RIVER BIOFILM BIOREMEDIATION

Environmental conditions significantly affect the species composition, microorganism growth, primary productivity, metabolism, and ecological function of periphyton. These

conditions include temperature, pH, intensity of solar radiation, water flow, salinity, concentration of available nutrients, and oxygen levels (Tang *et al*., 2023).

The composition of microorganisms can be altered due to changes in water flow, which may cause some organisms to detach or add others to the biofilm. This factor facilitates the exchange of matter between the periphyton and the external environment (Shangguan *et al*., 2015). The temperature and intensity of solar radiation influence the biofilms' growth. The temperature requirement for each type of microorganism can vary from 10 to 35 °C (Courtens *et al*., 2016). A high ratio of carbon to nitrogen (C/N) can lead to the proliferation of heterotrophic bacteria. In contrast, a low nitrogen to phosphorus (N/P < 16) ratio usually indicates nitrogen limitation, which can favor the growth of species capable of nitrogen fixation, such as certain cyanobacteria (Liepina-Leimane *et al*., 2024). Additionally, dissolved oxygen is a critical factor affecting the diversity and activity of bacteria, particularly those involved in the oxidation, nitrification, and denitrification of ammonia (Fitzgerald *et al*., 2015).

Physical factors can influence the bioremediation of contaminants by periphytic biofilms. One of the most critical factors is pH, as it impacts the growth of microorganisms and the activity of enzymes (Bhunia *et al*., 2022). Temperature also plays a crucial role, impacting periphyton's growth and metabolism. Specifically, temperature influences the activity of enzymes, thereby affecting the metabolic rate of periphyton biota. Additionally, temperature plays a crucial role in determining the structure of the microbial community, especially for bacteria. Different dominant communities in periphyton exhibit distinct responses to temperature (Mu *et al*., 2020). Therefore, the optimal pH and temperature for the specific microorganisms forming the biofilm must be carefully optimized to achieve better results in bioremediation processes.

Nutrient availability also plays a vital role in the bioremediation of contaminants mediated by periphytic biofilms. Nitrogen is the most significant macronutrient for periphyton, making up about 10% of the dry mass of algae. The presence of nitrogen affects the structure of the microbial community. Studies indicate nitrogen is vital for periphyton growth and metabolism, but excessive nitrogen can hinder periphyton diversity. In addition to nitrogen, carbon and phosphorus also play essential roles in periphyton growth and community structure (Tang *et al*., 2023).

Sunlight, salinity, and contaminants' physicochemical properties also impact bioremediation. Photosynthetic bacteria in periphyton require appropriate light radiation for their growth and, consequently, for contaminant removal. Light, either natural sunlight or artificial light, influences the rate of degradation by periphyton. Therefore, proper light

radiation is crucial for effective periphyton growth and contaminant removal (Carles *et al.*, 2021).

The impact of discharging effluent from effluent treatment stations (ETEs) on periphytic biofilms that form in these areas is a crucial point to consider. Several microorganisms can be carried from ETEs and thus cause effects on the microbial community of rivers and biofilms. These microorganisms can inhabit and alter the community's composition, directly or indirectly, through species interactions. This can contribute to changes in respiratory profiles. Additionally, microbial communities released into wastewater should be viewed as a potential stressor for receiving streams, like other stressors such as nutrients, micropollutants, or increased temperature (Carles *et al.*, 2022).

One way to improve the performance of natural biofilms in removing contaminants is through cell immobilization. Immobilizing biofilms on various substrates is a modern and efficient approach, as it enhances their activity and makes them more resilient to environmental disruptions (Chen *et al.*, 2015; Shabbir *et al.*, 2017b). Immobilization consists of adsorbing microorganisms onto a support matrix, such as gels, sponges, or porous materials, leading to the entrapment or incorporation of microorganisms in these materials. This process helps regulate the biomass's shape, enhancing cell density, production rate, and product yield (Wu, 2017).

Shabbir *et al.* (2017a, 2018) demonstrated that immobilized biofilms (epiphyton, metaphyton, and epilithon) in bioreactors can completely decolorize and biodegrade both azo dyes and crystal violet. The process involves initial bioabsorption followed by biodegradation, converting dyes into non-toxic aliphatic compounds, highlighting the strong potential of immobilized periphyton for industrial-scale wastewater treatment.

3.7 CURRENT CHALLENGES AND FUTURE HORIZON

Growing concern for environmental sustainability has stimulated the search for efficient and low-impact remediation strategies. In this context, biofilm-based technologies show potential for environmental management, combining natural self-purification processes with applications in engineered systems.

Water purification performed by natural biofilms in these environments may seem limited when considering biomass levels compared to a biological reactor. However, it is well-known that biofilms are ubiquitous and grow on all solid surfaces within a river, forming on rocks, branches, sand, and even river sediments. Thus, the self-cleaning process promoted by

biofilms is significant and deserves to be studied, both to understand its mechanisms and to optimize it. The challenge, in this case, is that although biofilms are effective in sorption and biodegradation, they remain dynamic communities whose performance depends on hydrology, pollution levels, and environmental conditions, making stability a critical factor.

Future directions point to optimizing bioprocess design for both in situ applications, river self-cleaning capacity and engineered bioreactors. Advances in genetic engineering, enzyme technology, and ecological mapping may enhance biofilm resilience and expand the spectrum of degradable contaminants. A detailed study of the microbial communities that compose biofilms is essential, as their taxonomic and functional diversity determines bioremediation efficiency. Understanding which microorganisms and metabolic pathways are involved in degradation processes can help identify key species, predict ecosystem responses to environmental stressors, and guide the development of targeted interventions. Integrating microbial ecology with process engineering therefore represents a critical step toward unlocking the full potential of biofilms in environmental management, and it also highlights an important research gap in this field. Furthermore, strategies such as bioaugmentation, biostimulation, and cell immobilization can significantly improve biofilm activity and scalability.

Recent research has increasingly reflected global concerns about climate change, emphasizing its impacts on biofilms (Cantonati *et al.*, 2024; Pacheco *et al.*, 2022; Romero *et al.*, 2018; Sentenac *et al.*, 2023). This emerging field offers significant opportunities for investigation, and upcoming studies are expected to provide valuable insights in the coming years.

Looking ahead, river biofilm-based systems can be developed as nature-based solutions for integrated water management. Constructed and floating wetlands already demonstrate how biofilms can be leveraged in scalable projects, while advanced biofilm bioreactors can be used to treat industrial effluents containing persistent pollutants such as dyes, pharmaceuticals, and pesticides. Furthermore, periphytic biofilms can function as bioindicators of pollution, supporting environmental management in rivers and reservoirs. By framing these biofilms as self-sustaining microecosystems, future research can bridge fundamental understanding with practical applications, positioning periphytic biofilms as a pillar of environmental biotechnology and ecological engineering.

3.8 CONCLUSION

Forming biofilms serves as a survival strategy for microorganisms, enabling them to withstand and tolerate harmful substances. Cells within a biofilm exhibit increased resistance to environmental fluctuations, making these structures particularly effective in detoxifying polluted environments. The interaction between biofilms and river water creates optimal conditions for natural attenuation processes, such as biodegradation and contaminant sorption. Notably, even at low contaminant concentrations, the adherence of biofilms to solid surfaces in rivers, both in sediments and in superficial areas, ensures a significant removal effect. As technological advancements progress, molecular studies on microbial diversity become increasingly feasible through the genomic analysis of microorganisms and their environments. This evolution paves the way for a deeper understanding of the structure and function of biofilm microbiomes in bioremediation and the establishment of genomic banks that can enhance the isolation of microorganisms and enzymes for biotechnological applications. Thus, research is being made to develop and identify contaminant removal techniques using periphytic biofilms, demonstrating their potential and offering new perspectives on their application.

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Chapter 4

"A raça humana está desafiada, mais do que nunca antes, a demonstrar nosso domínio, não sobre a natureza, mas sobre nós mesmos."
Rachel Carson

"The human race is challenged more than ever before to demonstrate our mastery, not over nature but of ourselves."
Rachel Carson

4 STUDY AREA CHARACTERIZATION AND CONTEXTUAL BACKGROUND

Two different locations were selected for this study to evaluate the potential of riverine biofilms in removing steroid hormones. These sites were chosen because of their contrasting environmental and geographic characteristics, which help to illustrate the variability and applicability of the study under different conditions. Additionally, it is important to note that the sampling points in the rivers were chosen based on three criteria: representativeness, relevance, and accessibility for placing and removing the samplers.

In Germany, the Alb River in Karlsruhe was selected for study, as it represents a temperate river system featuring both urban and semi-natural sections. In Brazil, the Barigui River, located in Curitiba, Paraná, was chosen because it flows through a subtropical region and is influenced by urban development and related human pressures. The selection of these two rivers facilitates a comparative analysis of biofilm behavior under different environmental conditions.

4.1 ALB RIVER – KARLSRUHE, GERMANY

The Alb River is approximately 55 kilometers long, originating in the northern Black Forest near the town of Bad Herrenalb and flowing westwards to meet the Rhine River. Along its course, the Alb River passes through a variety of landscapes and urban settings, including spa towns, small villages, industrial areas, and environmentally protected areas. In addition, the Alb River plays an ecological and hydrological role in the Karlsruhe region, hosting a rich diversity (Wasser 3.0, 2025).

Karlsruhe is a city located in southwestern Germany, in the Upper Rhine Valley. The city has an area of 173.4 km² and a population of approximately 308,707 inhabitants (Stadt

Karlsruhe, 2023). The topography is mostly flat, with an average elevation of 115 meters above sea level, though elevations increase noticeably toward the southeastern part of the city. According to the Köppen-Geiger climate classification, Karlsruhe has a warm temperate climate (Cfb), characterized by year-round humidity and warm summers (Pace *et al.*, 2025).

The Alb River was selected to choose a river with moderate anthropogenic pressures and seasonality, thus contributing to the understanding of how these factors influence the biofilm structure and the capacity to remove contaminants. Therefore, two sampling sites were chosen, one upstream and one downstream in relation to the river, capturing environmental gradients along the course. The upstream site (UPS; 48°58'4.23"N, 8°23'53.71"E) and the downstream site (DWS; 49°2'6.01"N, 8°19'46.03"E) reflect areas with different land use and occupation influences. A map illustrating the precise location of these sampling points, together with representative photographs of the site, is presented in Figure 4.1.

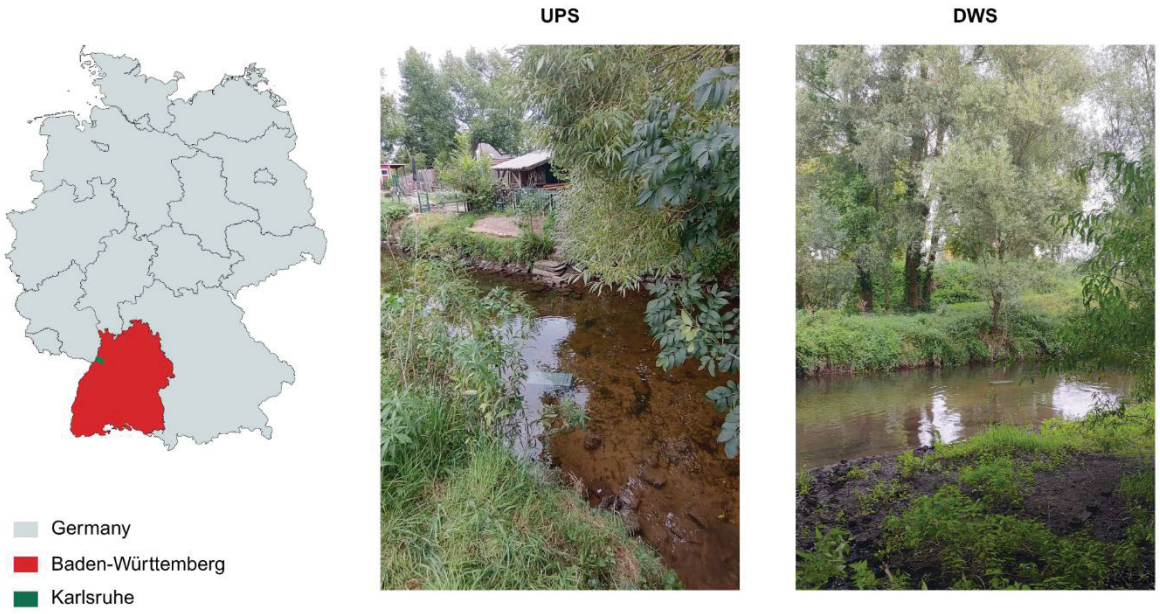
In Karlsruhe, the land cover is diverse. According to data from the 2018 Urban Atlas, reclassified based on the National Land Cover Database (NLCD), urbanized areas occupy 42.8% of the territory, mainly concentrated in the central areas. Forests, which account for 32.7% of the area, are most prevalent in the northern district of Waldstadt, while agricultural land (including crops and fields) accounts for 18.9% and predominates in the surrounding rural areas (Pace *et al.*, 2025).

The Upper Rhine Valley, where Karlsruhe is located, is known for experiencing intense heat stress due to regional climatic conditions, including orographic influences that lead to warm, humid air, high solar radiation, and low wind speeds, traits consistent with the Cfb climate classification (Gangwisch; Saha; Matzarakis, 2023). Between 1980 and 2010, the average annual temperature in Karlsruhe was 11.03 °C. Notably, the city is among the warmest in Germany and held the national temperature record of 40.2 °C (recorded in 2003) until 2018 (Kunz *et al.*, 2022).

Another important detail is that upstream of the DWS sampling point, there is a discharge from a combined sewer overflow (CSO). Combined sewer systems (CSS) are common in many European cities. These systems are designed to collect both dry weather flow (DWF), which includes domestic sewage, industrial discharges, and infiltration water, and stormwater runoff. Under normal conditions, this combined flow is directed to a WWTP. However, during heavy rainfall events, when the volume of water exceeds the system's transport capacity, the excess flow is released untreated into nearby water bodies through CSOs. These overflow events can impact the receiving environment, as they often contain a mixture of sanitary wastewater, pollutants from surface runoff, and resuspended sediments and biofilms

from within the sewer system. The magnitude and frequency of such discharges depend on several factors, including rainfall patterns, the design and storage capacity of the sewer system, and the treatment plant's intake limits (Quaranta *et al.*, 2022).

FIGURE 4.1 – STUDY AREA IN GERMANY.



SOURCE: The author (2025).

4.2 BARIGUI RIVER – CURITIBA, PARANÁ, BRAZIL

The Barigui River is 67 km long and drains a watershed of 279 km², that is predominantly urban. Its area covers three municipalities in Paraná, Brazil: Almirante Tamandaré (120 km²), Curitiba (144 km²) and Araucária (15 km²). The drainage pattern of the basin is primarily dendritic, following the region's natural topography (Froehner; Martins, 2008). The Upper Barigui River Basin has a karst aquifer, which serves as a source of drinking water for the local population. Environmental protection zones have been established to protect the springs and the aquifer. However, urban development and industrial activities along the river threaten water quality (Marques *et al.*, 2024).

The basin's estimated population in 2020 was approximately 860,000 inhabitants. While most of the area is served by separate sanitary sewer collection systems, many households remain unconnected to the sewer network, with only 57% of domestic wastewater receiving treatment (SNIS, 2023). According to the Köppen climate classification, the region

has a Humid Subtropical (Cfb) climate, characterized by mild summers and no distinct dry season. Annual precipitation averages approximately 1500 mm, while temperatures typically vary between 16.1°C and 18°C (Froehner; Martins, 2008).

The vegetation near the Barigüi River presents limited riparian forest cover, with denser patches of vegetation found mainly in the northern part of the watershed. The forested areas are composed mainly of subtropical species, including *Araucaria angustifolia*, Bracatinga (*Mimosa scabrella*), and a mixture of tropical and subtropical forest vegetation. In the northern part of the watershed, the land use is predominantly rural, with scattered urban development, including the city of Almirante Tamandaré. In contrast, the central portion of the watershed, encompassing parts of Curitiba, is largely urbanized, characterized mainly by residential areas, as well as commercial and service-related activities (Froehner *et al.*, 2010).

Two sampling points were selected in the upper basin of the river (Figure 4.2). Site BA1 (25°18'45.466"S, 49°17'43.959"W) is located 1.5 km downstream from the Barigui Water Treatment Plant (WTP), which supplies drinking water to the population of Almirante Tamandaré. The plant has an operational capacity of 200 L s^{-1} (SANEPAR, 2015). Site BA2 (25°21'30.0"S, 49°16'53.8"W) is located further downstream, just below the discharge point of the São Jorge WWTP. This WWTP releases approximately 48 L s^{-1} of treated effluent, processed through an anaerobic upflow sludge blanket (UASB) reactor followed by a coagulation and flotation system for phosphorus removal, into a river with a base flow of about 347 L s^{-1} (SNIRH, 2017). The São Jorge WWTP is licensed to discharge effluent with a maximum chemical oxygen demand (COD) of 125 mg L^{-1}, a biochemical oxygen demand (BOD) of 50 mg L^{-1}, and a flow rate of 530.10 m^3 h^{-1} (Lima, 2023). The treatment plant achieves an average BOD removal efficiency of approximately 85%, operating with an influent flow of 48.3 L s^{-1}, an influent load of 1,797.9 kg BOD per day, and an effluent load of 269.7 kg BOD per day (SNIRH, 2017).

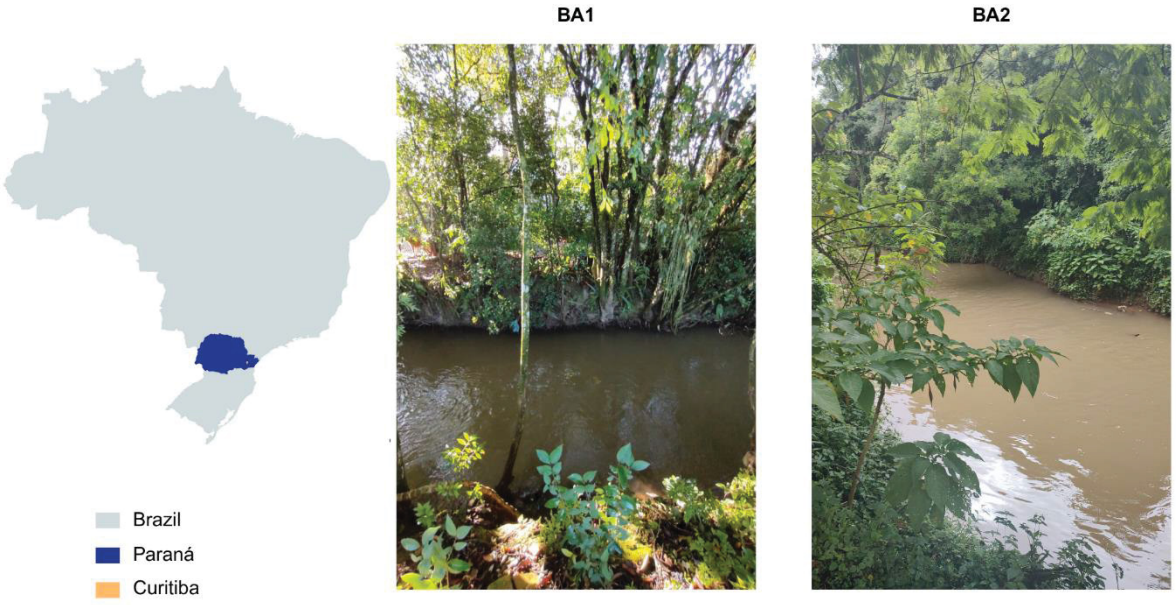
The two sampling points selected for this study are in the part of the river classified as Class 3, as defined by Brazilian CONAMA Resolution No. 357/2005, and are therefore subject to the maximum and minimum concentration limits specified for surface water contaminants. The study area presents a mixed land use and occupation profile, comprising residential, commercial, and small-scale agricultural activities, as well as patches of natural vegetation.

Agriculture and livestock dominate in the state's rural areas, with soybean, wheat, and pasture farming, alongside agroindustry (SEMA, 2010). Urban expansion in the RMC has increased pressure on water sources and floodplains, aggravating water quality problems for public supply. Economically, the region is driven by industry, commerce, and construction,

accounting for most of the formal employment. Agriculture focuses on potato, tobacco, yerba mate, and bean cultivation, while poultry, swine, and cattle farming are also significant (Paraná, 2015).

The region's high population density and economic activities have led to water quality degradation. The main source of pollution comes from WWTPs that cannot fully treat the pollutant load, resulting in the discharge of inadequately treated sewage into water bodies (Ide *et al.*, 2017; Mizukawa *et al.*, 2019).

FIGURE 4.2 – STUDY AREA IN BRAZIL.



SOURCE: The author (2025).

The following two chapters (Chapters 6 and 7) present studies conducted in the two regions previously described. These chapters explore the potential of natural river biofilms developed in each location for the removal of steroid hormones.

Chapter 5

"Eu fui ensinada que o caminho do progresso não é nem rápido, nem fácil."
Marie Curie

"I was taught that the path of progress is neither swift nor easy."
Marie Curie

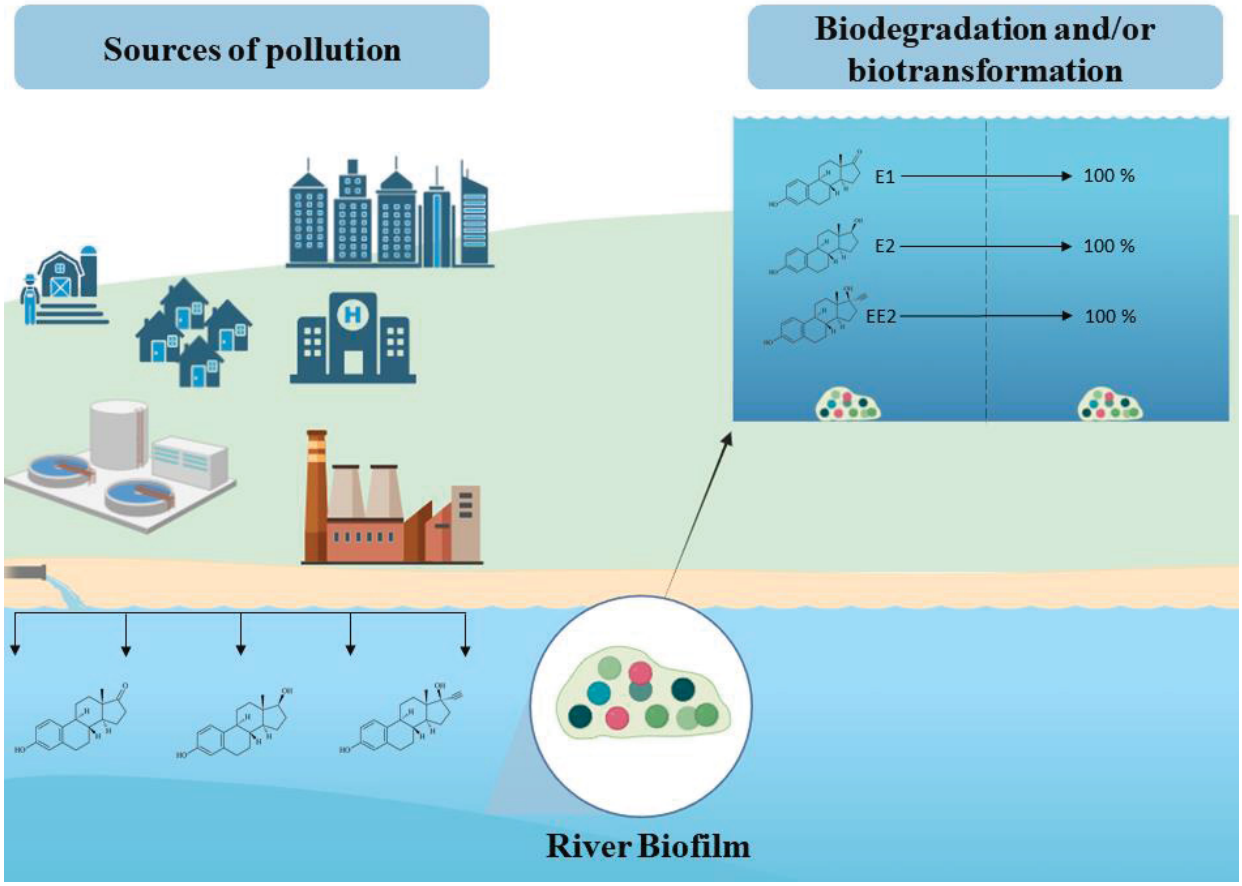
5 NATURAL RIVER BIOFILMS FOR ESTROGEN REMOVAL IN AQUATIC ENVIRONMENTS

Abstract

Estrogens, present in trace amounts in aquatic environments, can significantly impact living systems, including river microbial communities. River biofilms, composed of diverse microorganisms, play a vital role in ecosystem functions such as nutrient cycling, food web dynamics, and self-purification. This study investigates the capacity of natural river biofilms to remove estrogenic steroids (10 µg L^{-1}), Estrone (E1), β-Estradiol (E2), and 17α-Ethynylestradiol (EE2), from aquatic environments. Water and biofilm samples from the Alb River in Karlsruhe, Germany, were used in microcosm experiments to remove estrogen under constant laboratory conditions. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses revealed rapid removal of the compounds, with 100% elimination achieved within 9 days, primarily through biodegradation and/or biotransformation. The bacterial community structure showed resilience to estrogen exposure, with Proteobacteria, *Bacteroidota, Actinobacteria, Firmicutes*, and *Deinococcota* as dominant phyla. At the genus level, *Exiguobacterium, Acinetobacter, Massilia, Deinococcus, Tabrizicola*, and *Hymenobacter* exhibited significant growth, suggesting their potential involvement in estrogen degradation. These findings highlight the crucial role of natural river biofilms in the removal of estrogenic steroid hormones, reinforcing their importance in river self-purification and potential applications in bioremediation strategies. They may also supplement and improve the existing modeling approaches of river self-purification.

Keywords: Bioaccumulation; Biodegradation; Biosorption; Micropollutants, Self-purification.

FIGURE 5.1 – GRAPHICAL ABSTRACT



SOURCE: The author (2025).

5.1 INTRODUCTION

The consumption of pharmaceuticals has increased exponentially worldwide, leading to environmental risks associated with their production and use, particularly regarding the contamination of water resources (Morin-Crini *et al.*, 2022). One concerning category of these drugs is endocrine-disrupting compounds (EDCs). Among EDCs, estrogens are frequently found in surface waters (Bilal *et al.*, 2019; He *et al.*, 2022; Ojoghoro; Scrimshaw; Sumpter, 2021; Zhang *et al.*, 2016).

Estrogens are essential to various physiological processes in vertebrates, such as osmoregulation, sexual maturation, reproduction, and stress responses. These compounds, derived from cholesterol, are low molecular weight lipophilic compounds (<270 g mol^{-1}) and can be classified as natural or synthetic (Ilyas; Van Hullebusch, 2020; Mpupa *et al.*, 2022). After administration, estrogens are excreted through urine and feces as metabolites, conjugates, or the unchanged active form (Torres *et al.*, 2021).

Due to inefficient removal in wastewater treatment plants (WWTPs), direct discharge of untreated sewage, leaching, and pharmaceutical industry effluents, these compounds persist in water bodies (Torres *et al.*, 2021). The problem is that these substances are not environmentally benign even at trace concentrations (μg L^{-1} or lower) (Bayode *et al.*, 2024). Long-term exposure to these micropollutants poses risks to aquatic organisms, such as fish and amphibians, and potentially to human health by disrupting endocrine system functions (Ilyas; Van Hullebusch, 2020; Kasonga *et al.*, 2021).

The European Union has recognized the risks posed by estrone (E1), 17β-estradiol (E2), and 17α-ethinylestradiol (EE2) by including them on its 1st and 2nd Watch Lists for water quality monitoring (EU, 2018; Glineur *et al.*, 2020). In response, efforts have been focused on identifying effective strategies for removing these substances from aquatic environments. Some of these strategies include advanced oxidation processes (Mouchtari *et al.*, 2023), radiation (Abusam *et al.*, 2024), adsorption (Davarnejad *et al.*, 2023; Frimodig; Haukka, 2023), membrane technologies (Imbrogno *et al.*, 2025; Lei *et al.*, 2025), and nature-based solutions (Ilyas; Van Hullebusch, 2020; Liyanage *et al.*, 2024).

One option for a nature-based solution is bioremediation. In aquatic ecosystems, microbial communities are essential for degrading and transforming organic pollutants. River biofilms, composed of algae, bacteria, and fungi embedded in an extracellular polymeric matrix (EPM), are key players in natural water purification processes. These biofilms adhere to various

substrates and contribute to biogeochemical cycles and the maintenance of aquatic ecosystem functions (Desiante; Minas; Fenner, 2021; Li *et al.*, 2023).

Biofilms exhibit high adaptability to environmental changes and can modify their structure in response to contaminant exposure (Shabbir *et al.*, 2020). Due to their heterogeneous nature, they can respond to ecosystem conditions, driven by rapid growth, species diversity, and physiological variation (Lima *et al.*, 2025). Studies have shown that biofilms can remove diverse pollutants, including dyes (Shabbir; Faheem; Wu, 2018), metals (Zhong; Zhao; Song, 2020), microplastics (Shabbir *et al.*, 2022), pesticides (Carles *et al.*, 2019), and pharmaceuticals (Liang *et al.*, 2024; Yan *et al.*, 2023).

Specifically, regarding estrogens, Gong *et al.* (2023) demonstrated that biofilm matrices can act as effective natural passive samplers and indicators of spatial and temporal variations in pollution. Other studies have reported the bioaccumulation and degradation of estrogens by natural biofilms (Biswas *et al.*, 2024; Shabbir *et al.*, 2022; Writer; Ryan; Barber, 2011). While the ability of river biofilms to degrade various micropollutants is well documented, little is known about the concurrent removal of multiple estrogens under environmentally realistic conditions and the underlying microbial mechanisms.

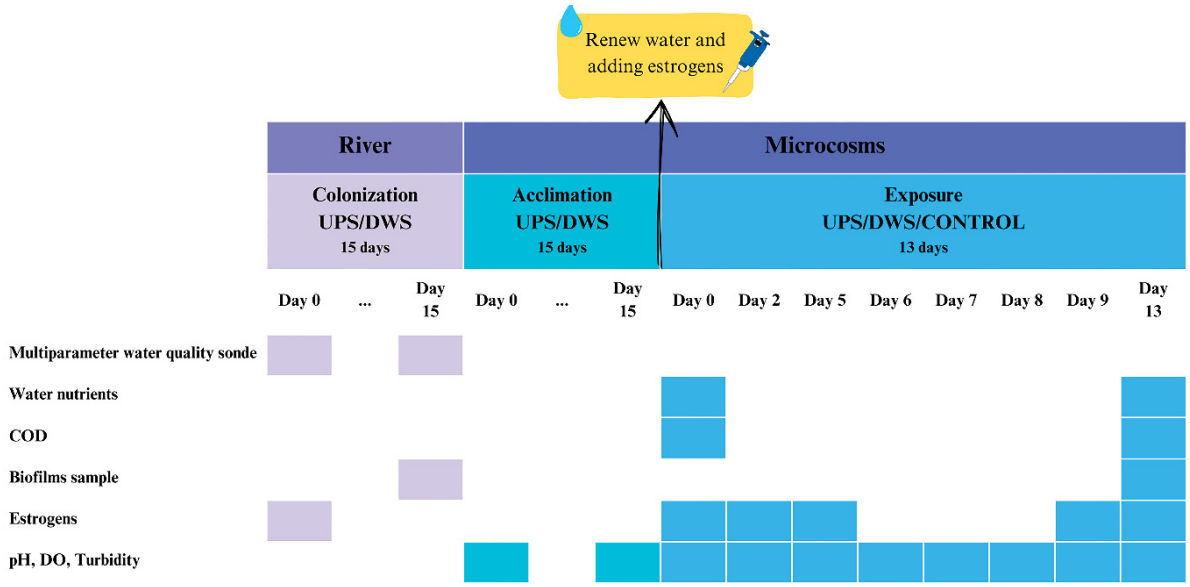
This study addresses this gap by evaluating the capacity of native river biofilms to remove estrone (E1), 17β-estradiol (E2), and 17α-ethinylestradiol (EE2). We aim to (i) elucidate the mechanisms involved in their removal, (ii) identify the key microbial groups responsible, and (iii) assess the role of biofilms as both indicators of river self-purification and agents of bioremediation. Elucidating these aspects may provide valuable insights for water resource management strategies and bioremediation applications.

5.2 MATERIALS AND METHODS

5.2.1 Experimental design

The experimental design included three phases: colonization period (15 days), acclimation (15 days), and exposure (13 days) (Figure 5.2).

FIGURE 5.2 – SCHEMATIC REPRESENTATION OF THE EXPERIMENTAL DESIGN AND TIMELINE, INCLUDING THE SAMPLING INTERVALS FOR VARIOUS PARAMETERS.



Note: UPS = Upstream site, DWS = Downstream site, CONTROL = control test conducted under the same conditions without adding biofilm. Multiparameter water quality sonde measurements = Temperature (°C), Dissolved Oxygen (mg L^{-1} O_2), pH, Electrical Conductivity (µS cm^{-1}), Total Dissolved Solids (mg L^{-1}), and Turbidity (NTU). Water nutrients = Orthophosphates (mg L^{-1} PO_4-P), Ammonia (mg L^{-1} NH_4-N), Nitrate (mg L^{-1} NO_3-N), and Nitrite (mg L^{-1} NO_2-N). COD = Chemical Oxygen Demand (mg L^{-1} O_2). Biofilm sample = Steroid hormone content analysis in the biofilm. Estrogens = Quantification of estrogens in the water. pH, DO (Dissolved oxygen), and Turbidity = Measurements conducted in the microcosms.

SOURCE: The author (2025).

5.2.1.1 Colonization

38 glass slides of 26 x 76 mm each (total surface area 0.15 m^2) were used as artificial surfaces and were installed as described in the supplementary material in the River Alb, Karlsruhe (Germany). Colonization of natural biofilms on these surfaces was carried out in late summer 2023 and late autumn 2023 at an upstream site (UPS, 48°58'4.23"N, 8°23'53.71"E) and a downstream site (DWS, 49° 2'6.01"N, 8°19'46.03"E) of the River Alb during 15 days. Water temperature, pH, dissolved oxygen (DO), turbidity, total dissolved solids, and conductivity were measured in the direct vicinity of the sampling locations using an EXO^2 multiparameter water quality sonde (YSI, Yellow Springs, OH). The glass slides with the grown biofilm were then removed from the river and transported to the laboratory for the microcosm experimental tests. Furthermore, river water was collected at both sampling points to be used in the microcosm experiments to create comparable conditions.

5.2.1.2 Acclimation

Two microcosms consisting of 12 L glass aquariums (rectangular parallelepiped length×width×height 30×20×20 cm) were used to determine the biodegradation potential of E1, E2, and EE2 in the laboratory. For the acclimation phase, the artificial surfaces with biofilm were transferred into microcosms (glass aquariums), each filled with 8 L of river water from the respective site. In one aquarium, the UPS-biofilm was placed and filled up with river water, while in the other one DWS-biofilm was placed and filled up with river water (38 colonized slides per aquarium). Aquarium pumps create water flow and aerate the systems.

5.2.1.3 Exposure

After two weeks of laboratory acclimation for the biofilms, the water was renewed, and 10 μg L^{-1} of E1, E2, and EE2 was introduced into the microcosms. The experimental conditions of the microcosm experiment were chosen to assess conditions as similar as possible to a natural environment. Additionally, an abiotic control test was conducted under the same conditions without adding biofilm.

Water samples (500 mL) from each microcosm were collected at 0, 0.04, 2, 5, 9, and 13 days to quantify E1, E2, and EE2 content. Samples at time 0.04 day were collected after 1 h of the initial spiking to allow homogeneous dispersion of the microcontaminant in the microcosm. Water samples were collected and filtered through an acetate and cellulose membrane (0.45 μm). At the beginning and end of the exposure experiment, additional water samples were collected to quantify nitrite, nitrate, phosphate, chemical oxygen demand (COD), and ammonia. At the end of the exposure experiment, biofilm samples were scraped from the glass slides, freeze-dried, and kept at -20 °C until further analysis.

5.2.2 Analytical Methodology

Contaminants were extracted from the water samplers (500 mL) by SPE. Briefly, the SPE Cartridges (CHROMABond C18 EC 3 ml, 200 mg) were preconditioned with 2 mL of methanol and 2 mL of ultrapure water. Extraction was carried out at an approximate flow rate of 15 mL min^{-1} through the cartridges. After drying the cartridges with a vacuum pump, they were frozen at 20 °C for further analysis. The elution was carried out with 6 mL of a mixture of methanol/acetonitrile (1:1 v/v). The eluents were evaporated under a stream of nitrogen gas

(N_2), and the residue was redissolved in 500 µL of acetonitrile. This procedure resulted in a 1000-fold preconcentration of the sample, which was subsequently analyzed by LC-MS/MS.

Using a two-step procedure, the contaminants were extracted from the freeze-dried biofilm samples (100 mg). In the first step, the samples were mixed with 5 mL of methanol and vortexed thoroughly. The mixture was then subjected to ultrasound treatment for 480 seconds, followed by centrifugation at 2000 rpm for 5 minutes. The supernatant was carefully collected. In the second step, the extraction process was repeated, and the supernatants from both steps were combined for analysis. The combined supernatants were analyzed by LC-MS/MS.

The concentrations of nitrite, nitrate, phosphate, chemical oxygen demand (COD), and ammonia were measured in the water samplers with photometric cuvette tests (LCK 341, LCK 339, LCK 349, LCK 1414, and LCK 304, Hach Lange, Berlin, Germany).

5.2.3 Removal and bioaccumulation

The mass distribution of E1, E2, and EE2 between water and biofilm at the end of the exposure experiment was calculated using the concentrations obtained for each microcontaminant after 13 days of exposure, as well as the volume of water (8 L) and biofilm mass in each microcosm. The following equations were used (Eqs. (1) – (4)) (Santos *et al.*, 2019):

$$\text{Removal (\%)} = 100 \times \frac{C_0 - C_t}{C_0} \quad (1)$$

$$\text{Contaminant remaining in water (\%)} = \frac{C_t \times V}{C_0 \times V} \times 100 \quad (2)$$

$$\text{Bioaccumulation (\%)} = \frac{C_b \times m_b}{C_0 \times V} \times 100 \quad (3)$$

$$\text{Degradation efficiency per area } (\mu g\ m^{-2} d^{-1}) = \frac{(C_0 - C_t) \times V}{A \times t} \quad (4)$$

Where C_0 is the concentration in the influent (µg L^{-1}), Ct is the concentration in the effluent at the time t (µg L^{-1}), t is the running time (days), V (L) corresponds to the volume of water used in the exposure experiment, C_b (µg g^{-1}) is the concentration of the corresponding

microcontaminant in biofilm, mb (g) corresponds to the mass of biofilm (in dry weight) and, A is the total surface area (m^2).

5.2.4 Identification of microorganisms

The total DNA from the late summer samples was purified from 500 mg, and then the 16S genes (for bacterial analysis) were amplified. Late-summer samples were selected for genetic analysis due to their higher biofilm biomass, which provided adequate DNA quantity and quality for reliable sequencing. The amplified fragments were subsequently sequenced on an Illumina NextSeq platform. The sequences were analyzed using the Qiime software to identify the microorganisms present in the samples and their respective percentages within the sample. The bioinformatics of raw data on microbial ecology was carried out by the GoGenetic© laboratory (Curitiba, Brazil).

5.3 RESULTS AND DISCUSSION

5.3.1 Water characterization

Water samples from the river were analyzed for water characterization, and the results are presented in Table S5.1. A notable difference between the two testing periods was the water temperature. During the late summer period, the temperature ranged from 13.9 to 16.0 °C, whereas in the late autumn period, it varied between 6.7 and 9.0 °C. These seasonal temperature variations can influence microbial diversity and community structure. For instance, lower temperatures may reduce microbial activity, potentially affecting the viability and composition of microbial populations in the aquatic environment (Mu *et al.*, 2020).

The analysis of hormone concentrations in the water samples revealed low levels, with the highest concentration of 0.041 μg L^{-1} (41 ng L^{-1}) of EE2 detected at the DWS point during late summer. The relatively low concentrations detected in water may be attributed to the poor solubility of unconsumed or free estrogens, which are more likely to adsorb to particles and/or undergo sedimentation, thereby reducing their presence in the aqueous phase (Torres *et al.*, 2021). In a review by Ciślak *et al.* (2023), the concentrations of E1 and E2 in surface water, groundwater, and sediments across European countries were reported to range from 0.019 to 1060 ng L^{-1} for E1 and from 0.053 to 5250 ng L^{-1} for E2. Their findings suggest that estrogen

concentrations in most environmental samples typically fall within the range of 0.1–10 ng L^{-1}, consistent with the low levels detected in this study.

At the start and end of laboratory tests, microcosms' water was analyzed for COD, phosphate, ammoniacal nitrogen, nitrate, and nitrite levels (Table S5.2). Additionally, dissolved oxygen (DO), pH, temperature, and turbidity were monitored to assess water quality during the experiment (Table S5.3). Nutrient availability (nitrogen and phosphorus), carbon sources, temperature, pH, and DO are critical for microbial growth. COD, an indicator of organic matter in water, indirectly supports microbial growth by providing carbon and energy sources (Islam *et al.*, 2019).

Therefore, aquatic environments rich in organic matter may provide favorable conditions for biofilm growth, utilizing this as substrate. Biofilms also actively consume organic matter from the water to sustain their growth. Higher COD levels in microcosms by the end of the tests (Late summer: UPS 7.870–9.547 mg L^{-1} O_2, DWS 6.470–7.254 mg L^{-1} O_2; Late autumn: UPS 4.730–9.550 mg L^{-1} O_2), except for the DWS microcosm in late autumn (11.900–7.570 mg L^{-1} O_2), suggest increased organic matter, potentially from biofilm activity or hormone breakdown (E1, E2, EE2).

Depending on environmental conditions, biofilms can produce or consume organic matter (Van Le *et al.*, 2023). Organic matter production by biofilms can occur through microorganisms capable of photosynthesis or chemosynthesis, converting inorganic compounds into organic matter. In the river and the microcosms, the biofilms had access to light, enabling the growth of photosynthetic microorganisms. However, the glass plates used for biofilm growth likely created dark microenvironments in certain areas of the artificial surface, restricting photosynthetic activity. In these shaded areas, primary production may have been reduced, influencing the balance between organic matter consumption and production.

Nitrogen in aquatic systems exists in various forms, including ammoniacal nitrogen, nitrite, and nitrate. Ammoniacal nitrogen, typically low in natural waters, can indicate pollution if elevated. Nitrite levels were low in microcosms and river samples (<0.007 mg L^{-1} NO_2-N), while ammonia showed minor variations (Late summer: UPS 0.012–0.021 mg L^{-1} NH_4-N, DWS 0.032–0.012 mg L^{-1} NH_4-N), except in the late autumn test (Late autumn: UPS 0.446–0.014 mg L^{-1} NH_4-N, DWS 0.119–0.010 mg L^{-1} NH_4-N), where a decrease coincided with increased nitrate (Late summer: UPS 2.550–1.258 mg L^{-1} NO_3-N, DWS 2.090–1.012 mg L^{-1} NO_3-N; Late autumn: UPS 0.067–1.780 mg L^{-1} NO_3-N, DWS 0.109–1.410 mg L^{-1} NO_3-N), suggesting nitrification. Phosphorus, essential for biofilm growth, exists as orthophosphate, polyphosphate, and organic phosphorus. High orthophosphate levels can cause eutrophication

and often indicate anthropogenic pollution (Wu, 2017). Orthophosphate levels have been low (~0.044 mg L^{-1} PO_4-P) and showed minimal changes.

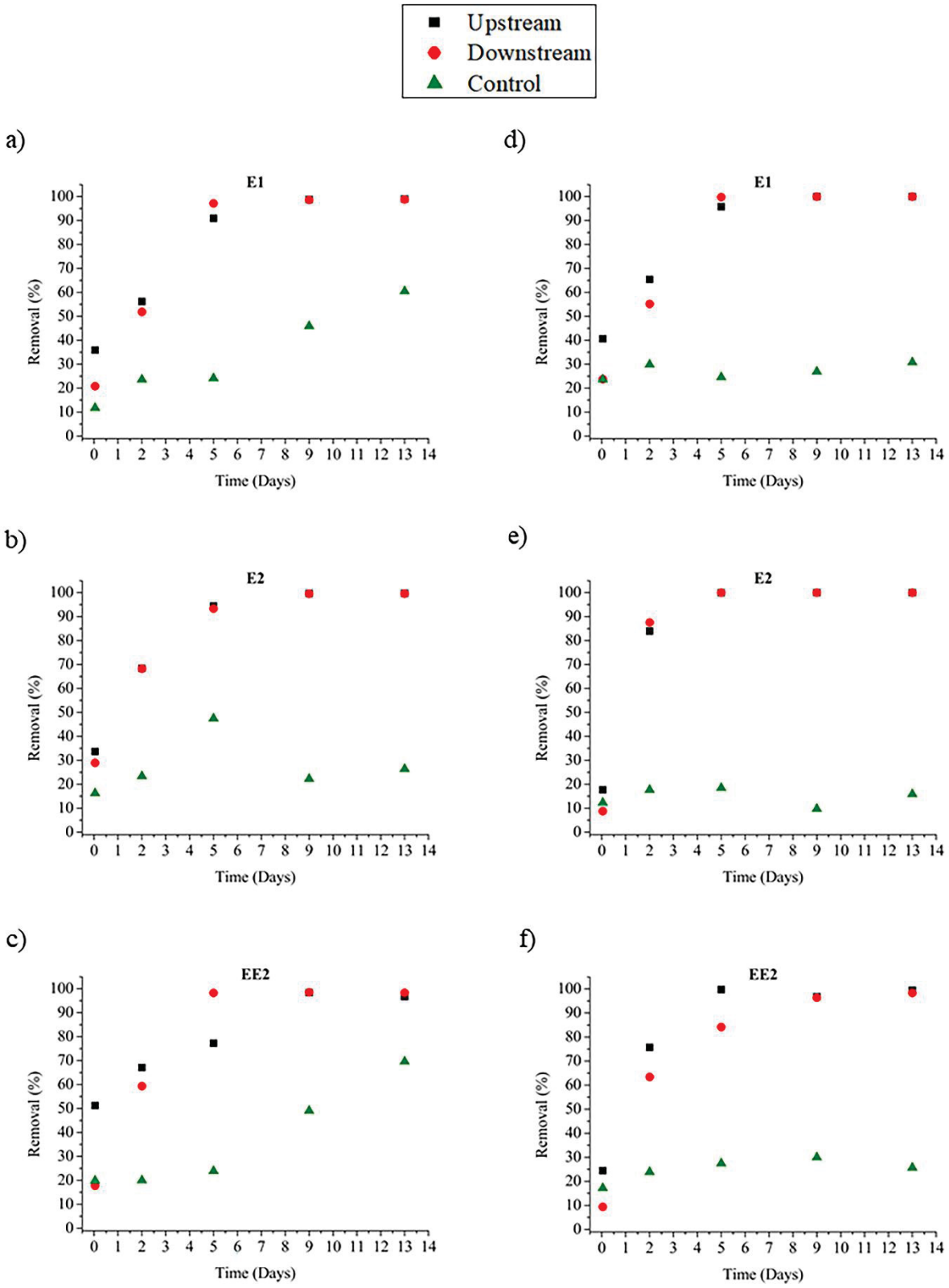
DO, crucial for aerobic microorganisms, remained stable in the late summer test (~10 mg L^{-1} O_2) but slightly decreased in late autumn (~8.56–8.98 mg L^{-1} O_2). The pH also showed no variations during the testing days (~7.5). In the UPS and DWS microcosms, water turbidity decreased from the first to the last day of testing (Late summer: UPS 2.5–0.51 NTU, DWS 10.90–0.72 NTU; Late autumn: UPS 1.39–0.37 NTU, DWS 1.82–0.55 NTU), likely due to the settling of suspended solids. In contrast, the control tests showed an increase in turbidity (Late summer control: 0.026–1.91 NTU; Late autumn: 0.29–0.52 NTU). This rise can be attributed to bacterial growth in the water and on aquarium surfaces. Although no biofilm was added to the control, bacterial proliferation became inevitable over time in the non-hermetic and non-sterile experimental setup. Collectively, these findings highlight the complex interactions between nutrients, organic matter, and microbial activity in aquatic systems.

5.3.2 Estrogen removal by river biofilms

The removal efficiency of estrogens (E1, E2, and EE2) during the exposure test is illustrated in Figure 5.3. The results show a consistent increase in removal over the first 5 days for both sampling periods and sampling points. By the ninth day, near-complete removal (~100%) was achieved for all target compounds. In the control test, maximum removal efficiencies reached 60% for E1 and 70% for EE2 during the late summer period. For the remaining compounds and conditions in the control test, removal efficiencies were below 49%. These findings highlight the effectiveness of river biofilms in removing E1, E2, and EE2 from polluted environments, with the control test (without biofilm) underscoring the importance of biological activity in hormone removal.

The removal trend for all compounds remained consistent throughout the tests. Seasonal variations, however, revealed a more rapid initial removal for E2 and EE2 during the late summer period, particularly within the first hour (0.04 h), which may be attributed to increased microbial activity under higher temperatures and greater biofilm biomass during this season. This finding highlights the potential influence of environmental conditions (temperature, light and nutrients) on the efficiency of micropollutant removal. Despite these seasonal effects, no big differences in removal efficiency were observed between the UPS and DWS sampling points.

FIGURE 5.3 – TIME SERIES OF AQUEOUS PHASE CONCENTRATIONS FOR COMPOUNDS E1, E2, AND EE2 DURING LATE SUMMER (a, b, AND c) AND LATE AUTUMN (d, e, AND f).



SOURCE: The author (2025).

One factor that may explain hormone removal in the controls is that, although the test was initially abiotic, prolonged exposure to light and aeration, combined with the non-hermetic nature of the aquaria, could promote bacterial growth on the glass and in the water. This microbial proliferation may contribute to hormone degradation, thereby aiding removal. Nonetheless, the presence of biofilms significantly accelerated and enhanced the removal of E1, E2, and EE2 from the water.

This removal capability arises from a combination of biological and physicochemical processes within the biofilm structure. Microorganisms in biofilms can metabolize and degrade estrogens, converting them into less active forms or eliminating them from the ecosystem. Moreover, the biofilm's extracellular matrix adsorbs hormones, enhancing their retention and subsequent removal from aquatic systems (Sentenac *et al.*, 2022). This remediation capacity is particularly important in polluted environments, where hormone concentrations can negatively impact aquatic ecosystems and human health (Ilyas; Van Hullebusch, 2020). Notably, all compounds were removed simultaneously, and no published study to date has reported the simultaneous removal of these three compounds from aqueous solutions by river biofilms.

5.3.3 Bioaccumulation of Estrogens

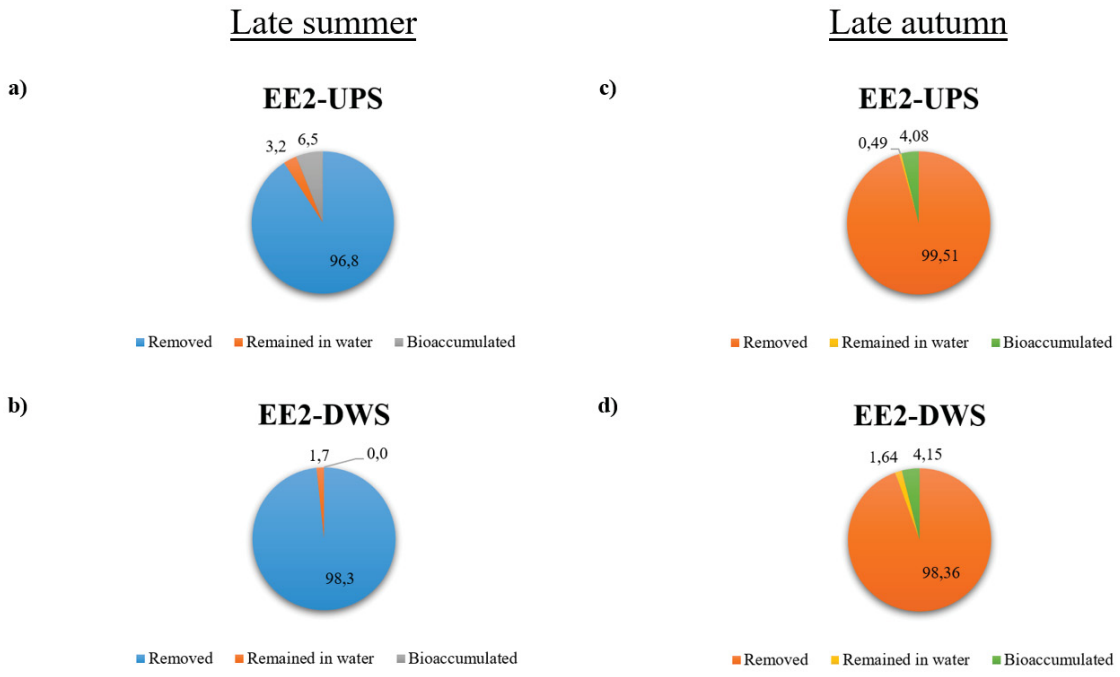
After the exposure test, the concentration of estrogens bioaccumulated in the biofilm biomass of the microcosms was quantified. After 13 days of exposure, only the steroid hormone EE2 showed measurable bioaccumulation. For the Late Summer samples, EE2 bioaccumulation was 0.191 $\mu g\ g^{-1}$ in the UPS sample and 0.000 $\mu g\ g^{-1}$ in the DWS sample. In the Late Autumn samples, EE2 bioaccumulation was 0.087 $\mu g\ g^{-1}$ in UPS and 0.111 $\mu g\ g^{-1}$ in DWS (Table S5.5). Figure 5.4 illustrates the distribution of EE2 after this period.

Estrogens can accumulate in biofilms through various mechanisms, including absorption by microbial cells, adsorption onto cell walls, or entrapment within the extracellular polymeric substance (EPS) matrix. The sorption of pharmaceutical compounds occurs primarily due to hydrophobic interactions between aliphatic and aromatic groups and lipid molecules in the EPS or microbial cell membranes, as well as electrostatic interactions between positively charged compounds and the negatively charged biofilm matrix (Tiwari *et al.*, 2017). The extent of this interaction depends on the physicochemical properties of each compound, such as hydrophobicity (log D), pKa, and charge distribution, which influence their affinity for the biofilm surface (Santos *et al.*, 2019).

Estrogens are hydrophobic substances (log D > 3) and exhibit a high potential for accumulation in aquatic organisms (He *et al.*, 2021). Given their physicochemical properties, it would be expected that organic microcontaminants with high log D values would exhibit bioaccumulation in biofilms. However, this was not observed in the present study. Table S5.4 summarizes the physicochemical properties of E1, E2, and EE2, including their log D values. Although EE2 does not have the highest log D (3.90), it exhibited the greatest accumulation potential. Another factor influencing bioaccumulation is the pKa of these compounds. At the experimental pH (7–8), estrogens predominantly exist in their neutral form, which may facilitate hydrophobic interactions with the biofilm matrix.

Despite these factors, the results indicate that bioaccumulation was not the predominant process in the removal of estrogens. Instead, biodegradation and/or biotransformation appears to have played a more marked role, suggesting that microbial activity within the biofilms was the primary mechanism responsible for the removal of these compounds. This is consistent with findings in the literature, which indicate that biodegradation is the main removal pathway for pharmaceutical contaminants in biological treatment systems (Ilyas; Van Hullebusch, 2020; Min *et al.*, 2018; Shabbir *et al.*, 2022; Tiwari *et al.*, 2017)

FIGURE 5.4 – MASS BALANCE ANALYSIS OF E1, E2, AND EE2 AFTER 13 DAYS OF BIOFILM EXPOSURE, EXPRESSED AS A PERCENTAGE OF THE INITIAL MASS OF ORGANIC CONTAMINANTS IN WATER. GRAPHS (a) AND (b) REPRESENT THE LATE SUMMER TEST, WHILE GRAPHS (c) AND (d) CORRESPOND TO THE LATE AUTUMN TEST.



SOURCE: The author (2025).

5.3.4 Community composition of the river biofilms

The structure of the microbial community was analyzed before the addition of E1, E2, and EE2, and after removal experiments, in order to evaluate the impact of these pollutants on the community profile of natural biofilms. It is important to note that late-summer samples were selected for genetic analysis due to their higher biofilm biomass, which provided adequate DNA quantity and quality for reliable sequencing. A total of 1679 species, 1418 genera, 688 families, 352 orders, 139 classes, and 56 phyla were obtained. The dominant phyla across all treatment groups included *Proteobacteria* (31.5%–42.9%), *Verrucomicrobiota* (0.7%–15.0%), *Planctomycetota* (0.4%–14.6%), *Bacteroidota* (2.2%–15.0%), *Acidobacteriota* (0.2%–8.9%), and *Actinobacteria* (4.7%–11.3%) (Figure 5.5a).

Exposure to estrogens led to marked changes in the biofilm community composition by day 13. Exposure to E1, E2, and EE2 increased the abundance of *Proteobacteria* at both sampling points (UPS: 31.5% to 42.9%; DWS: 34.8% to 40.3%), as well as *Bacteroidota* (UPS: 9.1% to 15.0%), *Actinobacteria* (UPS: 4.7% to 8.4%) and *Chloroflexota* (UPS: 1.3% to 4.5%) at the UPS site. These shifts in microbial composition may have contributed to the enhanced degradation of E1, E2, and EE2. However, the most substantial increases were observed in *Firmicutes* and *Deinococcota* at the DWS site, which rose from 1.6% to 42.0% and 0.0% to 4.8%, respectively.

In contrast, the exposure resulted in a notable reduction in the relative abundance of *Verrucomicrobiota* (UPS: 15.0% to 10.5%; DWS: 4.6% to 0.7%), *Planctomycetota* (UPS: 14.6% to 4.7%; DWS: 7.1% to 0.4%), *Bacteroidota* (DWS: 6.8% to 2.2%), *Acidobacteriota* (UPS: 6.4% to 3.9%; DWS: 8.9% to 0.2%), and *Actinobacteria* (DWS: 11.3% to 8.4%).

These results suggest that *Proteobacteria* populations recovered following exposure to E1, E2, and EE2. A similar pattern was observed for *Bacteroidota* and *Actinobacteria* at the UPS site. *Proteobacteria* and *Actinobacteria*, commonly found in river sediments, are frequently observed in biofilm communities derived from such environments (Ding *et al.*, 2024; Li *et al.*, 2025). Previous studies have highlighted that *Proteobacteria* and *Bacteroidota* are typically the dominant phyla in stream biofilm bacterial communities (Battin *et al.*, 2016). Notably, *Proteobacteria* are also recognized as the most prevalent phylum in wastewater treatment plants (WWTPs), where they play a key role in the biodegradation of both organic and inorganic contaminants (Yuan; Li; Zhong, 2020). A study evaluating the effects of E1 exposure on river biofilm communities similarly reported an increase in the abundance of certain *Proteobacteria*, suggesting their potential role in adapting to E1 contamination. The

authors further emphasized that these findings provide valuable insights into strategies for E1 removal by biofilms (Zhang, F. *et al.*, 2021).

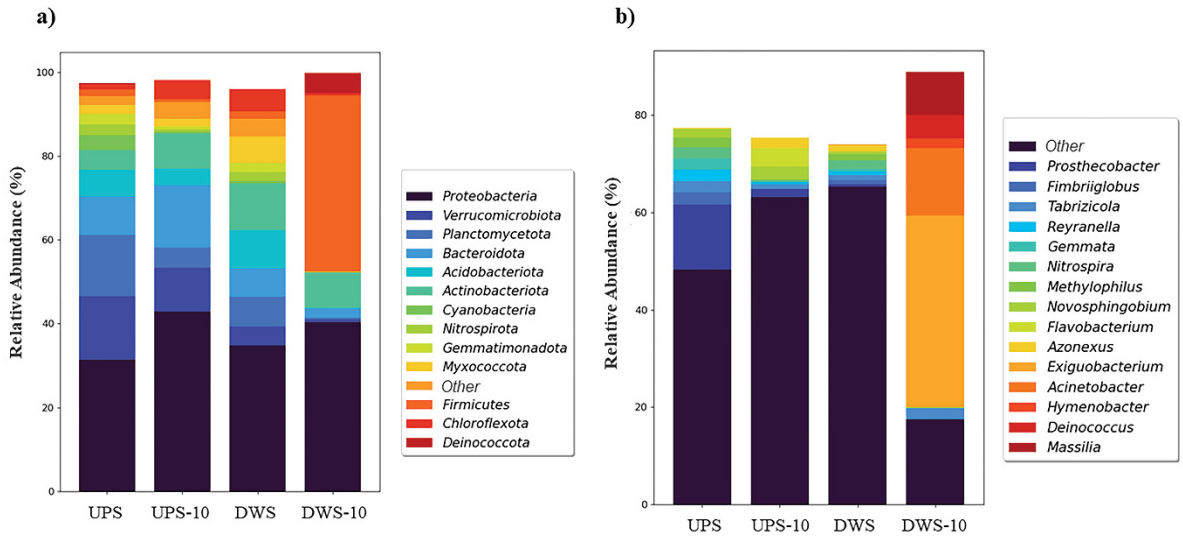
The substantial increase in the abundance of *Firmicutes* and *Deinococcota* at the DWS site demonstrates the resilience and adaptability of these phyla to stress conditions following exposure to estrogens. The difference in response between the UPS and DWS sites may be attributed to the downstream site's proximity to combined sewer overflows (CSO), which can influence the bacterial composition of the biofilms. CSOs affect biological communities in receiving waters by releasing microorganisms present in wastewater (Perry *et al.*, 2024), contributing to changes in the microbial composition of biofilms at the DWS site. A study by Carles *et al*. (2022) evaluated how microorganisms originating from wastewater affected river biofilm communities. The study concluded that microbial communities released in the effluent should also be considered a potential stressor for receiving rivers, alongside other stressors such as nutrients, micropollutants, or increased temperature. In the same study, the authors reported that *Firmicutes* have been frequently detected in the effluent and downstream river biofilm of WWTPs, as well as in biofilters used to treat urban wastewater. This further supports the role of WWTP-derived microorganisms in shaping microbial community responses to environmental stressors.

At the genus level, the UPS samples were dominated by *Prosthecobacter* (13.4%), *Fimbriiglobus* (2.5%), *Tabrizicola* (2.4%), *Reyranella* (2.3%), *Gemmata* (2.3%), *Nitrospira* (2.2%), *Methylophilus* (2.1%), and *Novosphingobium* (1.8%) (Figure 5.5b). Following hormone exposure, the relative abundance of most of these genera decreased, except for *Novosphingobium*, which increased to 2.6%. Additionally, after exposure, *Flavobacterium* (3.8%) and *Azonexus* (2.1%) exhibited increased abundances in the UPS samples.

In the DWS samples, the most abundant genera before hormone exposure were *Nitrospira* (1.7%), *Methylophilus* (1.3%), *Azonexus* (1.2%), *Tabrizicola* (0.9%), and *Fimbriiglobus* (0.9%). However, a marked shift in the bacterial community composition was observed post-exposure. *Exiguobacterium* increased dramatically from 0.0% to 39.3%, while *Acinetobacter* rose from 0.0% to 13.8%. Other genera that showed substantial increases included *Massilia* (from 0.0% to 8.8%), *Deinococcus* (from 0.0% to 4.8%), *Tabrizicola* (from 0.9% to 2.3%), and *Hymenobacter* (from 0.0% to 2.0%). Among the bacterial genera that increased at the DWS site, both due to hormone exposure and the proximity of the site to CSOs, some are considered potentially pathogenic and may exhibit antimicrobial resistance, as is the case with the genus *Acinetobacter* (He *et al.*, 2025).

These findings highlight a pronounced response of the bacterial community to hormone exposure, with certain taxa exhibiting resilience or adaptability. The results further confirm that natural biofilms harbor a diverse array of functional microorganisms capable of purifying polluted water bodies, underscoring the need for further exploration of their potential applications in water treatment strategies. Additionally, our findings indicate that biofilm composition reflects the negative impact of CSOs on microbial biodiversity. This is particularly important, as biofilms form the foundation of the aquatic food chain, and such changes can disrupt the entire ecological balance of this system.

FIGURE 5.5 – RELATIVE ABUNDANCE OF BACTERIAL COMMUNITIES DURING THE LATE SUMMER PERIOD AT THE PHYLUM (a) AND GENUS (b) LEVELS.



SOURCE: The author (2025).

5.3.5 The role of biofilms in river self-purification processes

Biofilms contribute to self-purification through abiotic and biotic mechanisms. To quantify the contribution of biofilms to estrogen removal, we calculated the degradation efficiency per unit area on day 9, when nearly 100% of E1, E2, and EE2 had been removed. The resulting degradation rate was 59 μg m^{-2} d^{-1}, providing a direct measure of the biofilm's capacity to degrade these contaminants per surface area. This value is approximate, considering only the glass plates as the growth surface for the biofilm. Nevertheless, this finding is crucial for understanding the ability of river biofilms to naturally remove these emerging contaminants, contributing to the self-purification process. Considering that studies indicate estrogen

concentrations in river water typically range from nanograms to micrograms per liter (Bayode *et al.*, 2024; Ciślak *et al.*, 2023; Kasonga *et al.*, 2021), our results suggest that the presence of biofilms in these environments enhances the self-purification process.

The results highlight the potential of biofilms as a sustainable strategy for mitigating estrogen pollution in aquatic environments. While biofilm-based remediation is cost-effective and environmentally friendly, its efficiency is dependent on environmental conditions (Das *et al.*, 2024). Optimizing these conditions to enhance biofilm-mediated biodegradation could improve removal efficiency. Strategies such as promoting biofilm development in targeted areas, selecting microbial communities with high biodegradation capacity, and integrating biofilm-based systems into wastewater treatment could strengthen nature-based solutions for microcontaminant control and ecosystem restoration (Mukhopadhyay; Duttagupta; Mukherjee, 2022).

In this context, in situ bioremediation strategies can be applied by enhancing biofilm functionality in impacted aquatic systems. One approach involves the placement of artificial surfaces (glass plates, porous ceramics, biodegradable polymers) at selected sites in the river, providing the colonization of adapted microbial communities. These surfaces facilitate the growth of natural biofilms capable of removing contaminants like estrogens through biological processes.

Other interventions include the development of floating biofilm reactors powered by solar energy. These systems incorporate submerged aerators beneath the floating structure to improve oxygenation and stimulate microbial activity, thereby enhancing the degradation capacity of biofilms (Zhang, Q. *et al.*, 2021). Additionally, bioaugmentation strategies, based on the introduction of microbial consortia with known degradation potential, can be implemented in polluted river stretches. Biostimulation techniques, which involve the adjustment of environmental variables such as nutrient availability, pH, or dissolved oxygen, may also boost the performance of indigenous biofilm communities.

By demonstrating the biodegradation potential of biofilms, this study paves the way for future research on factors that may hinder or enhance this process. Although laboratory and pilot-scale studies emphasize the promise of biofilm-based approaches, large-scale field applications remain underexplored. Expanding research on these technologies could facilitate their integration into water management strategies, enhancing contaminant removal in both natural and engineered systems.

5.4 CONCLUSIONS

This study assessed the removal of estrogens E1, E2, and EE2 by river biofilms from two sites, Upstream and Downstream, of the Alb River in southern Germany across two different seasons. Our findings provide key insights into the self-purification potential of natural biofilms, addressing the research questions posed in our study:

I. Which mechanisms do biofilms employ to remove these compounds from water?

Our results demonstrate the rapid elimination of E1, E2, and EE2 from the aqueous phase, with 51.3% of EE2 removed within the first hour and ~100% of all compounds eliminated within nine days. Microcosm experiments revealed that microbial biodegradation and/or biotransformation played a dominant role in estrogen removal, while bioaccumulation contributed minimally to the process.

II. Which microorganisms are involved in this process?

Our study indicates that *Proteobacteria*, *Bacteroidota,* and *Actinobacteria* populations recovered after exposure to estrogens. Additionally, the substantial increase in *Firmicutes* and *Deinococcota* abundance at the DWS site highlights the resilience and adaptability of these phyla under estrogen-induced stress. At the genus level, *Exiguobacterium* showed a striking increase, while *Acinetobacter, Massilia, Deinococcus, Tabrizicola,* and *Hymenobacter* also exhibited notable growth, suggesting their potential involvement in estrogen degradation.

III. How can these findings contribute to understanding biofilms as indicators of river self-purification and potential bioremediation agents?

Our results reinforce the critical role of biofilms in the natural purification of aquatic ecosystems. The simultaneous removal of E1, E2, and EE2, which are frequently detected in water bodies, highlights the potential of river biofilms as efficient, nature-based solutions for mitigating estrogen pollution.

Furthermore, to our knowledge, this is the first study to report the simultaneous biodegradation of E1, E2, and EE2 in natural river biofilms. These findings provide novel insights that can inform future water resource management strategies and the development of biofilm-based approaches for contaminant removal in aquatic environments.

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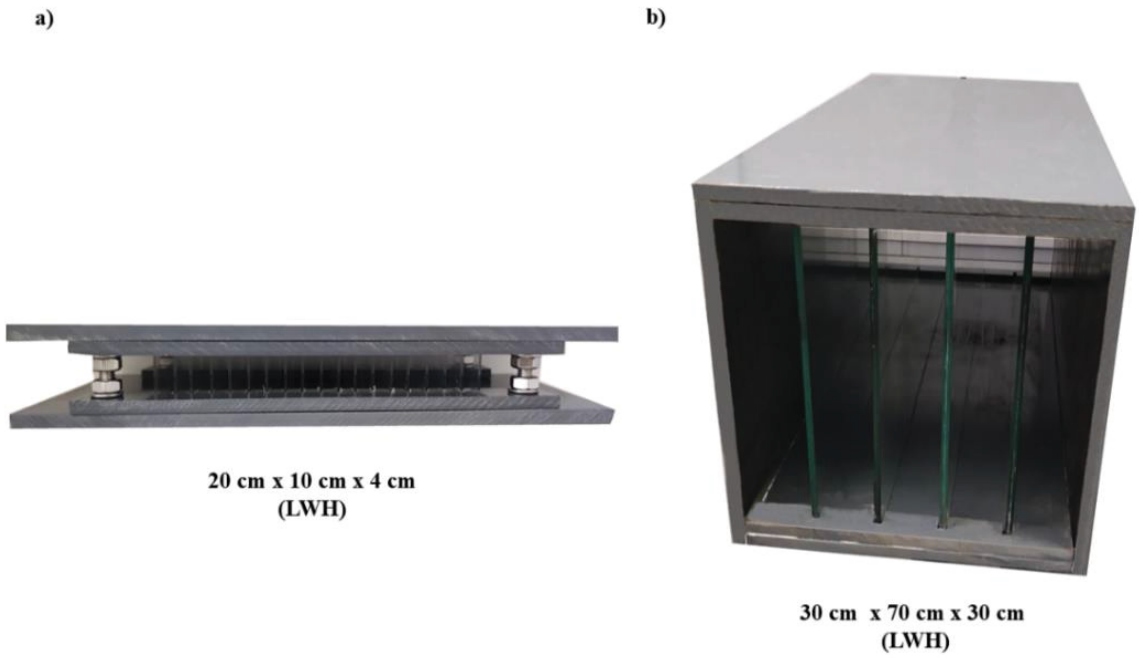
5.5 SUPPLEMENTARY MATERIAL

Materials and Methods

Artificial substrate

The artificial substrates used as samplers for natural biofilm growth in the river were microscope slides, made of glass (26 x 76 mm). The slides were attached to samplers made of Polyvinyl Chloride (PVC) developed for this purpose (Figure 5.6a). Each sampler consisted of 19 slides and two samplers were placed at each point. The samplers were attached to another, larger sampler, produced similarly, containing four large glass plates, each measuring 70 x 30 cm, with a total surface area of 1.68 m² (Figure 5.6b).

FIGURE 5.6 – BIOFILM SAMPLERS. (LWH = length x width x height).



SOURCE: The author (2025).

Results

TABLE S5.1 – WATER QUALITY PARAMETERS OF THE ALB RIVER.

Period	Date	Field site	T (°C)	DO (mg L^{-1} O_2)	EC (µS cm^{-1})	TDS (mg L^{-1})	pH	Turbidity (NTU)	E1 (µg L^{-1})	E2 (µg L^{-1})	EE2 (µg L^{-1})
Late Summer	28.09.2023	UPS	13.86	9.54	195.5	161	7.89	0.68	0.019	0.016	0.012
	28.09.2023	DWS	15.33	8.35	246.2	196	7.52	1.09	0.016	0.014	0.041
	11.10.2023	UPS	14.91	10.10	192.7	155	8.26	0.54	-	-	-
	11.10.2023	DWS	16.03	9.53	250.4	196	8.44	1.94	-	-	-
Late Autumn	29.11.2023	UPS	6.87	11.55	165.8	108	8.34	3.10	0.006	0.001	0.001
	29.11.2023	DWS	6.70	10.97	190.9	124	8.15	4.05	0.008	0.003	0.002
	12.12.2023	UPS	8.84	10.77	186.8	121	7.50	1.82	-	-	-
	12.12.2023	DWS	9.01	10.80	186.8	163	7.11	1.85	-	-	-

T = Water temperature, DO = Dissolved Oxygen, EC = Electrical Conductivity, TDS = Total Dissolved Solids.

TABLE S5.2 – WATER QUALITY PARAMETERS.

Period	Time	Field site	COD (mg L^{-1} O_2)	Orthophosphates (mg L^{-1} PO_4-P)	Ammonia (mg L^{-1} NH_4-N)	Nitrate (mg L^{-1} NO_3-N)	Nitrite (mg L^{-1} NO_2-N)
Late Summer	Day 0	UPS	7.87	0.042	0.012	2.550	0.007
	Day 0	DWS	6.47	0.060	0.032	2.090	0.007
	Day 13	UPS	9.54	0.024	0.021	1.258	-
	Day 13	DWS	7.25	0.047	0.012	1.012	-
Late Autumn	Day 0	UPS	4.73	0.022	0.446	0.067	-
	Day 0	DWS	11.90	0.031	0.119	0.109	-
	Day 13	UPS	9.55	0.048	0.014	1.780	0.004
	Day 13	DWS	7.57	0.081	0.010	1.410	0.002

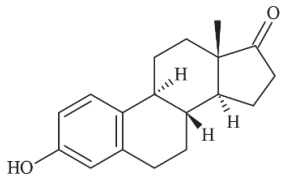
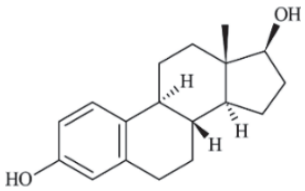
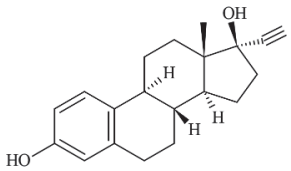
COD = Chemical Oxygen Demand.

TABLE S5.3 – PARAMETERS MEASURED IN THE MICROCOSMS DURING THE EXPERIMENTAL DESIGN.

Period	Time	Upstream			Downstream			Control		
		pH	DO	Turbidity	pH	DO	Turbidity	pH	DO	Turbidity
Late Summer	Day 0	8.01	10.36	2.56	8.04	10.27	10.90	7.48	10.55	0.26
	Day 2	7.89	10.49	0.63	8.34	10.47	0.63	7.54	10.69	0.54
	Day 5	8.04	10.39	0.67	8.19	10.40	0.52	7.49	10.82	1.19
	Day 6	8.04	10.55	0.59	8.20	10.66	0.69	7.79	10.78	1.50
	Day 7	8.03	10.53	0.60	8.19	10.60	0.71	7.78	10.70	1.60
	Day 8	8.01	10.47	0.61	8.18	10.51	0.70	7.81	10.70	1.74
	Day 9	8.00	10.51	0.87	8.15	10.50	1.00	7.79	10.68	2.51
	Day 12	8.05	10.40	0.64	8.09	10.43	0.87	7.65	10.50	2.01
	Day 13	7.92	10.41	0.51	8.12	10.44	0.72	7.95	10.48	1.91
		pH	**DO**	**Turbidity**	**pH**	**DO**	**Turbidity**	**pH**	**DO**	**Turbidity**
Late Autumn	Day 0	7.50	9.48	1.39	7.11	10.08	1.82	7.12	9.80	0.29
	Day 2	7.25	9.04	0.52	7.51	8.97	0.93	7.35	9.12	0.43
	Day 5	7.20	10.00	0.45	7.45	9.20	0.49	7.28	9.02	0.54
	Day 6	7.22	9.60	0.64	7.48	9.35	0.44	7.29	9.36	0.39
	Day 7	7.45	8.90	0.48	7.76	8.89	0.29	7.43	8.96	0.48
	Day 8	7.48	8.86	0.49	7.75	8.80	0.33	7.40	8.99	0.51
	Day 9	7.25	8.30	0.63	7.68	8.74	0.38	7.51	8.85	0.44
	Day 13	7.16	8.16	0.37	7.57	8.75	0.55	7.61	9.10	0.52

DO (mg L^{-1} O_2), Turbidity (NTU).

TABLE S5.4 – RELEVANT PHYSICOCHEMICAL PROPERTIES OF THE EVALUATED STEROIDS.

Hormone	Structure	Molecular formula	Molecular weight (g mol^{-1})	Water solubility at 25 °C (mg L^{-1})	pKa at 25 °C	Log K_{ow}[a]	Log D[b]
Estrone (E1)		$C_{18}H_{22}O_2$	270.37	147	10.91	3.13	4.30
17β-estradiol (E2)		$C_{18}H_{24}O_2$	272.39	82	10.40	3.94	3.70
17α-ethinyles tradiol (EE2)		$C_{20}H_{24}O_2$	296.41	116	10.50	4.15	3.90

[a] Octanol-water partition coefficient; [a] Octanol-water partition coefficients reported for pH 8.0.

TABLE S5.5 – MEASURED CONCENTRATIONS OF E1, E2, AND EE2 BIOACCUMULATED IN BIOFILM BIOMASS ON DAY 13, EXPRESSED IN µg g^{-1} OF DRY WEIGHT.

Period	Field site	E1 (µg g^{-1})	E2 (µg g^{-1})	EE2 (µg g^{-1})
Late Summer	UPS	0.00	0.00	0.191
	DWS	0.00	0.00	0.000
Late Autumn	UPS	0.00	0.00	0.087
	DWS	0.00	0.00	0.111

Chapter 6

"A história da vida na Terra tem sido uma história de interação entre coisas vivas e seus ambientes."
Rachel Carson

"The history of life on earth has been a history of interaction between living things and their surroundings."
Rachel Carson

6 EXPLORING THE EFFECTS OF ESTROGENIC STEROID HORMONES ON BACTERIAL COMMUNITIES IN RIVER BIOFILMS

Abstract

Steroid hormones released through human activities are increasingly found in aquatic environments. Even in low amounts, these substances can disturb natural systems. One of the most affected components are river biofilms, thin layers of microorganisms that grow on submerged surfaces. These biofilms have an important role: they help cycle nutrients, break down pollutants, and serve as water quality indicators. In this context, this study evaluated the capacity of natural biofilms to remove four steroid hormones, estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2), and progesterone (PRO) in microcosms, and assessed their effects on bacterial community structure. Biofilms efficiently removed E1, E2, and EE2, reaching maximum removal rates of 97.4% (after 4 days), 93.8% (after 3 days), and 88.2% (after 6 days), respectively. PRO was also removed less efficiently (48.7% in 3 days). Hormone exposure affected biofilms differently depending on their origin. Upstream biofilms showed reduced diversity and richness, indicating sensitivity, while those near a wastewater treatment plant exhibited increased diversity and evenness, suggesting resilience and potential adaptation to micropollutants. The dominant phyla across treatments were *Proteobacteria* and *Firmicutes*. Notably, genera such as *Alloprevotella* declined sharply, while *Paenibacillus* increased after exposure. These results support the dual role of river biofilms as both natural barriers and biological indicators of steroid hormone contamination. The study contributes to the understanding of microbial responses to emerging pollutants and underscores the relevance of biofilms in the natural attenuation and potential bioremediation of urban rivers.

Keywords: Biodegradation; Sampler; Endocrine-disrupting compounds; Environmental microbiology.

6.1 INTRODUCTION

One of the most pressing concerns in environmental sciences is ensuring water quality for human use and ecosystem integrity. Pollutants from diverse sources persist in aquatic environments, raising serious concerns about their impacts on aquatic life (Torres *et al.*, 2021). The continuous release of microcontaminants into river systems results in periodic exposure of organisms to these harmful substances.

Among the microcontaminants are steroid hormones, which include natural and synthetic compounds capable of interfering with endocrine functions. Steroid hormones such as estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2), and progesterone (PRO) have been detected in aquatic environments (Barcellos, 2014; Goeury *et al.*, 2022; Ide *et al.*, 2017; Machado *et al.*, 2014; Torres *et al.*, 2015). Their presence is alarming due to their potential to cause adverse effects on aquatic organisms and human health (Rodrigues *et al.*, 2025; Zhang *et al.*, 2016). These substances exhibit persistent physicochemical properties, such as lipophilicity, bioaccumulation, and low molecular weight, which favor their environmental dispersion and persistence (Ilyas; Van Hullebusch, 2020; Mpupa *et al.*, 2022). Even at trace concentrations, ranging from milligrams per liter (mg L^{-1}) to nanograms per liter (ng L^{-1}), they can act as potent endocrine disruptors (Zhang *et al.*, 2016).

Estrone (E1), 17β-estradiol (E2), and 17α-ethinylestradiol (EE2) are natural steroid hormones excreted by animals and humans through feces and urine. EE2, in contrast, is a synthetic estrogen widely used in oral contraceptives and hormone replacement therapy for menopause (Ojoghoro; Scrimshaw; Sumpter, 2021). Its primary application is in the formulation of birth control pills, which contain between 30 and 50 μg of EE2 per tablet (Torres *et al.*, 2021).

Most of the conventional drinking water treatment plants (WTPs) and wastewater treatment plants (WWTPs) are not designed to remove these compounds with a low concentration completely (Bayode *et al.*, 2024; Du *et al.*, 2020; Shabbir *et al.*, 2022). Additionally, estrogenic compounds can reach surface and groundwater bodies through various pathways, including rainfall, surface runoff, slurry irrigation, land application of manure or biosolids, and discharges from hospitals and pharmaceutical industries (Bilal; Barceló; Iqbal, 2021; Du *et al.*, 2020). Studies have demonstrated that steroid hormones can affect multiple cellular systems, particularly reproduction-related ones. They have been associated with disorders such as reproductive dysfunction, infertility, and hormone-related cancers, including

breast, testicular, and prostate cancers (Ojoghoro; Scrimshaw; Sumpter, 2021; Vilela; Bassin; Peixoto, 2018).

In this context, river biofilms represent a promising biological matrix for assessing the environmental impact of steroid hormones. Natural biofilms (microbial communities attached to surfaces in aquatic environments) consist of algae, fungi, bacteria, protozoa, and other organisms embedded in a self-produced matrix of extracellular polymeric substances (EPS) (Battin *et al.*, 2016). These biofilms play a crucial role in aquatic ecosystems by contributing to photosynthetic oxygen production, organic matter degradation, primary production, nutrient cycling, acting as sensitive bioindicators of environmental pollution, and by degrading contaminants present in the water (Li *et al.*, 2023; Makk *et al.*, 2024; Marques *et al.*, 2024; Mishra *et al.*, 2022).

Unlike isolated microbial species, biofilm communities possess a complex structural organization that increases their resilience to environmental stressors such as microcontaminants. This resilience is often achieved through adaptive shifts in metabolic pathways and community composition (Wang *et al.*, 2023). For instance, exposure to E1 has been shown to alter microbial co-occurrence networks within periphytic biofilms, with increases in certain *Proteobacteria* taxa suggesting possible adaptive mechanisms (Zhang *et al.*, 2021).

Lima *et al.* (2025) emphasize the need to investigate the synergistic effects of environmental factors on biofilms. Their study highlights the importance of hydrological variables (especially flow/flux and biofilm biomass) as key indicators of stressor-response relationships. Metrics such as species richness and community composition are also relevant and merit further investigation. Moreover, analyses of metabolic and physiological activity, coupled with advanced molecular tools such as microbiome, metagenomics, and transcriptomics, are recommended to deepen understanding of biofilm responses at the genetic level.

Despite the increasing attention to this topic, significant knowledge gaps remain regarding the effects of steroid hormones on fluvial biofilm communities. This study aims to evaluate the adverse effects of four representative steroid hormones (E1, E2, EE2, and PRO) on natural river biofilm communities. Furthermore, we investigate the potential of these communities to biodegrade the hormones and assess the ecological consequences of their presence in fluvial systems. We hypothesize that biofilms primarily remove steroid hormones through biodegradation, a process potentially accompanied by reduced microbial diversity. Specifically, our objectives were (1) to quantify the removal efficiency of each hormone by the

biofilm and (2) to determine whether environmentally relevant concentrations of steroid hormones reduce the abundance of sensitive taxa in river biofilm communities. As far as we know, no previous study has thoroughly examined structural alterations in river biofilms under combined exposure conditions to Estrone (E1), 17β-estradiol (E2), and 17α-ethinylestradiol (EE2).

6.2 MATERIAL AND METHODS

6.2.1 Cultivation and collection of biofilms

Before sampling, 44 glass slides measuring 26 x 76 mm each (total surface area 1738.88 cm^2) were placed as artificial surfaces in the Barigui River, Curitiba, Brazil. The slides were attached to compact samplers made of wood and PVC developed by this research. The samplers were connected to another larger sampler, produced similarly, containing four large glass plates (Lima *et al.*, 2025; Marques *et al.*, 2024; Reichert *et al.*, 2021).

The colonization of natural biofilms on these compact samplers occurred in February 2024 at two sites BA1 (25°18'45.466"S, 49°17'43.959"W) and BA2 (25°21'30.0"S, 49°16'53.8"W) of the river, for 15 days. The first site (BA1) is located downstream from the Barigui water treatment plant (WTP), which supplies drinking water to the people of Almirante Tamandaré. The second site (BA2) is downstream of the São Jorge WWTP. The WWTP uses an Upflow Anaerobic Sludge Blanket (UASB) reactor with a treatment capacity of 70 L s^{-1}, followed by a physicochemical process (SANEPAR, 2022).

Water temperature, pH, dissolved oxygen (DO), and conductivity were measured near the samplers in the coastal zone using a multiparameter water quality sonde (HANNA HI9829) at both sites in the river. Following this, the samplers with the grown biofilm were removed from the river and transported for use in experimental tests in microcosms. Additionally, water was collected from both river sites for use in the microcosms.

6.2.2 Exposure experiments

Two microcosms consisting of 15 L glass aquariums (rectangular parallelepiped) were used in the laboratory to determine the biodegradation potential of E1, E2, EE2, and PRO. For the acclimation phase, the artificial substrates with biofilm were transferred into microcosms (glass aquariums), each filled with 10 L of river water from the respective site. One aquarium

received BA1 biofilms and river water, while the other received BA2 biofilms and river water (44 colonized slides per aquarium). Aquarium pumps were placed in each microcosm to simulate water flow and aerate the system. After two weeks of laboratory acclimation for the biofilms, the water was renewed, and 25 µg L-1 of E1, E2, EE2, and PRO was introduced into the microcosms. The experimental conditions of the microcosm experiment were chosen to assess conditions as similar as possible to a natural environment. After 15 days of testing, the aquarium water was renewed and fortified with 50 µg L-1 of E1, E2, EE2, and PRO. Additionally, an abiotic control test was conducted under the same conditions without adding biofilm.

6.2.3 Water and biofilm sampling

Water samples (3 mL) from each microcosm were collected at 0, 0.04, 1, 2, 3, 4, 5, 6, 10, and 15 days to quantify E1, E2, EE2, and PRO content. Samples at time 0.04 day were collected after one hour of the initial spiking to allow homogeneous dispersion of the microcontaminant in the water of the microcosm. Water samples were collected and filtered through syringe filters with 25 mm x 0.45 µm PTFE membranes and 25 mm x 0.22 µm. At the end of both exposure experiments (25 and 50 µg L-1), biofilm samples were scraped from the glass slides and kept at −20 °C until further analysis.

6.2.4 Analytical Methodology

E1, E2, EE2, and PRO were detected and quantified in the water samples using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Freeze-dried biofilm samples (100 mg) were extracted using a two-step procedure. In the first step, the samples were mixed with 5 mL of methanol and vortexed thoroughly. The mixture was then subjected to ultrasound treatment for 480 seconds, followed by centrifugation at 2000 rpm for 5 minutes. The supernatant was carefully collected. The extraction process was repeated in the second step, and the supernatants from both steps were combined for analysis. LC-MS/MS analyzed the combined supernatants.

6.2.5 Bacterial community analyses

The biofilm samples were used for total DNA extraction using the Soil Fecal DNA extraction kit (Zymo). The 16S genes (V3V4 region for bacterial analysis) were amplified from this DNA. The amplified fragments were then sequenced on an Illumina NextSeq platform, and the sequences were analyzed using Qiime software to identify the microorganisms present in the samples and their respective percentages. Alpha diversity was assessed to evaluate species richness and evenness within samples.

6.3 RESULTS AND DISCUSSION

6.3.1 Water characterization

The physicochemical parameters measured on three different days (Table 6.1) at two sites of the Barigui River revealed that the BA2 field site exhibits a higher pollution load compared to BA1. The average DO at BA1 was 6.69 mg L^{-1} O_2, while at BA2, it was 4.93 mg L^{-1} O_2. The average turbidity was 12.48 NTU at BA1 and 21.23 NTU at BA2. Temperature, electrical conductivity, and pH showed no significant differences between the two field sites. Lower DO values may indicate organic pollution and environmental degradation. High turbidity levels can hinder light penetration in the water, impair photosynthesis in autotrophic organisms, and suggest surface runoff, effluent discharge, or erosive processes, also serving as indicators of diffuse or point-source pollution. This result was expected, as BA2 is located downstream of a WWTP, and it is common for water bodies to exhibit increased concentrations of various chemical compounds after the discharge of treated effluents (Kramer *et al.*, 2018; Montagner *et al.*, 2019).

TABLE 6.1 – MEASUREMENTS AT THE SAMPLING LOCATIONS.

Field site	Date	T (°C)	DO (mg L^{-1} O_2)	Conductivity (mS cm^{-1})	pH	Turbidity (NTU)	E1 (µg L^{-1})	E2 (µg L^{-1})	EE2 (µg L^{-1})	PRO (µg L^{-1})
BA1	07.03.2024	20.53	7.53	0.25	7.30	12.80	-	-	-	-
	22.03.2024	20.83	5.54	0.27	7.24	15.10	-	-	-	-
	05.04.2024	21.21	7.00	0.30	6.87	9.55	0.09	0.00	2.01	1.10
BA2	07.03.2024	21.01	6.34	0.26	7.29	19.30	-	-	-	-
	22.03.2024	21.12	3.42	0.29	7.29	33.90	-	-	-	-
	05.04.2024	21.73	5.03	0.31	7.49	10.50	0.15	0.00	0.80	1.10

T = temperature, DO = dissolved oxygen.

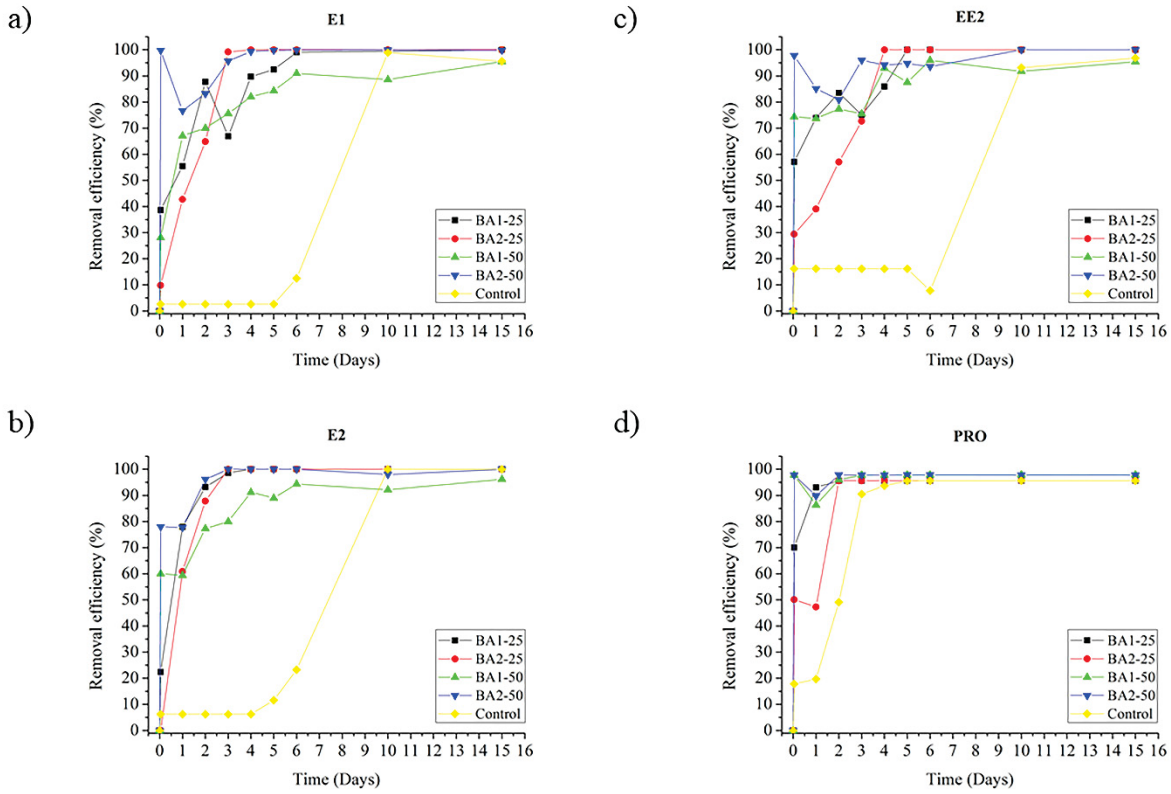
Steroid hormone concentrations measured in water samples were generally low. The highest concentration detected was 2.01 µg L^{-1} of EE2 at BA1, followed by progesterone

(PRO), which showed a 1.10 µg L^{-1} concentration at both sampling sites. The low levels detected in water could be explained by the limited solubility of unmetabolized or free hormones, which are prone to adhering to suspended particles or settling out as sediment, thus diminishing their concentration in the water column (Torres *et al.*, 2021).

6.3.2 Removal efficiency by biofilms

The removal efficiency of natural river biofilms for E1, E2, EE2, and PRO showed a rapid initial increase under all conditions (Figure 6.1), indicating the active role of biofilms in contaminant biodegradation and/or biosorption. This rapid uptake may be attributed to high microbial metabolic activity, microbial communities capable of degrading these compounds, or the availability of binding sites within the biofilm matrix for contaminant sorption (Saini *et al.*, 2023).

FIGURE 6.1 – REMOVAL EFFICIENCY OF HORMONES E1, E2, EE2, AND PRO BY NATURAL RIVER BIOFILMS.



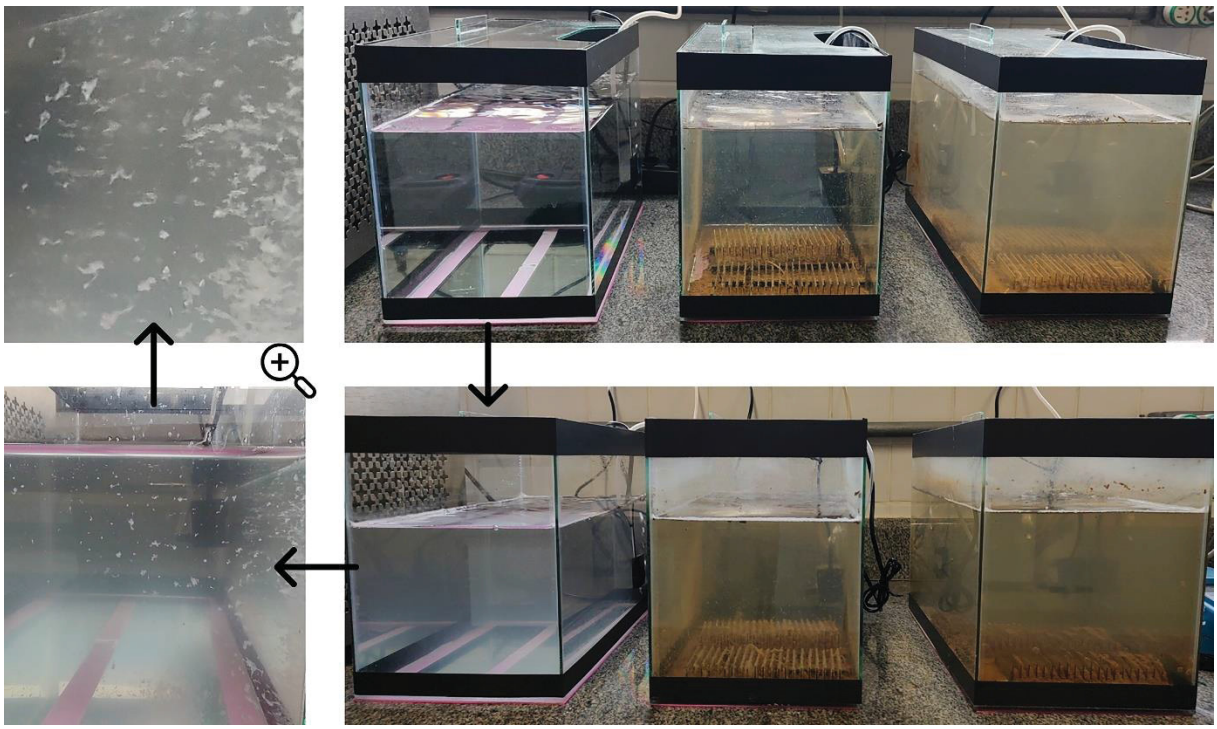
Note: BA1-25 = experiment with water from river site BA1 and 25 µg L^{-1} of hormones; BA2-25 = experiment with water from river site BA2 and 25 µg L^{-1} of hormones; BA1-50 = experiment with water from river site BA1 and 50 µg L^{-1} of hormones; BA2-50 = experiment with water from river site BA2 and 50 µg L^{-1} of hormones; Control = experiment without biofilm addition.

SOURCE: The author (2025).

All biofilm conditions (biotic) reached over 95% removal by the 15th day of exposure. E2 exhibited the highest efficiency, achieving 100% removal by the third day of the experiment in both initial concentrations for the BA1 biofilm. PRO showed similar removal levels in both the control (without biofilm addition) and biofilm treatments, suggesting that its removal may be largely due to spontaneous or abiotic processes.

The control samples showed a noticeable increase in removal efficiency for the other steroid hormones after 10 days. This outcome may be attributed to the compounds' half-lives, air oxidation, photodegradation, and other abiotic removal mechanisms. Moreover, the microcosm controls were not hermetically sealed, allowing air exchange that likely promoted the growth of suspended bacteria in the water. This uncontrolled microbial proliferation may have also contributed to the degradation of the compounds. Figure 6.2 illustrates this microbial growth, both suspended in the water and adhered to the glass surfaces of the aquaria, which is also evidenced by the increased water turbidity. Nonetheless, the presence of biofilms significantly accelerated and enhanced the removal of E1, E2, and EE2 from the water.

FIGURE 6.2 – MICROCOSMS FROM THE CONTROL, BA1, AND BA2 CONDITIONS, HIGHLIGHTING THE DIFFERENCE IN THE CONTROL MICROCOSM FROM THE BEGINNING TO THE END OF THE EXPERIMENT. THE INCREASED TURBIDITY AND VISIBLE SURFACE GROWTH INDICATE THE PROLIFERATION OF SUSPENDED BACTERIA IN THE CONTROL OVER TIME.



SOURCE: The author (2025).

To highlight the removal potential of biofilms, the maximum removal values were calculated by subtracting the removal observed in the control from that in the biofilm treatments on the day of peak removal, as shown in Table 6.2. Data up to the sixth day of testing were considered for this comparison. Natural river biofilms were able to remove E1, E2, and EE2 with maximum removal rates up to 97.4% (after 4 days), 93.8% (after 3 days), and 88.2% (after 6 days), respectively. PRO was also removed, although at a low level, 48.7% in 3 days, and just in the BA2 with the highest initial concentration.

TABLE 6.2 – MEXIMUM STEROID HORMONES REMOVAL BY NATURAL RIVER BIOFILMS.

Exposure	Field site	E1		E2		EE2		PRO	
		MR	Time	MR	Time	MR	Time	MR	Time
25 µg L^{-1}	BA1	86.5	6	93.8	4	83.8	5	0	6
	BA2	97.4	4	93.8	3	83.8	4	0	6
50 µg L^{-1}	BA1	78.5	6	71.1	6	88.2	6	0	6
	BA2	87.4	6	93.8	3	81.6	0	48.7	3

Note: E1 = estrone; E2 = 17β-estradiol; EE2 = 17α-ethinylestradiol; PRO = progesterone; BA1 = experiment with water from river site BA1; BA2 = experiment with water from river site BA2; MR = Maximum removal resulting from biofilms (%); Time = The exposure time reaching maximum removal (Days).

After exposure to two concentrations of steroid hormones, the amount of compound bioaccumulated in the final biofilm biomass was quantified. In the BA1 biofilm, 2.41 µg L^{-1} of E1 and 0.74 µg L^{-1} of PRO were bioaccumulated, corresponding to 3.2% and 1.0% of the total added amounts, respectively. In contrast, in the BA2 biofilm, only PRO showed measurable bioaccumulation (0.67 µg L^{-1}), corresponding to 0.9%. These results suggest that biodegradation was the main removal mechanism for E1, E2, EE2, and PRO by natural river biofilms. This conclusion is consistent with previous studies reporting degradation as the primary pathway for contaminant removal by river biofilms (Liang *et al.*, 2024; Yan *et al.*, 2023).

Bioaccumulation of steroid hormones in biofilms typically occurs through absorption by microbial cells, adsorption onto cell walls, or retention within the EPS matrix, driven by hydrophobic and electrostatic interactions. These processes depend on the physicochemical properties of the compounds, such as log D and pKa (Santos *et al.*, 2019). Although estrogens (E1, E2 and, EE2), due to their hydrophobicity (log D > 3), have an affinity for the biofilm matrix (He *et al.*, 2021), the results indicate that biodegradation, rather than bioaccumulation, was the dominant removal mechanism, highlighting the role of microbial activity over passive retention.

Regarding differences between biofilms from the two sampling sites (BA1 and BA2), the exposure tests did not reveal substantial variation in removal efficiency. This indicates that both biofilms possess a high capacity to remove these contaminants, regardless of the differing environmental conditions at the two locations.

These findings confirm that natural river biofilms effectively remove steroid hormones from aquatic environments, primarily through biodegradation. However, exposure to these compounds may also influence the structure and composition of the microbial communities within the biofilms, which can, in turn, affect their ecological functions and long-term treatment efficiency. Therefore, the following section explores how the bacterial community structure was altered following exposure to E1, E2, EE2, and PRO.

6.3.3 Bacterial community

The microbial community structure was analyzed before (BA1-A and BA2-A) and after the addition of E1, E2, EE2, and PRO to assess the impact of these pollutants on the natural biofilm community composition. In total, 1665 species, 1265 genera, 578 families, 298 orders, 118 classes, and 44 phyla were identified. The dominant phyla across all treatment groups were *Proteobacteria* and *Firmicutes*, encompassing more than 2300 taxa in total.

According to the phyla (Figure 6.3a), exposure to the steroid hormones led to notable shifts in the biofilm community structure at both tested concentrations (25 and 50 μg L^{-1}). At sampling site BA1, there was a marked increase in the relative abundance of *Proteobacteria* (from 48.7% to 62.6% at 25 μg L^{-1} and 66.2% at 50 μg L^{-1}), *Planctomycetota,* and *Bdellovibrionota.* Conversely, *Firmicutes, Bacteroidota, Acidobacteriota, Chlamydiota*, and *Dependentiae* decreased substantially.

At site BA2, a different pattern emerged: while *Proteobacteria, Firmicutes*, and *Bacteroidota* declined, there was an increase in *Actinobacteriota, Planctomycetota, Acidobacteriota, Bdellovibrionota, Chlamydiota*, and *Dependentiae*. These shifts suggest that microbial community responses to estrogenic compounds vary depending on local environmental conditions and the synergistic influence of other variables acting on the ecosystem.

Although a slight reduction in *Proteobacteria* was observed at BA2 after exposure, it remained the dominant phylum, followed by *Firmicutes, Actinobacteriota*, and *Bacteroidota*. These findings are consistent with previous reports indicating that *Proteobacteria* and *Actinobacteriota*, frequently present in river sediments, are commonly found in biofilm

communities from such environments (Ding *et al*., 2024; Li *et al*., 2025). Moreover, *Proteobacteria* and *Bacteroidota* are widely recognized as dominant phyla in stream biofilms (Battin *et al*., 2016), with *Proteobacteria* playing key roles in organic and inorganic pollutant degradation in wastewater treatment systems (Yuan *et al*., 2020). Similarly, a study on E1 exposure has reported increased *Proteobacteria* abundance in river biofilms, underscoring their adaptive potential and relevance for pollutant removal strategies (Zhang *et al*., 2021).

The observed variation in response between the BA1 and BA2 sites may be linked to the downstream site's proximity to a WWTP, which can affect the bacterial composition of biofilms. Carles *et al.* (2022) investigated the influence of wastewater-derived microorganisms on river biofilm communities and concluded that microbial communities introduced via treated effluent should be regarded as additional stressors to receiving rivers, alongside nutrients, micropollutants, and elevated temperatures. Their findings also highlighted the frequent detection of *Firmicutes* in WWTP effluents, downstream river biofilms, and biofilters treating urban wastewater, underscoring the role of WWTP-associated microorganisms in shaping microbial responses to environmental stress. *Firmicutes* are widely recognized as dominant bacterial taxa in anaerobic bioreactors (Li *et al*., 2019).

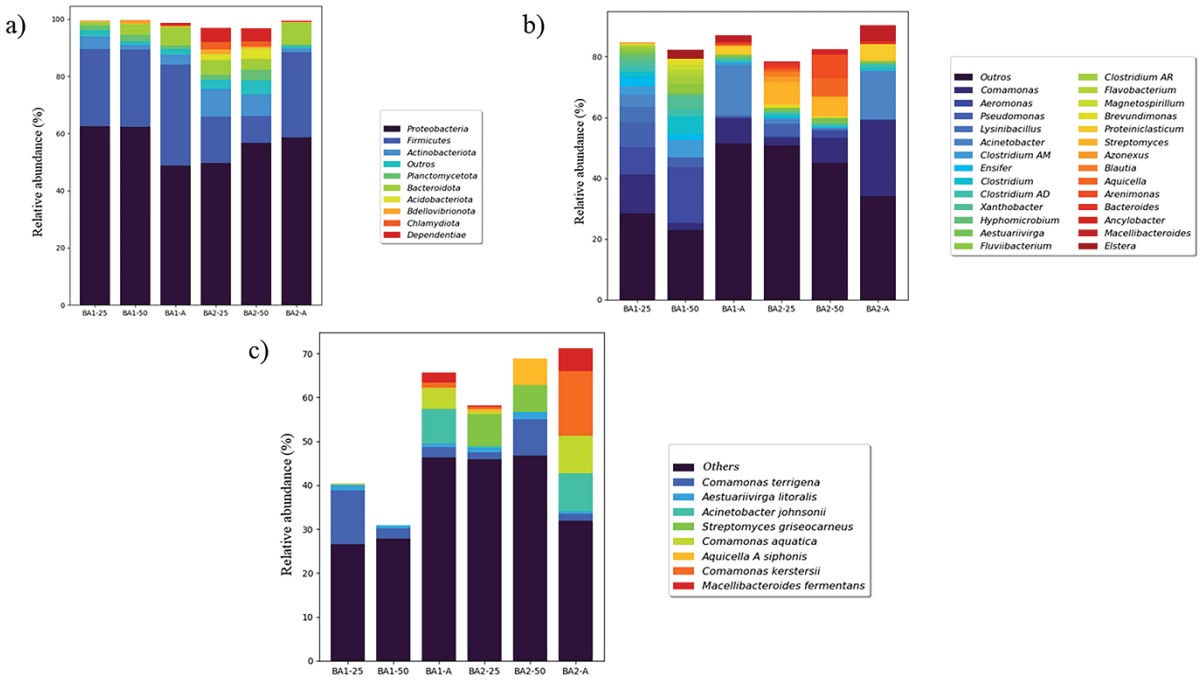
At the genus level, BA1-A samples were primarily dominated by *Acinetobacter* (16.4%) and *Comamonas* (8.3%) (Figure 6.3b). Following exposure to steroid hormones, the relative abundance of *Acinetobacter* declined, whereas *Comamonas*, *Aeromonas*, *Pseudomonas*, *Lysinibacillus*, *Clostridium*, and *Ensifer* increased. In BA2-A samples, the most dominant genera before hormone exposure were *Comamonas* (25.1%) and *Acinetobacter* (15.7%). However, a marked shift in the bacterial community structure was observed after exposure, characterized by increased *Streptomyces, Arenimonas*, and *Aquicella*, along with decreased *Comamonas* and *Acinetobacter*.

Comamonas terrigena and *Aestuariivirga litoralis* were consistently present from the beginning to the end of the experiments (Figure 6.3c). In the BA1 samples, after adding 25 µg L^{-1} and 50 µg L^{-1}, there was marked selectivity, with *Comamonas terrigena* predominating. In contrast, in the BA2 samples, other species such as *Streptomyces griseocarneus* and *Aquicella siphonis* dominated the biofilm following the introduction of contaminants. *Comamonas terrigena, Aestuariivirga litoralis*, and *Aquicella siphonis* belong to the *Proteobacteria* phylum, while *Streptomyces griseocarneus* belongs to *Actinobacteriota*.

These results suggest that exposure to E1, E2, EE2, and PRO may negatively affect the structure of bacterial communities within the biofilms, primarily through selective pressure, leading to a shift in community composition. Bacteria capable of using steroid hormones as a

carbon source or through alternative metabolic pathways are more likely to survive and become dominant under such conditions.

FIGURE 6.3 – RELATIVE ABUNDANCE OF BACTERIAL READS IN BIOFILM SAMPLES BEFORE AND AFTER CONTAMINANT EXPOSURE AT THE (A) PHYLUM, (B) GENUS, AND (C) SPECIES LEVELS.



Note: BA1-25 = biofilm from river site BA1 after exposure to 25 µg L^{-1} of hormones; BA1-50 = biofilm from river site BA1 after exposure to 50 µg L^{-1} of hormones; BA1-A = biofilm from river site BA1 before hormone exposure; BA2-25 = biofilm from river site BA2 after exposure to 25 µg L^{-1} of hormones; BA2-50 = biofilm from river site BA2 after exposure to 50 µg L^{-1} of hormones; BA2-A = biofilm from river site BA2 before hormone exposure.

SOURCE: The author (2025).

6.3.4 Diversity and richness of biofilms

Biofilms, as clusters of microorganisms, can adapt to external disturbances by changing their community composition. The structural stability of these microbial communities is closely linked to their functional resilience. Because many disruptions influence alpha diversity, it is commonly used as a metric to describe and compare microbial community structures in ecological analyses (Li *et al.*, 2023).

The evenness and richness can evaluate the diversity of microbial biofilms, and in the present study, this has been determined by the Chao1, Simpson, Fisher, Shannon, and Pielou indices (Table 6.3). A total of approximately 1501 to 4385 OTUs were identified across all samples. As shown in Table 6.3, the sample from BA1 before the addition of contaminants

(BA1-A) had the highest OTUs, followed by the samples from BA2 after the addition of the contaminants (BA2-50 and BA2-25). These results suggest that, at the BA1 site, the addition of steroid hormones reduced microbial richness and diversity. In contrast, the opposite pattern was observed at the BA2 site, where an increase in species richness was detected following exposure to the hormones.

The Chao1 index, which estimates community richness, corroborated the OTUs results, confirming the same biodiversity trend. The Simpson index, which reflects species dominance (with lower values indicating higher diversity), revealed greater dominance in the samples before steroid hormone exposure (BA1-A and BA2-A) and lower dominance in the BA2 samples following exposure to steroid hormones (BA2-25 and BA2-50).

At site BA1, the BA1-A exhibited relatively high species dominance (Simpson = 0.034) and moderate evenness (Pielou = 0.700). Following the addition of steroid hormones, a marked reduction in richness and diversity was observed, as reflected by the decreases in Chao1, Fisher, and Shannon indices. This trend was particularly evident in the BA1-50 sample (OTUs = 1501; Chao1 = 1501.3; Fisher = 247.61), suggesting a potential inhibitory effect of higher hormone concentrations on the microbial community. These findings indicate that the exposure to steroidal compounds at BA1 likely suppressed microbial diversity, possibly favoring a few resistant or more competitive taxa.

In contrast, the response at site BA2 was notably different. Although the BA2-A displayed lower diversity (Shannon = 5.00), reduced evenness (Pielou = 0.651), and higher dominance (Simpson = 0.032), the addition of steroid hormones led to an increase in both diversity and community uniformity, especially in BA2-25 (Simpson = 0.004; Shannon = 6.77; Pielou = 0.822). These results suggest that the microbial community at BA2, located downstream of a wastewater treatment plant, may be more resilient or pre-adapted to the presence of micropollutants such as steroid hormones. One plausible explanation is that BA2 initially harbored a dominant taxon, possibly Comamonas kerstersii, that limited the proliferation of other species. Hormone exposure may have selectively reduced the abundance of this dominant species, alleviating competitive pressure and allowing for the emergence of a more diverse and evenly distributed microbial community.

TABLE 6.3 – DIVERSITY STATISTICS OF RIVER BIOFILM FOR THE DIFFERENT STEROID HORMONE CONCENTRATION EXPOSURES.

Condition	Sequences	OTUs	Chao1	Simpson	Fisher	Shannon	Pielou
BA1-A	396.026	4385	4393	0,034	762,92	5,87	0,700
BA1-25	422.663	3473	3479	0,019	564,58	5,69	0,698
BA1-50	142.518	1501	1501	0,023	247,61	5,18	0,708
BA2-A	282.486	2175	2178	0,032	354,23	5,00	0,651
BA2-25	311.652	3753	3757	0,004	633,53	6,77	0,822
BA2-50	386.783	4129	4133	0,015	686,66	6,14	0,737

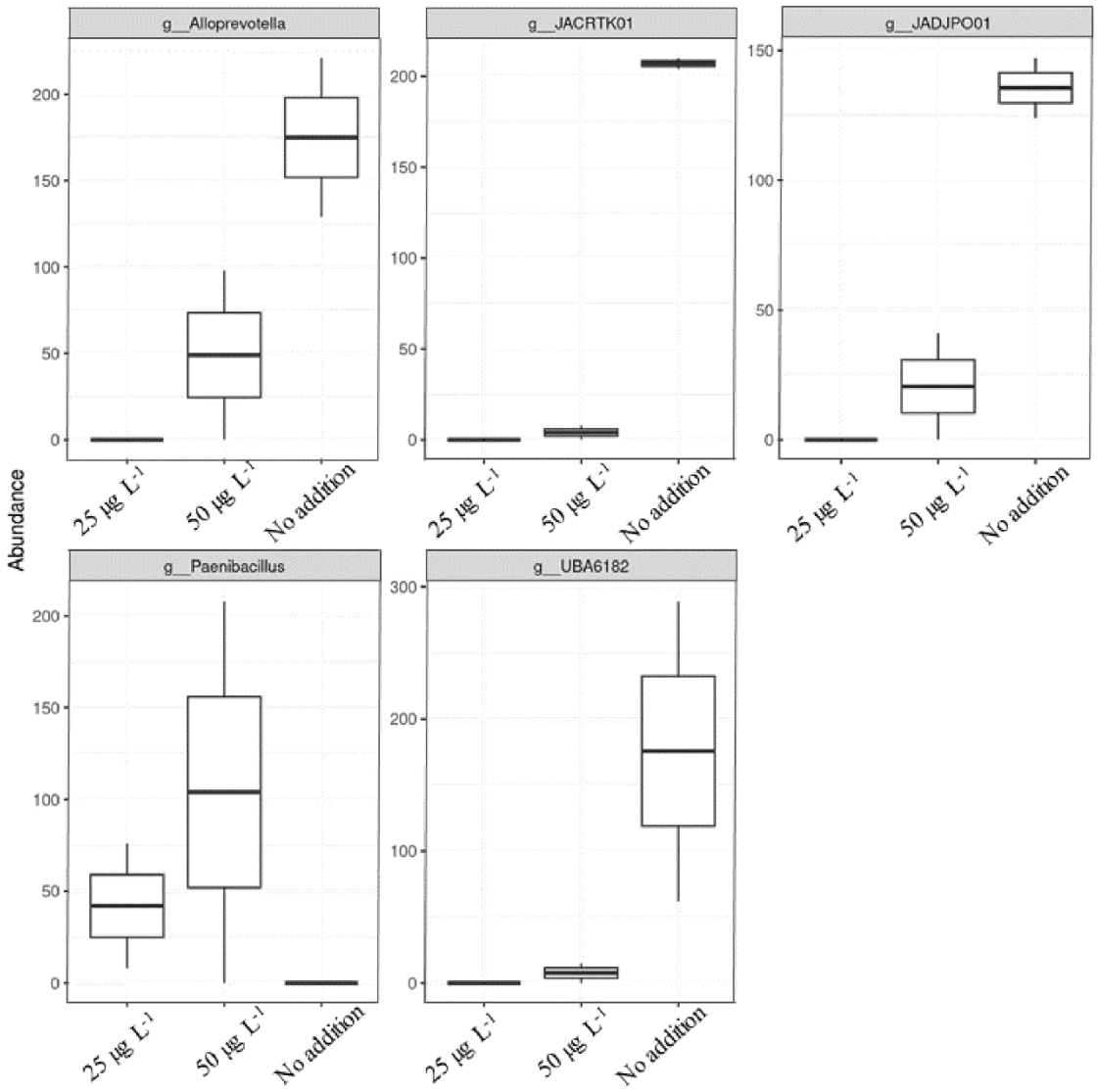
As previously mentioned, site BA2 is located downstream of a WWTP, a factor that can influence the environmental conditions at the sampling location. Effluents discharged from WWTPs often contain a complex mixture of nutrients (such as nitrogen and phosphorus), organic matter, and micropollutants that are not entirely removed during treatment. These inputs can alter the physical-chemical characteristics of the receiving water body and lead to shifts in biofilm structure and function by favoring specific microbial taxa capable of tolerating or metabolizing these compounds (Carles *et al*., 2021; Desiante *et al*., 2021). This, in turn, may influence community diversity, resilience, and ecological interactions within the biofilm.

Thus, as a complex and dynamic system, biofilms can undergo structural and compositional changes upon contaminant exposure (Fernandes *et al*., 2020). In this study, these changes may have led to either an increase or a decrease in microbial richness and diversity, depending on the sampling point (BA1 or BA2).

The analysis of bacterial genera with statistically significant differences in abundance among treatments identified five taxa (Figure 6.4): Alloprevotella (Bacteroidota), JACRTK01 (Firmicutes), JADJPO01 (Actinobacteriota), Paenibacillus (Firmicutes), and UBA6182 (Firmicutes). Steroid hormone exposure may induce selective pressure on microbial communities, leading to the decline of sensitive taxa and the enrichment of more resistant or metabolically versatile groups.

We observed that Alloprevotella, JACRTK01, JADJPO01, and UBA6182 were abundant in the no-addition group but showed a marked decrease after exposure to 25 and 50 $\mu g\ L^{-1}$ of steroid hormones. Among these genera, however, Alloprevotella and JADJPO01 exhibited a slight increase in abundance at the 50 $\mu g\ L^{-1}$ concentration, suggesting a possible bacterial regrowth after adaptation to the contaminants. In contrast, JACRTK01 and UBA6182 appeared sensitive to hormone exposure, showing a consistent decline in abundance. On the other hand, the genus Paenibacillus showed increased abundance after exposure to E1, E2, EE2, and PRO, indicating a potential stimulation of growth in the presence of these compounds.

FIGURE 6.4 – DIFFERENTIAL ABUNDANCE PLOTS OF BACTERIAL GENERA THAT SHOWED STATISTICALLY SIGNIFICANT DIFFERENCES AMONG THE TREATMENTS 25 µg L^{-1}, 50 µg L^{-1}, AND THE CONTROL (NO ADDITION).



SOURCE: The author (2025).

These results demonstrate that exposure to steroid hormones is associated with the replacement of susceptible taxa by hormone-tolerant groups, which may possess metabolic pathways for hormone transformation or resistance mechanisms (Zhang *et al.*, 2021). This shift suggests that microbial adaptation occurs primarily through community restructuring rather than through the short-term evolution of novel degradative pathways.

6.3.5 Environmental relevance

This was the first study investigating the removal potential of river biofilms involving simultaneously E1, E2, EE2, and PRO at environmentally relevant concentrations and the effects caused on the biofilm microbiome. We demonstrate that natural river biofilms are effective in removing steroid hormones, achieving over 95% removal of E1, E2, and EE2 within 15 days. In addition, biofilms partially removed PRO at a lower efficiency (48.7%). This indicates the multi-compound removal capability of biofilms, which is critical given the complex mixtures of emerging contaminants commonly found in real aquatic environments. These findings highlight the role of river biofilms as natural attenuation barriers, contributing to river self-purification and the maintenance of ecosystem health.

The low bioaccumulation rates observed and high removal efficiencies suggest that microbial biodegradation is the primary mechanism involved. Biodegradation is environmentally preferable to bioaccumulation, as it reduces the potential for biomagnification across trophic levels and often results in the formation of less toxic or more biodegradable transformation products.

The results demonstrate that biofilms are not passive structures but ecologically active components that influence biogeochemical cycles and directly affect water quality. This positions natural biofilms as valuable forms of ecological infrastructure that could be integrated into environmental management strategies, such as nature-based solutions for water quality improvement.

The differential response between biofilms from BA1 and BA2 also suggests that communities previously exposed to wastewater effluents may develop greater tolerance or adaptive capacity to micropollutants. This knowledge is essential for understanding natural attenuation processes in impacted environments. Furthermore, the study identified microbial genera with potential bioremediation capabilities, including *Comamonas, Pseudomonas, Streptomyces,* and *Aeromonas*, which showed increased abundance following hormone exposure. These taxa may be utilized in biofilm-driven methods to remediate steroid hormone-polluted waters

Finally, the observed shifts in bacterial diversity and composition suggest biofilms may serve as sensitive bioindicators of environmental contamination. Their use in ecological monitoring could enhance detecting and evaluating micropollutant impacts in freshwater ecosystems.

6.4 CONCLUSIONS

In this study, we collected biofilms from two sites along a river and employed a combination of exposure experiments, chemical analyses, and biological assessments to evaluate the removal potential of E1, E2, EE2, and PRO by natural river biofilms. The biofilms demonstrated high removal efficiencies for the E1, E2, and EE2 steroid hormones. Additionally, distinct microbial community responses to hormone exposure were observed. Biofilms from sites near a wastewater treatment plant exhibited increased diversity and evenness, suggesting possible adaptation to micropollutants. Certain taxa, such as *Paenibacillus*, increased in abundance following exposure, indicating potential for targeted biotechnological applications. Overall, our findings support the role of river biofilms as effective bioremediators of micropollutants, reinforcing their ecological significance in freshwater systems and their relevance to monitoring and treatment strategies addressing emerging contaminants.

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Chapter 7

"A primeira coisa sobre o empoderamento é entender que você tem o direito de estar envolvida. A segunda é que você tem contribuições importantes a fazer, e a terceira é que você tem de se arriscar para fazer essas contribuições."
Mae Jemison

"The first thing about empowerment is understanding that you have the right to be involved. The second is that you have something important to contribute, and the third is that you have to take the risks to contribute it."
Mae Jemison

7 INTEGRATION OF FINDINGS

This chapter presents the main results of the thesis, the contributions of the results, and suggestions for future research. During the development of this thesis work, three scientific articles were produced, which in a complementary manner demonstrate the potential of river biofilms in the bioremediation of steroid hormones.

The first article consisted of a literature review that highlighted the multifunctional role of river biofilms in self-purification and bioremediation processes in aquatic environments. The review revealed that natural biofilms have been reported to remove several pollutants, including copper, pesticides, pharmaceuticals, microplastics, dyes, and nutrients. The main removal mechanisms reported were biosorption, bioaccumulation, biodegradation, and biotransformation.

The second article evaluated the simultaneous removal of the steroid hormones E1, E2, and EE2 by biofilms cultivated and collected at two points in the Alb River, Germany. The results demonstrated that the hormones were completely removed in nine days, with rapid initial removal. Microbiological analyses showed the presence of resilient bacterial genera, such as *Exiguobacterium*, *Acinetobacter*, *Massilia*, *Deinococcus*, *Tabrizicola*, and *Hymenobacter*, which presented adaptations to the presence of these compounds. In addition, it was found that bioaccumulation was not a relevant factor in the removal of the hormones, indicating that biodegradation and/or biotransformation was the main mechanism involved.

The third article also evaluated the simultaneous removal of the estrogenic hormones E1, E2, and EE2, with the inclusion of PRO, which belongs to the progestogen class, all of

which are classified as steroid hormones. In this study, biofilms were cultivated and collected from the Barigui River, Curitiba, Brazil. The biofilms showed high efficiency in the removal of E1, E2, and EE2, with rates ranging from 95% to 100%. For the hormone PRO, the maximum removal efficiency was 48%. Among the microbial genera associated with these results were *Comamonas*, *Aeromonas*, *Pseudomonas*, *Streptomyces*, *Arenimonas*, and *Aquicella*. Statistical analyses showed that exposure to steroid hormones results in a shift from sensitive to tolerant taxa, probably due to their ability to degrade these compounds or resist their effects.

Based on the results obtained in all tests, it was possible to affirmatively answer the research question: "Can microorganisms present in natural river biofilms be used for the bioremediation of emerging contaminants in these ecosystems?" Likewise, the initially proposed hypotheses were validated: (i) natural river biofilms can remove the emerging contaminants E1, E2, EE2, and PRO from aquatic environments, and (ii) the main mechanism involved in the removal of these compounds is biodegradation and/or biotransformation.

From the extraction and quantification of the hormones adsorbed on the biofilm biomass, it was observed that sorption was minimal or nonexistent for some compounds. Control tests were conducted to exclude alternative removal mechanisms, such as photodegradation, oxidation, evaporation, or other abiotic processes. The control tests demonstrated removal, but notably lower rate than the removal containing the biofilms. Therefore, from this set of results, it is concluded that biofilm-mediated biodegradation was the predominant mechanism in the removal of steroid hormones.

Using microbiome analyses, we found that biofilms from Brazil and Germany had similar bacterial phyla but distinct genera. This indicates that although aquatic environments in different regions support communities with similar overall taxonomic structure at higher levels (such as phylum), there are more specific functional and adaptive differences at lower taxonomic levels (such as genus).

These differences observed at the genus level reflect responses that may be due to local environmental conditions, influenced by factors such as effluents and microorganisms carried by WWTPs discharges (Carles *et al.*, 2021, 2022; Carles; Artigas, 2020), global warming stressors (Yang *et al.*, 2025), land use (Trindade; Dunck, 2025), micropollutants (Tlili *et al.*, 2017), among others. These results suggest that the composition of the genera present in biofilms is shaped by local environmental selection, that is, by the selective pressure of the environment in which they developed. Furthermore, although these biofilms present distinct bacterial genera, they may perform similar ecological functions, such as hormone biodegradation (E1, E2, EE2, and PRO). Both the biofilms collected from the Alb River, known

for its clearer and better-preserved water, as well as those from the Barigui River, which is more affected by human activities and pollution, were effective in removing steroid hormones.

7.1 PERSPECTIVES FOR APPLICATIONS AND FUTURE RESEARCH

Aiming at the application of river biofilms on a larger scale, bioreactors can be developed. Among the different types of bioreactors, spiral tubular bioreactors have been described as efficient for wastewater treatment, as they provide a larger contact surface between the biofilms and the effluent. In addition, the Hydraulic Retention Time (HRT) can be easily adjusted by modifying the flow rate or the length of the tube (Ma *et al.*, 2018; Shangguan *et al.*, 2015).

Liu *et al.* (2018) developed a bioreactor called Fiber Periphyton Bioreactor (FPBR) and evaluated the feasibility of using fiber supports to immobilize periphytic biofilms in large-scale applications. The authors achieved immobilization through electrochemical interactions between photoautotrophic microorganisms (which carry a negative charge) and positively charged fiber supports, demonstrating the advantages and use of this type of bioreactor.

Cai *et al.* (2021), using a tubular bioreactor, demonstrated that the inclusion of an aeration system can generate water flow in the tubular structure, enhancing the bioremediation capacity of biofilms in urban rivers. The mixing promoted by aeration increases the probability of pollutants coming into contact with biofilms and creates a dissolved oxygen gradient, which favors coupled nitrification-denitrification reactions. These structural characteristics influence the abundance and diversity of planktonic microbial communities and improve the nutrient self-purification capacity of degraded urban rivers.

Therefore, as application suggestions, these biofilms can be used both in controlled systems and in natural environments. In WWTPs, biofilms can compose a tertiary or quaternary treatment stage, through the use of biofilters or bioreactors in which solid supports are colonized by biofilms collected from natural environments or enriched in the laboratory.

In impacted natural environments, *in situ* bioremediation using natural biofilms represents a viable option. Strategies such as the installation of artificial surfaces (e.g., glass plates, porous materials, or biodegradable polymers) submerged in strategic locations in the river can serve as a surface for the formation or inoculation of adapted natural biofilms, thus promoting the removal of hormones through biological processes. Another *in situ* alternative is the construction of solar-powered floating biofilm reactors, where solar energy is used to

operate a submerged aerator beneath the floating platform, improving oxygenation and microbial activity in the system (Zhang *et al.*, 2021).

Furthermore, bioaugmentation practices involving the introduction of selected microbial consortia from biofilms with proven hormone degradation capacity can be implemented in polluted river reaches. Biostimulation is another option and can be achieved by modulating environmental parameters, such as nutrient input or controlling pH and dissolved oxygen levels, to promote the activity of existing biofilm communities.

Another approach to river biofilms is to use them as biological indicators for steroid hormones and emerging contaminants. By monitoring the responses of microbial communities, we can assess sanitation measures, identify sources of pollution, and monitor environmental recovery.

The results presented in this thesis act as drivers for the implementation of Nature-Based Solutions (NbS). According to the International Union for Conservation of Nature (IUCN), NbS are defined as "actions to protect, conserve, restore, sustainably use and manage natural or modified ecosystems to effectively address societal challenges, while enhancing human well-being, ecosystem services, resilience and biodiversity". NbS, and therefore the results of this thesis, also support the Sustainable Development Goals (SDGs) by providing integrated strategies to respond to societal challenges such as climate change mitigation, natural disaster management, and sustainable development (Debele *et al.*, 2023).

As a suggestion for future studies, new research can be carried out on the stability and resilience of the biofilm community under long-term variable environmental and seasonal conditions. Microorganisms, such as bacteria that have shown resilience and activity in the biodegradation process, can be isolated and studied through genetic engineering approaches. This can support the development of microbial consortia with high specificity for the degradation of steroid hormones. Investigations into the metabolic pathways involved in the biodegradation of these compounds, as well as the application of omics tools (such as metagenomics, metatranscriptomics, and metabolomics), can explain these biochemical mechanisms. Future studies could also explore the hormone that did not exhibit high removal rates in this study, providing a broader understanding of the system's limitations and potential improvements. Finally, studies can explore the integration of biofilms in hybrid technologies that combine biological and physicochemical processes to evaluate their applicability in water and wastewater treatment for the removal of emerging contaminants.

7.2 GENERAL CONCLUSION

The results of this thesis demonstrate an innovative approach for the simultaneous bioremediation of a mixture of steroid hormones (E1, E2, EE2, and PRO) in aquatic environments. To date, the removal of various hormones by river biofilms has not been documented, highlighting the uniqueness of this research. The findings reveal that biodegradation is the primary mechanism for removal. Analysis of the microbial communities showed that samples from geographically distinct environments displayed similar phyla but different genera, with both exhibiting potential for degrading these compounds. These results underscore the role of biofilms as nature-based solutions (NbS), applicable in both wastewater treatment systems and *in situ* remediation strategies.

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APPENDIX I – CATALOG SHEET OF THE SCIENTIFIC ARTICLE INCLUDED IN CHAPTER 3 OF THE THESIS

Exploring periphytic biofilms as nature's cleanup crew for contaminated surface waters

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APPENDIX II – CATALOG SHEET OF THE SCIENTIFIC ARTICLE INCLUDED IN CHAPTER 5 OF THE THESIS

Natural River Biofilms for Estrogen Removal in Aquatic Environments

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APPENDIX III – CATALOG SHEET OF THE SCIENTIFIC ARTICLE INCLUDED IN CHAPTER 6 OF THE THESIS

Exploring the Effects of Estrogenic Steroid Hormones on Bacterial Communities in River Biofilms

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APPENDIX IV – GENETIC ANALYSIS OF MICROORGANISM IDENTIFICATION (CHAPTER 5)

FIGURE A1 – RELATIVE ABUNDANCE OF BACTERIAL COMMUNITIES DURING THE LATE SUMMER PERIOD AT THE PHYLUM LEVEL

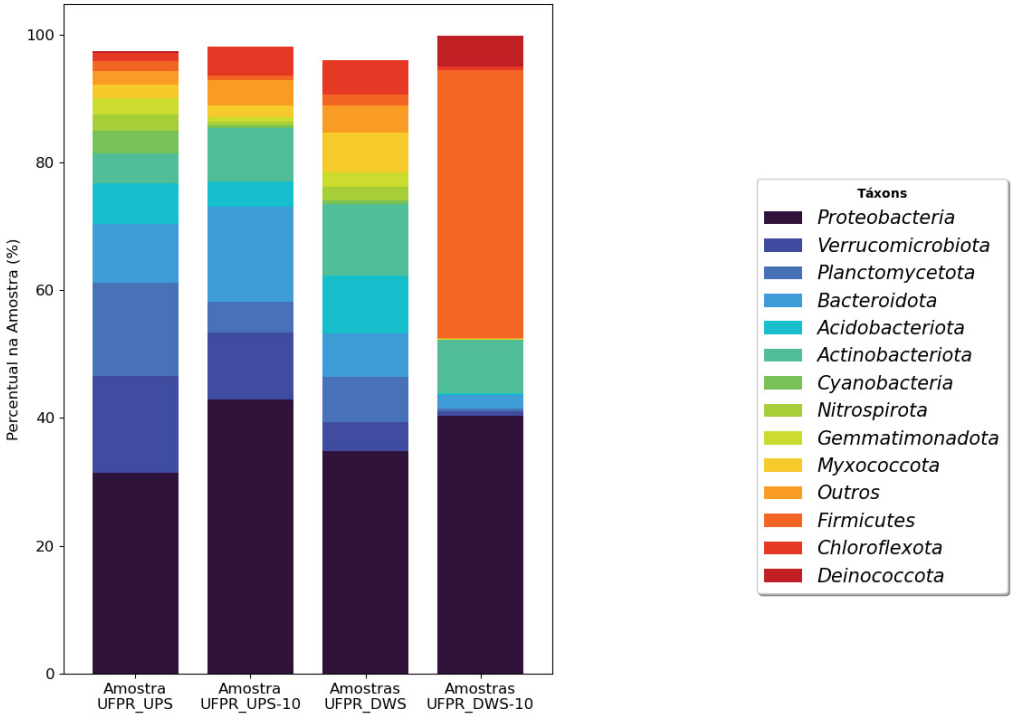


FIGURE A2 – RELATIVE ABUNDANCE OF BACTERIAL COMMUNITIES DURING THE LATE SUMMER PERIOD AT THE CLASS LEVEL

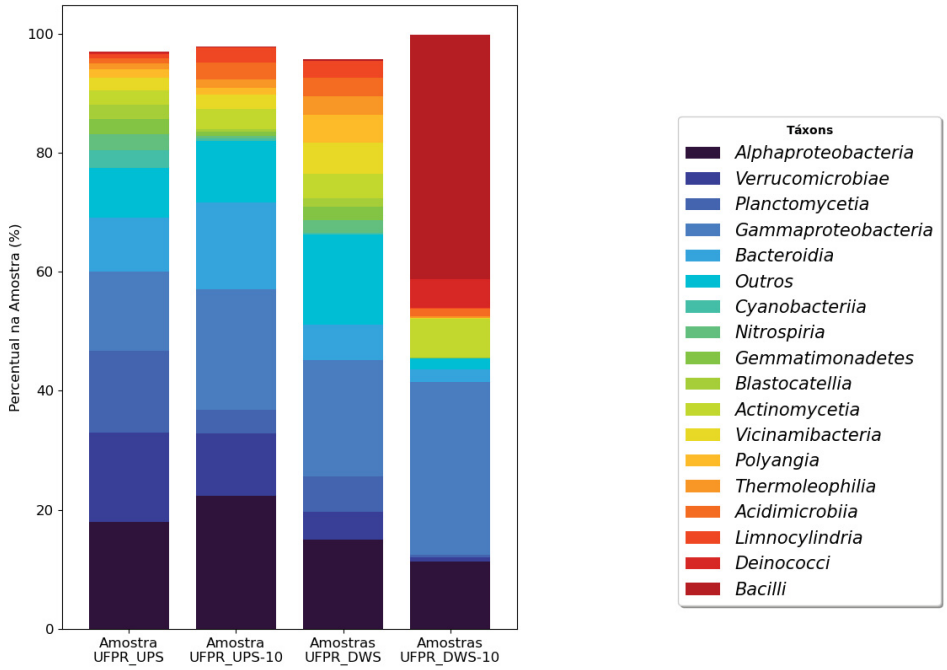


FIGURE A3 – RELATIVE ABUNDANCE OF BACTERIAL COMMUNITIES DURING THE LATE SUMMER PERIOD AT THE ORDER LEVEL

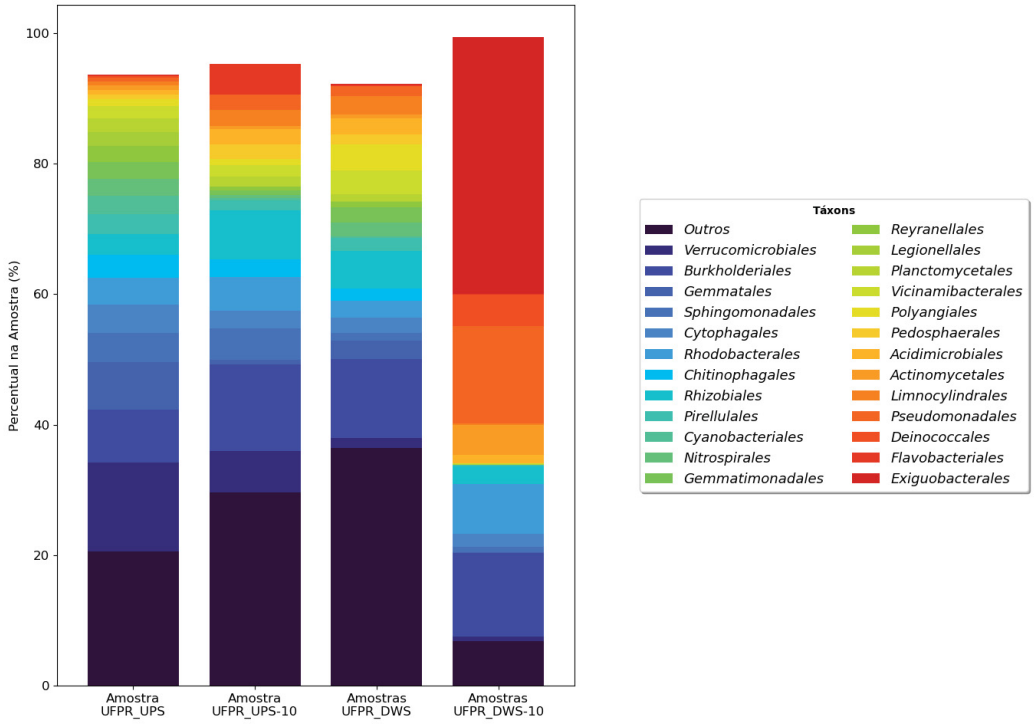


FIGURE A4 – RELATIVE ABUNDANCE OF BACTERIAL COMMUNITIES DURING THE LATE SUMMER PERIOD AT THE FAMILY LEVEL

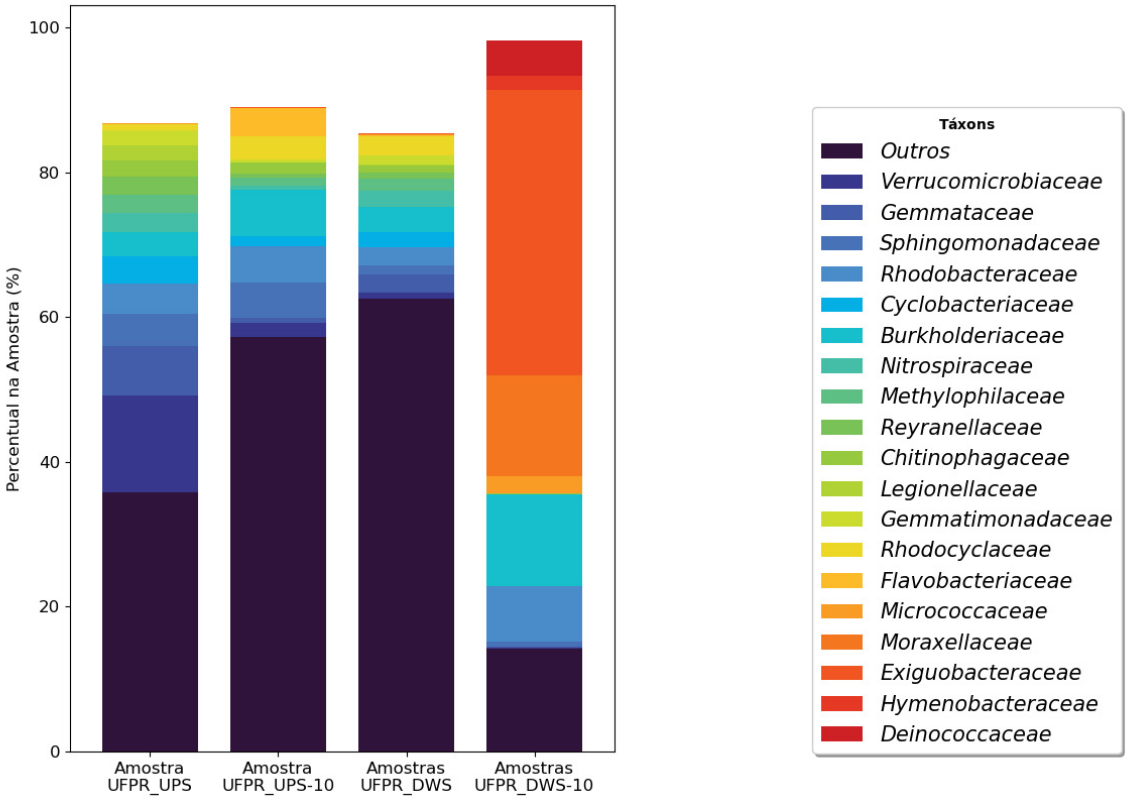


FIGURE A5 – RELATIVE ABUNDANCE OF BACTERIAL COMMUNITIES DURING THE LATE SUMMER PERIOD AT THE GENUS LEVEL

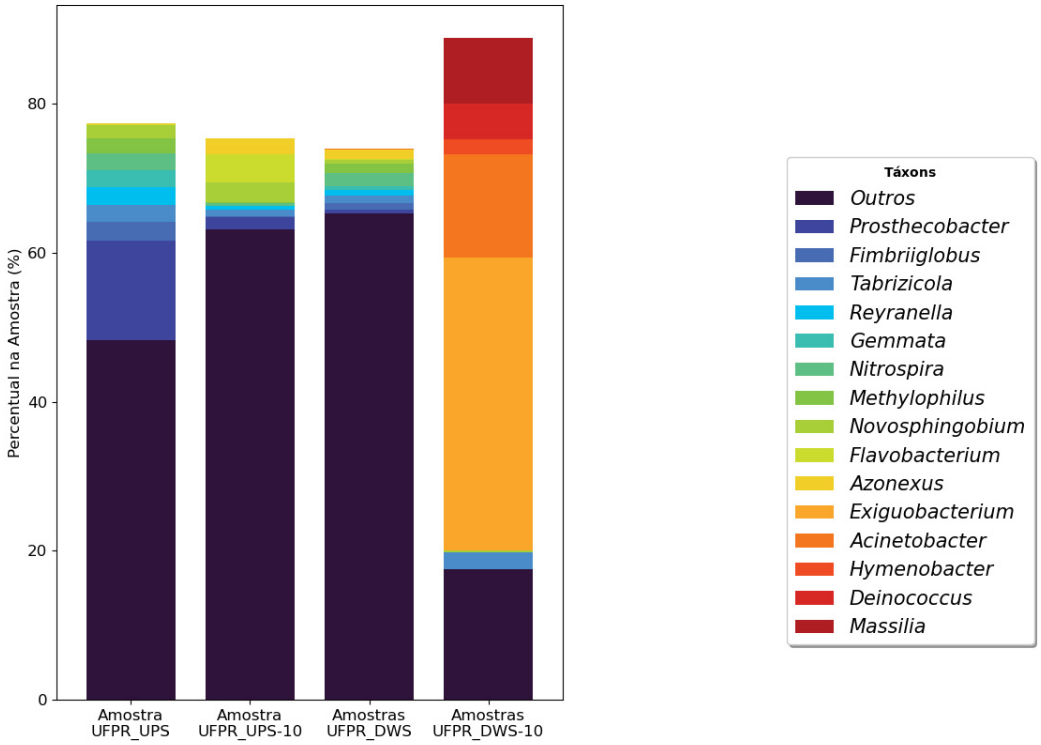
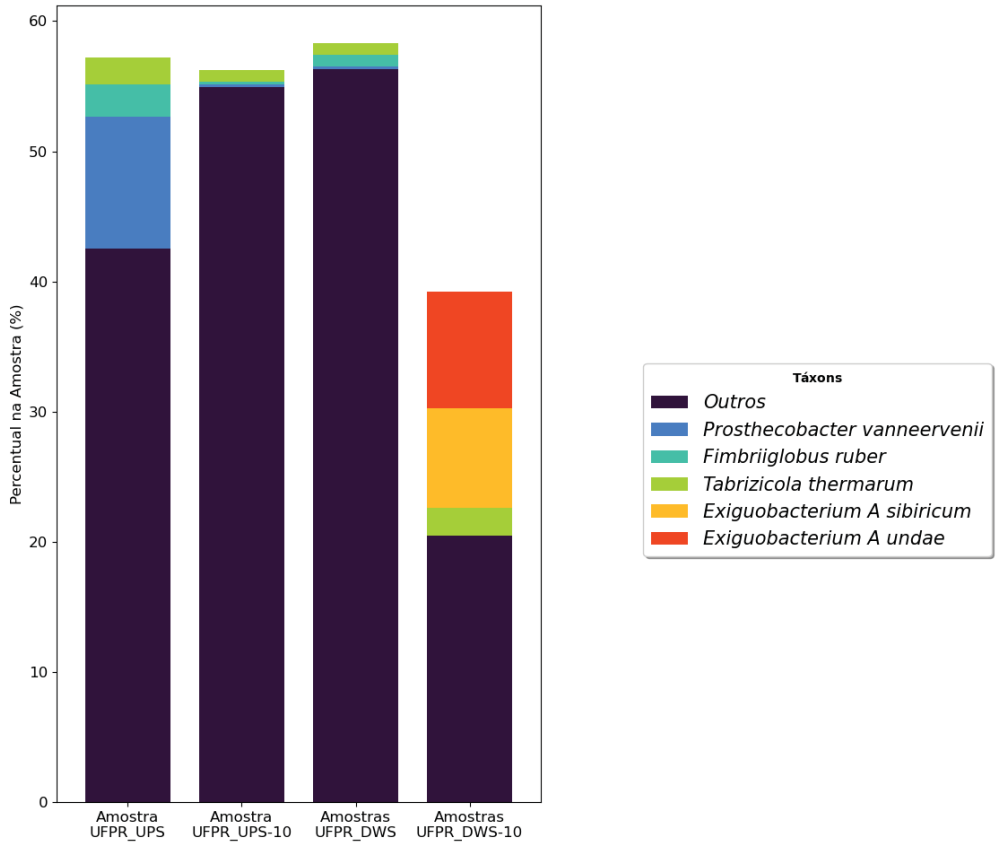


FIGURE A6 – RELATIVE ABUNDANCE OF BACTERIAL COMMUNITIES DURING THE LATE SUMMER PERIOD AT THE SPECIES LEVEL



APPENDIX II – GENETIC ANALYSIS OF MICROORGANISM IDENTIFICATION (CHAPTER 6)

FIGURE A7 – RELATIVE ABUNDANCE OF BACTERIAL READS IN BIOFILM SAMPLES BEFORE AND AFTER CONTAMINANT EXPOSURE AT THE PHYLUM LEVEL.

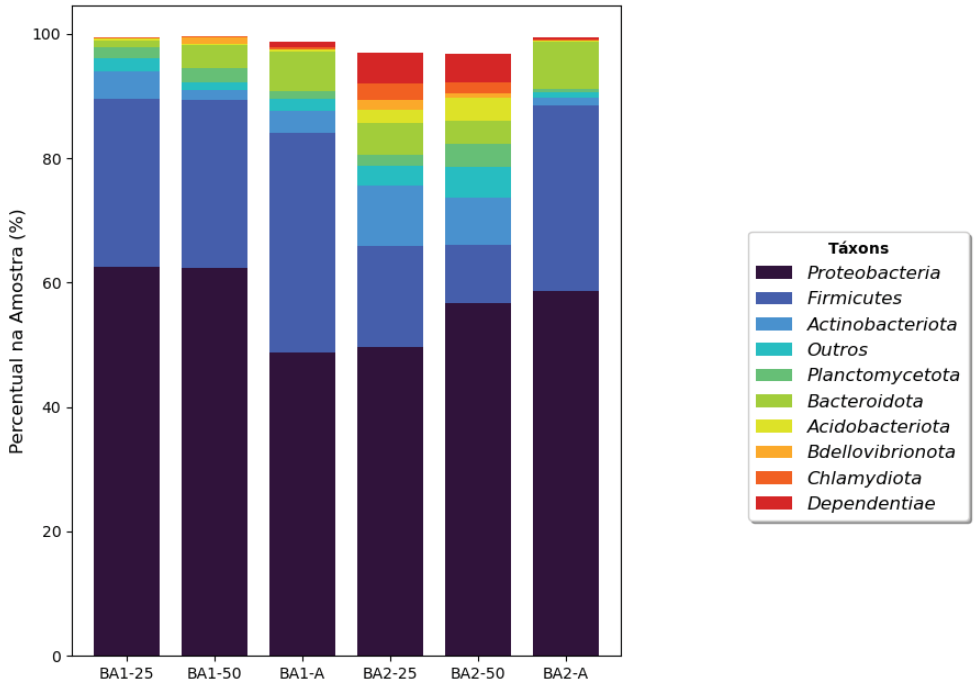


FIGURE A8 – RELATIVE ABUNDANCE OF BACTERIAL READS IN BIOFILM SAMPLES BEFORE AND AFTER CONTAMINANT EXPOSURE AT THE CLASS LEVEL.

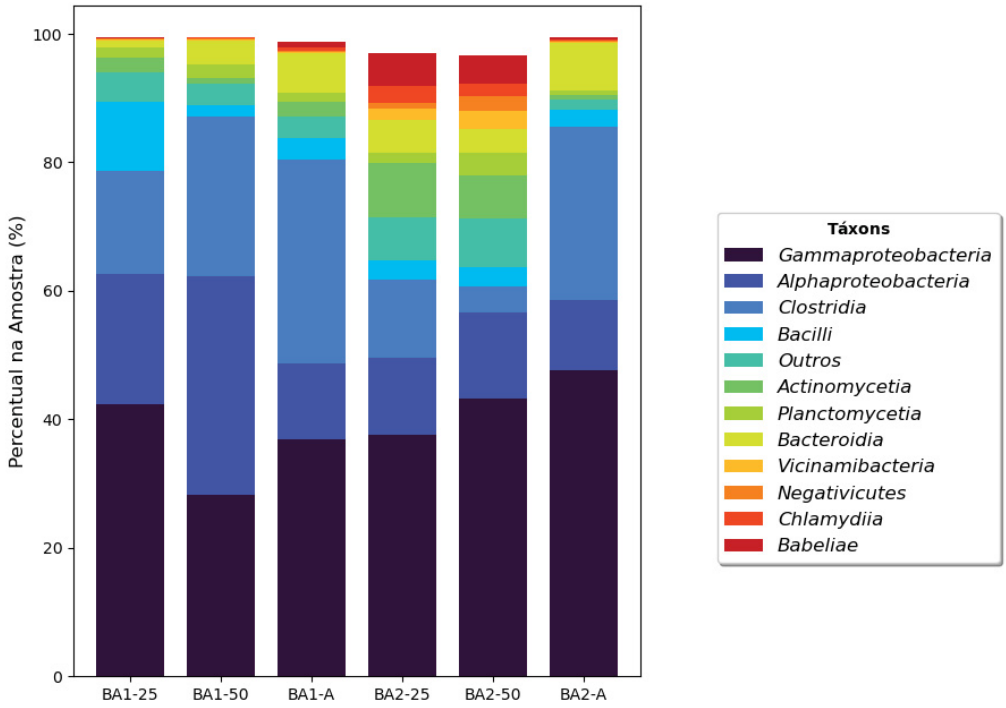


FIGURE A9 – RELATIVE ABUNDANCE OF BACTERIAL READS IN BIOFILM SAMPLES BEFORE AND AFTER CONTAMINANT EXPOSURE AT THE ORDER LEVEL.

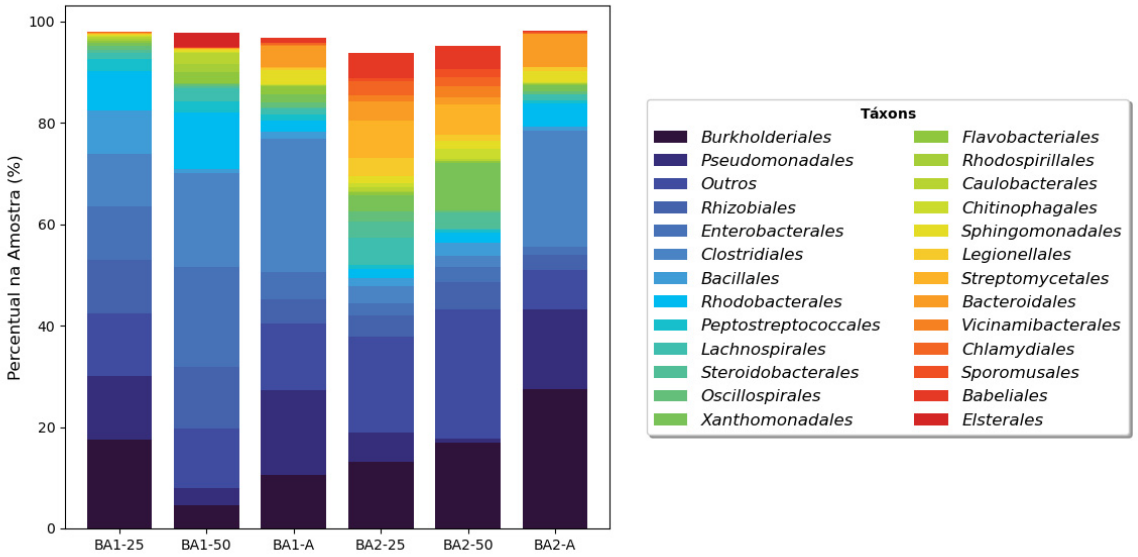


FIGURE A10 – RELATIVE ABUNDANCE OF BACTERIAL READS IN BIOFILM SAMPLES BEFORE AND AFTER CONTAMINANT EXPOSURE AT THE FAMILY LEVEL.

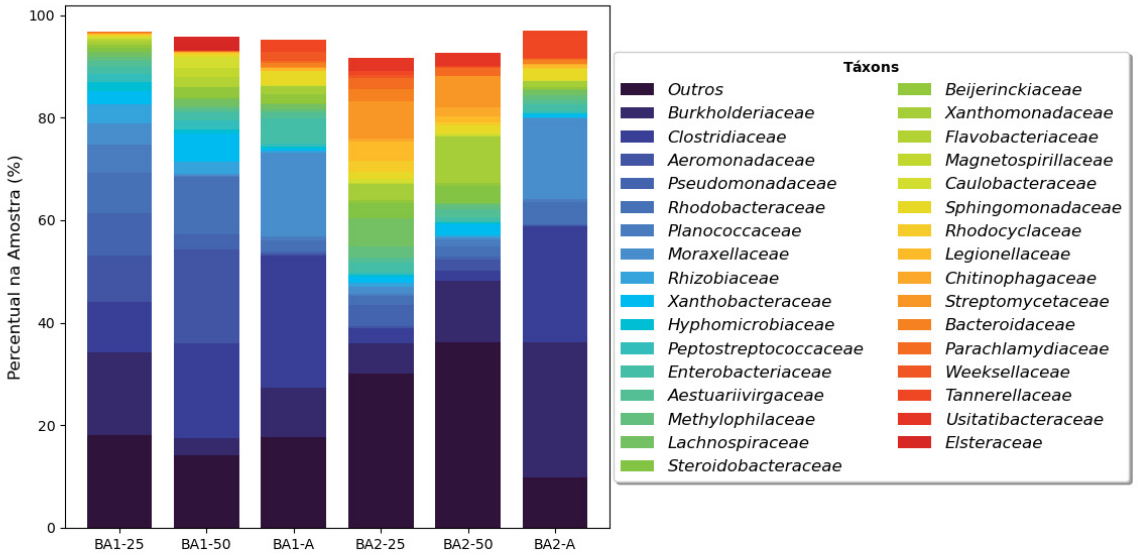


FIGURE A11 – RELATIVE ABUNDANCE OF BACTERIAL READS IN BIOFILM SAMPLES BEFORE AND AFTER CONTAMINANT EXPOSURE AT THE GENUS LEVEL.

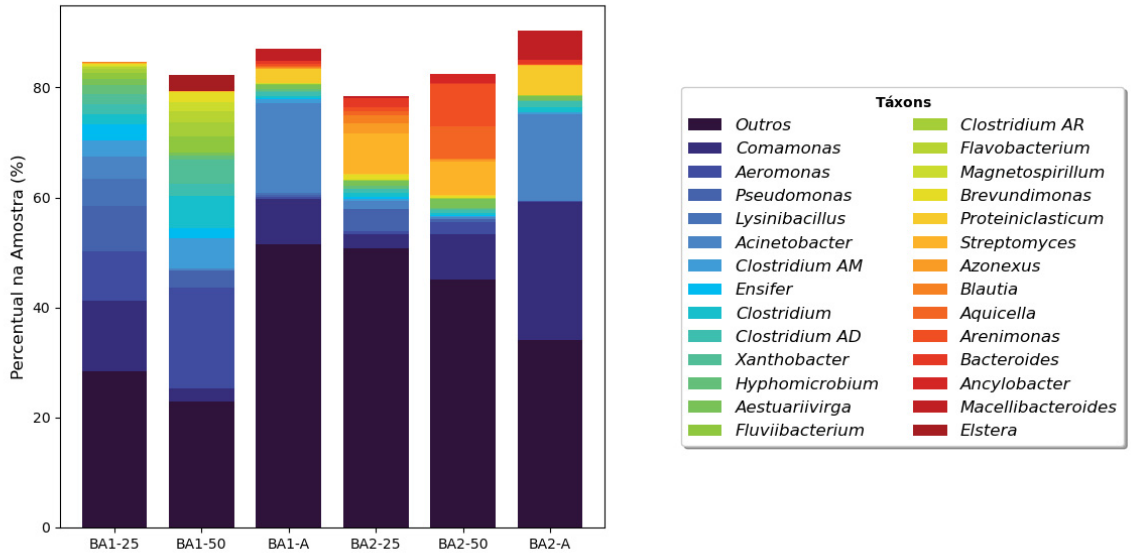


FIGURE A12 – RELATIVE ABUNDANCE OF BACTERIAL READS IN BIOFILM SAMPLES BEFORE AND AFTER CONTAMINANT EXPOSURE AT THE SPECIES LEVEL.