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Biodegradation of natural and synthetic steroid hormones by river biofilms: Impacts on bacterial community structure

Biodegradação de hormônios esteroides naturais e sintéticos por biofilmes de rios: Impactos na estrutura das comunidades bacterianas

Tatiani Andressa Modkovski[1], *Luis Otávio Miranda Peixoto*[1], *Juliane Ribeiro das Chaves*[1], *Gabriel Helmer Baer*[2], *Rafaela Imoski*[2], *Ludmila da Cruz Souza*[2], *Samanta Teixeira Medeiros*[2], *Barbara Alves de Lima Nawate*[2], *Charles Windson Isidoro Haminiuk*[2], *Júlio César Rodrigues de Azevedo*[2]

ABSTRACT

Steroid hormones released into the environment by human activities are increasingly found in aquatic environments. Even at low concentrations, they can disrupt natural systems. One of the most affected components is river biofilms, thin layers of microorganisms that grow on submerged surfaces. They play an important role in nutrient cycling, pollutant degradation, and act as indicators of water quality. This study evaluated the ability of natural biofilms from two sites to remove four steroid hormones: natural estrogenic compounds estrone (E1) and 17β-estradiol (E2), synthetic estrogenic compound 17α-ethinylestradiol (EE2), and progesterone (PRO), a natural progestogen. Microcosm assays demonstrated the efficient removal of E1, E2, and EE2, with maximum removal rates of 97.4 (4 days), 93.8 (3 days), and 88.2% (6 days), respectively. PRO was also removed, but with lower efficiency (48.7% in 3 days). The predominant mechanism used by biofilms for hormone removal was biodegradation. Hormone exposure affected biofilms differently depending on their origin. Biofilms collected upstream showed reduced diversity and richness, indicating sensitivity. In contrast, those collected near a wastewater treatment plant had higher diversity and evenness, suggesting resilience and possible adaptation to micropollutants. The predominant phyla were *Proteobacteria* and *Firmicutes*, with a reduction in *Alloprevotella* and an increase in *Paenibacillus* following exposure. These results highlight the dual role of river biofilms as natural barriers and

RESUMO

Hormônios esteroides liberados no ambiente por atividades humanas são cada vez mais encontrados em ambientes aquáticos. Mesmo em baixas concentrações eles podem desestabilizar sistemas naturais. Um dos componentes mais afetados são os biofilmes fluviais, finas camadas de microrganismos que crescem em superfícies submersas. Eles desempenham papel importante na ciclagem de nutrientes, decomposição de poluentes e atuam como indicadores da qualidade da água. Este estudo avaliou a capacidade de biofilmes naturais de dois locais na remoção de quatro hormônios esteroides: estrogênicos naturais estrona (E1) e 17β-estradiol (E2), estrogênico sintético 17α-etinilestradiol (EE2) e progesterona (PRO), um progestágeno natural. Ensaios em microcosmos mostraram remoção eficiente de E1, E2 e EE2, com taxas máximas de 97,4 (quatro dias), 93,8 (três dias) e 88,2% (seis dias). A PRO também foi removida, mas com menor eficiência (48,7% em três dias). O mecanismo predominante utilizado pelos biofilmes para a remoção dos hormônios foi a biodegradação. A exposição aos hormônios afetou os biofilmes de maneira diferente, dependendo de sua origem. Os biofilmes coletados a montante apresentaram redução na diversidade e riqueza, indicando sensibilidade. Em contraste, aqueles coletados próximos a uma estação de tratamento de efluentes apresentaram maior diversidade e uniformidade, sugerindo resiliência e possível adaptação a micropoluentes. Os filos predominantes foram *Proteobacteria* e *Firmicutes*, com redução de *Alloprevotella* e aumento de *Paenibacillus* após a exposição. Esses resultados destacam o duplo papel dos biofilmes

[1]Universidade Federal do Paraná – Curitiba (PR), Brazil.

[2]Universidade Tecnológica Federal do Paraná – Curitiba (PR), Brazil.

Corresponding author: Tatiani Andressa Modkovski – Avenida Coronel Francisco Heráclito dos Santos, 100 – Jardim das Américas – CEP: 81531-980 – Curitiba (PR), Brazil. E-mail: tatianiandressa@gmail.com

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biological indicators of contamination by steroid hormones, as well as reinforce their relevance in natural attenuation and the potential for bioremediation in urban rivers.

Keywords: bacterial resistance; bioremediation; emerging contaminants; endocrine-disrupting; sampler.

de rios como barreiras naturais e indicadores biológicos de contaminação por hormônios esteroides, além de reforçarem sua relevância na atenuação natural e o potencial para biorremediação em rios urbanos.

Palavras-chave: resistência bacteriana; biorremediação; contaminantes emergentes; desreguladores endócrinos; amostrador.

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 these pollutants, particular attention has been given to steroid hormones such as estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2), and progesterone (PRO), which have been detected in aquatic environments (Machado et al., 2014; Torres et al., 2015; Ide et al., 2017; Barcellos et al., 2019; Goeury et al., 2022). Their presence is alarming due to their potential to cause adverse effects on aquatic organisms and human health (Zhang et al., 2016; Rodrigues et al., 2025). These substances exhibit persistent physicochemical properties, such as lipophilicity, bioaccumulation, and low molecular weight, which favor their environmental dispersion and persistence (Ilyas and 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).

In fact, 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 (Vilela et al., 2018; Ojoghoro et al., 2021).

However, 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 (Du et al., 2020; Shabbir et al., 2022; Bayode et al., 2024). 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 (Du et al., 2020; Bilal et al., 2021).

Given these limitations, it becomes essential to explore alternative matrices for monitoring and mitigation. 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, and nutrient cycling; acting as sensitive bioindicators of environmental pollution; and by degrading contaminants present in the water (Mishra et al., 2022; Li et al., 2023; Makk et al., 2024; Marques et al., 2024).

Moreover, 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) emphasized 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.

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, (2) to evaluate the biodegradation and bioaccumulation of steroid hormones within biofilm communities, (3) to characterize changes in the bacterial community structure, including shifts in dominant taxa and overall microbial diversity, and (4) 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 E1, E2, EE2, and PRO.

Materials and methods

Cultivation and collection of biofilms

Before sampling, 44 glass slides measuring 26 × 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 (Reichert et al., 2021; Marques et al., 2024; Lima et al., 2025). At each site, two compact samplers containing 22 slides each were installed, for a total of 44 slides per site. This setup provided sufficient surface area to allow significant biomass growth.

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 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.

Exposure experiments

Two microcosms consisting of 15 L glass aquariums (rectangular parallelepipeds) 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 the microcosms, 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. A control microcosm, containing distilled water and the hormones but no biofilm, was also prepared.

The hormone concentrations used in this study were selected to be above the average concentrations typically reported in aquatic environments. In the specific study region, concentrations in surface waters reported by other authors ranged from 0.92 to 2.40 µg L^{-1} for E1, 1.42–210 µg L^{-1} for E2, 1.48–5.80 µg L^{-1} for EE2, and around 0.45 µg L^{-1} for PRO (Machado et al., 2014; Ide et al., 2017; Barcellos et al., 2019). Therefore, our choice aimed to represent a worst-case scenario to better assess the potential ecological risks.

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 days were collected after 1 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 × 0.45 µm PTFE membranes and 25 mm × 0.22 µm. At the end of both exposure experiments (25 and 50 µg L^{-1}), the total biofilm was scraped from the glass slides and stored at −20°C until further analysis.

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.

For the chromatographic analyses, the Cromatógrafo líquido com detecção por espectrometria de massas (LCMS-8045) was equipped with Biphenyl 2.1 × 100 mm, 3µm column.

The mobile phases were water with 1 mM NH_4Ac (A) and MeOH (B) at a constant flow ratio of 0.3 mL min^{-1}. The parameters of the method were as follows: gradient mode (0 min: 85%B; 5 min: 90%B; 5.01 min: 90%B; 8 min: 85%B; 12 min: 85%B), injection volume of 5 µL, and temperature of 40°C. Calibration curves for quantification were prepared in water over a concentration range of 10–70 µg L^{-1}. Method performance was evaluated using quality control parameters, resulting in limits of detection (LOD) and quantification (LOQ) as follows: E1, 2.33 and 7.05 µg L^{-1}; E2, 2.91 and 8.82 µg L^{-1}; EE2, 1.95 and 5.91 µg L^{-1}; and PRO, 2.41 and 7.29 µg L^{-1}.

Bacterial communityaanalyses

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. Additionally, one biofilm sample from each site not exposed to hormones (BA1-A and BA2-A) was included as a control for comparative purposes. The analyses were carried out by GoGenetic© Laboratory (Curitiba, Brazil).

Alpha diversity was assessed using operational taxonomic units (OTUs), Chao1, Shannon, Simpson, Fisher, and Pielou indices to evaluate species richness and evenness within samples. Differential abundance analysis between sample groups was performed using the standard error of the log2-fold change (lfcSE) to identify significantly enriched taxa.

Results and discussion

Water characterization

The physicochemical parameters measured on three different days (Table 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). 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).

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 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).

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 2 illustrates this microbial growth, both suspended in the water and adhering 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.

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 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 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.

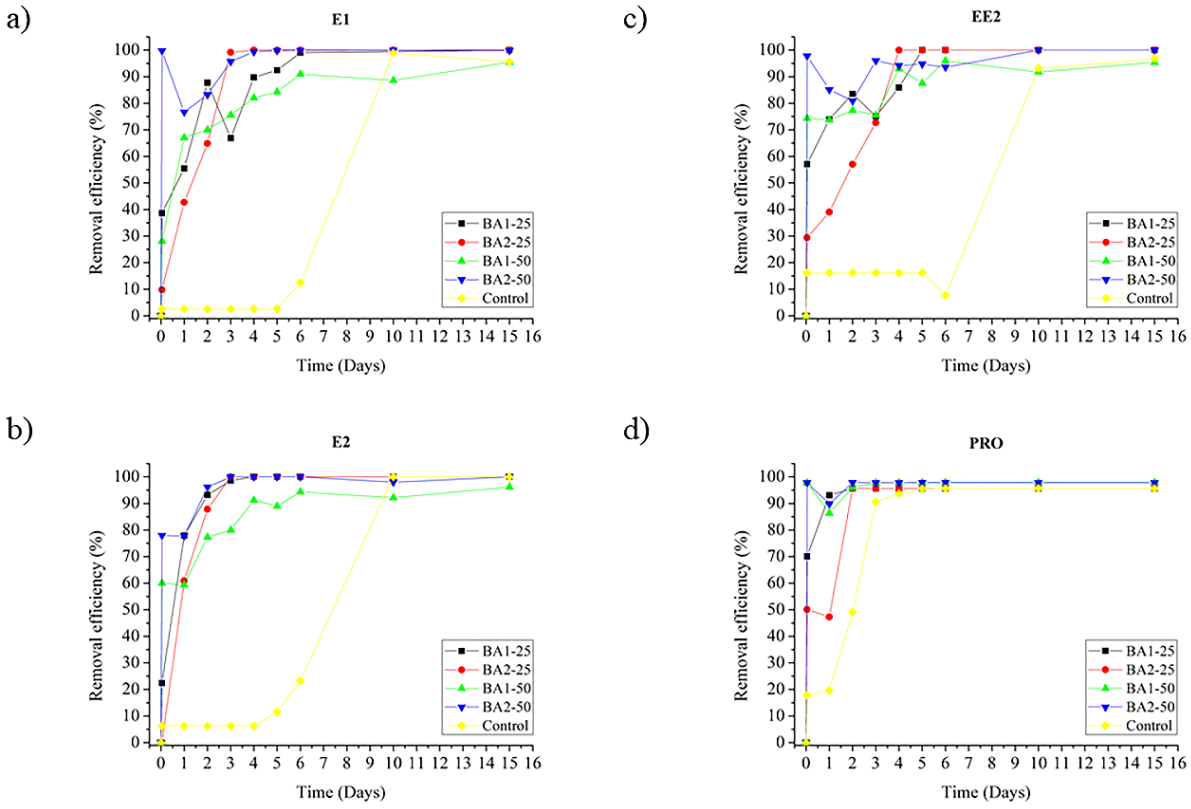

Figure 1 – Removal efficiency of hormones E1, E2, EE2, and PRO by natural river biofilms.
E1: estrone; E2: 17β-estradiol; EE2: 17α-ethinylestradiol; PRO: progesterone; BA1-25: experiment with water from river site BA1 and 25 μg L^{-1} of hormones; BA2-25: experiment with water from river site BA2 and 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.

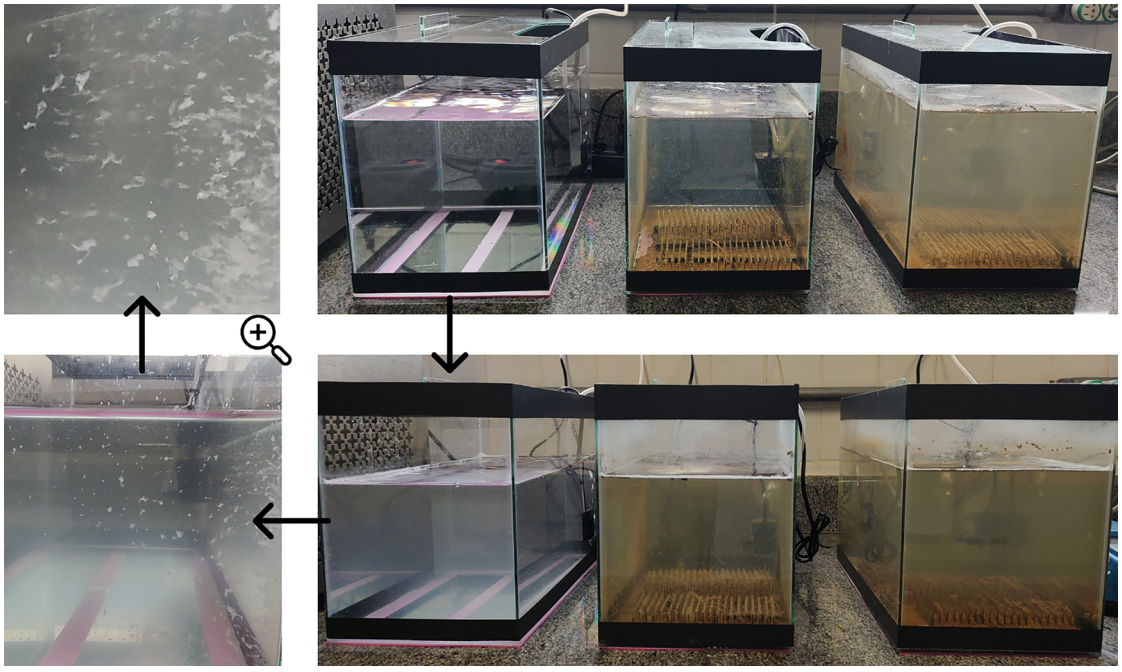

Figure 2 – Microcosms from the control, BA1, and BA2 conditions, highlighting the difference in the control microcosm from the beginning to the end of the experiment. The increased turbidity and visible surface growth indicate the proliferation of suspended bacteria in the control over time.

Table 2 – Maximum steroid hormone 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

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 (Yan et al., 2023; Liang et al., 2024).

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 biofilm samples possess a high capacity to remove these contaminants, regardless of the differing environmental conditions at the two locations.

Although the transformation products of steroid hormones were not analyzed in this study, our findings on biofilm-mediated removal remain relevant, as they demonstrate the capacity of microbial communities to respond to these compounds. It is known that biodegradation may lead to complete mineralization or partial transformation, generating metabolites that can persist in the environment or bind to matrix components such as humic acids (Kiel and Engesser, 2015). While these pathways contribute to the reduction of the parent contaminant, further studies addressing the identity and potential toxicity of transformation products can be valuable to complement our results and provide a more comprehensive understanding of biofilm-mediated hormone degradation.

Finally, the findings of this study on steroid hormone removal efficiency confirm that natural river biofilms 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.

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, 1,665 species, 1,265 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 2,300 taxa in total.

According to the phyla (Figure 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. A study conducted by Viancelli et al. (2023) also reports that bacterial metabolic behavior in response to E2 is species-dependent, and that the growth of some species is further influenced by the presence of other taxa within the community.

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 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 3C). In the BA1 samples, after adding 25 µg L^{-1} and 50 µg L^{-1}, there was marked selectivity, with *C. 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. *C. terrigena*, *A. litoralis*, and *Aq. siphonis* belong to the *Proteobacteria* phylum, while *Strep. 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. Although this metabolic characteristic can increase pollutant removal when associated with beneficial microorganisms or microorganisms from wastewater treatment systems, it also presents ecological risks if pathogenic species are stimulated under these conditions (Viancelli et al., 2023).

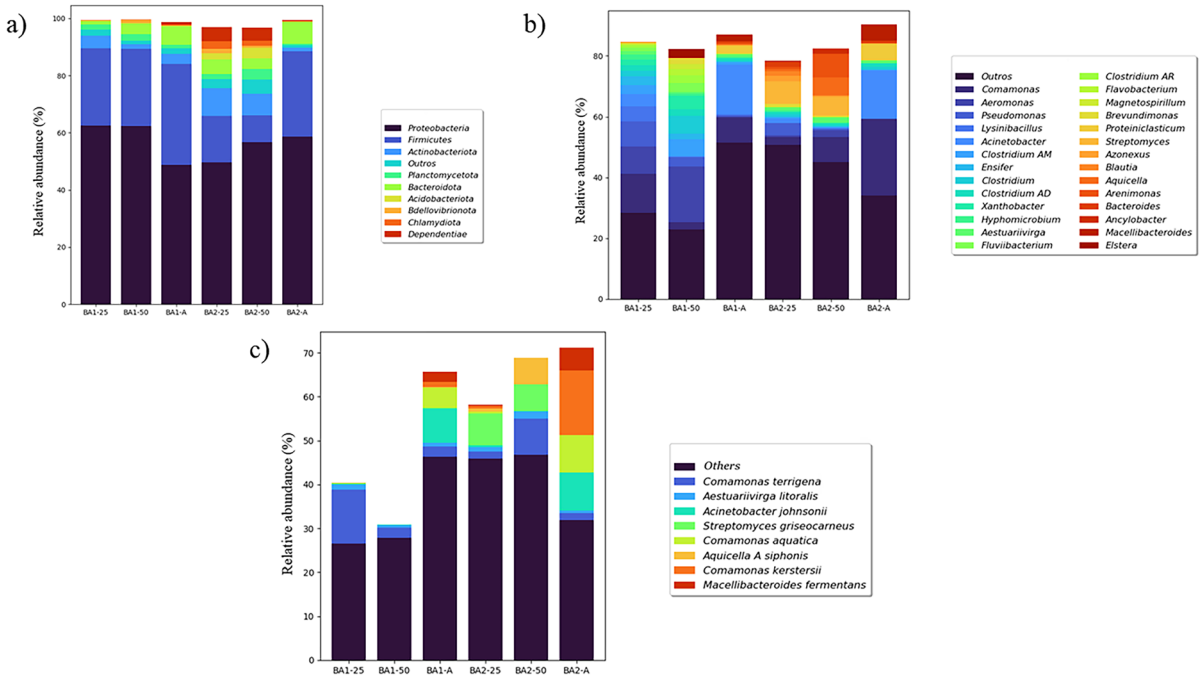

Figure 3 – Relative abundance of bacterial reads in biofilm samples before and after contaminant exposure at the (A) phylum, (B) genus, and (C) species levels. BA1-25: biofilm from river site BA1 after exposure to 25 µg L^{-1} of hormones; BA1-50: biofilm from river site BA1 after exposure to 50 µg L^{-1} 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.

The bioremediation of steroid hormones by microbial communities can occur through two distinct pathways: metabolic degradation, in which microorganisms utilize the compounds as sources of carbon and/or energy, and cometabolic degradation, in which enzymes produced by the microorganisms incidentally transform the compounds without providing a direct energetic advantage. Cometabolism depends on the presence of a primary substrate capable of inducing the expression of the necessary enzymes (Boopathy, 2000).

Among the enzymes involved, peroxidases and laccases are particularly promising due to their efficiency in catalyzing the transformation of organic pollutants into less harmful compounds. These enzymes function under environmentally favorable conditions, such as neutral pH and ambient temperature, require only mild oxidants (oxygen for laccases and hydrogen peroxide for peroxidases) and present practical advantages such as reduced sludge production and straightforward application and control (Méndez et al., 2016; Brugnari et al., 2021).

In recent decades, numerous bacterial species have been identified as producers of laccases. In particular, members of the genera *Pseudomonas* and *Streptomyces*, detected in our river biofilms, have been reported to secrete these enzymes (Kaur et al., 2023; Rahman et al., 2024). Therefore, one of the potential mechanisms contributing to steroid hormone removal in these biofilms may be the extracellular production of laccases and other oxidative enzymes by these microorganisms, leading to the biodegradation of these compounds.

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 3). A total of approximately 1501–4385 OTUs were identified across all samples. As shown in Table 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 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

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.

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 physico–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 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.

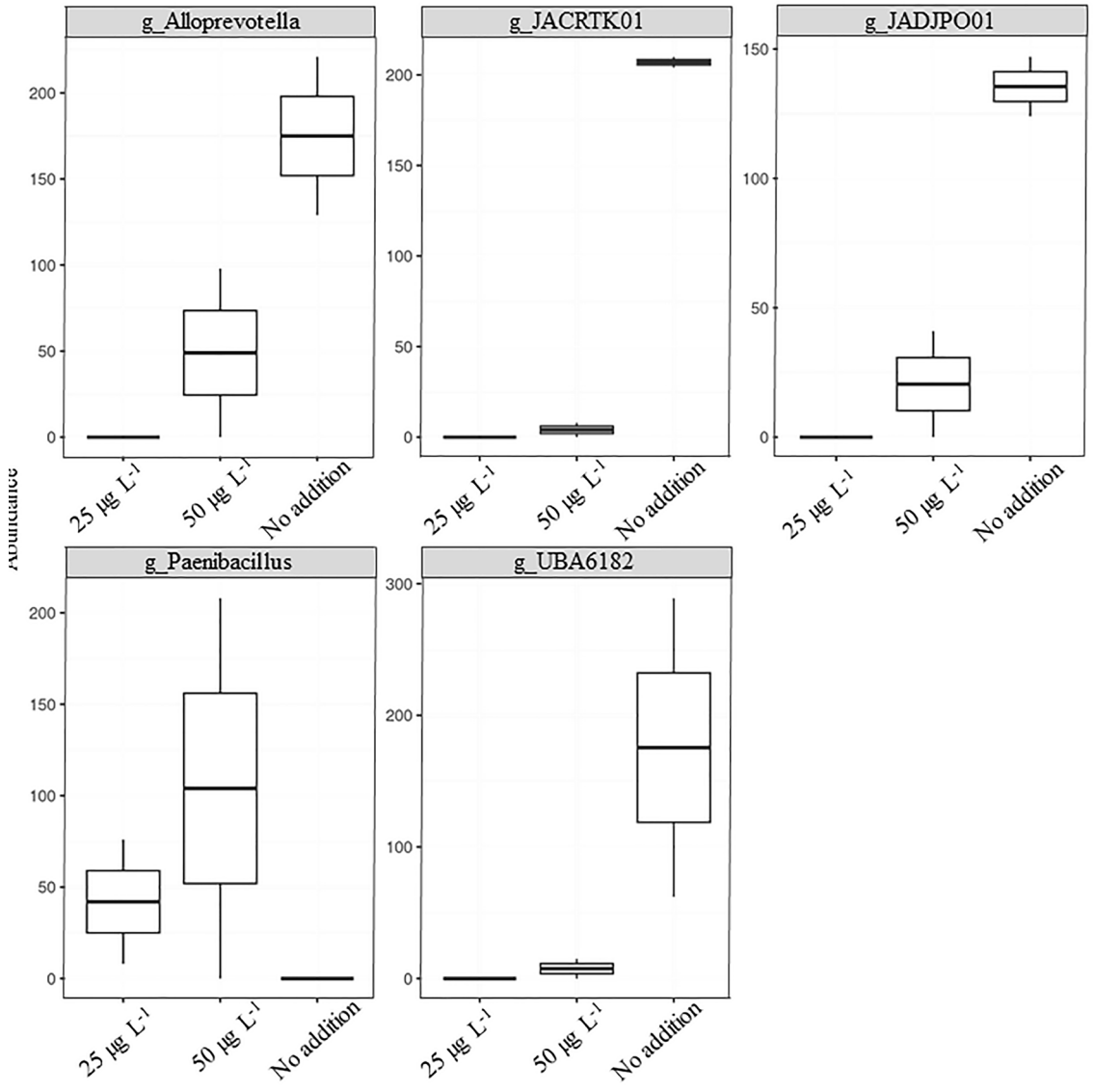

Figure 4 – Differential abundance plots of bacterial genera that showed statistically significant differences among the treatments 25 µg L^{-1}, 50 µg L^{-1}, and the control (no addition).

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 µg L^{-1} of steroid hormones. Among these genera, however, *Alloprevotella* and *JADJPO01* exhibited a slight increase in abundance at the 50 µg 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.

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.

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 (micrograms per liter) and the effects caused on the biofilm microbiome. The results indicate 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 the detection and evaluation of micropollutant impacts in freshwater ecosystems. Future research should also consider the influence of climate change on microbial biofilms, as extreme hydrological events are expected to become more frequent and intense in urban areas. Floods, for example, not only mobilize pollutants but also alter microbial communities, increasing the risk of spreading pathogens and waterborne diseases (Fonseca et al., 2021). In this context, our findings provide an important foundation for future work, as they reveal how biofilm-associated microorganisms respond to environmental stressors, such as steroid hormones.

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 biodegradation 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. Some microbial taxa, such as *Paenibacillus*, showed increased abundance following exposure, indicating potential biotechnological relevance, though further studies are needed to confirm practical applications.

Furthermore, our results highlight the potential use of river biofilms as a basis for bioreactors in the remediation of micropollutants and underscore their value in monitoring urban rivers for emerging contaminants. 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.

Authors' Contributions

Modkovski, T.A.: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing — original draft. **Peixoto**, L.O.M.: formal analysis, validation, writing — review and editing. **Chaves**, J.R.: investigation, validation, writing — review and editing. **Baer**, G.H.: investigation, validation, writing — review and editing. **Imoski**, R.: formal analysis, methodology, validation, writing — review and editing. **Souza**, L.C.: investigation, validation, writing — review and editing. **Medeiros**, S.T.: investigation, validation, writing — review and editing. **Nawate**, B.A.L.: investigation, methodology, validation, writing — review and editing. **Haminiuk**, C.W.I.: conceptualization, supervision, validation, writing — review and editing. **Azevedo**, J.C.R.: conceptualization, funding acquisition, methodology, project administration, resources, supervision, validation, writing — review and editing.

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