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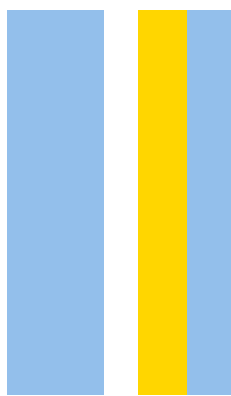
TOXICOLOGIA E CONTAMINAÇÃO AMBIENTAIS

Microbial degradation of two halogenated pharmaceuticals for the recovery of natural aquatic environments

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Resumo

O crescimento exponencial da população mundial e a urbanização têm colocado pressão tanto nos recursos naturais como no ambiente. A poluição antropogénica, incluindo contaminantes de preocupação emergente, como compostos farmacêuticos, está a gerar grande preocupação, uma vez que as estações convencionais de tratamento de água / águas residuais não são eficientes a degradar os compostos farmacêuticos, resultando na sua contínua libertação para o ambiente. Estuários são ecossistemas cruciais, sendo que estes estão sob um crescente stress antropogénico. Para mitigar o impacto da contaminação, métodos sustentáveis como a biorremediação devem ser considerados para o restauro destas áreas ecologicamente importantes.

Desta forma, este estudo teve como objetivo avaliar o potencial de culturas microbianas autóctones, enriquecidas a partir do rizosedimento de plantas de sapal presentes no estuário do Rio Lima, na remoção de bezafibrato (Bzf) e paroxetina (Prx), usando métodos dependentes e independentes de cultivo. Para tal, foi realizado um processo de enriquecimento durante 20 semanas (10 ciclos de 2 semanas), usando sedimento do estuário do rio Lima como inóculo, dopado com 1 mg L^{-1} de Bzf ou Prx. As culturas resultantes foram cultivadas nos meios plate count agar (PCA) e meio mínimo de sais minerais (MM) dopado com os respetivos fármacos. As estripes bacterianas isoladas foram posteriormente purificadas e criopreservadas numa solução a 25 % de glicerol misturado com solução salina (0,85 %) ou com meio líquido PCA. A identificação taxonómica foi realizada através da sequenciação do gene 16S rRNA, utilizando primers universais (27F; 1492R). Após o isolamento da comunidade cultivável, cada cultura enriquecida foi mantida num estado ativo, durante 22 semanas (12 ciclos de 2 semanas) para acompanhar as alterações temporais na estrutura das comunidades microbianas e avaliar a eficiência da remoção dos dois compostos farmacêuticos ao longo do tempo. A caracterização dos sedimentos iniciais e das comunidades microbianas enriquecidas foi realizada através da sequenciação das regiões V4-V5 do gene 16S rRNA, utilizando os primers 515YF e Y926R-jed. Durante a experiência, foram observadas elevadas eficiências de remoção de Bzf e Prx ($> 90 \%$), revelando o potencial degradador das comunidades microbianas autóctones. Os resultados também mostraram que a escolha do composto farmacêutico para os enriquecimentos, seja Prx ou Bzf, teve um impacto distinto na composição da comunidade microbiana. Estas descobertas fornecerão uma compreensão mais profunda da comunidade microbiana associada à degradação de Bzf e Prx, facilitando assim o desenvolvimento de tecnologias de remediação adequadas para implementação em ambientes contaminados com compostos farmacêuticos.

Palavras-chave: Biorremediação, produtos farmacêuticos, culturas enriquecidas, microrganismos autóctones.

Abstract

The world's exponential growth in population and urbanization have strained natural resources and the environment. Anthropogenic pollution, including contaminants of emerging concern, such as pharmaceuticals, is raising major concern, as conventional water/wastewater treatment plants poorly degrade pharmaceutically active compounds, resulting in their continuous release into the environment. Estuaries are crucial ecosystems under increasing anthropogenic stress. To mitigate contamination's impact, sustainable bioremediation methods should be considered for restoring these ecologically important areas. Thus, this study aimed to evaluate the potential of autochthonous microbial cultures, enriched from salt marsh plants rhizosediment within the Lima River estuary, to remove bezafibrate (Bzf) and paroxetine (Prx), using culture independent and dependent methods.

For that an enrichment process was conducted for 20 weeks (10 cycles of 2 weeks), using sediment of Lima River Estuary as inoculum, doped with 1 mg L^{-1} Bzf or 1 mg L^{-1} Prx. The resulting cultures were cultivated on plate count agar (PCA) and mineral-salts medium (MM) containing the respective pharmaceuticals. Bacterial strains isolated were subsequently purified and cryopreserved in 25 % of glycerol mixed with saline solution (0.85 %) or liquid PCA media. Taxonomic identification was achieved by sequencing the 16S rRNA gene, using universal primers (27F; 1492R). After the isolation of the culturable bacterial strains, each enriched culture was kept in an active state for more 22 weeks (11 cycles of 2 weeks each), to follow temporal changes in the microbial community structure and evaluate the removal efficiency of each pharmaceutical over time. Characterization of the initial sediments and enriched microbial communities was performed by high-throughput sequencing of the V4-V5 hypervariable region of the 16S rRNA gene, using the primers 515YF and Y926R-jed. During the experiment, high removal efficiency of Bzf and Prx ($> 90 \%$) were observed, revealing the degrading potential of autochthonous microbial communities obtained from estuarine sediments. Results also showed that the choice of the pharmaceutical compound for the enrichments, whether it was Prx or Bzf, had a distinct impact on the composition of the microbial community. These findings will offer deeper insights into the microbial community associated to the degradation of Bzf and Prx, thereby facilitating the development of effective bioremediation technologies suitable for implementation in impacted environments.

Keywords: Bioremediation, pharmaceuticals, enriched cultures, autochthonous microorganisms.

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Notation and Glossary

AFFF	Aqueous Film-Forming Foam
ASV	Amplicon Sequence Variant
Bzf	Bezafibrate
CECs	Contaminants of Emerging Concern
DAD	Diode Array Detector
EAOPs	Electrochemical Advanced Oxidation Processes
EC ₅₀	Half maximal effective concentration
HDL	High-Density Lipoprotein
HPLC	High Performance Liquid Chromatography
MM	Mineral-salt Medium
MPs	Microplastics
NCBI	National Center for Biotechnology Information
NMDS	Non-metric Multidimensional Scaling
NOEC	No Observed Effect Concentration
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
PCA	Plate Count Agar
PPARs	Peroxisome Proliferator-Activated Receptors
Prx	Paroxetine
PTSD	Post-Traumatic Stress Disorder
SPE	Solid-Phase Extraction
SDGs	Sustainable Development Goals
SMBR	Submerged Membrane Bioreactor
WFD	Water Framework Directive
WHO	World Health Organization
WL	Watch List
WWTPs	Wastewater Treatment Plants

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1. Introduction

1.1. Framing and presentation of the work

Although world population growth has not been as marked as it was in late 1960, it is still happening, and since 2010 the world population has gone from 7 billion to 7.8 billion (Gu et al., 2021). On top of this phenomenon, which by itself puts pressure on the environment, we are experiencing urbanization. Urbanization encompasses the movement of individuals from rural to urban areas, a phenomenon that, when is not properly controlled, can impose adverse environmental impacts (Pauchard & Barbosa, 2013). Currently, more than half of the global population resides in urbanized areas, and by 2050 is expected that this number will be at least 70 % (Cui & Shi, 2012; Zhao et al., 2018). The impact of urbanization holds substantial significance, entailing habitat destruction, noise and light pollution, thermal disturbances, and chemical (organic or inorganic) contamination (Fanelli et al., 2019; Kempenaers et al., 2010; Pimonsree et al., 2022).

Pharmaceuticals are contaminants of emerging concern (CECs), having a polar nature, diverse structures, and ionizable groups (Quesada et al., 2019). With around 4000 active ingredients divided into 24 therapeutic classes (Quesada et al., 2019), they encompass categories like antibiotics, antidepressants, and analgesic hormones (Balu et al., 2022). According to Banker et al. (2002), pharmacology serves as a versatile, cost-effective, and preferred method for treating several medical conditions. These compounds play a vital role in modern science, in both human and veterinary medicine, to treat, or prevent different diseases (Balu et al., 2022; Fernandes et al., 2021; Petrović et al., 2003). With the rise of generic pharmaceuticals and reduced manufacturing costs, consumption has surged (Quesada et al., 2019), leading to substantial daily usage (Petrović et al., 2003). However, the variety and different dosages in each country makes the data collection on drug consumption more challenging (Hernández-Tenorio et al., 2022; Kock et al., 2023). The insufficient removal of pharmaceuticals and their metabolites during wastewater treatment, can lead to their entry into the environment and ultimately into the drinking water supply (Kümmerer, 2009, 2010), underscoring the importance of monitoring pharmaceuticals in the environment (Carmona et al., 2014; Miarov et al., 2020).

The persistence of pharmaceuticals in the environment, coupled with their expanding production and usage, as well as continuous low-level environmental input, has emerged as a concern within the scientific community (Fernandes et al., 2021; Kock et al., 2023). Consequently, it is crucial to proactively eliminate pharmaceutical compounds from the environment or from other potential sources of contamination before their release into the

environment. Municipality and domestic wastewaters flow into wastewater treatment plants (WWTPs) to reduce pollution before the effluent release into the environment, making WWTPs vital for pollutant removal (Quadros et al., 2010). On the other hand, some industries, for example agricultural, automotive, or oil and gas production, benefit from having facilities capable to deal with their own wastewaters (Patwardhan, 2017). They, in turn, if compliant with effluent discharge standards are able to dispose them without upsetting the ecological balance (Myburgh et al., 2019; Patwardhan, 2017). However, WWTPs are not designed to eliminate pharmaceutical compounds, and they constitute one of the major sources of pharmaceuticals in the environment (Alygizakis et al., 2016; Kümmerer, 2010; Lindholm-Lehto et al., 2015; Mejías et al., 2021). Additionally, pharmaceuticals and their metabolites can enter the environment through various means, such as, improper disposal of expired drugs, hospital discharges, pharmaceutical manufacturing wastewater, contamination of manure, sewage sludge and landfill leachates (Fernandes et al., 2020; Gaw et al., 2014). Currently, pharmaceuticals are commonly detected in surface water, groundwater, sediments, among others, with concentrations ranging between ng L^{-1} to $\mu\text{g L}^{-1}$ (Hernández-Tenorio et al., 2022).

To address this problem, emerging techniques such as electrochemical advanced oxidation processes (EAOPs) (Lozano et al., 2022), nanofiltration and reverse osmosis (Nayak et al., 2022; Radjenović et al., 2008), or application of activated carbon (Finn et al., 2021) have been widely explored to remove pharmaceuticals from the wastewater. However, as noted by the same authors, further research is required to assess the removal of pharmaceuticals and their metabolites at real concentrations. Another technique, the submerged membrane bioreactor (SMBR), incorporates a submerged membrane module within a bioreactor to achieve approximately 90 % removal of substances like acebutolol, estrone, and caffeine (Tiwari et al., 2021). In conclusion, ongoing efforts are seeking to enhance pharmaceutical's removal efficiency, but more studies are essential as none of the existing methods provide consistent 100 % removal. In addition, pharmaceuticals physicochemical properties, even within the same class, can cause different interactions with these emerging techniques, influencing their removal efficiency (Liu et al., 2023).

Bioremediation can be an environmentally friendly approach to remove pharmaceuticals. The process involves the use of specialized microorganisms and/or plants to remove the contaminants from the contaminated areas, providing a sustainable remediation method for restoring the natural conditions (Chibuike et al., 2014; Dixit et al., 2015). These organisms possess the capability to detoxify or break down the organic pollutants into less hazardous forms through their metabolic pathways, using the pollutant as sources of energy and carbon (Jabbar et al., 2022). In comparison to conventional methods, bioremediation

emerges as a more eco-friendly, minimally invasive, cost-effective, and long-lasting solution (Dzionek et al., 2016). Furthermore, the planet is facing a significant biodiversity crisis. Sustainable Development Goals (SDGs) were presented seeking to ensure the Earth's environmental sustainability. Consequently, it is imperative to advance with innovative research and methodologies to safeguard wildlife while aligning with the SDGs' objectives.

Bearing all of this mind, the objective of this study was to evaluate the potential of autochthonous microbial consortia, enriched from salt marsh plants rhizosediment within the Lima River estuary, to remove bezafibrate (Bzf) and paroxetine (Prx), using culture independent and dependent methods. With both approaches it was possible to assess the shifts and dynamics of the microbial community structure during the enrichment process and at the same time, isolate the potential degrading microorganisms that are present in the obtained enriched cultures.

1.2. Thesis outline

This thesis is organized into five chapters, starting with the Introduction (Chapter 1), where an initial overview and structure of the work is provided. Chapter 2, titled 'Context and State of the Art,' offers a more comprehensive exploration of the underlying problem motivating this study, along with an in-depth discussion of the topics covered within this thesis, including the role of estuaries and salt marsh plants, urbanization impacts in the environment, emerging contaminants and pharmaceuticals, bioremediation and bioaugmentation as a solution to recover the contaminated environments, among others. Chapter 3 outlines the materials and methods employed in conducting this research. The outcomes and their corresponding analyses are presented in Chapter 4, focusing on the laboratory experiments and their results. In Chapter 5, the major conclusions drawn from this study are synthesized and critically evaluate the research's accomplishments. This section also identifies the objectives that were achieved and outlines potential avenues for future research.

2. Context and state of the art

2.1. Urbanization impacts on ecosystems and contaminants of emerging concern

Urbanization involves the migration of people from rural to urban areas, a process that, if not properly managed, can have harmful effects on the environment (Pauchard & Barbosa, 2013). The impact of urbanization on urban aquatic ecosystems is not a novel concept and it has been thoroughly studied by the scientific community (O'Driscoll et al., 2010; Pauchard & Barbosa, 2013; Salerno et al., 2018; Todd et al., 2019; Wang et al., 2020; Zhang et al., 2018; Zheng et al., 2020). As urban populations increase, so does the pressure on these ecosystems (Taheran et al., 2018). The consequences of urbanization are significant and encompass habitat destruction, noise, light, and thermal pollution, along with chemical contamination (Fanelli et al., 2019; Kempenaers et al., 2010; Pimonsree et al., 2022). Additionally, the water quality in urban watersheds is significantly affected by the discharge of wastewater effluents from WWTPs, which contain a complex combination of biologically active organic chemicals (Bai et al., 2018; Barber et al., 2013). Consequently, roughly 90 % of water supplies in urban areas can become contaminated (Geissen et al., 2015).

Contaminants of emerging concern (CECs) constitute a diverse group of compounds commonly found in everyday products. Pharmaceuticals, per- and poly-fluoroalkyl substances (PFAS), synthetic musks, food additives, personal care and veterinary products are some examples of these organic contaminants (Aminot et al., 2021; Catherine et al., 2019; Nakata et al., 2012; Propp et al., 2021). While WWTPs are primarily designed to remove organic loads, nutrients, suspended solids, and select trace elements (Kosek et al., 2020) they are not specifically designed to remove CECs, thus contributing to their presence in the environment (Golovko et al., 2021), with concentrations ranging from $\mu\text{g L}^{-1}$ to ng L^{-1} (Samal et al., 2022). CECs are released from WWTPs into the environment without significant removal, leading to their gradual presence/accumulation in sediments, soil, surface and groundwater, and seas (Brumovský et al., 2017; Choy et al., 2017; Suárez et al., 2008; Tondera et al., 2018). The continuously release of CECs into the environment, results in their progressive accumulation, causing adverse effects on several organisms (Suárez et al., 2008; Tondera et al., 2018; Wilkinson et al., 2017). Additionally, conventional treatment technologies face significant challenges in remediating CECs (Söregård et al., 2019).

The Water Framework Directive (WFD) is a comprehensive legislative framework aimed at achieving and maintaining good water quality across Europe's freshwater bodies, including rivers, lakes, groundwater, and coastal waters. WFD was created by the European Parliament & Council within the Directive 2000/60/EC. Due to the widely usage of products regarded as CECs, and their concentrations found in freshwaters or wastewaters, the European Parliament & Council implemented the Directive 2013/39/EU amending the previous Directives 2000/60/EC and 2008/105/EC, which lacked the inclusion of CECs as priority substances within the scope of water policy, with the aim of regulating the contamination of aquatic environments by these compounds. The Watch List (WL) is a mechanism introduced by the WFD to monitor and address emerging pollutants, including certain pharmaceuticals, that may pose a potential risk to aquatic ecosystems and human health. The initial WL was first issued in Decision 2015/495/EU and subsequently updated in Decision 2018/840/EU. In 2020, a newly revised WL was published in Decision 2020/1161/EU, encompassing 18 CECs which was again revised in 2022. The Decision 2022/1307/EU determined that the available information concerning 16 CECs of the 18 included in the WL in the prior decision was insufficient, leading to a recommendation for their retention on the WL. Furthermore, 10 additional CECs were identified as viable contenders for inclusion in the WL. The presence of CECs on the WL does not automatically trigger regulatory action, but it does initiate a process of risk assessment. The European Commission, in collaboration with relevant agencies and experts, evaluates the available scientific data to assess the environmental and human health risks associated with these substances. If the risk assessment indicates that a substance poses a significant risk to the aquatic environment or human health, it may be considered as a priority substance in future updates to the WFD. This would lead to stricter regulatory controls and measures for its management and reduction in water bodies.

2.2. Estuaries – ecological significance and anthropogenic impacts

Estuaries, characterized by the dynamic convergence of freshwater inflows and saltwater intrusions, stand as essential ecosystems at the interface of terrestrial and marine environments. These ecosystems are renowned for their intricate ecological dynamics and, concurrently, face intensifying anthropogenic pressures (Gillanders et al., 2011; Kennish, 2002, 2024).

Estuaries, often referred to as “nurseries of the sea”, are recognized for their fundamental roles in supporting diverse biota, as their intricate habitat provides sanctuary

for juvenile stages of numerous marine species, fostering growth and development (Van Niekerk et al., 2020). This, in turn, bolsters critical fisheries, underpinning global food security and sustaining livelihoods (Childs et al., 2015). Simultaneously, anthropogenic development exerts significant stress on them. Human activities such as urbanization, industrialization, and pollution, have increasingly compromised the integrity of these delicate habitats (Fernandes et al., 2017; Gillanders et al., 2011; Jones et al., 2011; Kennish, 2002; Kennish, 2023). If alterations in habitat indeed result in reduced biodiversity, this factor alone could instigate transformations in the structure and functionality of ecosystems, including nutrient cycling, productivity, and food provision (Kennish, 2023).

Many estuaries feature expansive salt marsh zones inhabited by a variety of salt-tolerant plant species, specifically halophytes. These plants, encompassing both native and invasive species, can thrive in environments with salt concentrations of approximately 0.2 M NaCl or even higher, often enduring recurrent flooding events (Lakshmanan & Robinson, 2013). Their ability to accumulate and remove contaminants such as metals (Anjum et al., 2014), and pharmaceuticals (Barreales-Suárez et al., 2021) from the soil presents substantial potential for phytoremediation.

Several organic and inorganic pollutants have been widely detected in different estuarine areas (Pérez-Fernández et al., 2020). In fact, following the rupture of a mining dam in Brazil, results obtained by Gabriel et al. (2020) reported a strong metal contamination in Rio Doce estuary, in which Cd, Cr, Pb, As, Cu, and Zn were found in the sediments. Consequently, over the following two years, potential ecological risks associated with harmful toxic effects on commercially significant estuarine organisms, such as crabs, shrimps, and fishes were observed. Furthermore, the presence of PFAS was assessed by Novak et al. (2023) in an Australian estuary, being detected in water samples from every sampling site with, a mean concentration of $0.329 \mu\text{g L}^{-1}$. These results were associated by the authors to the use of aqueous film-forming foam (AFFF) to fight fires in the commercial airport and the defence base. Moreover, the global issue of coastal systems, including estuaries, pollution by microplastics (MPs) is widely recognized. Several authors have studied the ingestion of MPs by aquatic organisms to evaluate the extent of contamination in transitional ecosystems. Bessa et al. (2018) selected three commercial fish species, namely *Dicentrarchus labrax*, *Diplodus vulgaris* and *Platichthys flesus* from Mondego estuary. The results revealed that 38 % of the 120 individuals sampled had ingested MPs, in which polyester, polypropylene, and rayon were the most dominant MPs. Besides, Van Cauwenberghe and Janssen (2014) estimated that a human consumes a vast number of mussels, ingesting 11,000 microplastics per year. This value was calculated using the

average amount of MPs found in two European commercially bivalves, namely *Mytilus edulis* and *Crassostrea gigas*.

The Lima River Estuary, situated in NW Portugal, as many other estuaries is situated near a large city (Viana do Castelo, Portugal) and represents an urban-industrialized estuarine environment facing multiple sources of disruption (Almeida et al., 2011; Sousa et al., 2006). The lower estuary contends with the impact of navigational and port activities, while the entire estuary is affected by diffuse pollution from diverse sources, such as, from agriculture, industrial and urban activities. The influx of agricultural runoff, urban wastewater, and industrial discharges, contributes for the release of nutrients and various substances such as pharmaceuticals into the estuary (Dias, 2018; Paíga et al., 2013). Additionally, the consistent dredging of the navigational channel poses the risk of remobilizing contaminants from the sediments into the water column (Almeida et al., 2011). This estuary features a substantial Eurosiberian salt marsh, characterized by a thriving salt meadow predominantly occupied by *Juncus maritimus*, as indicated by Rede Natura (2000). Additionally, *Phragmites australis*, a plant species frequently encountered in the area, is a subject of debate among some authors since this species has been classified as invasive (Almeida et al., 2011; Blossey et al., 2020).

2.3. Pharmaceuticals in the environment

Pharmaceuticals encompass a diverse group of compounds, both natural and synthetic, typically polar molecules that exhibit variations in their functional groups and structural features, often characterized by multiple ionizable groups (Quesada et al., 2019). Additionally, pharmaceuticals comprise approximately 4,000 active ingredients and can be categorized into 24 distinct therapeutic classes, such as antibiotics, antidepressants, analgesic hormones, antiepileptics, lipid-lowering drugs, β -blockers, antiulcer drugs and antihistamines (Quesada et al., 2019). Pharmaceuticals play a vital role in contemporary science, used in both human and veterinary medicine (Balı et al., 2022; Petrović et al., 2003). They are essential in our society in the treatment, and prevention of various diseases (Balı et al., 2022).

With the beginning of generic pharmaceuticals production and availability, and reduced manufacturing costs, their affordability has increased, leading to a consistent rise in consumption (Quesada et al., 2019). Consequently, these drugs are now being consumed in substantial quantities on a daily basis (González Peña et al., 2021). However, due to regional disparities in lifestyle and accessibility, variations exist in both dosages and the

types of compounds used, making it exceptionally challenging to gather comprehensive data on global pharmaceutical consumption (Quesada et al., 2019). (Samal et al., 2022)

Pharmaceuticals, including their active metabolites, can enter in the environment through WWTPs effluent discharges, improper disposal of expired drugs, discharges from healthcare facilities, pharmaceutical manufacturing wastewater, application of manure and sludge containing pharmaceutical residues as organic fertilizers, and the leaching of pharmaceuticals from landfills (Fernandes et al., 2020; Gaw et al., 2014). Pharmaceuticals and/or their metabolites are frequently detected in wastewater from sewage treatment plants and surface water, with concentrations ranging from ng L^{-1} to $\mu\text{g L}^{-1}$ (Hernández-Tenorio et al., 2022; Liu et al., 2015). In addition, and raising concerns about these compounds, several authors have reported levels of pharmaceuticals found in drinking water supplies. In fact, Wu et al. (2015) reported low concentrations levels of three benzodiazepines in drinking water in Shanghai. While, Zuccato et al. (2000) observed concentrations up to 23.5 ng L^{-1} of the anti-depressant diazepam, in water samples collected from the main water system of Lodi. A study conducted in Valencia, where mineral and tap water were tested for the presence of pharmaceuticals, revealed that concentrations of ibuprofen (39 ng L^{-1}), naproxen (11 ng L^{-1}), propylparaben (9 ng L^{-1}) and butylparaben (28 ng L^{-1}) were found (Carmona et al., 2014). In addition, carbamazepine ($1 - 21 \text{ ng L}^{-1}$), diclofenac ($4 - 209 \text{ ng L}^{-1}$), ibuprofen ($5 - 836 \text{ ng L}^{-1}$), ketoprofen ($9 - 952 \text{ ng L}^{-1}$), and naproxen ($2 - 129 \text{ ng L}^{-1}$) were found in a lake in Finland (Lindholm-Lehto et al., 2015). Fonseca et al. (2021) conducted a study to screen for the presence of pharmaceuticals in Rio Tejo, where amoxicillin (70.48 ng L^{-1}), venlafaxine (38.86 ng L^{-1}), carbamazepine (254.62 ng L^{-1}) and alpha-hydroxyalprazolam (172.38 ng L^{-1}) were detected with the highest mean concentrations. Furthermore, concentrations of carboxyibuprofen (1227 ng L^{-1}), diclofenac (241 ng L^{-1}), hydroxyibuprofen (287 ng L^{-1}), ibuprofen (222 ng L^{-1}), and naproxen (178 ng L^{-1}) were found in seawater samples from the North Portuguese coast Lolić et al. (2015). Vazquez-Roig et al. (2012) reported the presence of pharmaceuticals in sediment samples collected Pego–Oliva Marshlands in Valencia, such as acetaminophen (15.1 ng g^{-1}), ciprofloxacin (7.3 ng g^{-1}), and fenofibrate (16.2 ng g^{-1}). This emphasizes the critical need for continuous monitoring of residual pharmaceuticals in the environment (Carmona et al., 2014; Miarov et al., 2020).

In addition to monitoring their concentrations, understanding the environmental impacts of pharmaceuticals holds paramount importance, as pharmaceuticals may cause adverse impacts in the receiving ecosystems (Samal et al., 2022). To understand and unravel their effects, it is vital to unveil their modes of action and metabolic pathways involved, as well as assess the potential impacts on non-target organisms (Christen et al.,

2010; Jjemba, 2018). Mezzelani et al. (2016) studied the effect of several non-steroidal anti-inflammatory drugs (NSAIDs), namely diclofenac ($25 \mu\text{g L}^{-1}$), ibuprofen ($25 \mu\text{g L}^{-1}$), and nimesulide ($25 \mu\text{g L}^{-1}$) in *Mytilus galloprovincialis* and reported that exposure to these compounds led to immune parameter disorders, early genotoxicity, regulation of lipid metabolism, oxidative stress, and neurotoxic effects. Furthermore, neurotoxic effects have been observed in organisms exposed to paracetamol, including in *Anguilla anguilla* ($5 - 3125 \mu\text{g L}^{-1}$) (Nunes et al., 2015), *Ruditapes philippinarum* ($0.25 - 25.00 \mu\text{g L}^{-1}$) (Nunes et al., 2017) and *M. galloprovincialis* ($20 - 200 \mu\text{g L}^{-1}$) (Solé et al., 2010). Moreover, other studies revealed that the exposure to paracetamol ($1.50 - 5.37 \text{ mg L}^{-1}$) (Daniel et al., 2019), acetaminophen ($84.4 \mu\text{g L}^{-1}$) and diclofenac ($0.08 - 18.4 \mu\text{g L}^{-1}$) (Oliveira et al., 2015) caused an inhibition of cholinesterase in *Daphnia magna* individuals. Another study conducted by Sousa and Nunes (2021) reported alterations in the swimming behaviour in both *D. magna* and *Daphnia longispina* upon exposure to paracetamol ($2.25 - 38.4 \text{ mg L}^{-1}$). Thus, the development of new remediation technologies to remove pharmaceutical from the environment or from other potential sources of contamination are needed.

2.3.1. Paroxetine and bezafibrate

Among the wide array of pharmaceutical compounds available, this work focused on two specific compounds: Prx and Bzf, two halogenated pharmaceuticals. These two compounds were selected as representatives of widely used pharmaceutical families, being detected in several environmental matrices.

Paroxetine (Prx) is a selective serotonin reuptake inhibitor (SSRI) antidepressant (Boyer & Feighner, 1992) commonly used to treat several mental health disorders, depression, generalized anxiety disorder, social anxiety disorder, obsessive-compulsive disorder, and post-traumatic stress disorder (PTSD) (Kowalska et al., 2021). This pharmaceutical has several commercial brands including Paxil, Aropax, Pexeva, Seroxat, Sereupin and Brisdelle, and it was first marketed in 1992 in U.S. (Kowalska et al., 2021; Nevels et al., 2016). Based on the available literature, Prx stands out as one of the most prescribed off-label drugs in routine clinical practice (Lu et al., 2023; Sharma & Dang, 2020).

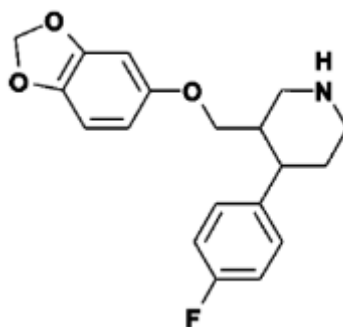


Figure 1 – Structure of the paroxetine molecule (Kowalska et al., 2021)

Prx increases serotonin levels in the brain, a neurotransmitter intricately tied to mood regulation, through the inhibition of its reuptake into nerve cells (Davis et al., 2016). Reports concerning the effects of SSRIs on non-target organisms, such as in several fish species, are on the rise, being often linked to the potential of SSRIs to influence the serotonergic system in fish, even when occurring at minimal concentrations (McDonald, 2017; Silva et al., 2015). According to several authors, several SSRIs can change the behaviour, physiology, and gene expression on several fish species (Puckowski et al., 2016). Specifically, *Danio rerio* embryos exposed to fluoxetine ($100\ \mu\text{g L}^{-1}$) suffered significant changes in 138 genes. Huang et al. (2020) linked this effect to mitochondrial dysfunction and subsequent neurodevelopmental effects. In addition, acute interperonial exposure to fluoxetine ($50\ \mu\text{g g}^{-1}$) was reported to reduce metabolic regulation in *Opsanus beta* (Amador et al., 2018). Venlafaxine ($100\ \mu\text{g L}^{-1}$) has been reported to reduce larval activity in *Danio rerio* (Tang et al., 2021), and cause changes in circadian rhythm of *Gambusia holbrooki* (Melvin, 2017). Furthermore, *Poecilia wingei* exposed to citalopram ($15\ \mu\text{g L}^{-1}$) suffered from anxiolytic effects, reflecting on behavioural changes affecting the survival chances of the individuals (Olsén et al., 2014). Other non-target organisms have been reported to be affected by SSRIs. Rodrigues et al. (2015) reported a decreased activity of acetylcholinesterase in *Carcinus maenas* when exposed to sertraline ($0.05 - 500\ \mu\text{g L}^{-1}$). Moreover, microalgae *Chlorella pyrenoidosa* growth was inhibited when exposed to fluoxetine, with a EC_{50} concentration of $0.464\ \text{mg L}^{-1}$ (Xie et al., 2022).

The effects of Prx have been studied, showing that Prx ($400 - 3200\ \text{ng L}^{-1}$) can significantly decrease *Danio rerio* swimming activity and disrupt their stress response when exposed to it (Ferreira et al., 2023). In addition, it was also shown that individuals of *Danio rerio* exposed to $25\ \mu\text{g L}^{-1}$ of Prx had a significantly lower production of hepatocytes (Moreira et al., 2022). Moreover, Prx at a concentration of $1.5\ \mu\text{g L}^{-1}$ revealed to pose cytotoxic effects, breaking DNA strands in hemocytes cells of *Mytilus edulis* (Lacaze et al., 2015). In a study conducted by Ishak et al. (2022), where nematode taxa were exposed to

a mixture of amitriptyline ($0.4 \mu\text{g L}^{-1}$) and Prx ($40 \mu\text{g L}^{-1}$) suffered an unbalanced sex-ratio, suggesting that the interaction of these two compounds affect the hormone system of nematodes. In another study, it was also demonstrated that the presence of Prx ($0.0650 \mu\text{g L}^{-1}$) induced adverse effects on the growth of the microalgae *Chlorella vulgaris* (Johnson et al., 2007). Furthermore, a study conducted by Li et al. (2022) revealed that Prx (10 ; 100 ; $1000 \mu\text{g L}^{-1}$) despite no significant alterations were observed in the overall diversity of macrobenthos community, exerted a discernible influence on benthic nitrogen-converting bacteria, suggesting that paroxetine directly affects the nitrogen conversion process by targeting these microbial populations. By indirectly influencing nitrogen transformation, Prx impacted the trophic transfer efficiency at higher trophic levels, including protozoa.

According to the World Health Organization (WHO), depression was the third-leading disease in 2008, and it is projected to claim the top position by 2030 (Malhi & Mann, 2018). Presently, around 350 million individuals worldwide grapple with depression (Organization, 2017), which has consequently fuelled a steady increase in the global consumption of antidepressants (Rose, 2017). Consequently, the presence of these compounds in the environment is expected to increase. Prx has been detected in several environments and wastewater effluents at different concentrations. Lu et al. (2023) reported Prx concentrations up to 69.9 ng L^{-1} in wastewater samples in the biggest province of China. Moreover, in a study conducted by Gros et al. (2012), Prx was detected in surface waters (40 ng L^{-1}), specifically in water samples collected from river Onyar (Girona) and seawater (between 3.3 ng L^{-1} to 14.2 ng L^{-1}) collected from the Mediterranean Sea. In addition, Osorio et al. (2016) was able to detect low levels of Prx (average of 0.66 ng L^{-1}) in other Iberian rivers. In addition, the same authors were able to detect Prx in the sediments from each river basin, in concentrations ranging between 0.05 ng L^{-1} to 0.76 ng L^{-1} . Furthermore, López-Serna et al. (2013) reported Prx concentrations up to 30.2 ng L^{-1} in the underlying groundwater of Barcelona.

Portugal has consistently reported elevated rates of antidepressant consumption, a trend that has endured since 2000 (Estrela et al., 2020). In fact, between 2010 and 2020, the dispensation of antidepressant packages in pharmacies increased by 83.5 %, being Prx one of the most frequently prescribed (Martins, 2021). In this context, Prx has been detected in surface waters, specifically in both Zebro and Lis rivers, with concentrations of 60.65 ng L^{-1} and 25.5 ng L^{-1} , respectively (Paiga et al., 2016; Palma et al., 2020). Furthermore, Prx has also been found in the sediments of Cávado (0.16 ng L^{-1}) (Dias, 2018), Douro ($< 1 \text{ ng L}^{-1}$) (Sousa et al., 2011), Leça (25.5 ng L^{-1}) (Paiga et al., 2016), and Lima (0.19 ng L^{-1}) (Dias, 2018) rivers.

Bezafibrate (Bzf) is a lipid-lowering agent from the fibrate group, primarily used to treat lipid-related conditions by activating peroxisome proliferator-activated receptors (PPARs), specifically PPAR- α , which leads to the breakdown of triglycerides and an increase in high-density lipoprotein (HDL or "good" cholesterol) (National Center for Biotechnology Information, 2023). Several studies have been conducted to understand the effects of fibrates on non-target organisms. These effects are often associated with the capacity of fibrates to primarily affect fish physiology, causing disturbances in cellular and tissue lipid metabolism (Blonç et al., 2023; Isidori et al., 2007). These metabolic disruptions, in turn, can disturb the timing and functioning of the reproductive axis and vitellogenesis, potentially leading to irregularities during larval development (Blonç et al., 2023). These developmental anomalies may subsequently influence typical swimming behaviours and, possibly, foraging performance (Blonç et al., 2023; Isidori et al., 2007). Specifically, in a study conducted by Quinn et al. (2011) where *Dreissena polymorpha* was exposed to gemfibrozil ($1000 \mu\text{g L}^{-1}$), the results revealed an increase in defence biomarkers, glutathione transferase and metallothionein and DNA damage after 96h of exposure. Guo et al. (2015) reported that exposure of *Ctenopharyngodon idella* to clofibrate (50 mg kg^{-1}) through dietary intake led to the upregulation of peroxisomes in both liver and heart mitochondria. Moreover, the same authors showed that that clofibrate exposure can impact the metabolic processes of both adult organisms and larvae and lead to significant developmental alterations in embryos, resulting in severe deformities and behavioural changes. In addition, Fernandes et al. (2011) reported that clofibrate (0.2427 mg L^{-1}) exposure caused an inhibitory effect upon CYP17 associated to the synthesis of androgens, in *Cyprinus carpio*.

Bzf is commonly prescribed to individuals with hyperlipidemia, to reduce the risk of cardiovascular events like heart attacks and strokes (Elkeles et al., 1998). Bzf can also be used to treat hypertriglyceridemia and mixed dyslipidemia (Arbel et al., 2016; Hirose et al., 2015; Kotani et al., 2010; Li et al., 2014). Moreover, Bzf has exhibited promising results to treat certain small cancers when combined with other drugs (Tanaka et al., 2022). Additionally, Bzf has been used to manage autoimmune-mediated liver disease, primary biliary cirrhosis, particularly when patients do not respond adequately to the traditional treatment (Tanaka et al., 2021).

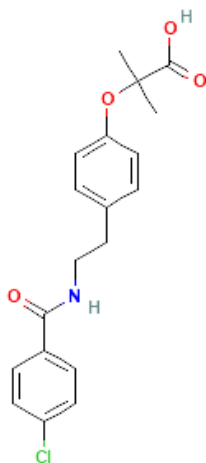


Figure 2 – Structure of the bezafibrate molecule (National Center for Biotechnology, 2023)

The effects of bezafibrate in non-target organisms has been addressed by the scientific community. Rivera-Jaimes et al. (2018) performed an ecotoxicological risk study using *Daphnia sp.*, fish, and algae to assess the sensitivity or adverse effects caused by the presence of Bzf. Results showed that, *Daphnia sp.* was the most sensitive species, having a no observed effect concentration (NOEC) of 0.02 mg L⁻¹ (Rivera-Jaimes et al., 2018). In contrast, both algae and fish revealed a higher resistance to Bzf, with NOEC concentrations of 60 and 5.3 mg L⁻¹, respectively. Zhang et al. (2020) reported that chronic exposures of *Ceriodaphnia dubia* and the *Brachionus calyciflorus* to Bzf, with effect concentrations EC₅₀ of 0.13 to 7.36 mg L⁻¹, caused a reproductive inhibition in these specific species. In addition, Bzf was reported to cause genotoxicity even at low concentrations, in *Danio rerio* (Rocco et al., 2010). In addition, the exposure of *D. rerio* to 1.7, 33 and 70 mg of BZF/g of food, revealed the potential of Bzf to act as an endocrine disruptor, influencing gonadal steroidogenesis and spermatogenesis affecting adult male individuals (Velasco-Santamaría et al., 2011). Furthermore, high Bzf concentrations (3 – 60 µg L⁻¹) increased *Phaeodactylum tricornutum* cell density, causing a shift from autotrophic to mixotrophic metabolism (Duarte et al., 2019a).

Over the years, Bzf has been detected in several environmental matrices. Specifically, Bzf was detected in surface waters (two water reservoirs and four rivers) in Brazil in concentrations up to 211.2 ng L⁻¹ (de Souza et al., 2021). Water samples collected from a river in England revealed concentrations of Bzf of 0.66 ng L⁻¹ (Petrie et al., 2016). Other study identified the presence of Bzf in Mexican surface waters at 2100 ng L⁻¹ (Rivera-Jaimes et al., 2018). Concentrations levels of Bzf up to 45 ng L⁻¹ were also detected in Spanish river Reconquista (Mastrángelo et al., 2022). The presence of Bzf has also been reported

in groundwaters. Groundwater collected in eastern suburb of Beijing revealed Bzf concentration of 7.63 ng L^{-1} (Ma et al., 2022). In addition, in groundwaters underlying Barcelona, Bzf was detected at 25.8 ng L^{-1} (López-Serna et al., 2013). Moreover, Bzf was reported to be present in an hospital effluent in Valencia (Spain) as high as 265 ng L^{-1} (Mendoza et al., 2015). Furthermore, Patel et al. (2019) reported that Bzf was detected in drinking water at concentrations ranging from 0.31 ng L^{-1} to 0.85 ng L^{-1} , with levels reaching up to 0.16 ng L^{-1} in the tap water.

Additionally, Portugal leads in the consumption of lipid-modifying agents, with Bzf ranking among the most commonly used (Blais et al., 2021). So, it is only natural that both Prx and Bzf have been previously identified in effluents from WWTPs in Portugal and in the environment (Paiga et al., 2016; Pereira et al., 2015; Silva et al., 2014). Bzf has also been detected in several estuaries and rivers of Portugal. In the Mondego (15.5 ng L^{-1}), and Tagus (11.86 ng L^{-1}) estuaries, concentrations of Bzf were detected, but were only found in only 2.78 % of the samples (Pereira et al., 2017). In a study conducted in Leça river, where several pharmaceuticals including Bzf were analysed, a maximum concentration of 770 ng L^{-1} of Bzf was reported (Gonçalves et al., 2013). In contrast, Antuã and Cértima rivers consistently showed Bzf concentrations below 10 ng L^{-1} , while the Leça River reached a maximum recorded concentration of 40 ng L^{-1} (Barbosa et al., 2018). In a study conducted by Reis-Santos et al. (2018), Bzf was found in 87.1 % of the samples collected in Tejo estuary, with an average concentration of 3.31 ng L^{-1} .

Bearing this in mind, it is essential to prevent the introduction of pharmaceuticals into the environment, to protect ecosystems and the long-term sustainability of the planet. The observed concentrations of these compounds in natural environments, along with their potential impacts on non-target organisms, emphasize the ongoing necessity to develop effective remediation techniques suited for application in contaminated environments.

2.4. Bioremediation and phytoremediation technologies for environmental restoration

There are several physical-chemical technologies that can be used to remediate polluted water bodies and soils, particularly for the elimination of metals. These methods encompass chemical precipitation, filtration, oxidation, reduction, ion-exchange, reverse osmosis, membrane technologies, among others (Dixit et al., 2015; Dursun et al., 2020; Sarker et al., 2023). Similarly, for the removal of organic pollutants, advanced oxidation

processes like photocatalysis, Fenton reactions, and ozonolysis, often complemented by sonolysis, are widely studied in WWTPs (Anandan et al., 2020; Dehghani et al., 2023). However, it is worth noting that these methods can exhibit limitations in terms of efficiency and cost-effectiveness, particularly when dealing with exceedingly low concentrations of contaminants.

Bioremediation is a process that uses microorganisms, plants, or enzymes to remove or neutralize pollutants and contaminants from soil and/or water matrices (Magan et al., 2022). Bioremediation is considered an eco-friendly and sustainable approach to environmental restoration, as it relies on natural biological processes to mitigate pollution. Additionally, this method offers cost-effectiveness by eliminating transportation expenses, as it can be applied directly to the targeted cleanup area (Chibuike et al., 2014; Saha et al., 2021).

Within bioremediation, there are different approaches, being the most common biostimulation and bioaugmentation (Adams et al., 2015). Biostimulation relies on the addition of essential nutrients and electron acceptors, that are typically present in limited quantities to enhance and stimulate the natural community from the contaminated site, which otherwise restrict microbial activity (Elektorowicz, 1994; Piehler et al., 1999; Rhykerd et al., 1999). In the other hand, bioaugmentation is based in the addition of specialized microorganisms with metabolic capacity to degrade the target pollutant in the contaminated site (Adams et al., 2015; Herrero & Stuckey, 2015; Van Der Gast et al., 2004). However, the bioaugmented bacteria has to compete with the indigenous bacterial communities for the available carbon sources (Herrero & Stuckey, 2015; Yang et al., 2016), and has to adapt to the environmental conditions imposed by the contaminated site. Both factors can be crucial for the success of the bioaugmentation strategy and the effective degradation of the target pollutant. To overcome these issues, the use of autochthonous bacteria offers distinct advantages since they can be enriched and/or isolated from the contaminated site (Huda et al., 2023). In fact, they can adapt more easily to the specific conditions of contaminated site, can possess a competitive edge within the microbial community (Feichtmayer et al., 2017) and it is expected fewer negative impacts to the local biodiversity (Fernandes et al., 2022; Kumar & Gopal, 2015). Thus, these advantages enhance their ability to thrive and be successful in the removal of the target compound (Kumar & Gopal, 2015). On the other hand, the use of allochthonous microorganisms can lead to disruption of the natural microbial dynamics and structure by increasing interspecies competition for nutrients and carbon sources (Raper et al., 2018). Moreover, their use can enhance the spreading of undesirable phenotypes (Raper et al., 2018; Žur et al., 2020). Furthermore, since they are not from the contaminated site, they may have a poor ability to compete with the natural

community and to adapt to the natural condition, compromising the removal of the pollutant (Žur et al., 2020).

The metabolic potential of microorganisms to degrade different organic pollutants, including pharmaceuticals have been reported by several authors. Cy et al. (2020) tested the biodegradation of ibuprofen (5 mg L^{-1}) using activated sludges from an hospital and municipal WWTPs. The results showed that under anaerobic conditions, no significant degradation was observed, while, under aerobic conditions, a degradation over 80 % of ibuprofen was observed. Moreover, the genera *Nocardia*, *Sphingomonas*, and *Variovorax* were associated to the ibuprofen degradation. In another study, biodegradation of diazepam (10 mg kg^{-1}) and oxazepam (10 mg kg^{-1}) was assessed using microbial communities present in reed bed affected by an WWTPs effluent (Crampon et al., 2021). Results demonstrated that after 15 days of incubation, more than 95 % of both compounds were removed, where the genus *Brevibacillus* was the most abundant in the biodegradation process of both compounds. In addition, Woźniak-Karczewska et al. (2018) studied the efficiency of two bacterial strains (*Ochrobactrum anthropic* and *Ochrobactrum intermedium*) to degrade piracetam (1 g L^{-1}). The results revealed to be promising, showing total degradation by *O. anthropic* after 46 h, and by *O. intermedium* after 28 h. In another study conducted by Wang et al. (2018), the degradation potential of *Acinetobacter sp.*, to degrade 2 mg L^{-1} and 30 mg L^{-1} of sulfamethoxazole, sulfadiazine, sulfamethazine, trimethoprim, triclosan, diclofenac, and carbamazepine, was assessed. Results showed high sulfonamides removal rates, in which sulfamethoxazole was completely removed within 5h, while sulfadiazine and sulfamethazine were totally removed after 10h of incubation. In addition, no significant removal efficiency for the remaining pharmaceuticals was observed. Wang et al. (2018) attributed these results to the fact that a specific enzyme is required to achieve degradation sulfamethoxazole, which is not capable to interact with the chemical structure of trimethoprim, triclosan, diclofenac, and carbamazepine. Furthermore, Yang et al. (2018) reported 45 % biodegradation of atrazine (5 mg L^{-1}) after 36 h by *Citricoccus sp.* strain, however, higher removal rates were achieved (<91 %) after an incubation period of 60 h. Xu et al. (2022) evaluated the degradation capability of 9 bacterial strains (2 strains of *Comamonas testosteroni*; *Pseudomonas putida*; *Acinetobacter calcoaceticus*; *Pseudomonas sp.*; *Rhodococcus sp.*; *Pseudomonas stutzeri*; *Buttiauxella sp.*; *Vibrio sp.*) to degrade a 0.1 mM of 4 polycyclic aromatic hydrocarbons, namely phenanthrene, anthracene, pyrene, and benzo[a]pyrene, individually and as a consortium. Results showed that 88.8 % phenanthrene was degraded by the bacterial consortium. In the other hand, the 9 individual bacterial strains achieved degradations between 65.6 % and 83.7 % (Xu et al., 2022). Moreover, 92.0 % of anthracene removal was achieved by both the individual strains

and the consortium. Furthermore, the same authors reported that, *Pseudomonas putida* and *Acinetobacter calcoaceticus* achieved higher removal rates of pyrene (around 87 %), while the consortium was able to remove 83.1 %. For benzo[a]pyrene, the authors reported 83.8 % removal efficiency by the consortium, in which removal efficiency by the 9 individual bacterial strains ranged between 52.1 % and 77.9 % (Xu et al., 2022).

Biodegradation of Bzf or Prx is not thoroughly documented. Sui et al. (2016) tested the effect of some parameters on the biodegradation of Bzf ($0.5 - 6 \text{ mg L}^{-1}$) by microbial community presented in an WWTP activated sludge. Results showed that Bzf removal efficiencies decreased with the increase of initial concentration. After 24 h, the communities exposed to 6 mg L^{-1} displayed 43 % of removal, while when exposed to 0.5 mg L^{-1} displayed removal efficiencies of 57 %. The effect of temperature in the removal efficiency was also tested, resulting in higher removal efficiencies (55 %) at 20°C compared to 14 % removal of Bzf when temperature decreased to 10°C . On the other hand, Duarte et al. (2019b) tested the degradation potential of Bzf and Prx (1 mg L^{-1}) by autochthonous enriched cultures, using Douro River estuary sediments and activated sludge from an WWTP as inoculum. Results showed, for both pharmaceuticals, a high removal from the culture medium ($>97\%$), that the authors mainly associated to microbiological processes. Following this study, Fernandes et al. (2020) assembled bacterial consortia using isolated bacterial strains obtained from Duarte et al. (2019b) enrichment cultures with Bzf and Prx. Results demonstrated that the bacterial consortia, composed by bacterial strains isolated from enriched cultures using estuarine sediment as inoculum, were able to remove more than 90 % of Prx and Bzf, in static conditions.

Despite the promising potential demonstrated by microorganisms to degrade / remove contaminants, some factors may influence their degradation ability. Specifically, elevated concentrations of contaminants can be toxic to the degrading microbial community, whereas low contaminant levels may inhibit the activation of bacterial degradation enzymes (Adams et al., 2015). Moreover, the bioavailability of contaminants, meaning the extent to which contaminants adsorb to solid surfaces or become entrapped by molecules within the contaminated medium or diffuse within soil or sediment can be a challenge (Chen & Ding, 2012; Gourlay-Francé & Tusseau-Vuillemin, 2013; Ren et al., 2018). Furthermore, inherent differences in the chemical structures of each compound can lead to differences in biodegradation rates since enzymatic reactions involved in biodegradation are chemically specific (Gómez-Consarnau et al., 2012). Temperature exerts a direct influence on the pace of microbial metabolic processes, thereby impacting microbial activity (Adams et al., 2015; Xu et al., 2022). Moreover, other physical or chemical parameters inherent to the contaminated environment can influence the degradation of the target pollutant (Adams et

al., 2015). Furthermore, the availability of primary carbon sources and nutrients for bacteria cell growth and division are needed to achieve degradation. Still, contaminants can be used by some bacteria as sole carbon sources (Shimizu, 2013), which can contribute to variances in different biodegradation rates for the same pharmaceutical. In addition, biodegradation rates may be improved by previous or long-term contact by the microbial populations with target contaminants, which can enhance the microbial ability to recognize the compound (Fernandes et al., 2021; Smulek et al., 2020). Bearing all of this in mind, microbial degradation potential can vary both in natural environments and in laboratory conditions. Deeper knowledge regarding these factors will allow better engineered microbial solutions to tackle environmental contamination and recover contaminated sites.

Phytoremediation, the use of plants and associate microorganisms to remove, accumulate, and/or degrade organic and inorganic pollutants has also gain attention (Fernandes et al., 2017a). Contaminated sites are remediated through the uptake or degradation of pollutants, primarily facilitated by the plant root system. This system offers an extensive surface area for the absorption and accumulation contaminants as well as of water and nutrients crucial for plant growth (Ecobiol, 2006). Moreover, combination of both phytoremediation and bioremediation processes can be also a suitable strategy for the removal of pharmaceuticals from contaminated sites as the microbial community that inhabits the plant's rhizosphere and rhizosediment can be stimulated by plant root exudates, enhancing the pollutant removal (Ecobiol, 2006; Feng et al., 2017; Fernandes et al., 2017a; Fernandes et al., 2017b; Pilon-Smits, 2005). Fernandes et al. (2015) tested the removal efficiencies of enrofloxacin ($100 \mu\text{g L}^{-1}$) of a system containing an association of a salt marsh plant (*P. australis*) with microorganism and unplanted systems. Results showed that the systems without sediment but containing *P. australis* achieved higher removal efficiencies of enrofloxacin, being only 5 % of the concentration detected in solution. On the other hand, 95 % of added concentration was still detected when analysing the solution of the unplanted system. In addition, systems with sediments showed removal rates of enrofloxacin higher than 95 % in planted systems, while in unplanted systems, lower removal rates were observed, until nutrients were added. In turn, the removal of enrofloxacin increase approximately to 65 %. Thus, phytoremediation can be used as a complementary technology along with other methods or as an alternative to traditional ones.

The potential of salt marsh plants to remediate contaminated sites has been thoroughly studied (Almeida et al., 2011; da Silva et al., 2014; Fernandes et al., 2017b; Oliveira et al., 2014; Windham et al., 2003) and showed that plant species like *Juncus maritimus*, *P. australis*, *Spartina patens*, *Triglochin striata*, and *Spartina alterniflora* have high potential to

be used in remediation technologies to recover estuaries contaminated with metals and hydrocarbons.

Recently, several studies showed the potential of *P. australis* plants to mitigate environments contaminated with CECs (Carvalho et al., 2012; Fernandes et al., 2015; Sauvêtre & Schröder, 2015). In fact, constructed wetlands, with *P. australis* in laboratory conditions, were able to remove between 94 % and 95 % of the initial concentration of enrofloxacin (Carvalho et al., 2012; Fernandes et al., 2015) and 75 % of tetracycline (Carvalho et al., 2012), two different veterinary antibiotics. Furthermore, Sauvêtre and Schröder (2015) showed that *P. australis* was also able to remove 90 % of the initial concentration (5 mg L⁻¹) of an antiepileptic drug (carbamazepine) from individual pots containing perlite and grown in semi-hydroponic conditions.

Bearing this in mind, both bioremediation and phytoremediation technologies revealed to be promising tools for the removal of pharmaceuticals from the environment and ecosystem recovery. However, further research is imperative to optimize these methods, expand their applicability, and address the evolving challenges posed by emerging pharmaceutical compounds in the environment.

3. Materials and methods

3.1. Reagents and materials

Prx and Bzf were obtained from Enzo Life Science and Sigma-Aldrich, respectively. All solvents and reagents used to perform analytical analysis were HPLC grade or equivalent.

To avoid contamination during the experiments, all the materials were sterilized. Biological sterilization was performed by autoclaving the material, in a 20 min cycle with a temperature of 120 °C. On the other hand, chemical decontamination was achieved by submerging the material in a chloride acid bath (10 % (v/v)) for 24 h, being afterwards washed with deionized water.

3.2. Sampling and assembly of the enriched slurries

In a previous study, *Phragmites australis* and sediment samples were collected in Lima River estuary, as described in detail by Cunha (2022). The sediment was used as inoculum to assemble an enrichment experiment. Briefly, 50 g of sediment were added to 1 L flasks containing 200 mL of a mineral-salts medium (MM) (full constitution in supplementary material). Each flask was doped with 1 mg L⁻¹ of the target pharmaceutical, namely Bzf or

Prx, and supplemented with 500 mg L⁻¹ of sodium acetate as co-substrate. The flasks were incubated in static and dark conditions, at room temperature (23 °C). This experiment was carried out for 10 cycles of 15 days each, during a total of 20 weeks (Figure 3). The enrichment process was conducted in triplicate for each pharmaceutical. In the end of each cycle, 5 mL from each enriched culture were collected and centrifuged at 3046 rpm (VWR MEGA STAR 600R) for 15 minutes. The resultant pellet was collected to characterize the enriched microbial community structure using next-generation sequencing (NGS), while the supernatant was collected to assess the removal of Prx and Bzf through high performance liquid chromatography (HPLC) with a diode array detector (DAD). The removal efficiency was calculated using the ratio between the concentration in the supernatant and the initial concentration of each pharmaceutical, this being the nominal value of 1 mg L⁻¹. All samples were storage at -20 °C until further analyses.

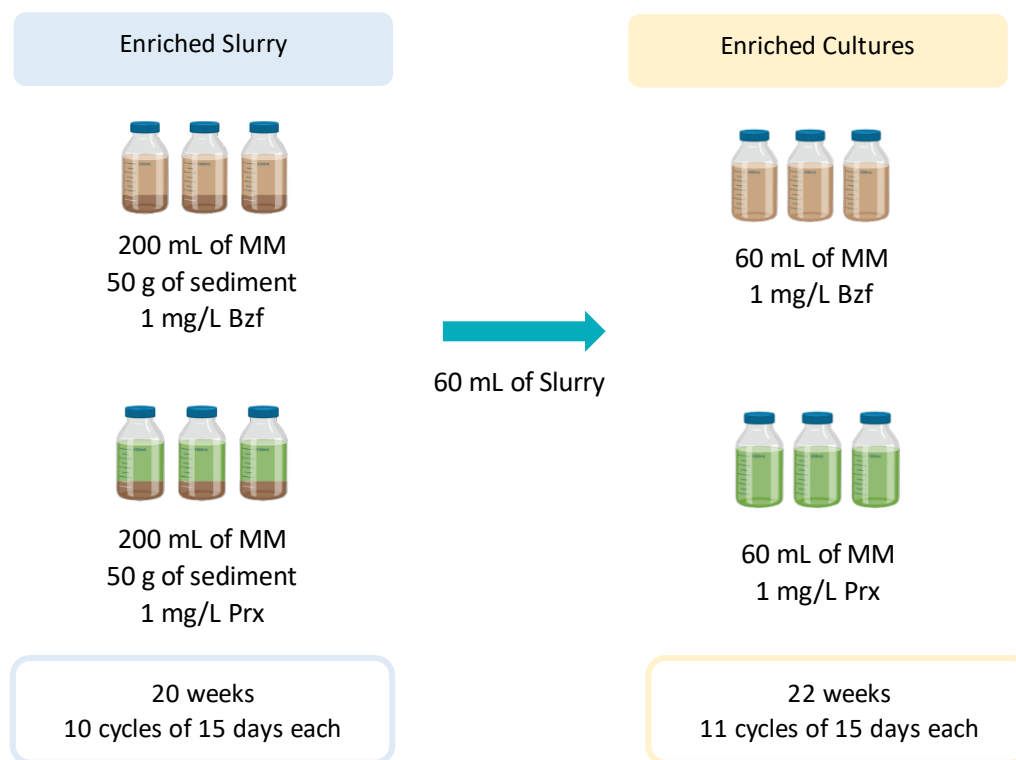


Figure 3 - Experimental design of the operation of the enriched slurry and the enriched culture maintenance.

3.3. Isolation of bacterial strains from the enriched cultures

In the end of 10th cycle, the enriched cultures were spread in several tenfold (up to 10⁻⁶) dilutions in Plate Count Agar (PCA) and MM doped with 1 mg L⁻¹ of either Prx or Bzf. After a 48 h incubation, at 28 °C, bacterial colonies that displayed distinct morphologies and characteristics were visually identified and purified through successive streaking on the same media plates where they initially appeared (Figure 4). After that, each bacterial strain was cryopreserved in 25 % of glycerol mixed with saline solution (0.85 %) or mixed with liquid PCA media and stored at -80 °C.

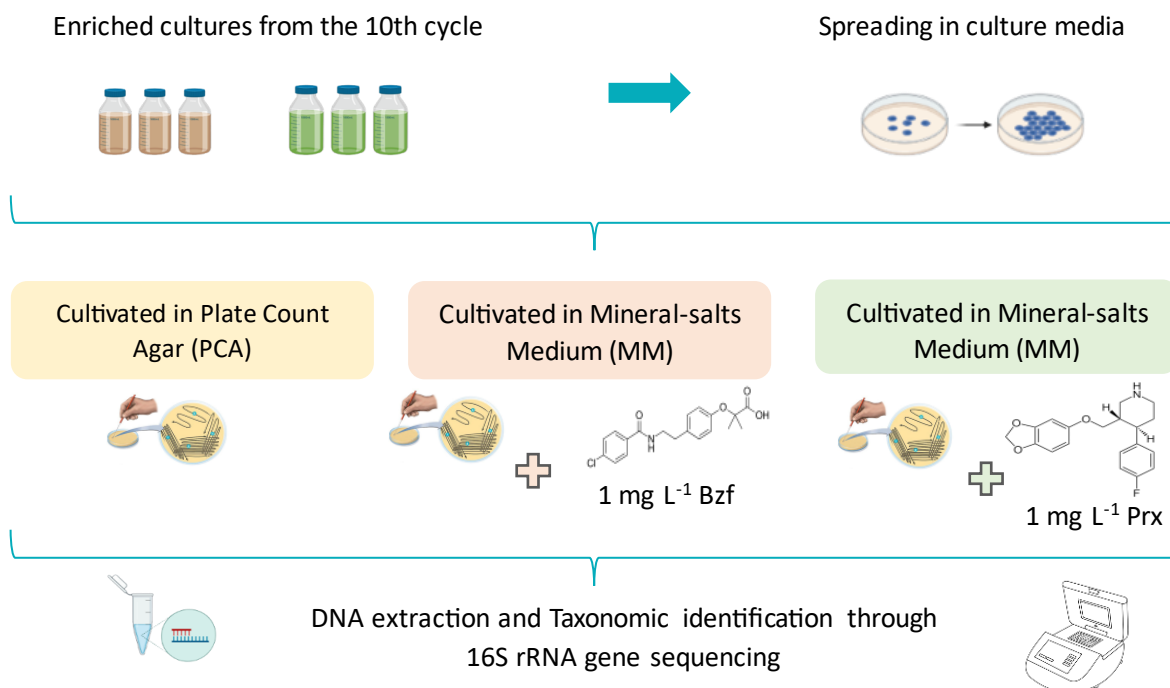


Figure 4 - Experimental design of the isolation and purification of bacterial strains from the enriched cultures.

3.4. Maintenance of the enriched cultures

After the isolation of the culturable strains of the microbial community, each enriched culture was maintained in an active state to continuing following and monitoring the temporal evolution of the microbial community structure as well as follow the removal efficiency of each pharmaceutical (Figure 3).

The bacterial cultures were maintained in triplicates (three replicates for Prx and three replicates for Bzf) in 250 mL flasks, in static and dark conditions, at room temperature. The maintenance was carried out for 11 cycles of 15 days each, during a total of 22 weeks. Cultures operation and maintenance as well as sampling was performed as described in the sub-chapter 3.2.

3.5. Taxonomic identification of purified bacterial strains

The taxonomic identification of the bacterial strains isolated involved a multi-step process, starting with the extraction of DNA from each isolate using the E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA), following the manufacturer's protocol. Then, the hypervariable regions V1–V9 of the 16S rRNA gene were amplified using a set of

universal primers, namely 27 F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT- 3'). For that, polymerase chain reaction (PCR) was performed in 10 µL aliquots, containing 1–3 µL of DNA template, 1 µL of each primer (2 mM), and 5 µL of MyTaq Mix 2x (Bioline, Meridian Bioscience). Additionally, to complete the required reaction volume, sterile water was added to the samples with 1-2 µL of DNA template. PCR program was performed as follows: initial denaturation at 95 °C for 2 min, followed by 30 cycles at 95 °C for 30 s, 55 °C for 90 s and 72 °C for 1 min, and a final extension at 72 °C for 7 min. PCR products were visualized in a 1.5 % electrophoresis agarose gel. Purification and sequencing were performed by GenCore at I3S (Instituto de Investigação e Inovação em Saúde), in Porto, Portugal. Raw sequences were then analysed using Geneious 2023.2.1 software (Biomatters Ltd, Auckland, New Zealand). and the resulting consensus sequences were submitted to GenBank for taxonomic identification (Nucleotide Blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) using both nucleotide collection (nr/nt) and rRNA/ITS database (16S ribosomal RNA sequences (Bacteria and Archaea). EzBioCloud database (Yoon et al., 2017) was also used to compare the results between different databases.

3.6. Microbial community characterization

DNA extraction of initial sediment and of each enriched culture from each cycle was performed using the DNeasy PowerSoil Pro Kit (QIAGEN, Merck KGaA, Darmstadt, Germany), following the protocol provided by the manufacturer. Quantification of the DNA samples was conducted using a Qubit™ 3.0 fluorometer (Invitrogen, Thermo Fisher Scientific) with Qubit™ dsDNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific).

Characterization of the initial sediments and enriched microbial consortia was assessed through high-throughput sequencing of the V4-V5 hypervariable region of the 16S rRNA gene using 515YF (5' - GTGYCAGCMGCCGCGGTAA - 3') and Y926R-jed (5' - CCGYCAATTYMTTTRAGTTT - 3') primers, at Genoinseq in Cantanhede, Portugal. The initial PCR reaction was performed using 2.5 µL of DNA template diluted in a total volume of 25 µL (1:10). The PCR was performed as follows: 3 min denaturation step at 95 °C, followed by 25 cycles of 98 °C for 20 s, 50 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min. Negative PCR controls were integrated into all amplification procedures. Subsequently, PCR products underwent one-step purification and normalization procedures using the SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al., 2017). Afterward, these purified products were pooled and subjected to paired-end

sequencing using the Illumina MiSeq® sequencer with the MiSeq Reagent Kit v3 (600 cycles), following the manufacturer's protocols (Illumina, San Diego, CA, USA) at the Genoinseq in Cantanhede, Portugal.

Sequence data processing was conducted at Genoinseq (Cantanhede, Portugal). Initially, raw reads were extracted from the Illumina MiSeq® System in fastq format. Subsequently, a quality-filtering step was implemented using PRINSEQ version 0.20.4 (Schmieder & Edwards, 2011) to eliminate sequencing adapters, trim bases with an average quality below Q25 within a 5-base window, and discard reads with fewer than 100 bases. The forward and reverse reads were then merged by overlapping paired-end reads through AdapterRemoval version 2.1.5 (Schubert et al., 2016), using default parameters.

To generate the amplicon sequence variant (ASV) table, DADA2 pipeline was applied to the sample dataset. This analysis was conducted using R software (version 4.1.0) (R Core Team, 2019) together with the *dada2* package (version 1.21.0). Taxonomic identification was performed using SILVA database, version 138.1 (Quast et al., 2013).

3.7. Downstream data analysis

Undesirable lineages, “Chloroplast”, “Mitochondria” and “Eukaryota” were excluded from the dataset. With R software (version 4.1.0), observed ASVs and Shannon alpha diversity indexes (Spellerberg & Fedor, 2003) were obtained using the package *phyloseq* (v1.38.0). Beta diversity was visualized using non-metric multidimensional scaling (NMDS), based on Bray–Curtis dissimilarities (Bray & Curtis, 1957) using the packages *phyloseq* (v1.38.0), *ggplot2* (v3.4.2), *vegan* (v2.6–4), *dplyr* (v1.1.2), *scales* (v1.2.1) and *reshape2* (v1.4.4) (R software). To analyse the significant differences in the microbial community structure between enrichments (Prx and Bzf) or over time, the Anosim test was conducted, using R version 4.1.0 and *vegan* (v2.6–4).

To analyse the taxonomic profile of the bacterial communities, a first processing step that involved transforming the data into relative abundance values was applied for each sample using the R package *phyloseq* (version 1.38.0). At phylum and genus level, only taxa with more than 2 % was considered.

3.8. Analytical methods

3.8.1. Solid phase extraction (SPE)

Since MM is composed essentially of mineral salts, a solid phase extraction (SPE) was performed to clean the matrix of the samples collected from the enriched cultures before chromatographic analysis. To start, 1 mL of each sample was previously diluted in 9 mL of double deionised water. The cartridges Oasis HLB (30 μ m, 3 cc, 60 mg) from Waters Corporation (Millford, MA, USA) were initially pre-conditioned by loading 5 mL of methanol, followed by 5 mL of deionized water using a vacuum manifold system (Supelco, Spain) coupled to a vacuum pump. Afterwards, the samples were loaded into the cartridges, followed by 5 mL of a mixed solution composed by H₂O/MeOH (95:5 v/v). Then, the cartridges were left to dry for 30 minutes, and eluted with 5 mL of a solution of methanol/formic acid (96:4 v/v). The eluted samples were evaporated for 3 hours at 45 °C using an evaporator (Rotational Vacuum Concentrator RVC 2-18 CDplus) (Christ, Germany) and resuspended in 0.5 mL solution of methanol/H₂O (70:30 v/v).

Tests were carried out to assess the retention rate of each pharmaceutical by the HLB cartridges. Known concentrations of Bzf and Prx were passed through the cartridges, following the same methodology. Retention rates values by the cartridges were 87 % (sd – 5.4 %) and 81 % (sd – 7.8 %) for Bzf and Prx, respectively and no correction of the concentration determined for the enriched cultures was done.

3.8.2. Assessment of the concentration of the selected pharmaceuticals

To assess the removal efficiency of the microbial communities, the concentration of the target pharmaceuticals, after the SPE process, were analysed after each cycle using an HPLC VWR/HITACH equipment (HPLC-DAD Elite LaChrom) coupled with a diode array detector (DAD) and a 150 mm x 4.6 mm C18 Kinetex column with a particle size of 2.6 μ m (Phenomenex, UK). The target pharmaceuticals were detected using a gradient of two eluents, water/formic acid (99:1 v/v) and acetonitrile (ACN), with a flow rate of 1 mL min⁻¹. The gradient was implemented as follows: 100 % of water/formic acid (99:1 v/v) for 2 min; 6 min until gradually reaching 100 % ACN; 2 min until gradually reaching 100 % of water/formic acid (99:1 v/v), maintaining this composition for 2 min. A volume of 20 μ L of each sample was injected into the system, being the outcome monitored at λ = 298 nm for Prx and at λ = 252 nm for Bzf. A calibration curve with aqueous standard solutions, ranging from 0.1 to 2.0 mg L⁻¹, of both Prx and Bzf was performed to quantify the compound. To do so, a known quantity of each compound was dissolved in methanol to prepare stock

standard solutions, which were then used to prepare standard solutions for each compound, using the same solvent of the reconstitution step.

3.9. Statistical analysis

Statistical analysis regarding the removal efficiency of Bzf and Prx was carried out using the software R version 4.0.0 (R Core Team, 2020). Significant differences among samples were evaluated through a parametric Student's t-test, using mean values and corresponding standard deviations of the replicates ($n = 3$). Significant differences were assumed for p-value below or equal to 0.05 (confidence level of 95 %).

Statistical analysis to assess the effects of each pharmaceutical and the temporal evolution in the prokaryotic community structure was carried out using the software R version 4.0.0 (R Core Team, 2020). Significant differences throughout time and effects of each pharmaceutical were evaluated through robust non-parametric hypothesis-test ANOSIM analysis (Analysis of (dis)similarities).

4. Results

4.1. Taxonomic identification of bacterial strains

A total of 46 bacterial strains were isolated from the two enriched cultures, started by Cunha (2022). This isolation was accomplished by using MM medium doped with the respective pharmaceutical (the same medium that was used for the enrichment process), and PCA, a non-selective medium, relatively rich in nutrients. Higher number of bacterial strains were isolated from PCA, in which, from the total of 46 bacterial strains, 30 were isolated from PCA. More specifically, 15 bacterial strains were isolated from the enriched slurry doped with Prx, and 15 bacterial strains were isolated from the enriched slurry doped with Bzf. In contrast, from the MM medium doped with the respective pharmaceutical, only 16 bacterial strains were isolated and purified, in which 9 bacterial strains were retrieved from the enriched culture doped with Bzf, and 7 bacterial strains were retrieved from the enriched culture doped with Prx (Table S1).

Taxonomic identification of the bacterial isolates through 16S rRNA gene sequencing showed that most of the bacterial strains belongs to the phylum Pseudomonadota (corresponding to near 87 %), in which bacterial strain from the phyla Actinomycetota and Bacteroidota were also retrieved (Figure 5).

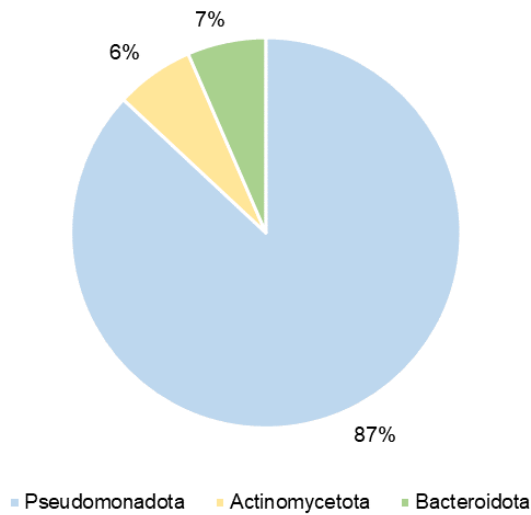


Figure 5 - Relative Abundance of bacterial phyla within the microbial cultures pre-enriched with Prx and Bzf.

At the genus level, the results showed that 13 different bacterial genera were isolated, being *Pseudomonas* the most abundant genus in the Bzf enriched cultures. Moreover, in the Prx enriched culture, the genera *Pseudomonas* and *Alcaligenes* were the most abundant (Figure 6).

The genera *Xanthobacter*, *Rhodococcus*, *Ancylobacter*, *Achromobacter* and *Kaistella* were only isolated from the Bzf enriched cultures. On the other hand, the genera *Comamonas*, *Chryseobacterium*, *Stenotrophomonas* and *Pseudochrobactrum* were only isolated from Prx enriched cultures. Moreover, the genera *Microbacterium* and *Brucella* were isolated in both enriched cultures.

Taxonomic analysis using the NCBI 16S ribosomal RNA sequences (Bacteria and Archaea) database revealed that bacterial isolate *Kaistella* sp. A22, affiliated with the phylum Bacteroidota, is a potential new specie since the similarities between this strain and the closest strains presented in the NCBI 16S ribosomal RNA sequences (Bacteria and Archaea) database is below 98.7 (Yarza et al., 2014).

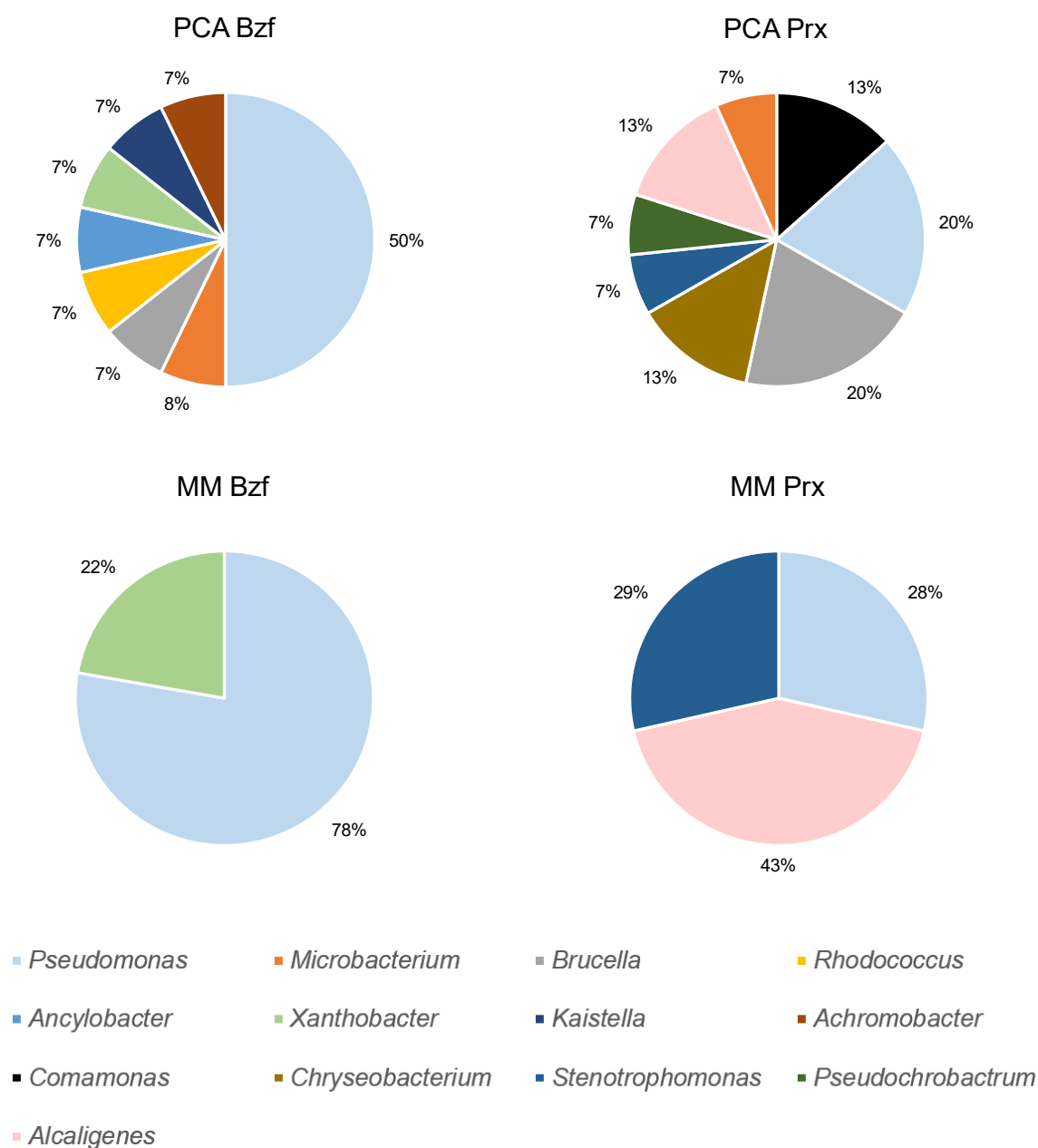


Figure 6 - Relative Abundance of bacterial genera within the microbial cultures pre-enriched with Prx and Bzf.

PCA Bzf – bacterial strains isolated in PCA media from the Bzf enriched cultures; PCA Prx – bacterial strains isolated in PCA media from the Prx enriched cultures; MM Bzf – bacterial strains isolated in MM media from the Bzf enriched cultures; MM Prx – bacterial strains isolated in MM media from the Prx enriched cultures.

4.2. Pharmaceutical removal from the enriched slurries and maintenance cultures

The concentration of Prx and Bzf in each enriched slurry and in each culture under maintenance, after each two-week cycle was assessed through HPLC-DAD. Although all

the replicas from each enriched slurries and maintenance cultures evolved independently of each other and may present different responses to the presence of the pharmaceuticals over time, statistical differences were calculated using the mean values. Nevertheless, the outcomes from individual replicates can be analysed and discussed separately.

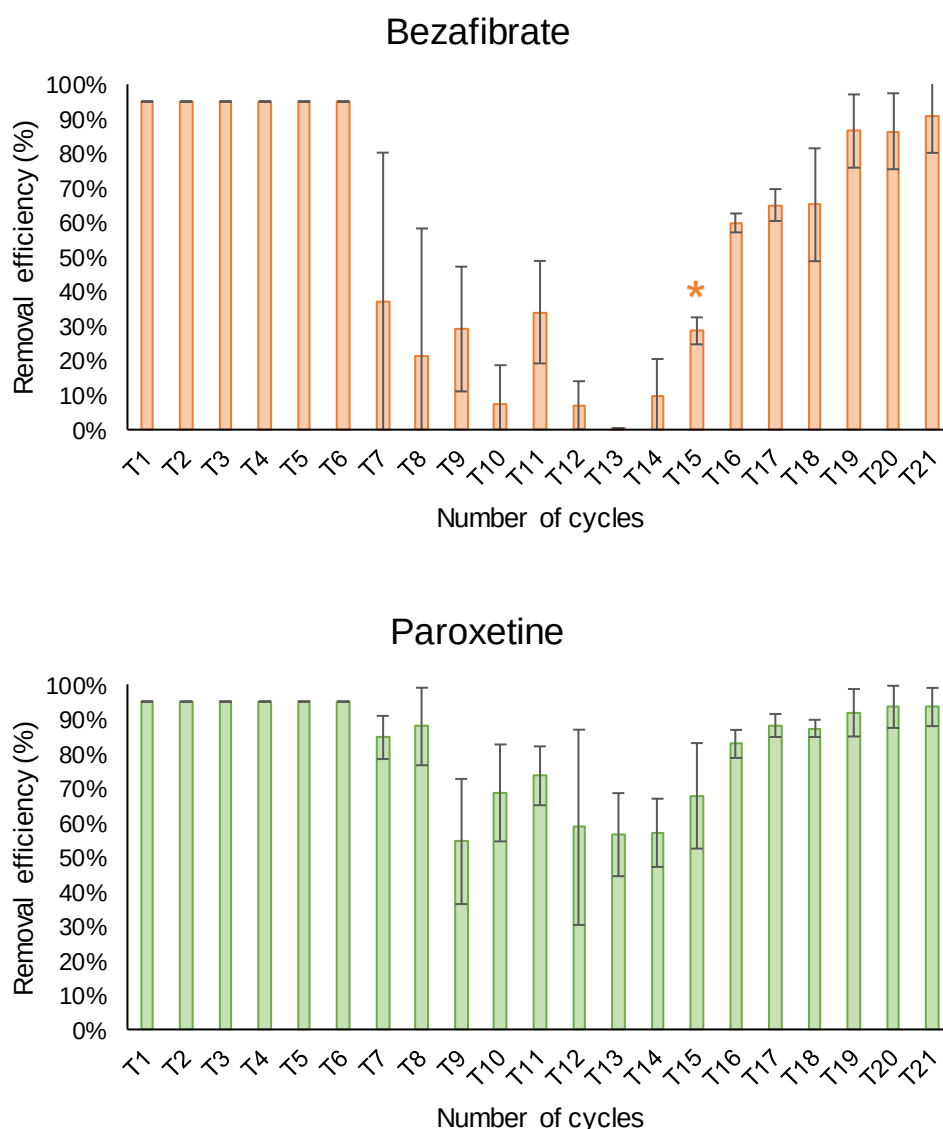


Figure 7 - Removal efficiency of Bzf and Prx by the enriched slurries (T1 - T10) and enriched cultures (maintenance T11- T21) derived from estuarine sediment, along the 21 cycles. Results are expressed as the mean of triplicates and error bars are relative to standard deviation. * – significant differences between the current cycle and the next ($p < 0.05$).

Regarding the enriched slurry, removal efficiency higher than 95 % were observed until the 6th cycle (Figure 7). It is important to note that considering the method's detection limit of 0.05 mg L⁻¹, it was assumed that the removal efficiency exceeded 95 %. During the transition from the 5th to the 6th cycle, an issue occurred in the laboratory involving the

malfunction of the air conditioning system, leading to an increase of the room temperature (from 23 °C to 28 °C – 30 °C, for 11 weeks). After this issue, a noticeable reduction in biomass within each flask was observed. Consequently, the removal of Bzf after the 6th cycle showed a decrease in efficiency, in which the removal efficiency dropped drastically. In fact, a major decrease was evident for the replicate 2 in the 7th cycle and for both replicates 1 and 2 of the 8th cycle (Figure 8). Comparing the behaviour demonstrated by each replica, it was observed that replica 3 exhibited a more robust response to the stress induced by change of temperature, between the 6th and 11th cycle, compared to the other two replicates, however, this replicate displayed a decrease on the removal efficiency in the cycle 12th (2nd cycle of maintenance) (Figure 8). After the 15th cycle, the enriched cultures were able to successfully readjust and significantly increase their removal efficiency ($p < 0.05$; Figure 7), in a gradually manner, reaching approximately 85 % removal in the final cycles (Figure 8).

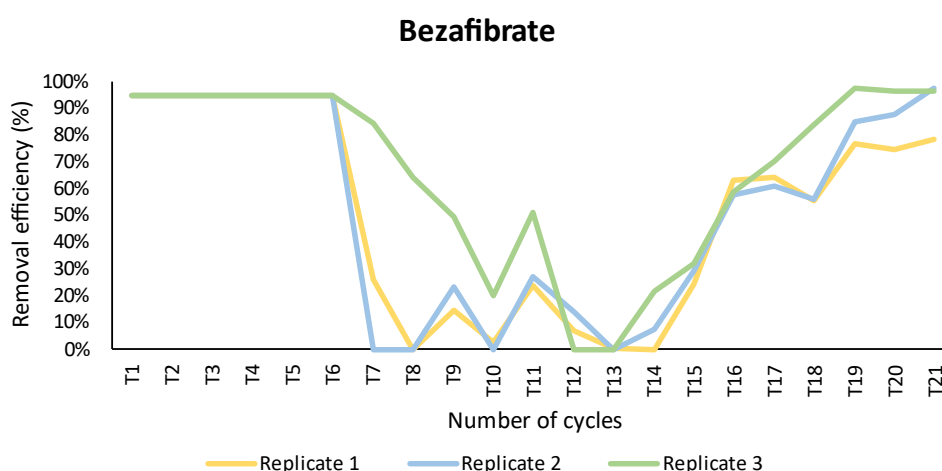


Figure 8 - Removal efficiency of Bzf by the enriched slurries (T1 - T10) and enriched cultures (maintenance T11- T21) derived from estuarine sediment, along the 21 cycles. Independent replicates are displayed in different colours.

Regarding Prx enriched slurries, removal efficiency surpassing 95 % were consistently observed up to the 6th cycle. As mentioned, considering the method's detection limit of 0.05 mg L⁻¹, it was assumed that the removal efficiency exceeded 95 %. As described above, an unexpected increasing of temperature had an impact on the enriched slurries, as a reduction in biomass within each flask was observed. The effects on the enriched slurries were only visible after the 8th cycle (Figure 9). In fact, all three replicas displayed high removal efficiencies (averaging around 85 %) until the end of 8th cycle. In the 9th cycle, a decrease on the removal efficiency was observed, with the lowest removal efficiency registered in the 12th cycle (2nd cycle of the maintenance). The most robust response to the

stress induced by the increase of temperature was displayed by the replicate 1, which registered removal efficiencies of 62 % and 55 % during 13th and 14th cycles of the maintenance cultures, respectively (Figure 9). Replicate 1 managed to successfully readjust and increase the removal efficiencies, consistently higher than 85 %, right after the 16th cycle of the enriched microbial cultures. As for the replicas 2 and 3, a slight loss in removal efficiency was observed during the 7th and 8th cycles, in which a major decrease on the removal efficiency (45 %) was observed in the 9th cycle. After this cycle, both replicas displayed an increase in removal efficiencies, apart from replicate 2 during the 12th cycle, managing to consistently achieve removal efficiencies higher than 84 % after the 17th cycle. The only exceptions were observed in the 12th cycle for replicate 2, where another major drop of efficiency was observed (Figure 9).

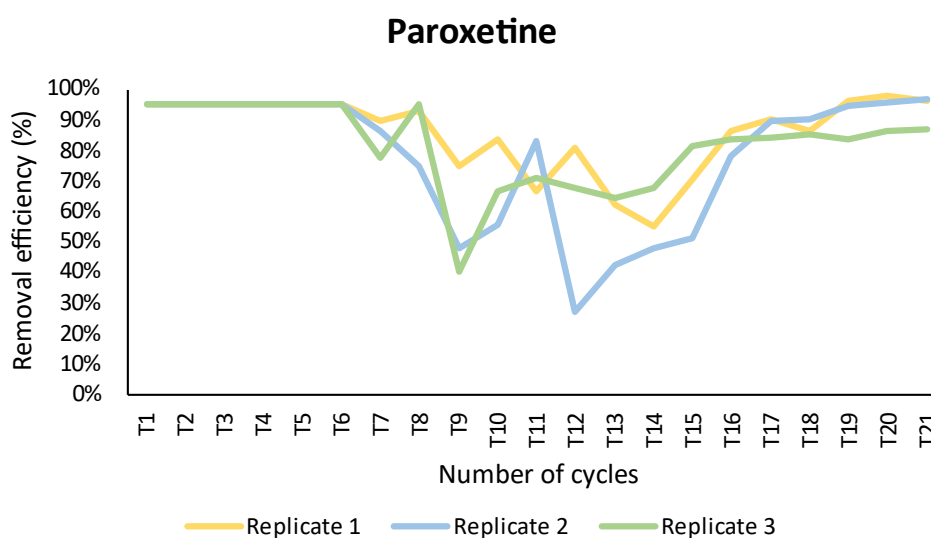


Figure 9 - Removal efficiency of Prx by the enriched microbial slurries and enriched microbial cultures derived from estuarine sediment, along the 21 cycles. Independent replicates are displayed in different graphics.

The results showed that both cultures were sensitive to the change of temperature. The response among replicates were different showing that, despite of being in the same conditions, the performance among replicates changed overtime. Moreover, the Bzf cultures displayed a higher decrease in the removal efficiency. This can be related with the microbial community composition, as different communities can display different responses to stress.

4.3. Prokaryotic community dynamics

The procaryotic community dynamics and structure during the enrichment process was assessed by NGS sequencing. The results presented in this chapter are until the 10th cycle (when was performed the bacterial isolation and started the maintenance period) as the NGS data regarding further cycles are still being processed (ongoing sequencing). With this analysis, it was possible to evaluate the dynamics of the enriched slurries during the enrichment process (10 cycles) and how Prx and Bzf can change and select the community structure.

In the available sequencing dataset (first 10 cycles, corresponding to the enriched slurries), the minimum read count per sample, which represents the number of sequences remaining after trimming, was 33,820. The mean read count per sample was 53,384, and the maximum read count observed in a single sample was 103,818. The comprehensive list of read counts per sample can be found in Table S2.

4.3.1. Alpha Diversity

To evaluate the alpha diversity, observed ASVs and Shannon index were calculated for each sample, as represented in Figure 10. The results revealed that initial sediment collected at the Lima River estuary presented a higher observed ASVs and the Shannon index comparing with both microbial slurries doped with Bzf or Prx, throughout the time. Results also showed that the presence of both pharmaceuticals decreased the abundance and diversity throughout time of experiment. This result was more evident in Shannon index, as for both enriched slurries, this index decreased in each cycle, while the Observed ASVs decreased in the 2nd and 4th cycles but stabilized after that. These results indicates that the presence of the pharmaceutical influenced the abundance and diversity of the community present in the sediment used in these experiments, pointing to a community selection overtime.

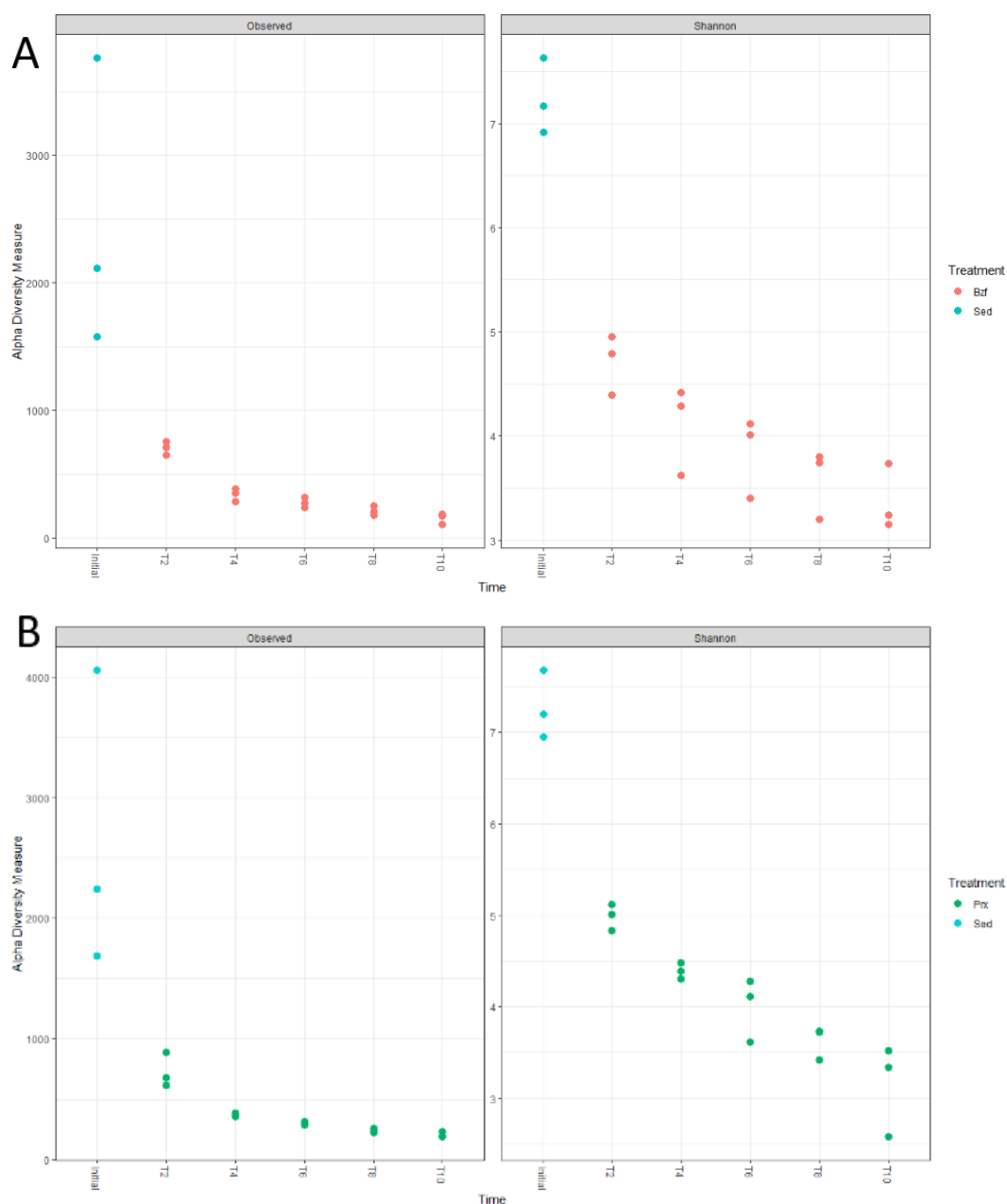


Figure 10 - Alpha diversity represented by the Observed ASVs and the Shannon diversity index for the initial sediments and the enriched slurries over time, when exposed to Bzf (A) or Prx (B) (cycles T2 – T10)

4.3.2. Beta Diversity

The enrichment process using different pharmaceuticals can lead to different changes in the procaryotic community. In addition, during an enrichment process, it is expected that changes may occur in the community since this process seeks to enrich the procaryotic community with the ability to degrade (or in some cases resist) to the imposed conditions. Thus, beta diversity in each enriched slurry was evaluated using non-metric

multidimensional scaling analysis (NMDS) with Bray-Curtis dissimilarity distances (Figure 11). NMDS analysis showed that, for both pharmaceuticals, a high community dissimilarity between the initial sediment collected at the Lima River estuary and the enriched slurry samples collected after each cycle. Moreover, the procaryotic community structure changed over time, a pattern that was observed for both enriched slurries. Furthermore, the results also showed that there were no clusters formed, not even between replicates of the same treatment, suggesting and confirming the independent evolution of the microbial communities (Figure 11).

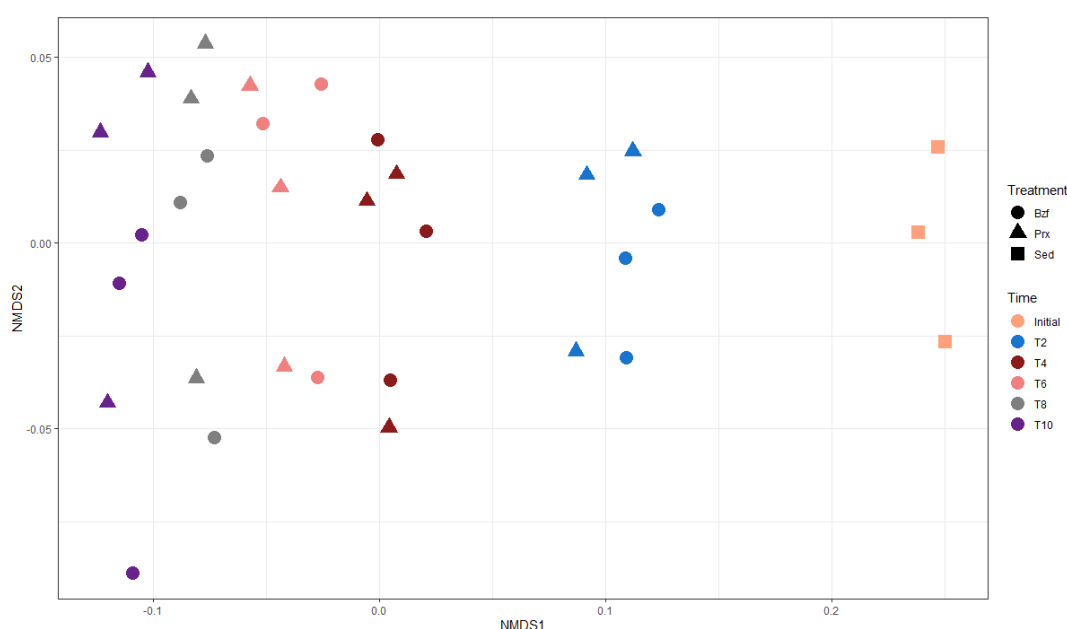


Figure 11 - Beta-diversity represented by a Non-metric Multidimensional Scaling (NMDS) plot with Bray-Curtis dissimilarity distances of the enriched slurries over time, when exposed to Bzf or Prx (cycles T2 – T10).

ANOSIM analysis (Analysis of (dis)similarities) (Clarke, 1993) was performed to assess the factors responsible for the changes in the procaryotic community structure. ANOSIM analysis showed that the pharmaceuticals used in this study had a significant effect on the shape of the procaryotic community of the sediments, as shown in the table 1. These findings strongly indicate that different pharmaceuticals induced a notable shift in the composition of the microbial communities. The ANOSIM test results were in accordance with the results obtained from both alpha and beta diversity analysis, revealing a slight but noteworthy statistical difference when comparing microbial communities at different cycles throughout the experiment (Table 1).

Table 1 - One-way ANOSIM analysis for the pharmaceutical and time effect on the prokaryotic community structure (9999 permutations).

Factor	R2	Pr (>F)
Pharmaceutical	0.9367	0.0002
Time	0.644	0.0001

4.3.3. Characterization of the prokaryotic communities

Taxonomic profile of the prokaryotic communities, in the initial sediment and in the enriched slurries throughout the experiment, was performed at phylum and genus level (or prior taxonomic attribution).

At a phylum level, the results showed that the phyla Pseudomonadota, Actinomycetota, Bacteroidota Verrucomicrobia, Planctomycetota, Desulfobacterota, Chloroflexi, and Acidobacteriota were presented in the initial sediment samples, with a relative abundance higher than 2 % (Figure 12 and 13). Regarding the Bzf enriched slurries, after only 2 cycles (4 weeks), the conditions imposed by the enrichment process had an effect on the procaryotic community structure. The phylum Pseudomonadota was the most dominant, followed by the phylum Bacteroidota (Figure 12). Moreover, Desulfobacterota and Bacillota phyla had a relative abundance greater than 2 % in all replicates in all cycles. Looking with more detail, the phyla Spirochaetota and Campylobacterota appeared in most replicates through time with a relative abundance greater than 2 %, in which Campylobacterota revealed to have a higher abundance with a longer exposure time (after 8th cycle). After the 4th cycle, Synergistota also appeared with a relative abundance higher than 2 %. In contrast with the sediment samples, Actinomycetota showed a relative abundance higher than 2 % only on replicate 3 of the 6th cycle and replicate 1 of the 10th cycle.

As for the Prx enriched slurries, after the first 2 cycles, the phylum Pseudomonadota was the most abundant, followed by the phylum Bacteroidota (Figure 13). As described for the Bzf slurries, the phyla Synergistota, Chloroflexi, and Spirochaetota were present in most of the replicates throughout time. Desulfobacterota and Bacillota phyla had a relative abundance higher than 2 % in all replicates of all the cycles. Once more, and in contrast with the sediment samples, Actinomycetota was only represented in replicate 2 of the 6th cycle and in replicates 1 of both 8th and 10th cycle.

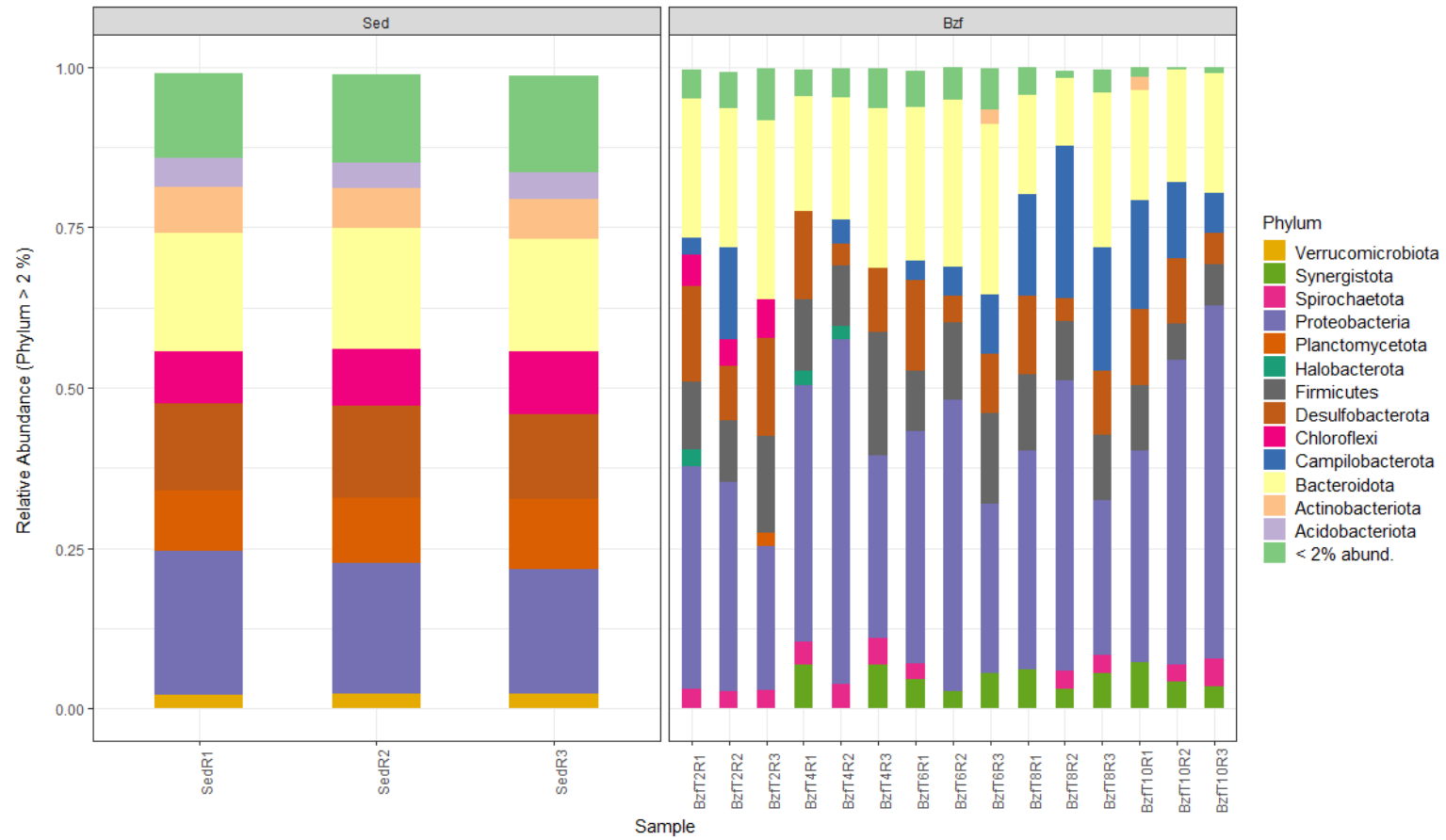


Figure 12 – Temporal evolution of the taxonomic profile at phylum level of the enriched slurries when exposed to BzF (relative abundance > 2 %, represented for the 2nd - 10th cycles).

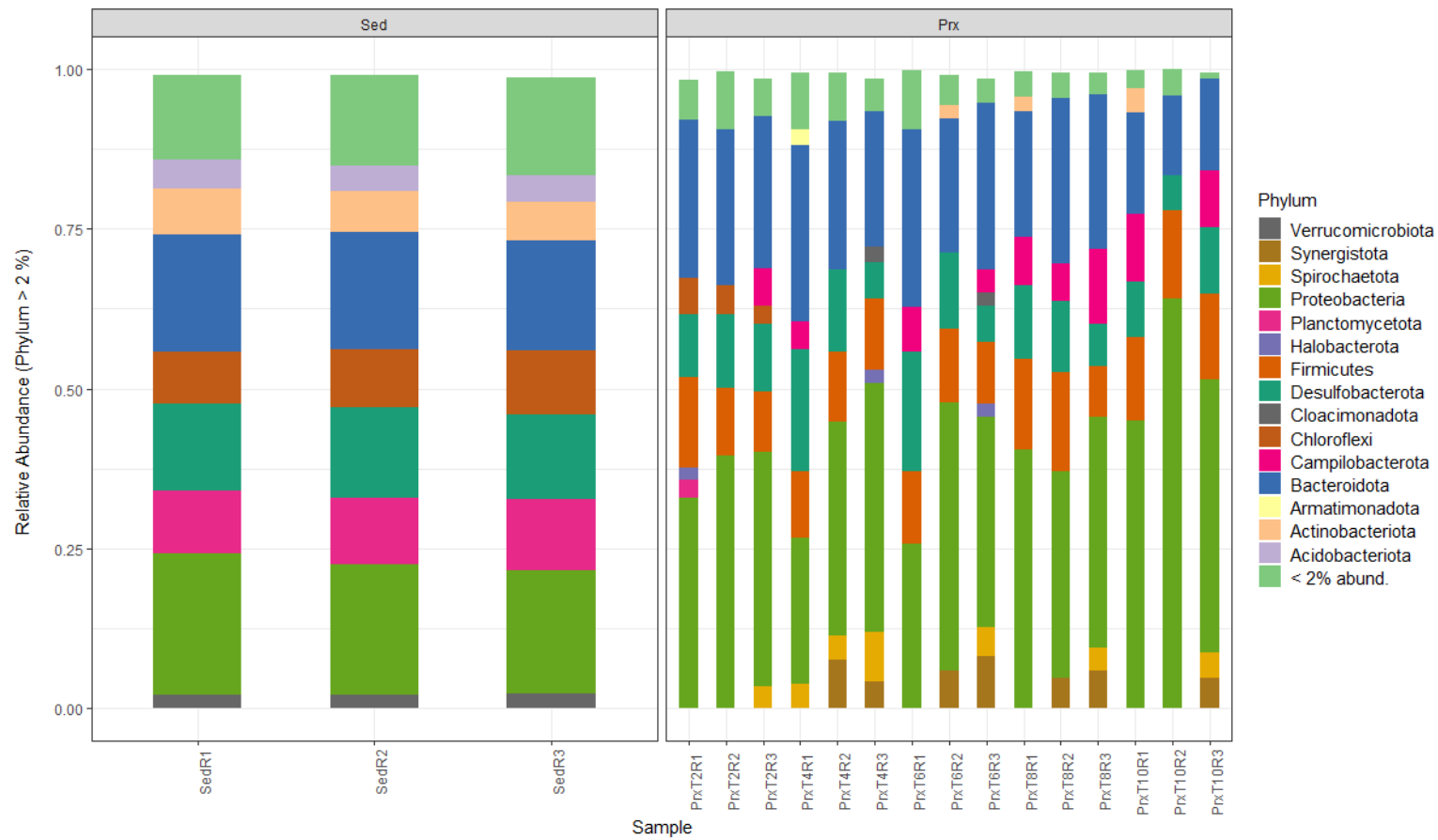


Figure 13 – Temporal evolution of the taxonomic profile at phylum level of the enriched slurries when exposed to Prx (relative abundance > 2 %, represented for the 2nd - 10th cycles)

Looking at a genus level, the results suggested that the presence of Bzf and Prx had an effect on the procaryotic community structure throughout time, being these communities very dynamic (Figure 14 and 15). The replicates of both enriches slurries showed different taxonomic profiles with a different evolution over time. Moreover, a significant percentage of the relative abundance was below 2 %. The initial sediment was composed mainly by genera *Woeseia* and *Illumatobacter*, which were present in the 3 replicates. In the Bzf enriched slurries, the genus *Arcobacter*, while initially underrepresented, demonstrated the capacity to proliferate and was one of the most dominant genera by the end of the 10th cycle (Figure 14). Moreover, both *Xanthobacter*, *Sphaerochaeta* and *Desulfovibrio* were consistently present in most replicates over time. Furthermore, after 4th cycle, the genus *Christensenellaceae* persisted in replicates 1 and 3 throughout most cycles (Figure 14).

Regarding the Prx enriched slurries, there was no clear pattern throughout time. The genus *Arcobacter* was consistently represented in most replicates across all cycles (Figure 15). The genus *Xanthobacter* appeared in both replicates 2 and 3 from 6th cycle until 10th cycle. Furthermore, *Lentimicrobium* and *Desulfovibrio* genera were present in most of the cultures over time. The genus *Methylobacillus* was only dominant in the replicate 1 of the 8th cycle and the genus *Alcaligenes* was only dominant in the replicate 2 of the 10th cycle. Moreover, the genus *Parapusillimonas* was present during the first 6th cycles while the genus *Christensenellaceae* was present in high abundance in the replicate 1 and 2 after 6th cycle. Furthermore, the genus *Stenotrophomonas* demonstrated the ability to thrive in early stages but registered a decrease in relative abundance throughout time (Figure 15).

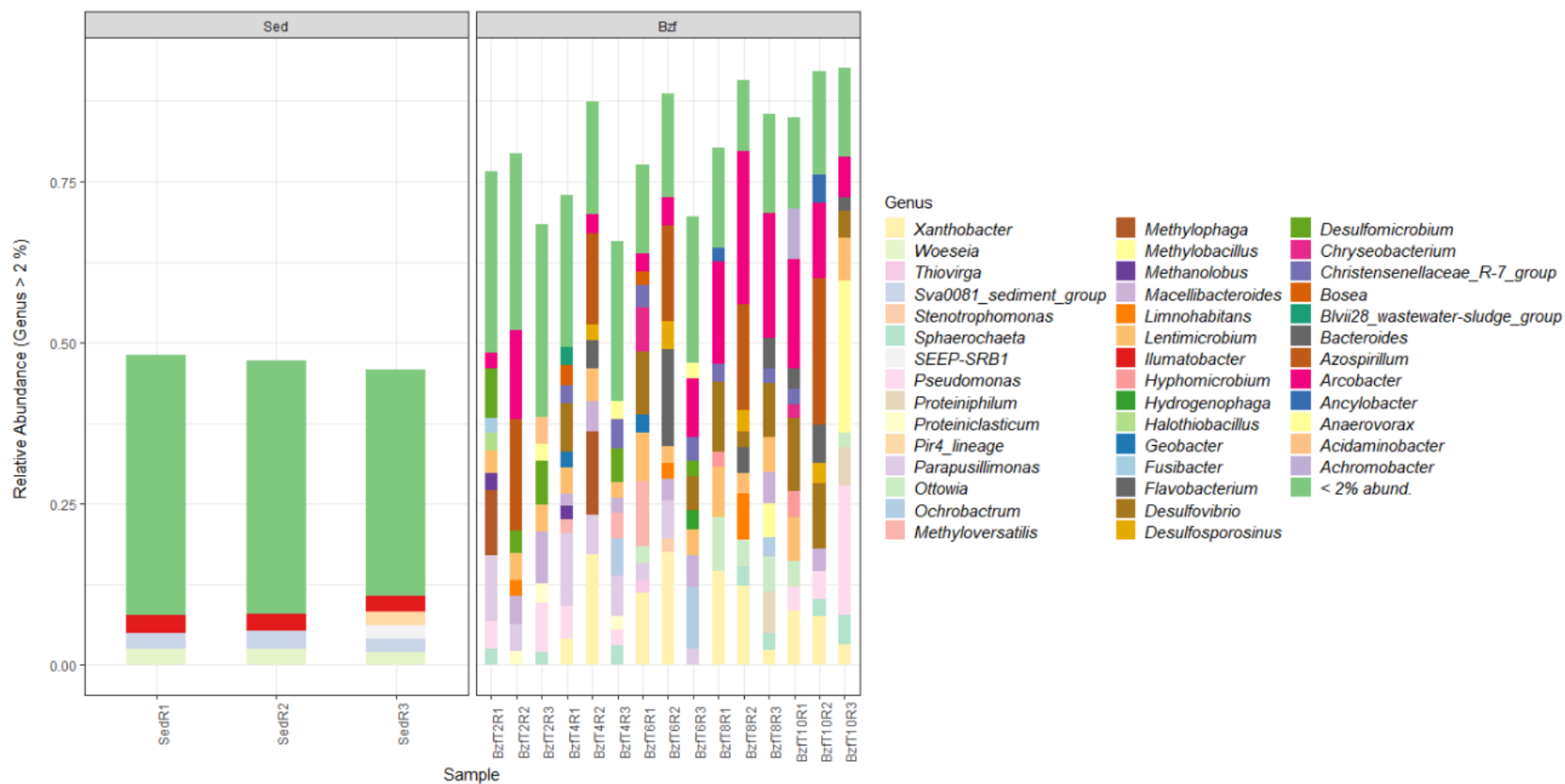


Figure 14 - Temporal evolution of the taxonomic profile at genus level of the enriched slurries when exposed to Bzf (relative abundance > 2 %, represented for the 2nd - 10th cycles).

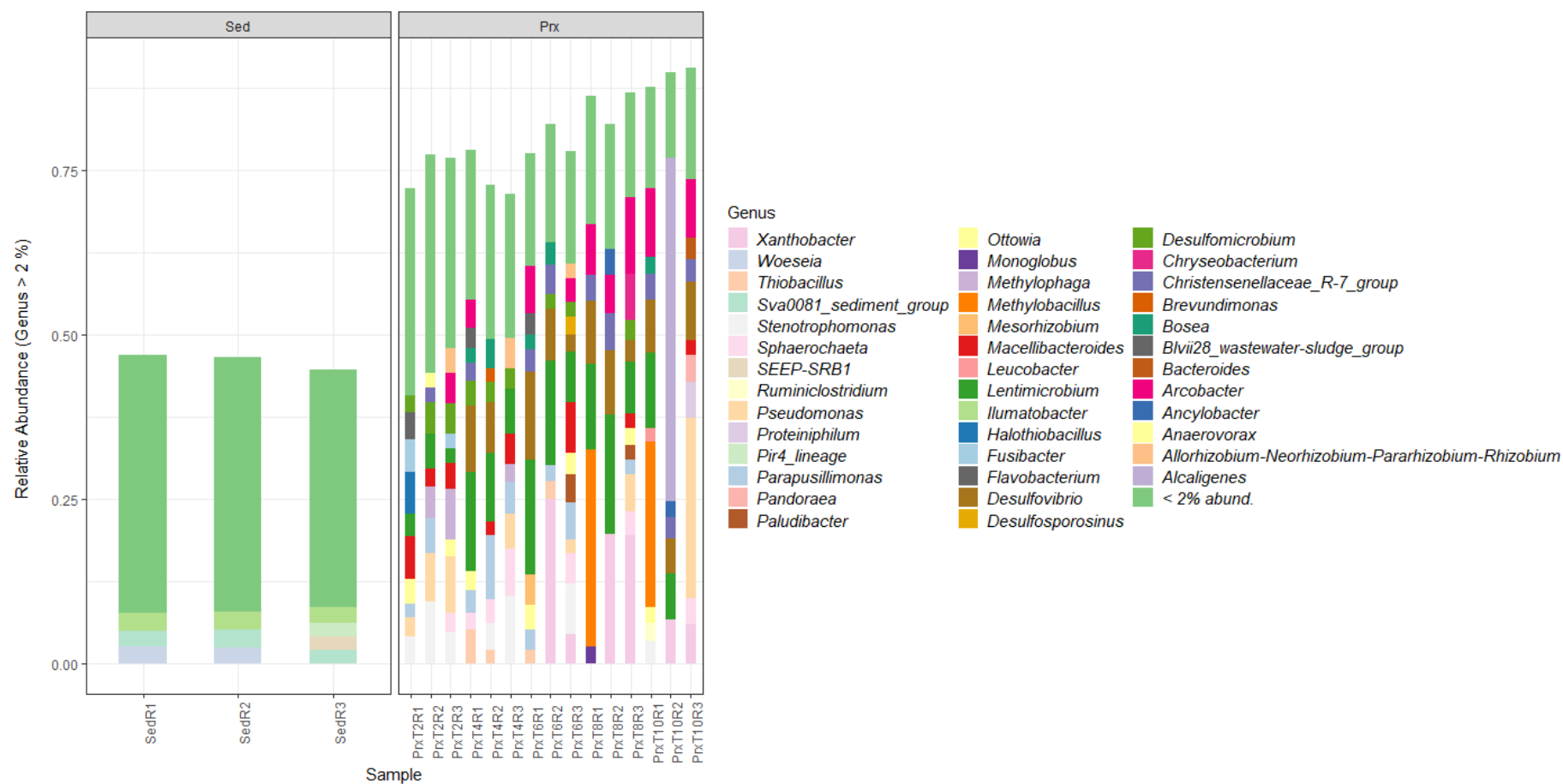


Figure 15 – Temporal evolution of the taxonomic profile at genus level of the enriched slurries when exposed to Prx (relative abundance > 2 %, represented for the 2nd - 10th cycles).

5. Discussion

The presence of pharmaceuticals in the environment, combined with their negative impacts in non-target organisms, has led to a growing demand for the development of new technologies to tackle pharmaceuticals contamination, while minimizing their adverse effects. As highlighted by Abatenh et al. (2017), the use of microorganisms can be crucial in the development of bioremediation strategies to recover contaminated environments. So, to develop these technologies, it is necessary to identify and isolate microorganisms with the metabolic capacity to degrade pharmaceuticals. A crucial step of this technology is the microbial enrichment, where the autochthonous microbial communities are exposed to the targeted compounds to selectively identify, purify, and cultivate the key degrading microorganisms.

Recognizing the importance of maintaining ecological integrity of the contaminated site, this study aimed to evaluate the potential of autochthonous microbial cultures, enriched from salt marsh plants rhizosediment within the Lima River estuary, to remove Bzf and Prx, seeking the development of new biotechnological tools to recover natural contaminated environments. With this objective in mind, two enriched slurries from a prior study (Cunha, 2022) were selected. The culturable bacterial community was isolated by spreading the enriched cultures from the 10th cycle onto two distinct solid media, PCA and MM containing Bzf and Prx. A total of 46 phenotypically distinct bacterial strains were effectively isolated from the two enriched slurries. Nonetheless, it is important to note that this figure may not represent the complete bacterial diversity within the enriched cultures. This limitation arises from the fact that only a fraction of bacteria can be cultured, and some bacterial strains might not thrive on solid media or in the culture media selected for this study (Vartoukian et al., 2010). However, the retrieved bacteria hold the greatest relevance in biotechnology because culturable bacteria can be harnessed to generate bacterial biomass for potential applications in on-site bioremediation of contaminated areas.

Different microbial enrichments, whether with Prx or Bzf, led to different microbial community composition, as exposure to different compounds resulted in the selection and isolation of different bacterial strains (Figure 5 and 6). Most of these strains belongs to the phylum Pseudomonadota (previously known as Proteobacteria, 86.96 %), being also isolated bacterial strains from the phyla Bacteroidota and Actinomycetota, in equal proportions (both 6.52 %). Pseudomonadota, a phylum known for its remarkable diversity, is recognized as a ubiquitous phylum in the environment (Leão et al., 2023; Li et al., 2023; Liu et al., 2022), being often found in marine and soil environments (von Mering et al., 2007). In biological wastewater treatments, this phylum often takes the lead (Cyzdik-Kwiatkowska

& Zielińska, 2016), having several species performing key roles in organic matter degradation and nutrient removal (Cyzdik-Kwiatkowska & Zielińska, 2016; Vasiliadou et al., 2018). Furthermore, it is also renowned for its involvement in the biodegradation of diverse pollutants, such as pesticides, hydrocarbons, and pharmaceuticals (Alexandrino et al., 2020; Alexandrino et al., 2017; Fernandes et al., 2020; Perdigão et al., 2021; Shahi et al., 2016; Vasiliadou et al., 2018). From the Bzf enriched cultures it was possible to isolate 9 different bacterial genera, while from the Prx enriched cultures, it was possible to isolate 8 different bacterial genera. In the Bzf enriched cultures, *Pseudomonas* (60.87 %) was the most abundant genus, while in the Prx enriched cultures, the genera *Alcaligenes* (22.73 %) and *Pseudomonas* (22.73 %) were the most abundant. Both *Pseudomonas* and *Alcaligenes* have demonstrated the ability to biodegrade pharmaceuticals, such as diclofenac (Mohamed et al., 2023) and fluoxetine (Palma et al., 2021). *Alcaligenes*, in particular, has emerged as a sustainable solution for the bioremediation of hospital wastewater, effectively removing pharmaceutical pollutants, including salicylic acid, naproxen, and diazepam (Rashid et al., 2022). *Pseudomonas*, on the other hand, has been reported by several authors to degrade acetaminophen, paracetamol, cefalexin, and sulfamethoxazole (De Gusseme et al., 2011; Jiang et al., 2014; Lin et al., 2015; Reis et al., 2014; Zhang et al., 2013). Concerning other genera isolated in this study, it has been demonstrated that the genus *Stenotrophomonas*, when combined with bacterial species from *Pseudomonas* genus and other bacterial genera, possesses the capability to bioremediate diclofenac (Nowak et al., 2023). Additionally, when partnered with *Rhizobium* (a genus not isolated in this study), it effectively eliminated both diclofenac and ibuprofen (Pápai et al., 2023). The genus *Brucella* has been reported as capable to degrade erythromycin (Zhang et al., 2017). Bacterial strains from the *Xanthobacter* genus, as reported by Chang et al. (2014), has the capacity to efficiently biodegrade tetracycline antibiotics. Strains from the genus *Comamonas*, isolated in this study, have also been noted for their ability to degrade tetracycline antibiotics, particularly oxytetracycline (Chen et al., 2019). Furthermore, *Achromobacter* genus has been associated by several authors to the biodegradation of sulfamethoxazole (Huang et al., 2012; Liang & Hu, 2019; Liang et al., 2020; Xue et al., 2019). *Microbacterium* has been associated degradation of ibuprofen present in a hospital wastewater (Show et al., 2023). A study conducted by Gauthier et al. (2010) revealed the potential of a bacterial strain belonging to the genus *Rhodococcus* to degrade sulfamethoxazole. Additionally, *Rhodococcus* have been reported as capable to degrade different hydrocarbons (Auffret et al., 2009; Song et al., 2011).

For the genera *Kaistella*, *Pseudochrobactrum*, and *Ancylobacter* it was not found studies reporting their potential to degrade pharmaceuticals. However, some of these bacterial strains were associated to the degradation of other organic pollutants. *Pseudochrobactrum* genus was associated to biodegradation of phenol (Mao et al., 2015). Lu et al. (2011) demonstrated the capacity of a genus *Ancylobacter* bacterial strain, isolated from contaminated soil, to degrade the herbicide metsulfuron-methyl. For *Kaistella* genus, no data regarding their ability to degrade organic compounds was found.

To our best knowledge, none of these bacterial genera were associated to the degradation of Prx or Bzf, being the exception the genera *Pseudomonas*, *Brucella* (previously known as *Ochrobactrum*) and *Microbacterium* (isolated from estuarine sediment), and *Achromobacter* (isolated from a wastewater activated sludge) (Fernandes et al., 2020). In the study conducted by Fernandes et al. (2020), an enrichment process with Prx and Bzf was conducted using estuarine sediment from Douro River Estuary as inoculum. From both enriched cultures, several bacterial strains were isolated, in which the genera *Pseudomonas*, *Bosea*, *Acinetobacter*, *Shewanella*, *Leadbetterella* and *Microbacterium* were isolated from the Prx enriched culture and the genera *Acinetobacter*, *Herminiimonas*, *Dyadobacter*, *Ochrobactrum*, *Leucobacter* and *Microbacterium* were isolated from the Bzf enriched culture. Comparing both studies, both enrichment processes selected different bacteria genera for the same pharmaceuticals, despite of both enrichments used estuarine sediment from two estuaries located in North of Portugal (Lima and Douro estuary).

Regarding the removal efficiency of Prx and Bzf performed by the enriched slurries and the enriched cultures under maintenance, the results demonstrated a high removal efficiency (exceeding 95 %) until the end of the 8th and 6th cycles, respectively. However, after each respective cycle, the removal rates significantly decreased to 54 % and 37 %, possibly due to sudden temperature fluctuations. It is important to note that this is currently a working theory as we are awaiting further results from the characterization of the enriched cultures from the 11th to 21st cycles to confirm this hypothesis. Regarding the problem of sudden temperature fluctuations, it is worth highlighting that replica 3 of the Bzf enriched slurry exhibited higher resilience compared to the other replicates. This was evident in its ability to maintain a Bzf removal efficiency of 85 %. In contrast, replicates 1 and 2 displayed Bzf removal efficiencies ranging between 0 – 26 %. On the other hand, replica 1 of the Prx enriched slurry exhibited higher resilience compared to the other replicates, maintaining a removal efficiency of 90 %, while replicates 2 and 3 showed Prx removal of 86 % and 78 %, respectively.

It is important to note that, despite of both enriched cultures had the same inoculum (estuarine sediment), each replica evolved independently during the course of this experiment, displaying diverse responses to the Prx and Bzf exposure. The absence of significant differences in removal efficiencies observed in this study can be attributed to the considerable variability within the enriched cultures, as reflected by standard deviations reaching up to 43 % in some cycles. To prevent this variability within the enriched microbial communities and fluctuations on the removal efficiencies, some authors defend conducting enrichment processes while selecting optimal communal microbial traits that focuses on the properties and functions of entire microbial communities, rather than individual species or traits (Arias-Sánchez et al., 2019; Swenson et al., 2000; Williams & Lenton, 2007; Xie et al., 2019), a technique called artificial ecosystem selection. This approach offers several benefits associated with diverse microbial communities, including enhanced productivity (Tilman et al., 2014), the utilization of a wider array of resources facilitated by interactions among community members (Cavaliere et al., 2017), and increased resilience in the face of environmental and ecological perturbations, such as exposure to anthropogenic contaminants, fluctuations in oxygen levels, or pH changes (Giri et al., 2020). This approach defends the successive selection of the cultures with the best degrading traits to seed the new generation of communities, until it is reached a robust and stable community with high removal efficiency (Borchert et al., 2021).

While certain studies have suggested efficient Prx removal from wastewater during treatment in WWTPs (Schultz & Furlong, 2008), it is important to note that the literature contains divergent findings, as other studies have reported variable removal efficiencies, ranging between 74.21% and 81.19 % (Silva et al., 2014). Similarly, the removal of Bzf by WWTPs has exhibited considerable variability, with removal efficiencies ranging from 10 % to 97 % in different studies (Collado et al., 2014; Jelic et al., 2011; Luo et al., 2014). Duarte et al. (2019b) conducted an enrichment process with Prx and Bzf, using estuarine sediment as inoculum. They reported removal efficiencies higher than 97 % for both pharmaceuticals, after the enrichment process. Fernandes et al. (2020) constructed two bacterial consortia using the bacterial strains isolated from the enriched cultures conducted by Duarte et al. (2019b) and, once more, removal efficiencies higher than 97 % for both pharmaceuticals were observed. Furthermore, it is worth noting that Prx degradation can also occur through abiotic processes like photodegradation (Duarte et al., 2019b). This factor could potentially influence our results, even though the flasks were maintained in darkness. In contrast, Bzf has been demonstrated to be stable and resistant to degradation by these factors (Duarte et al., 2019b; Luo et al., 2014; Trovó et al., 2008). During the enrichment process, it is possible to select a community that has the ability to degrade the target pharmaceutical or

a community that has some resilience to the stress induced by the presence of the pharmaceuticals. Thus, after the isolation of the potential degrading bacterial strain, it is necessary to ascertain their potential to degrade the compound. Thus, it is necessary to assemble a consortium using these isolated strains, in the presence of the selected pharmaceutical as the sole carbon source or using a co-substrate, to assess if the isolated bacterial strains are the ones responsible for the degradation of the target compound.

Microorganisms are valuable bioindicators since they offer a sensitive, versatile, and cost-effective means of assessing environmental pollution and its impacts on ecosystems. Their ability to respond quickly to changes, their wide distribution, and genetic diversity make them essential for environmental monitoring and assessment (Bouchez et al., 2016). Despite some variability in microbial communities structure have been associated to abiotic factors, such as salinity, pH or temperature and latitudinal gradients of biodiversity (Fierer & Jackson, 2006; Fuhrman et al., 2008), some other authors report that anthropogenic contamination can impact them on a larger scale (Sun et al., 2012). Wang and Tam (2011) reported that a microbial culture originating from a marine sediment revealed a decrease in bacterial diversity when exposed to concentrations of polycyclic aromatic hydrocarbons (PAHs). Metals have been thoroughly linked to shifts in microbial communities, negatively impacting the evolution of these communities (Li et al., 2021; Luo et al., 2018; Song et al., 2018). Another study reported that ibuprofen was responsible for a reduced bacterial biomass in a microbial enriched culture samples derived from an activated sludge (Davids et al., 2017). Furthermore, Aguilar-Romero et al. (2020), Kraigher et al. (2008), Li and Jin (2009) and Zhang et al. (2016) reported that different classes of pharmaceuticals (such as anti-inflammatory and bacteriostatic antibiotics) cause shifts and losses in the diversity of enriched bacterial communities. These findings are in accordance with the results obtained in this study, since the presence of both Bzf and Prx not only changed the composition and structure of the enriched communities (Figure 11), but also decreased the abundance and diversity (Figures 10A and 10B). The presence of different pharmaceuticals might explain the shift observed in each enriched cultures, as different communities were selected. Wei et al. (2020) pointed out that the microbial community composition can be notably influenced by the supplied carbon source. Bacterial metabolism exhibits high flexibility and adaptability, with distinct bacterial strains harbouring different metabolic capabilities encoded within their genetic material (Passalacqua et al., 2016). Having said this, the capacity of bacteria to metabolize carbon from different molecules hinges upon their genetic and enzymatic machinery (Gómez-Consarnau et al., 2012).

In this study, a second carbon source was supplied, specifically sodium acetate. The addition of a second carbon source, especially if this compound is easily degradable, can

lead to the appearance or dominance of certain microorganisms known as "cheaters" or "cross-feeders." These opportunistic microorganisms exploit cooperative or mutualistic interactions within the microbial community, benefiting from shared resources (Smith & Schuster, 2019) or they can obtain nutrients or energy by consuming metabolic byproducts generated by other community members without actively participating in the primary metabolic processes responsible for the production of these byproducts (Pande & Kost, 2017). The proliferation of cheaters can significantly influence the dynamics of microbial communities. Previous studies have indicated that once the population of cheater organisms surpasses a certain threshold, there is a potential for the complete obstruction of resource access by the rest of the community. This obstruction can ultimately result in a decline in community function (Stump et al., 2018). Furthermore, cheaters have the potential to disrupt cooperative relationships and reduce the overall benefits of cooperation, potentially weakening the community's resilience (Sun et al., 2019). Understanding these dynamics is crucial in the development of bioremediation tools as during the enrichment process, these "cheaters" or "cross-feeders" can suppress the degrading community, threatening the selection of the key degrading organisms.

The prokaryotic community structure analysis revealed that the enriched slurries doped with either Bzf or Prx were dominated by Pseudomonadota phylum, followed by the phylum Bacteroidota. This trend was observed in all replicas throughout time. At genus level, the results showed no clear pattern in the temporal evolution of the taxonomic profiles present in the enriched slurries doped either Bzf or Prx. Despite no clear domination observed, genera *Arcobacter* and *Xanthobacter* were present in most cycles of the Bzf enriched slurries. On the other hand, the genera *Lentimicrobium* as well as *Xanthobacter* were abundant in most replicas of the Prx enriched slurries. In addition, genus *Methylobacillus* managed to display a high relative abundance in replicate 1 of the 8th and 10th cycle.

Most of the genera identified during this study have demonstrated degradation potential of anthropogenic contaminants in other studies. Specifically, *Sphaerochaeta* genera was reported to be dominant in an modified electrode with palladium and was responsible for dehalogenation of the anti-inflammatory drug diclofenac (Xu et al., 2018). The genus *Desulfovibrio* was associated to the decrease of ciprofloxacin and sulfamethoxazole, in constructed wetland systems (Martins et al., 2018; Ouyang et al., 2021). Martins et al. (2018) also reported that the genus *Arcobacter* was responsible for the biodegradation of both ciprofloxacin and 17 β -estradiol. *Lentimicrobium*, a well-established genus in the communities of this study, was reported to have the capacity to remove trimethoprim and oxytetracycline (Liang et al., 2019; Wei et al., 2022). Lastly, Yan et al., (2022) reported the degradation of amoxicillin by a specie from *Bosea* genus (Yan et al., 2022). Other genera

that presented relative abundances > 2 % in this study have been linked to degradation of other anthropogenic contaminants, such as crude oil or other organic compounds. *Parapusillimonas* was reported to be part of a microbial community capable to achieve maximum degradation of crude oil (Singh & Pandya, 2015). A novel bacterium isolated by Pang et al. (2021) from a sludge sample, identified as belonging to the genus *Ottowia* was associated to the degradation of phenylacetic acid. *Methylobacillus* genus was reported to be capable to biodegrade anthropogenic contaminants, such as endocrine disruptor dibutyl phthalate and triclosan (Kumar & Maitra, 2016; Thelusmond et al., 2019). Li et al. (2019) suggested that the interaction between the genera *Desulfomicrobium* and *Comamonas* revealed potential to degrade nitrogen heterocyclic compounds, such as synthetic dyes. The genus *Thiobacillus* was reported to be involved in the removal of ciprofloxacin, azithromycin and oxytetracycline (Chen et al., 2019; Wang et al., 2019). *Macellibacteroides* genus, that belong to the phylum Bacteroidota was associated to the removal of ciprofloxacin (Martins et al., 2018).

The isolation process was conducted in the 10th cycle of the enrichment process. Most of the isolated bacterial strains were not found in the prokaryotic community, in relative abundances higher than 2 %. In fact, the genus *Pseudomonas*, one of the most abundant in the culture dependent methods, was punctually detected with relative abundances higher than 2 %, being most of the times, below this threshold. The same happened with the genera *Microbacterium*, *Brucella*, *Starkeya*, *Rhodococcus*, *Kaistella*, *Comamonas*, *Chryseobacterium*, and *Pseudochrobactrum*. This can occur since many bacteria are not culturable. As previously mentioned, the culture media selected for this study can benefit the growth of some bacterial strains and cannot be the most suitable to isolate some of the culture bacteria that are represented in the prokaryotic community of each enriched slurry. Furthermore, the communities with high relative abundance are not necessary the most relevant, as some rare communities or communities with lower abundances can displayed crucial roles in the removal of the target pharmaceuticals. The equilibrium within the community and how this communities interacts are still underexplored and more studies are needed to understand how this can influence the prokaryotic community's response to the presence of the contaminant and how this affects its removal.

This work showed the potential of microorganisms, enriched from natural environments, to degrade two different pharmaceuticals. Nevertheless, deeper knowledge is crucially needed to understand the reasons behind the extreme variations in removal efficiencies throughout the entire study, in order to obtain more robust bioremediation techniques capable to tackle events of contamination in natural environments. In addition, more studies

are needed to understand the role the bacterial strains isolated in this study in the degradation of Prx and Bzf, alone and in a consortium.

6. Conclusion

The results obtained in this study revealed the potential of autochthonous microbial communities enriched from the rhizosediment of Lima river estuary to remove the two target pharmaceuticals, Prx and Bzf. Specifically, high removal efficiencies (>95 %) were observed for both compounds at the initial cycles of the enrichment process. The following loss in efficiency associated to the air conditioning system failure and sudden increase of temperature, affected the removal efficiency however, the microbial communities in study were able to bounce back from an adverse effect, readjusting and regaining consistently higher removal efficiencies in the final cycles.

The culture dependent methods allowed the isolation of 46 bacterial strain, within 13 different genera, in which the *Pseudomonas* genus was the most abundant. Looking to the microbial composition, most of these bacterial genera were not present in the community of each enriched slurry, with relative abundance of 2 %, despite of being present when we look to the community composition with lower relative abundance (relative abundance <2 %).

Moreover, prokaryotic community analysis showed that the presence of Bzf or Prx influenced the community as well as that both enriched slurries communities changed over time. Furthermore, in both enriched slurries, the phylum Pseudomonadota was the most abundant among the prokaryotic communities, showing its remarkable versatility. On the other hand, no clear domination by any genera was observed during the enrichment process.

These results offer a deeper insight into the microbial community associated to the degradation of Bzf and Prx and can contribute for the development of effective bioremediation technologies to recover natural environments.

7. References

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8. Supplementary material

SM 1 - Mineral-salts medium (MM) constitution

The Mineral-salts medium (MM) contains the following composition **(per litre of deionized water)**:

- 2.7 g of di-Sodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$)
- 1.4 g of Potassium dihydrogen phosphate (KH_2PO_4)
- 0.5 g of Ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$)
- 0.2 g of Magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)
- 10 mL of trace elements solution with the following composition, per litre:
 - 12.0 g of Disodium ethylenediaminetetraacetate dihydrate ($\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$)
 - 2.0 g of Sodium hydroxide (NaOH)
 - 0.4 g of Manganese (II) sulfate tetrahydrate ($\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$)
 - 0.4 g of Zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$)
 - 0.5 mL of concentrated Sulfuric acid (H_2SO_4)
 - 10.0 g of Sodium sulfate (Na_2SO_4)
 - 0.1 g Sodium molybdate dihydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$)
 - 2.0 g of Iron (II) sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)
 - 0.1 g of Copper (II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)
 - 1.0 g of Calcium chloride (CaCl_2)

Table S1 - Taxonomy identification of bacterial strains isolated from each treatment obtained by sequencing of the V1-V9 region hypervariable of the 16S rRNA, using as inoculum estuarine sediment (Lima river estuary). PCA Bzf – Strains from the enriched cultures doped with bezafibrate from the enriched cultures cultivated in PCA; PCA Prx – Strains doped with paroxetine cultivated in PCA; MM Bzf – Strains from the enriched cultures doped with bezafibrate cultivated in MM doped with bezafibrate; MM Prx – Strains from the enriched cultures doped with paroxetine cultivated in MM doped with paroxetine.

Incubation Condition	Isolation Medium	Isolated Strains	Taxonommic Identification				GenBank Accession Number
			Closest Relative	Phylum	Query Cover (%)	Identity (%)	
Static Conditions	PCA Bzf	A1	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.93	OR326625
		A2	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.93	OR326626
		A4	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.93	OR326627
		A6	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.93	OR326628
		A7	<i>Pseudomonas sp.</i>	Pseudomonadota	100	100	OR326629
		A8	<i>Microbacterium sp.</i>	Actinomycetota	100	100	OR326630
		A9	<i>Brucella sp.</i>	Pseudomonadota	100	99.78	OR326631
		A10	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.86	OR326632
		A12	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.86	OR326633
		A13	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.86	OR326634
		A15	<i>Rhodococcus sp.</i>	Actinomycetota	100	100	OR326635
		A17	<i>Ancylobacter sp.</i>	Pseudomonadota	100	99.85	OR326636
		A21	<i>Xanthobacter sp.</i>	Pseudomonadota	100	99.93	OR326637
		A22	<i>Kaistella sp.</i>	Bacteroidota	100	97.24	OR326638
		A23	<i>Achromobacter sp.</i>	Pseudomonadota	100	99.93	OR326639
	MM Bzf	C1	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.93	OR326640
		C2	<i>Pseudomonas sp.</i>	Pseudomonadota	99	99.86	OR326641

		C3	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.36	OR326642
		C4	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.72	OR326643
		C6	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.79	OR326644
		C7	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.86	OR326645
		C8	<i>Xanthobacter sp.</i>	Pseudomonadota	100	99.93	OR326646
		C9	<i>Xanthobacter flavus</i>	Pseudomonadota	100	100	OR326647
		C10	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.86	OR326648
	PCA Prx	B1	<i>Comamonas sp.</i>	Pseudomonadota	100	99.71	OR326649
		B2	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.93	OR326650
		B4	<i>Brucella rhizosphaerae</i>	Pseudomonadota	100	99.85	OR326651
		B5	<i>Comamonas sp.</i>	Pseudomonadota	100	99.71	OR326652
		B7	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.86	OR326653
		B8	<i>Chryseobacterium taeanense</i>	Bacteroidota	100	99.86	OR326654
		B12	<i>Stenotrophomonas sp.</i>	Pseudomonadota	100	99.64	OR326655
		B14	<i>Brucella rhizosphaerae</i>	Pseudomonadota	100	99.7	OR326656
		B16	<i>Chryseobacterium sp.</i>	Bacteroidota	100	98.99	OR326657
		B17	<i>Pseudochrobactrum sp.</i>	Pseudomonadota	100	99.85	OR326658
		B21	<i>Alcaligenes sp.</i>	Pseudomonadota	100	99.93	OR326659
		B23	<i>Microbacterium sp.</i>	Actinomycetota	100	99.93	OR326660
		B25	<i>Brucella rhizosphaerae</i>	Pseudomonadota	100	99.78	OR326661
		B26	<i>Alcaligenes sp.</i>	Pseudomonadota	100	99.93	OR326662
		B29	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.78	OR326663
	MM Prx	D1	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.79	OR326664
		D2	<i>Alcaligenes sp.</i>	Pseudomonadota	100	99.86	OR326665
		D3	<i>Alcaligenes sp.</i>	Pseudomonadota	100	99.78	OR326666
		D4	<i>Pseudomonas sp.</i>	Pseudomonadota	100	99.57	OR326667
		D5	<i>Stenotrophomonas sp.</i>	Pseudomonadota	100	99.51	OR326668

		D7	<i>Alcaligenes sp.</i>	Pseudomonadota	100	99.93	OR326669
		D9	<i>Stenotrophomonas sp.</i>	Pseudomonadota	100	99.65	OR326670

Table S2: Total number of sequences obtained for each sample after sequencing (Input - raw sequences), and number of sequences after each DADA2 treatment: after filtering out low-quality sequencing reads (Filtered), trimming the reads to a consistent length (Denoised) and after the removal of the chimeras (Nonchim)).

bp lenght trimms	Sample	Input	Filtered	DenoisedF	DenoisedR	Merged	Nonchim
TruncLen (260,180)	BzfT2R1	104395	84592	76892	78632	57833	53551
	BzfT2R2	99212	78987	70425	72358	56203	51526
	BzfT2R3	83713	68239	60252	62252	47856	45304
	BzfT4R1	90535	73225	69300	70172	52544	49405
	BzfT4R2	91533	75748	72259	73293	62730	53169
	BzfT4R3	105795	86506	81694	82636	63097	58399
	BzfT6R1	81314	67758	65566	66253	49524	43433
	BzfT6R2	114959	95704	93005	93682	83211	68522
	BzfT6R3	101200	83721	80212	80968	62799	56540
	BzfT8R1	86930	72568	70368	71091	58103	49974
	BzfT8R2	102112	85321	83604	84184	76197	58425
	BzfT8R3	111703	94179	91185	92344	73826	63274
	BzfT10R1	105893	89754	87600	88434	76557	59390
	BzfT10R2	72165	58222	56545	56952	51643	43063
	BzfT10R3	127473	110327	108477	109403	98037	68150
	PrxT2R1	106567	85233	75513	77478	56523	53727
	PrxT2R2	100120	79979	72064	73727	51516	47875
	PrxT2R3	78271	64298	58052	59624	43460	40570
	PrxT4R1	64209	52538	48800	49894	37484	35337
	PrxT4R2	82327	67483	63782	64568	46023	41974
	PrxT4R3	104523	87275	84191	85123	69178	61996
	PrxT6R1	99879	80700	77402	78207	59304	54263
	PrxT6R2	88778	73465	71240	71984	56930	50995
	PrxT6R3	98662	81783	78528	79503	65098	57396
	PrxT8R1	61995	52054	50641	51160	39359	33820
	PrxT8R2	85155	69672	67006	67556	52671	46366
	PrxT8R3	100728	85273	83278	83893	72489	58354
	PrxT10R1	98763	83438	81786	82289	65796	52084
	PrxT10R2	110654	89145	88011	88344	76334	62768
	PrxT10R3	92342	77991	75986	76812	68544	52872
	SedR1	119679	95509	72992	77161	54879	50773
	SedR2	89987	72006	52969	56827	37734	34572
	SedR3	239969	189054	151702	158347	111981	103818

SEDE ADMINISTRATIVA

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

FACULDADE DE CIÊNCIAS

