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Bioremediation of Pharmaceuticals by Autochthonous Microorganisms in Estuarine Environment

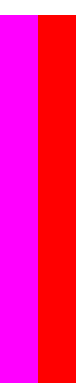
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**Bioremediation of Pharmaceuticals by Autochthonous
Microorganisms in Estuarine Environment**

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ABSTRACT

Emerging contaminants refers to a group of micropollutants found in the environment at trace concentrations ($\mu\text{g/L}$ or ng/L). These contaminants are not currently covered by existing water-quality regulations, although they could represent a threat to the ecosystem and for human health and safety. Pharmaceuticals are indispensable to ensure human quality of life; however, they are a representative group of emerging contaminants. In fact, a great portion of pharmaceuticals is normally excreted from human body in parental form or as metabolites, which could be loaded into the aquatic environment when the removal process in the wastewater treatment plant (WWTP) is not efficient.

Microbial communities have the ability to metabolize a wide variety of contaminants and are known to be able to colonize contaminated environments. Towards this, the present work aimed to develop bioremediation techniques that can stimulate the intrinsic capacity of autochthonous microbial communities to degrade pharmaceuticals in an urban estuary and associated WWTP. As representatives of extensively used pharmaceuticals, this study was focused on an antidepressant drug, Paroxetine, and on a lipid regulator, Bezafibrate. Two experiments were carried out using microorganisms originated from different sources: (i) sediments from the Douro estuary and (ii) activated sludge from a local WWTP.

The first experiment consisted in enrichment cultures of mixed *consortia* of autochthonous microorganisms with capacity to grow in the presence of the selected pharmaceuticals, obtained from samples collected at the two sources. The enrichment was carried out in batch mode, under different incubation conditions (agitation vs. static), by exposing the cultures to the different pharmaceuticals (1 mg/L) in co-metabolism with acetate (500 mg/L). The biodegradation potential was monitored at the end of each feeding cycle (2 weeks) by analyzing both pharmaceuticals in solution (by High Performance Liquid Chromatography). In the case of Paroxetine, fluoride ion release was also followed as an indicator of defluorination of the molecule (through ion selective analysis). Microbial biomass growth was estimated by optical density of cultures (by spectrophotometry at 600 nm). Microbial community was characterized by Automated Ribosomal Intergenic Spacer Analysis at the end of first (2 weeks) and last (14 weeks) feeding cycle. Important changes occurred in the microbial community structure, influenced by time of experiment, incubation conditions and pharmaceuticals presence. Indeed, incubation conditions affected not only the bacterial community structure, but also the efficiency of pharmaceuticals removal that was initially lower under agitation than under static conditions. At the end of the experiment, results showed high removal of the pharmaceuticals from solution (>97%) due to biotic

processes. However, in case of paroxetine, removal was also due to adsorption and abiotic processes, however, defluorination only occur in the presence of microorganisms.

The second study aimed to evaluate the capacity of isolated strains derived from the enrichment cultures obtained in the first experiment, re-united in new *consortia* to degrade the selected pharmaceutical to which they were previously exposed. Thus, five sets of microcosms were assembled: two *consortia* derived from Paroxetine enrichment under static conditions, of which one was obtained from estuarine sediment and the other from activated sludge from WWTP, and three *consortia* derived from Bezafibrate enrichment, two under static conditions, of which one was obtained from estuarine sediment and the other from activated sludge, and the last obtained from activated sludge microcosms under agitation conditions. Results showed that despite different acclimation times, high removal efficiency of pharmaceuticals (>97%) was obtained in three of the five studied *consortia*. Moreover, *consortia* from estuarine sediment was more efficient in removing both studied pharmaceuticals. In the case of paroxetine, microorganisms present in *consortia* were able to promote fluoride anion release. Complementary analysis in terms of identification of the isolated strains will allow to understand which are the microorganisms present in *consortia*, responsible for bezafibrate and paroxetine degradation.

This work emphasizes the potential of autochthonous microorganisms from estuarine environment and associated WWTP, for application in bioremediation techniques, namely autochthonous bioaugmentation for pharmaceuticals removal.

Keywords: Bioremediation; Bezafibrate; Paroxetine; microbial community; autochthonous microorganisms

RESUMO

Contaminantes emergentes são um grupo de micropoluentes encontrados no ambiente em concentrações vestigiais ($\mu\text{g/L}$ or ng/L). Estes contaminantes não se encontram atualmente sob regulação da qualidade da água, contudo podem representar uma ameaça para o ecossistema, saúde e segurança humana. Os fármacos são indispensáveis para assegurar qualidade de vida ao Homem; contudo, são um grupo representativo de contaminantes emergentes. De facto, uma grande porção dos fármacos é excretada do corpo humano na forma do composto parental ou como metabolito, podendo atingir o meio aquático quando a sua remoção nas estações de tratamento de água residuais (ETAR) não é eficiente.

As comunidades microbianas têm a capacidade de metabolizar uma grande variedade de contaminantes, sendo reconhecidas pela sua capacidade de habitar em ambientes contaminados. O presente trabalho tem como objetivo desenvolver técnicas de biorremediação que consigam estimular a capacidade intrínseca de comunidades microbianas autóctones para degradar compostos farmacêuticos em estuário urbano e ETAR associada. Como representantes de fármacos extensamente consumidos, este estudo foca-se num fármaco antidepressivo, Paroxetina e em um regulador lipídico, Bezafibrato. Duas experiências foram desenvolvidas, utilizando microrganismos com origem em duas fontes diferentes: (i) sedimentos do estuário do Douro e (ii) lamas ativadas de uma ETAR local.

A primeira experiência consistiu no enriquecimento de culturas do consórcio de microrganismos autóctones com capacidade de crescer na presença dos fármacos selecionados, obtidos a partir de amostras recolhidas nas duas fontes. O enriquecimento foi desenvolvido sob diferentes condições de incubação (agitação vs. estático), com a exposição das culturas aos diferentes fármacos (1 mg/L) em co-metabolismo com acetato (500 mg/L). O potencial de biodegradação foi monitorizado no fim de cada ciclo (2 semanas) através da análise da presença do fármaco em solução (por Cromatografia Líquida de Alto Desempenho). No caso da Paroxetina, a libertação do ião fluoreto também foi seguida como um indicador da defluorinação da molécula (através de análise seletiva do ião). O crescimento da biomassa microbiana foi estimada por medição da densidade ótica das culturas (por espectrofotometria a 600 nm). A comunidade microbiana foi caracterizada por Análise de Espaçador Intergénico Ribossômico Automatizado no final do primeiro (2 semanas) e último (14 semanas) ciclos. Alterações importantes ocorreram a nível da estrutura da comunidade, influenciadas pelo tempo da experiência, condições de

incubação e presença dos fármacos. De facto, as condições de incubação afetaram não só a estrutura da comunidade bacteriana, como também a eficiência de remoção dos fármacos que por sua vez foi mais baixa em condições de agitação do que em condições estáticas. No final da experiência, os resultados demonstraram elevadas eficiências de remoção dos fármacos de solução (>97%) devido a processos bióticos. Contudo, no caso da paroxetina, a remoção também foi baixa devido a processos abióticos e de adsorção, contudo, a defluorinação apenas foi verificada na presença de microrganismos.

O segundo estudo pretendeu avaliar a capacidade de estirpes de bactérias isoladas derivadas das culturas de enriquecimento obtidas na primeira experiência, reunidas num novo consórcio, para degradar os fármacos selecionados, aos quais tinham sido previamente expostas. Assim, cinco consórcios foram obtidos: dois derivaram do enriquecimento com Paroxetina sob condições estáticas, sendo que um deles teve origem nos sedimentos estuarinos e o outro nas lamas ativadas de ETAR. Os outros três consórcios derivaram do enriquecimento com Bezafibrato, dos quais dois foram obtidos do enriquecimento sob condições estáticas, um com origem nos sedimentos estuarinos e o outro nas lamas ativadas, e o terceiro foi obtido de microcosmos de lamas ativadas sob condições de agitação. Os resultados demonstraram, que apesar dos diferentes tempos de aclimatização, elevadas eficiências de remoção (>97%) foram obtidas em três dos cinco consórcios estudados. Além disso, os consórcios obtidos a partir de sedimentos estuarinos demonstraram ser mais eficientes na remoção dos dois fármacos em estudo. No caso da Paroxetina, os microrganismos presentes no consórcio foram capazes de promover a libertação do anião de fluoreto. Análises complementares em termos de identificação das estirpes isoladas vão permitir perceber quais os microrganismos presentes em cada consórcio responsáveis pela degradação de Paroxetina e Bezafibrato.

Este trabalho enfatiza o potencial de microrganismos autóctones presentes em ambiente estuarino e ETAR associada para a aplicação em técnicas de biorremediação, nomeadamente bioaumento autóctone para remoção de fármacos.

Palavras-Chave: Biorremediação; Bezafibrato; Paroxetina; comunidade microbiana; microrganismos autóctones

CONTENTS

Agradecimentos.....	i
Abstract.....	ii
Resumo	iv
Abbreviations And Acronyms.....	viii
Figure List	viii
Table List.....	xi
1. Introduction.....	13
1.1. Background.....	13
1.2. Pharmaceutical Compounds.....	15
1.2.1. Bezafibrate	15
1.2.1. Paroxetine	17
1.3. Microbial communities and Bioremediation	19
1.4. Contaminated Sites	20
1.4.1. Douro estuary	21
1.5. Objectives	22
2. Methods.....	24
2.1. Experiments	24
2.1.1. Reagents and materials.....	24
2.1.2. First experiment.....	24
2.1.3. Second Experiment	25
2.1.4. Microcosms Operation	27
2.2. Analytical Determinations	27
2.2.1. Microbial Growth	27
2.2.2. Pharmaceuticals Removal from solution	27
2.2.3. Fluoride Anion Release	28
2.2.4. Bacterial community structure	28
2.3. Statistical Analysis	29
3. Bioremediation of Bezafibrate and Paroxetine by Microorganisms from a Wastewater Treatment Plant and Estuarine Sediments	31
3.1. Results	31
3.1.1. Pharmaceuticals Removal	31
3.1.1.1. Bezafibrate	31
3.1.1.1.1. Cultures enrichment.....	31
3.1.1.1.2. Abiotic Degradation and Adsorption Potential.....	32
3.1.1.2. Paroxetine	33

3.1.1.2.1. Enrichment culture	33
3.1.1.2.2. Abiotic Degradation and Adsorption Potential.....	33
3.1.2. Fluoride Anion Release	35
3.1.3. Biomass Growth	36
3.1.4. Bacterial Richness and Diversity	38
3.1.5. Bacterial Community Structure	40
3.2. Discussion	44
3.3. Conclusions	49
4. Bioremediation Efficiency of Microorganisms Isolated from Pharmaceutical Enriched Consortia.....	51
4.1. Results	51
4.1.1. Isolated bacterial strains	51
4.1.1. Pharmaceuticals Removal	52
4.1.1. Fluoride Anion Release	54
4.2. Discussion	54
4.3. Conclusions	57
5. Conclusions and Future Perspectives	59
References	60

ABBREVIATIONS AND ACRONYMS

Abs	Absorbance
AFL	ARISA Fragment Length
ANOVA	Analysis of Variance
ANOSIM	Analysis of Similarity
ARISA	Automated Ribosomal Intergenic Spacer Analysis
AS	Activated Sludge
Bzf	Bezafibrate
DDD	Defined Daily Doses
DNA	Deoxyribonucleic Acid
ES	Estuarine Sediments
HPLC	High-Performance Liquid Chromatography
II	Initial Inoculum
ITS	Internal Transcribed Spacer
K_D	Distribution Coefficient
K_{ow}	Octanol-Water Partition Coefficient
MDS	Multidimensional Scaling
MM	Mineral-salt medium
MM-pharmaceutical	Mineral-salt medium Enriched with Pharmaceutical
Na_2PO_4	Disodium Phosphate
NaF	Sodium Fluoride
OC	Organic Carbon
OD	Optical Density
OECD	Organization for Economic Co-Operation and Development
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
pK_a	Acid Dissociation Constant
Prx	Paroxetine
rpm	Rotations Per Minute
rRNA	Ribosomal Ribonucleic Acid
tRNA	Transfer Ribonucleic Acid
SSRI(s)	Selective Serotonin Reuptake Inhibitor(s)
TISAB	Total Ionic Strength Adjustment Buffer
WHO	World Health Organization
WWTP(s)	Waste Water Treatment Plant(s)

FIGURE LIST

<i>Figure 1 – Pharmaceuticals fate in the environment [Adapted from: Halling-Sørensen et al. (1998)]</i>	14
<i>Figure 2 - Reported contaminants detected at Douro estuary [Adapted from: Mucha et al., 2003, Mucha et al., 2004, Ribeiro et al., 2009, Gravato et al., 2010, Madureira et al., 2010, Rocha et al., 2012, Waszak et al., 2014]</i>	21
<i>Figure 3 – Sampling sites at north bank of douro river</i>	24
<i>Figure 4 - Scheme of microcosms setup</i>	25
<i>Figure 5 - Scheme of microcosms set up with isolated strains</i>	26
<i>Figure 6 - Schematic example of microcosms operation, culture renewal and sampling</i> ..	27
<i>Figure 7 - Removal percentages (average and standard deviation, n=3) of Bzf from solution in microcosms with both inocula (Green – Activated Sludge, Blue – Estuarine Sediment), under both incubation conditions (A – Static, B – Agitation)</i>	32
<i>Figure 8 - Removal percentages (average and Standard Deviation, n=3) of Prx from solution in microcosms with both inocula (Green – Activated Sludge, Blue – Estuarine Sediment), under both incubation conditions (A – Static, B – Agitation)</i>	34
<i>Figure 9 - Removal percentages (average and standard deviation, n=3) of Prx from solution in adsorption controls for both sterilized inocula (Green – Activated Sludge, Blue – Estuarine Sediment), under both incubation conditions (A – Static, B – Agitation)</i>	35
<i>Figure 10 - Optical density (Abs at 600nm), measured over time, of cultures of activated sludge enriched with Bzf (Blue), Prx (Green) or without pharmaceutical addition (control) (Yellow) under both incubation conditions. A – Static, B – Agitation</i>	37
<i>Figure 11 - Optical density (Abs at 600nm), measured over time, of cultures of estuarine sediment enriched with Bzf (Blue), Prx (Green) or without pharmaceutical addition (control) (Yellow) under both incubation conditions. A – Static, B – Agitation</i>	38
<i>Figure 12 - Bacterial richness along experiment time. II – Initial inoculum; C - Control, Bzf – Bezafibrate treatment; Prx – Paroxetine treatment; WWTP – Inoculum from WWTP sludge; ES – Inoculum from estuarine sediment. a – significant differences comparing with respective control ($p < 0.05$); b – significant differences among same treatment, at different time ($p < 0.05$); c – significant differences among same treatment, with different incubation conditions ($p < 0.05$); d – significant differences between microcosm and initial samples ($p < 0.05$)</i>	39
<i>Figure 13 - Bacterial diversity along experiment time. II – Initial inoculum; C - Control, Bzf – Bezafibrate treatment; Prx – Paroxetine treatment; WWTP – Inoculum from WWTP sludge; ES – Inoculum from estuarine sediment. a – significant differences comparing with respective control ($p < 0.05$); b – significant differences among same treatment, at different time ($p < 0.05$); c – significant differences among same treatment, with different incubation</i>	

conditions ($p < 0.05$); d – significant differences between microcosm and initial samples ($p < 0.05$)40

Figure 14 - Hierarchical clustering based on Bray-Curtis similarities from ARISA fingerprints of bacterial communities inoculated from activated sludge (A and B) and estuarine sediment (C and D) after 2 weeks (A and C) and 14 weeks (B and D) of experiment. S – Static; Ag – Agitation; C – Control; B – Bezafibrate treatment; P – Paroxetine treatment.....41

Figure 15 - Multidimensional scaling (MDS) ordination based on Bray-Curtis similarities from ARISA fingerprints of bacterial communities originated from estuarine sediments. A – Effect of incubation conditions; B – Effect of time. Ag – Agitation; S – Static; II – Initial Inoculum; C – Control; B – Bezafibrate treatment; P – Paroxetine treatment; 2 and 14 correspond to sample time in weeks, 0 is applicable to initial sediment42

Figure 16 - Multidimensional scaling (MDS) ordination based on Bray-Curtis similarities from ARISA fingerprints of bacterial communities originated from activated sludge. A – Effect of incubation conditions; B – Effect of time. Ag – Agitation; S – Static; II – Initial Inoculum; C – Control; B – Bezafibrate treatment; P – Paroxetine treatment; 2 and 14 correspond to sample time in weeks, 0 is applicable to initial sludge42

Figure 17 - Removal percentage of Bzf in the consortia of isolates, over time. B1 – Estuarine Sediments, Static; B2 – Activated Sludge, Static; B3 - Activated Sludge, Agitation. a – different from other consortia at same period ($p < 0.05$)53

Figure 18 - Removal percentage of Prx in the consortia of isolates, over time. P1 – Estuarine Sediments, Static; P2 – Activated Sludge. Static. a - different between consortia at same period ($p < 0.05$)53

Figure 19 - Percentage of defluorination in the consortia of isolates, over time, in Prx microcosms. P1 – Estuarine Sediments, Static; P2 – Activated Sludge. a – Different between consortia, at same period ($p < 0.05$)54

TABLE LIST

<i>Table 1 - Relevant physicochemical properties of Bezafibrate</i>	<i>15</i>
<i>Table 2 - Occurrence of Bezafibrate in different environmental compartments.....</i>	<i>16</i>
<i>Table 3 - Relevant physicochemical properties of Paroxetine</i>	<i>18</i>
<i>Table 4 - Occurrence of Paroxetine in different environmental compartments.....</i>	<i>18</i>
<i>Table 5 - Percentage of defluorination percentage (mean and standard deviation, n=3) in microcosms enriched with Paroxetine, over time</i>	<i>36</i>
<i>Table 6 - Two-way crossed ANOSIM test for different time of exposure (week 2 and week 14), incubation conditions (static vs. agitation) and treatment (C – Control; Bzf – Bezafibrate; Prx – Paroxetine) effect, based on ARISA results from bacterial communities inoculated from activated sludge (AS) and estuarine sediment (ES)</i>	<i>43</i>
<i>Table 7 - Bacterial strains isolated from the different consortia in PCA media (Blue) and MM-pharmaceutical media (Yellow). (x) indicates the presence and (-) the absence. P1 – Paroxetine, Estuarine Sediments, Static; P2 – Paroxetine, Activated Sludge, Static; B1 – Bezafibrate, Estuarine Sediments, Static; B2 – Bezafibrate, Activated Sludge, Static; B3 – Bezafibrate, Activated Sludge, Agitation</i>	<i>52</i>

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. BACKGROUND

Until the beginning of the last decade of 20th century the concern about environmental pollution was caused by the presence of persistent organic pollutants and metals, mostly produced by the industrialization that occurred until that period (Petrović *et al.*, 2003). However, the rapid advances of sensitive analytical techniques allowed to analyze more complex environmental matrix, targeting the attention to previously undetected substances in aquatic environment (Richardson and Bowron, 1985, Farré *et al.*, 2008). These substances were denominated as “emerging contaminants”, being their concentration in the environment not covered by water-quality regulations, potentiating their widespread and potential threats to ecosystems. This group includes a variety of products used daily, including pharmaceuticals, personal care products, steroids and hormones, drugs of abuse, surfactants, flame retardants, industrial additives and agents, and gasoline additives, without discarding their transformation products (Petrović *et al.*, 2003, Farré *et al.*, 2008). Despite the fact that the environmental concentrations of these compounds are not regulated, their constant introduction in the environment can cause harm to living organisms, irrespective of their recalcitrance (Petrović *et al.*, 2003).

Pharmaceuticals are a large group of organic chemicals, included in emerging contaminants, extensively used in human and veterinary medicine. For that, they raise a special concern regarding their environmental presence since they are created to be biologically active, having a specific mode of action (Fent *et al.*, 2006). According to their purpose, veterinary or human medicine, pharmaceuticals can be transported and distributed in the environment by different routes, depending on their physicochemical properties (e.g. water solubility and polarity) (Fig. 1) (Halling-Sørensen *et al.*, 1998, Cunningham *et al.*, 2004, Farré *et al.*, 2008). Depending on their application (e.g. fish farms, therapeutic treatment), veterinary pharmaceuticals can enter the environment through different routes. On the other hand, human pharmaceuticals, after being metabolized, are expelled in the parental form and as metabolites in urine and feces, entering in wastewater treatment plants (WWTPs) (Richardson and Bowron, 1985, Halling-Sørensen *et al.*, 1998, Farré *et al.*, 2008). In WWTP some of these compounds can be removed by biodegradation or retained in sludge. Still, some compounds can be hydrophilic, thus remaining in soluble phase, and can be considered recalcitrant substances (Richardson and Bowron, 1985). Since conventional WWTP were initially not designed to remove emerging contaminants, these facilities are now considered one of the major sources of human pharmaceuticals to surface

waters, affecting aquatic organisms if still biologically active (Richardson and Bowron, 1985, Halling-Sørensen *et al.*, 1998, Petrović *et al.*, 2003, Fent *et al.*, 2006, Farré *et al.*, 2008). Furthermore, transport of pharmaceuticals to surface and ground water can occur when sludge from WWTP and manure from feedstock industry are applied on fields as fertilizers, due to their abundant content in organic matter. Indeed, potentially adsorbed pharmaceuticals can affect soil and water organisms, through leaching or runoff of contaminated soil (Halling-Sørensen *et al.*, 1998, Fent *et al.*, 2006, Farré *et al.*, 2008).

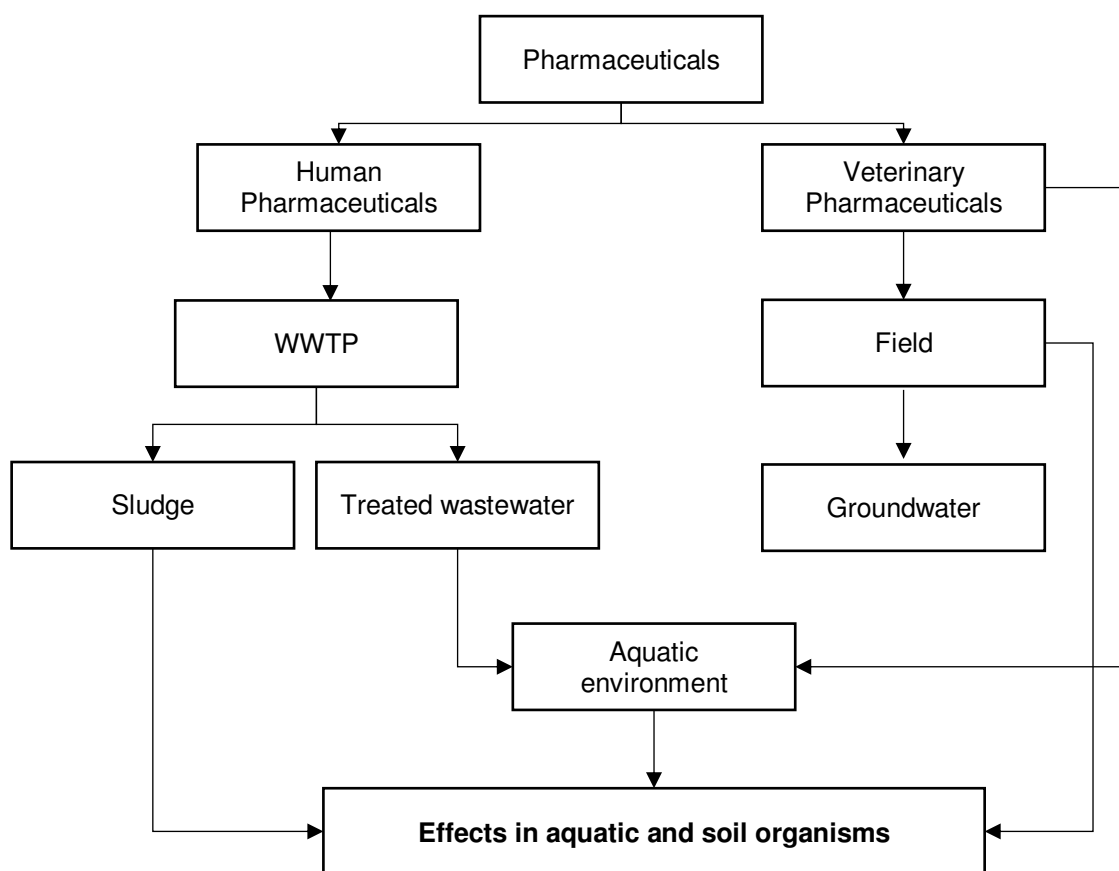


Figure 1 – Pharmaceuticals fate in the environment [Adapted from: Halling-Sørensen *et al.* (1998)]

In recent years, a great concern about antibiotics emerged among the scientific community, due to an increase in resistant bacteria in the environment. However, other classes of compounds should not be ignored, especially polar compounds, as acid pharmaceuticals, because they have a natural capacity to resist WWTP treatments, arising as a potential risk in drinking water supply (Petrović *et al.*, 2003).

The present research is focused on pharmaceutical compounds, which are representatives of two extensively consumed therapeutic classes: a lipid regulator, Bezafibrate (Bzf), and an antidepressant, Paroxetine (Prx).

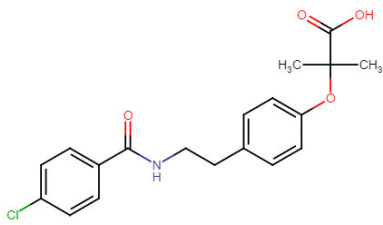
1.2. PHARMACEUTICAL COMPOUNDS

1.2.1. Bezafibrate

The increase in population with obesity and increased diagnosis and treatment promoted an exponential prescription and consumption of cholesterol-lowering pharmaceuticals (OECD, 2013). Indeed, in some countries that belong to the Organisation for Economic Co-operation and Development (OECD), the consumption of these classes of pharmaceuticals more than tripled from 2000 to 2011. In Portugal, the consumption of cholesterol-lowering pharmaceuticals increased from 19 defined daily doses (DDD) per 1000 inhabitants per day in 2000, to 88 DDD per 1000 inhabitants per day in 2011 (OECD, 2013).

Bzf is a commonly acidic pharmaceutical prescribed to treat hyperlipidemia, hypercholesterolemia and mixed hyperlipidemia (Tang *et al.*, 2014), holding a chlorine atom in its molecule, linked to a benzene ring. According to its physicochemical properties (Table 1), the acidity profile of Bzf is determinant for its environmental fate, since at environmental conditions anionic form of Bzf is favored (Luo *et al.*, 2014).

Table 1 - Relevant physicochemical properties of Bezafibrate

Molecular Structure		
		
Molecular Formula	C ₁₉ H ₂₀ ClNO ₄	PubChem
Molecular Weight	361.8194	PubChem
Acid Dissociation Constant (pKa)	3.6	Tang <i>et al.</i> (2014)
Partition coefficient (Log K_{ow})	4.25	Petrie <i>et al.</i> (2013)
Organic Carbon normalized sorption coefficient (Log K_D)	0.033	Ternes <i>et al.</i> (2007)
Water solubility (25 °C) (mg/L)	7.9	Petrie <i>et al.</i> (2013)
Henry's Law Constant (atm·m³/mol)	2.12x10 ⁻¹⁵	Petrie <i>et al.</i> (2013)

Although the partition coefficient suggests that adsorption to sediments or sludge would be likely to occur ($\text{Log } K_{ow} > 4$), the more accurate prediction takes in consideration K_{ow} and $\text{p}K_a$. In this way, the balance between these chemical characteristics indicates that Bzf can be removed by adsorption or remain in water phase, depending on environmental physicochemical parameters (Luo *et al.*, 2014).

Bzf is considered to have a relatively high rate of excretion from human body (40-69%), commonly being detected in influents of WWTP. In here, its removal is not always well-established. Lara-Martín *et al.* (2014) reported that Bzf present in the influent of a WWTP was completely removed during treatment, while Jelic *et al.* (2011) stated that only 14% of Bzf was removed during treatment at WWTP. On the other hand, Lindqvist *et al.* (2005) has reported removal efficiencies in a range of 15-100%. Thus, Bzf removal at WWTP was considered as a “Moderate Removal” (Luo *et al.*, 2014).

Several studies reported the presence of Bzf in WWTP, through influents (Lindqvist *et al.*, 2005, Luo *et al.*, 2014, Pereira *et al.*, 2015) (Table 2). Radjenović *et al.* (2009) reported a concentration range of 1900–29800 ng/L in effluent of primary treatment, while Lindqvist *et al.* (2005) reported the presence of Bzf in discharge point of WWTP (4-24 ng/L) and downstream from the discharge point (3 ng/L). Despite being sporadically detected in sediments or suspended solids, main concern arises from the presence of Bzf on discharge point of WWTP and its presence on surface water. Results in published literature suggest that inconsistent removal rates lead to the presence of Bzf in surface waters in European countries at a concentration range of 25-3100 ng/L (WHO, 2012). In Portugal, Gonçalves *et al.* (2013) reported the presence of Bzf in surface water, at a concentration range of 256-770 ng/L.

Table 2 - Occurrence of Bezafibrate in different environmental compartments

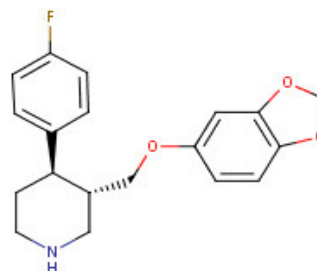
	Concentration (ng/L)	Reference
WWTP influent	3500	Luo <i>et al.</i> (2014)
	35.6-6000	Pereira <i>et al.</i> (2015)
WWTP effluent	5.8-324	Silva <i>et al.</i> (2011)
	4-24	Lindqvist <i>et al.</i> (2005)
	40-24000	Pereira <i>et al.</i> (2015)
Superficial Water	2.01-25.5	Silva <i>et al.</i> (2011)
	3-4	Lindqvist <i>et al.</i> (2005)
	256-770	Gonçalves <i>et al.</i> (2013)
	Concentration (ng/g)	
Suspended solids	1.17-203	Silva <i>et al.</i> (2011)
Sediments	0.5	Silva <i>et al.</i> (2011)

Bzf was classified as a high priority emerging contaminant, since it can have a significant impact in environmental health (GWRC, 2009). Toxicity effects indicated that this pharmaceutical compound can be harmful to aquatic life. Studies of ecotoxicology developed by Blaise *et al.* (2008) demonstrated that species from decomposers to secondary consumers can be negatively affected by the presence of Bzf in aquatic environment, either in acute or chronic exposition. *Vibrio fischeri* can suffer toxic effects at a concentration >15.6 mg/L, at acute tests, having its luminescence inhibited, while *Pseudokirchneriella subcapitata* can have growth inhibited at concentration >31.3 mg/L, at chronic toxicity tests. Micro-invertebrates *Hydra attenuata* and *Thamnocephalus platyurus* were also demonstrated to be sensitive to Bzf presence at concentrations of 5.3 and >62.5 mg/L, respectively. And for *T. platyurus* mortality was observed at the reported concentration (62.5 mg/L). Acute cytotoxicity test also demonstrated that primary hepatocytes of *Oncorhynchus mykiss* were damaged at concentration >362 mg/L.

Nevertheless, one should be aware that these concentrations are much higher than those normally found in the environment.

1.2.1. Paroxetine

Antidepressant pharmaceuticals had their consumption increased in most OECD countries. This can be related with the earlier diagnostic and consequently, the intensity and duration of treatment. Despite that, the milder forms of depression, as anxiety, can extend the treatment time, increasing the consumption over time. In Portugal, a rise of 20% in consumption was verified between 2007 and 2011 (OECD, 2013). Prx is an antidepressant pharmaceutical, that belongs to selective serotonin reuptake inhibitors (SSRIs), being one of the most prescribed drug to treat anxiety and depression (Brown *et al.*, 2015). Table 3 represents a brief resume of some important characteristics of Prx. The high value of Log K_{ow} may suggest that Prx can adsorb to sediments and organic matter. In fact, in environmental conditions, Prx is found commonly in the cationic form, which can potentiate the formation of a complex with sediments or organic carbon (OC) (negatively charged) (Kwon and Armbrust, 2008, Brown *et al.*, 2015, Semblante *et al.*, 2015). Furthermore, its distribution coefficient (K_D) indicates that Prx can be either extremely adsorbed or just slightly, especially with pH increasing (Brown *et al.*, 2015). Prx has not raised a high attention regarding its toxic effects because it is mostly excreted as metabolites either in feces and urine, and only 3% are expelled in parental form (Cunningham *et al.*, 2004).

Table 3 - Relevant physicochemical properties of Paroxetine**Molecular Structure**

Molecular Formula	C ₁₉ H ₂₀ FNO ₃	PubChem
Molecular Weight	329.365403 g/mol	PubChem
Acid Dissociation Constant (pKa)	9.6	Cunningham <i>et al.</i> (2004)
Partition coefficient (Log K_{ow})	3.6	PubChem
Organic Carbon normalized sorption coefficient (K_D)	131-5067	Brown <i>et al.</i> (2015)
Water solubility (25 °C) (mg/L)	6804	Cunningham <i>et al.</i> (2004)
Henry's Law Constant (atm·m³/mol)	<10 ⁻⁸ to 10 ⁻¹²	Cunningham <i>et al.</i> (2004)

Several studies have reported Prx presence in effluents, being also detected in surface water (Table 4). Concentration of 90 ng/L of Prx in surface water (Wu *et al.*, 2009) and 150-386 ng/L at WWTP effluents (Collado *et al.*, 2014) have been reported. However, due to its physicochemical behavior, it has a great tendency to be retained in organic matter (Brown *et al.*, 2015). Indeed, Wu *et al.* (2009) has reported that Prx was present in biosolids from a local WWTP in a concentration of 87 ng/g.

Table 4 - Occurrence of Paroxetine in different environmental compartments

	Concentration (ng/L)	Reference
WWTP influent	270	Lara-Martín <i>et al.</i> (2014)
	16	Verlicchi <i>et al.</i> (2012)
	9.1-9.7	Schlüsener <i>et al.</i> (2015)
WWTP effluent	150-386	Collado <i>et al.</i> (2014)
	16	Verlicchi <i>et al.</i> (2012)
	2.3-2.4	Schlüsener <i>et al.</i> (2015)
Superficial Water	90	Wu <i>et al.</i> (2009)
	Concentration (ng/g)	
Biosolids	87	Wu <i>et al.</i> (2009)

Daphnids are sensible to Prx at 50 µg/L and 490 µg/L, in chronic and acute toxicity tests (Brown *et al.*, 2015). Toxic effects of Prx on *Mytilus edulis* were also studied. Prx lead to loss in cell viability at 15 mg/L and to DNA strand breaks in hematocytes at concentration

of 1.5 µg/L (Lacaze *et al.*, 2015). In this study, genotoxic impact of Prx was associated to its cytotoxic effect. Furthermore, Prx was considered one of the most toxic compounds of the study. Also, Prx was detected in fish tissue (Gelsleichter and Szabo, 2013). Thus, Prx can have an important impact in the ecosystem, because it can be present in the environment, causing effects in organisms and bioaccumulating.

1.3. MICROBIAL COMMUNITIES AND BIOREMEDIATION

Natural ecosystems functioning is dependent on microorganism's activity, since they are primary producers, decompose organic matter, participate in nutrient cycles and are considered natural attenuators (Altieri, 1999, Stenuit *et al.*, 2008). Microorganisms have a key role in soil and water purification processes, although the presence of a diverse and abundant microbial community is essential to promote an effective response to the different contamination agents (Oladele, 1999, Allison and Martiny, 2008). Several laboratory studies (Carvalho *et al.*, 2006, Yu *et al.*, 2006, Zhu *et al.*, 2007, Barra Caracciolo *et al.*, 2013, Ribeiro *et al.*, 2013, Abed *et al.*, 2014, Dey *et al.*, 2016, Sheu *et al.*, 2016, Wang *et al.*, 2016) reported the efficient degradation of different pollutants by microbial communities or single bacterial strains, obtained from known contaminated sites.

Bioremediation is a cost-effective tool that uses microbial *consortia* or bacterial strains to remove pollutants from the environment and remediate contaminated environments. It emerged in environmental biotechnology field and has been increasingly studied and applied in real situations (Stenuit *et al.*, 2008, Juwarkar *et al.*, 2010). Bioremediation techniques include natural attenuation, biostimulation and bioaugmentation. While natural attenuation corresponds to the natural remediation capacity of a microbial community present in a contaminated site to control the spread of contamination, biostimulation requires the addition of nutrients or electron acceptors to stimulate the intrinsic degradation capacity of bacteria. Bioaugmentation is characterized by the inoculation of contaminated sites with strains or microbial *consortia* with biodegrading capacities in sites where natural attenuation does not exist, where microorganisms do not have degradative capacity, or is too slow (Stenuit *et al.*, 2008, Hosokawa *et al.*, 2009, Juwarkar *et al.*, 2010). This technique has three different approaches: i) application of indigenous microorganisms, ii) application of exogenous microorganisms and iii) application of genetically modified microorganisms (Hosokawa *et al.*, 2009, Juwarkar *et al.*, 2010). Application of genetically modified microorganisms can cause negative impacts on the environment, and so its application involves strict rules. Moreover, bioaugmentation technique involving the application of exogenous microorganisms can present disadvantages, since effects on natural microbial community and environment are

unknown, and exogenous microorganisms are most of the times incapable to compete with indigenous microorganisms (Hosokawa *et al.*, 2009). The use of indigenous microorganisms - autochthonous bioaugmentation - can be more efficiency, since the use of microorganisms from the specific contaminated sites, can decrease the period of acclimatization and adaption to the autochthonous conditions.

However, bioremediation techniques require an extensive knowledge on physicochemical properties of the pollutant, as well as on the microbial community regarding its structure, phenotypic potential, function and environmental interactions (Stenuit *et al.*, 2008). Fingerprinting approaches, as Automated Ribosomal Intergenic Spacer Analysis (ARISA), is a cost-effective and rapid method to study complex communities in a variety of ecosystems or controlled experiments, though fingerprint does not inform about taxonomic profile. However, consistencies between microbial structures generated from ARISA fingerprint and more in-depth methods, as 454-pyrosequencing, were reported (Stenuit *et al.*, 2008, van Dorst *et al.*, 2014). ARISA targets the intergenic transcribed spacer (ITS), a region with great taxonomic resolution that allows verifying genome diversification and evolutionary relationship (Ettoumi *et al.*, 2013).

To better understand the response of a microbial community to a specific pollutant, it is necessary to study their interactions. Laboratory microcosms are recognized as ecosystem models, since they contain natural biotic communities under controlled environmental conditions (e.g. temperature and light). Confined environment of microcosms allows to establish a causal relationship between the pollutant and its effects on the studied community (Barra Caracciolo *et al.*, 2013), that would not be possible in natural ecosystem due to the interference of non-controlled environmental factors.

1.4. CONTAMINATED SITES

In literature, the relation between environmental contamination and anthropogenic activity is well-established. In fact, the increase of hazardous waste sites around the world is proportional to increase of population. Despite some of the pollutants being natural compounds (e.g. fossil fuels, metals), human activity has a key role in the contamination of the environment by these compounds, as well as by emerging contaminants.

As previously reported, WWTPs are described as pharmaceuticals contamination source, since these compounds have a tendency to accumulate in sludge or be release into the surface water (Farré *et al.*, 2008). When in surface water, pharmaceuticals can be distributed in the ecosystem, reaching estuarine ecosystems. Thus, bacterial communities present in both systems can interact with these substances.

Knowing that autochthonous bacterial communities with natural remediation capacities can be present in contaminated sites, the collection of these microorganisms can be useful for the development of bioremediation techniques, such as autochthonous bioaugmentation, with the application of specific strains or microbial *consortia* in contaminated sites.

1.4.1. Douro estuary

The Douro River is an international river, flowing 930 Km since its source in Sierra de Urbión, Spain, until the river mouth in Oporto city, Portugal. Aside from being the third longest river in the Iberian Peninsula, its watershed is the largest in the Iberian Peninsula (98000 km²). Over its flow, Douro River receives water from several natural effluents both in Spain and in Portugal. Douro river estuary is considered an urban estuary since approximately 550 000 inhabitants occupy both banks. Douro estuary receives effluents from eight WWTPs, either directly or indirectly, in which two of them discharge to the estuary. These two WWTPs treats wastewater residues from an equivalent of 370 000 inhabitants (Madureira *et al.*, 2010).

In the last decades, Douro River estuary has been indicated as a highly contaminated environment (Fig. 2) due to the high anthropogenic pressure that it undergoes. Several studies reported the presence of metals (Mucha *et al.*, 2003, Mucha *et al.*, 2004, Gravato *et al.*, 2010), endocrine disruptors (Ribeiro *et al.*, 2009, Rocha *et al.*, 2012), polycyclic aromatic hydrocarbon (Gravato *et al.*, 2010), pesticides (Waszak *et al.*, 2014) and pharmaceuticals (Madureira *et al.*, 2010) in Douro estuary.

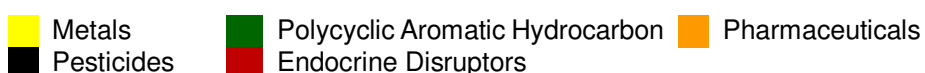


Figure 2 - Reported contaminants detected at Douro estuary [Adapted from: Mucha *et al.*, 2003, Mucha *et al.*, 2004, Ribeiro *et al.*, 2009, Gravato *et al.*, 2010, Madureira *et al.*, 2010, Rocha *et al.*, 2012, Waszak *et al.*, 2014]]

1.5. OBJECTIVES

The present work aimed to explore the potential of autochthonous microorganisms, from Douro estuary sediments and from activated sludge of a WWTP discharging into the estuary, for bioremediation of pharmaceuticals. As representatives of extensively used pharmaceuticals, an antidepressant drug (Prx) and a lipid regulator (Bzf) were chosen. Two experiments were separately conducted to understand the biodegradation capacity of microbial communities from the two origins.

The first experiment was carried out in microcosms inoculated with estuarine sediment or activated sludge, doped with one of the selected pharmaceutical compounds (Bzf or Prx), in batch mode, with different agitation conditions. This experiment aimed to evaluate changes in microbial community structure caused by the presence of the pharmaceutical compound and the effects of incubation conditions and time. It also aimed to understand the involvement of these microorganisms in pharmaceuticals removal/degradation from the medium.

The second study aimed to evaluate the capacity of isolated strains derived from the enrichment culture in the first experiment, re-united in *consortia* to degrade the selected pharmaceutical to which they were previously exposed.

This dissertation is divided in 5 chapters. The first one consists of a general introduction and literature review of the theme. The second chapter is dedicated to the material and methods. In the third and fourth chapters are presented the results, discussion and conclusions of the first and second experiment, respectively. The last chapter is dedicated to the general conclusions and future directions.

CHAPTER 2

METHODS

2. METHODS

2.1. EXPERIMENTS

2.1.1. Reagents and materials

Paroxetine from Enzo Life Sciences, and Bezafibrate, methanol, acetonitrile and formic acid (98%) were acquired from Sigma-Aldrich. All remaining reagents were analytical grade or equivalent.

All material used in both experiments were decontaminated or sterilized in order to avoid chemical and/or biological contaminations. Chemical decontamination was performed by immersing the material in a chloride acid bath for 24 h, being further washed with deionized water and placed at a warm house (40 °C) to remove water present on the material. Biological sterilization was performed by autoclaving the material (120 °C, 20 min).

2.1.2. First experiment

The samples to be used as inocula were collected in the Autumn (October, 2015) in two different compartments: sediments from the Douro estuary and activated sludge from a local WWTP (Fig. 3). The collection was performed under sterile conditions and transported to the laboratory in the dark. The study consisted in two experiments in parallel, each one for the different type of sample.

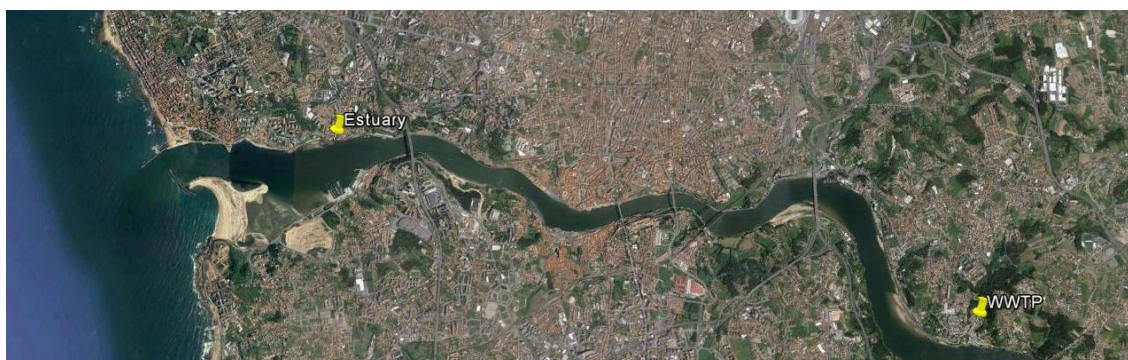


Figure 3 – Sampling sites at north bank of douro river

Once in the laboratory, a portion of each initial sample was frozen at -20 °C for further analysis (DNA extraction for microbial community analysis).

The remaining amounts were used for microcosms assembling. For this, samples (10 mL of activated sludge from WWTP or 10 g of estuarine sediment) were placed in flasks

of 250 mL, containing four portions of mineral-salt medium (MM) (40 mL). This medium contained essential macro and micro nutrients, as inorganic salts and a buffer (Na_2PO_4), which provide the suitable conditions for metabolic activity, and consequently microorganisms growth (Amorim *et al.*, 2014). Microcosms were then doped with Bzf or Prx (1 mg/L). For that a proper amount of a methanolic solution (prepared by dissolving a known amount of each compound in methanol) of each compound was added to the medium.

Microcosms were supplemented with sodium acetate (500 mg/L) to promote microorganisms' metabolic activity, generating a co-metabolism with the pharmaceutical compound.

For each type of inoculum and compound tested, additional microcosms were assembled to identify some of the processes responsible for the pharmaceuticals removal. Adsorption controls were assembled, consisting in sterilized samples (by autoclaving) dissolved in MM, whereas the abiotic controls consisted only in sterilized MM, both doped with one pharmaceutical compound. Moreover, microcosms without addition of pharmaceutical compounds were assembled, acting as microbiological control, to evaluate how the microbial community adapted to incubation conditions.

Microcosms were setup in triplicates and incubated at room temperature, in the dark. The incubation differed in terms of agitation conditions, with part of the microcosms in static conditions and the other in constant agitation (130rpm) (Fig. 4).

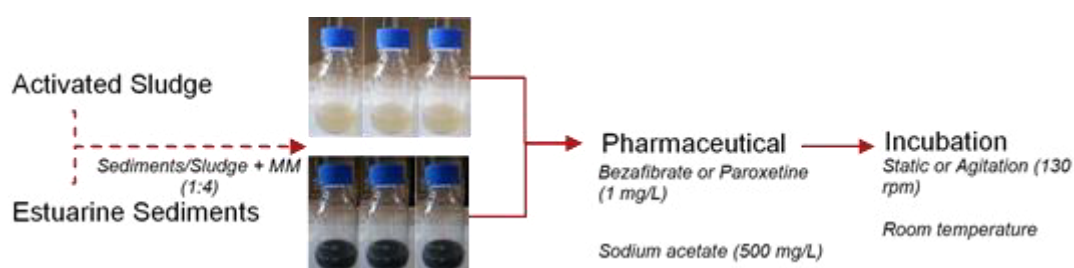


Figure 4 - Scheme of microcosms setup

To preserve the different *consortia* obtained at the end of the experiment, triplicates from each treatment were combined in a sterile tube. The *consortia* of all experiments were then preserved in glycerol (85%) at $-80\text{ }^\circ\text{C}$, for future reactivation.

2.1.3. Second Experiment

Based on the results obtained in first part of the study, three *consortia* of Bzf enrichment and two from Prx enrichment were isolated. Prx *consortia* isolation and

purification was performed for both inocula, but only for the cultures in static conditions (P1 and P2, corresponding to estuarine sediments and activated sludge, respectively). Regarding Bzf, isolation was performed for both inocula for the cultures in static conditions (B1 and B2, corresponding to estuarine sediments and activated sludge, respectively), and for one culture from activated sludge under agitation conditions (B3). For that, several dilutions were prepared from the selected *consortia*, being inoculated following spread-plate method into plate count agar (PCA) media and MM enriched with the corresponding pharmaceutical (1 mg/L) and sodium acetate (500 mg/L), solidified by the addition of agar (15 g/L) (Fig. 5).

After analyzing abundance and diversity of strains in plate media, through morphologic analysis, strains were purified in the respective medium by streak-plate method. Isolated strains were preserved in glycerol (85%) and at -80 °C, and a sample of biomass was collected and frozen in sterile ultra-pure water for future DNA extraction and sequencing.

To verify the efficiency of the isolated strains to degrade the pharmaceutical compound tested previously, isolated strains were inoculated in liquid MM (40 mL), doped with the respective pharmaceutical (1 mg/L) and supplemented with sodium acetate (500 mg/L) (Fig. 5). For that, an equivalent portion of biomass was collected from the respective plate, until a final Optical density (OD) ($\lambda=600$ nm) between Abs=0.450-0.500.

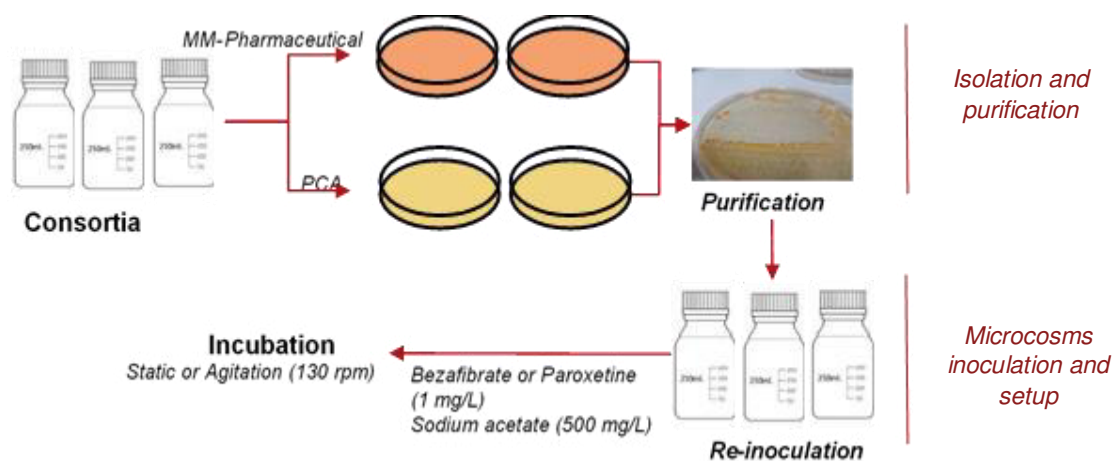


Figure 5 - Scheme of microcosms set up with isolated strains

The experiment was assembled in triplicates and incubated in conditions similar to the previous experiments, i.e., *consortia* obtained from microcosms in agitation in the first experiment were maintained in agitation, and *consortia* from static conditions in first part were maintained in static conditions.

2.1.4. Microcosms Operation

Microcosms experiment was carried out for 7 feeding cycles of 2 weeks each in first experiment and 3 feeding cycles of 2 weeks in the second experiment. First feeding cycle started in the first day of the experiment, while the following feeding cycles started in the last day of the preceding (Fig. 6). During this process, microcosms were fed with sodium acetate (500 mg/L) twice a week, and to ensure the presence of oxygen in the microcosm each culture was transferred to a new sterilized flask, also twice a week.

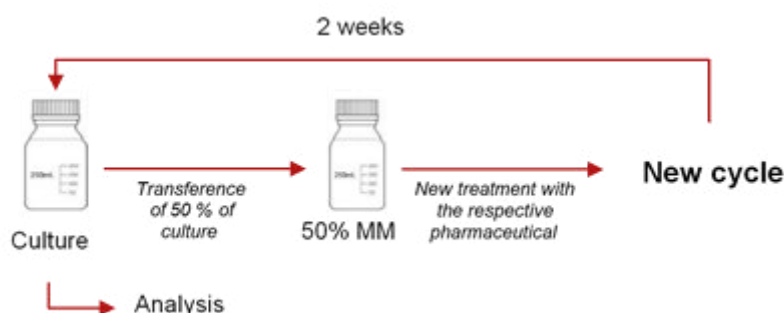


Figure 6 - Schematic example of microcosms operation, culture renewal and sampling

Upon the end of each feeding cycle, 50% of each culture was transferred to a new microcosm vessel containing 20 mL of MM, being doped with the respective pharmaceutical compound, in the same dosage. The remaining solution was collected to determine pharmaceuticals removals in both experiments and microbial growth and the impact on the microbial community (2nd and 14th week), in the first experiment. Oxygen content in solution was measured in the end of the first experiment using an electrode for dissolved oxygen.

2.2. ANALYTICAL DETERMINATIONS

2.2.1. Microbial Growth

Microbial growth was estimated by measuring the OD by spectrophotometry (VWR V-1200 spectrophotometer) at 600 nm at the beginning and at the end of each feeding cycle. In the beginning of the feeding cycle, a sample of the new culture was collected in order to follow microbial growth over the feeding cycle, while a sample of culture was collected from the remaining solution for future analyses.

2.2.2. Pharmaceuticals Removal from solution

To determine each pharmaceutical compound concentration, the solution collected at the end of each cycle was transferred into amber glass vials and centrifuge (at 2500 rpm).

The supernatant was then collected for further analysis by high-performance liquid chromatography (HPLC). Compounds analyses were carried out in a HPLC Beckam Coulter equipment (System Gold) provided with a diode array detector (module 168) and an automatic sampler (module 508). Compounds separation was performed in gradient mode with two eluents, water /formic acid, 99:1 (v/v) and acetonitrile (both previously degassed in an ultrasonic bath), with a flow rate of 1 mL/min, in a 100 mm x 4.6 mm Kinetex 2.6 μ m C18 column (Phenomenex, UK). Samples were injected in a volume of 50 μ L and detector signal was monitored at λ =252 nm for Bzf and at λ =298 nm for Prx. For compounds quantification, a calibration curve with aqueous standard solutions (0.1 to 2 mg/L) of each compound was used. Each solution was prepared from stock standard solution prepared by dissolving a known amount of each compound in methanol.

2.2.3. Fluoride Anion Release

The quantification of fluoride anion concentration in solutions from Prx treatments was carried out using a fluoride ion-selective electrode (CRISON GLP21). A calibration curve was prepared using aqueous standard solutions (0.001; 0.0025; 0.005; 0.01; 0.02; 0.1; 0.2 and 1 mM NaF). To minimize interferences, 10% of a total ionic strength adjustment buffer (TISAB II) solution was added to each sample.

2.2.4. Bacterial community structure

DNA was extracted from initial inocula (0.4-0.6 g) and from microcosms (solid phase) at the end of the first (2 weeks) (0.2-0.6 g) and last (14 weeks) (0.1-0.3 g) feeding cycles, using MO BIO PowerSoil kit. The efficiency of DNA extraction was monitored in electrophoresis in gel agarose 1.5%.

Bacterial community structure was evaluated by ARISA, a rapid and effective technique that allows assessing microbial community diversity, being particularly useful for studies with spatial and temporal scales (Fisher and Triplett, 1999). This analysis requires PCR amplification of total DNA of the intergenic spacer region (ITS) (16S-23S) of rRNA operon genes. The intergenic region may encode tRNAs specific on the bacterial species, acting as an indicator of heterogeneity and also providing taxonomic resolution (Fisher and Triplett, 1999, Brown *et al.*, 2015).

DNA amplification used a set of primers specific for bacterial communities, ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3') (Cardinale *et al.*, 2004). PCRs were performed in duplicate reactions of 25 μ L, containing 0.4 μ M of both primers, 0.2 mM of dNTP's, 2.5 U Taq DNA polymerase, 1 mg/mL bovine serum albumin and 2-3.5 mM MgSO₄. PCR products were visualized by electrophoresis in

gel agarose 1.5%, and purified using a purification kit (UltraClean 15 DNA Purification Kit, MO BIO Laboratories Inc.), being the purified product quantified by Quant-it HsDNA assay kit Qubit fluorometer (Invitrogen). Sample fragments were run on a ABI3730 XL genetic analyzer at STABVIDA Sequencing Facilities (Lisbon, Portugal).

2.3. STATISTICAL ANALYSIS

In each treatment, triplicates were analyzed and treated in separate, being the average and standard deviation values calculated for each treatment, at the different periods.

ARISA fragment lengths were evaluated by Peak Scanner™ version 1.0 Software (Applied Biosystems). Data were transferred to an excel sheet and transformed in a matrix of aligned fragments, to be analyzed in PRIMER 6 software package (version 6.1.11). Fragments with fluorescence units inferior to 50 were rejected, being considered “background noise”. Fragments bellow 200 bp were not considered because they are considered to be too short ITS for bacteria. Bacterial richness and diversity indexes were calculated using Primer 6 software (version 6.1.13) to address the ecological description of community, wherein peaks number was representative of species number and peak height represented relative abundance of each bacterial species.

To evaluate the community structure, the matrix was normalized using presence/absence pre-treatment function and samples were analyzed using Bray–Curtis similarity method, and then examined using a hierarchical cluster analysis. A multidimensional scaling (MDS) was obtained using default parameters with a minimum stress of 0.01 to generate a configuration plot based on percentage similarity. Microbial community similarity was evaluated through an analysis of similarity (two-way crossed ANOSIM, based on Bray-Curtis similarity), using PRIMER 6 software. This analysis is a permutation-based hypothesis statistical test, equivalent to univariate ANOVA, which tests for differences between groups of (multivariate) samples from different factors or experimental treatments (Danovaro *et al.*, 2006).

Statistic tests were performed using commercial software Statistica, version 13, Dell Inc. (2015). For pharmaceuticals concentrations, defluorination rates, bacterial richness and diversity significant differences among samples were calculated through a non-parametric analysis, and significant differences ($p < 0.05$) were detected by Kruskal-Wallis ANOVA multiple comparisons test.

CHAPTER 3

BIOREMEDIATION OF BEZAFIBRATE AND PAROXETINE BY MICROORGANISMS FROM A WASTEWATER TREATMENT PLANT AND ESTUARINE SEDIMENTS

3. BIOREMEDIATION OF BEZAFIBRATE AND PAROXETINE BY MICROORGANISMS FROM A WASTEWATER TREATMENT PLANT AND ESTUARINE SEDIMENTS

3.1. RESULTS

The maintenance of all microcosms was performed under sterilized conditions, being incubated at room temperature (20°C) and in the dark. Microcosms' incubation only differed in agitation conditions. Microcosms with the same pharmaceutical compound were incubated in static conditions and under agitation conditions (1300 rpm). Agitation conditions influenced the oxygenation of the microbial cultures, however the presence of oxygen in microcosms was also influenced by flask renewal twice a week. Indeed, in microcosms under agitation conditions, oxygen dissolved in solution had higher concentration values (8.67-9.37 mg/L) than in microcosms under static conditions (4.74-5.13 mg/L).

The results reported in this section are in terms of microbial community structure and pharmaceutical removal from solution. The latest includes results from microbial cultures as well as results regarding pharmaceuticals adsorption potential to sediments and cells and degradation/removal by abiotic processes. Complementary analyses of fluoride anion released in experiments with Prx are also reported.

3.1.1. Pharmaceuticals Removal

Pharmaceuticals removal from microbial cultures was evaluated by analyzing samples of the liquid portion of the microcosms for both inocula. These data are complemented with analysis of abiotic and adsorption controls solution. The experiment had the duration of 14 weeks, adsorption potential experiment was only performed for 10 weeks, due to the complete removal of solid particles and cells by renewal cycles, making unfeasible the continuation of the trial.

3.1.1.1. Bezafibrate

3.1.1.1.1. Cultures enrichment

Removal percentages of Bzf from solution (Fig. 7) showed that incubation conditions had a greater effect in removal efficiency than the origin of the inocula. Indeed, in static conditions (Fig. 7A) Bzf showed high removal rates over time, never being detected in solution (<0.03 mg/L), in microcosms containing activated sludge or estuarine sediments,

leading to estimated removal above 97%. In agitation conditions (Fig. 7B) different responses according to the inoculum origin and experimental time were observed. Actually, after two weeks of the experiment Bzf was only partial removed from solution: 30 ± 7 % and 86 ± 3 % for activated sludge and estuarine sediments, respectively. Removal efficiency improved over time, reaching a steady state, with no compound detection in solution (<0.03 mg/L), after six weeks of the experiment, in case of microcosms inoculated with activated sludge, or after 8 weeks of experiment, in case of microcosms inoculated with estuarine sediments.

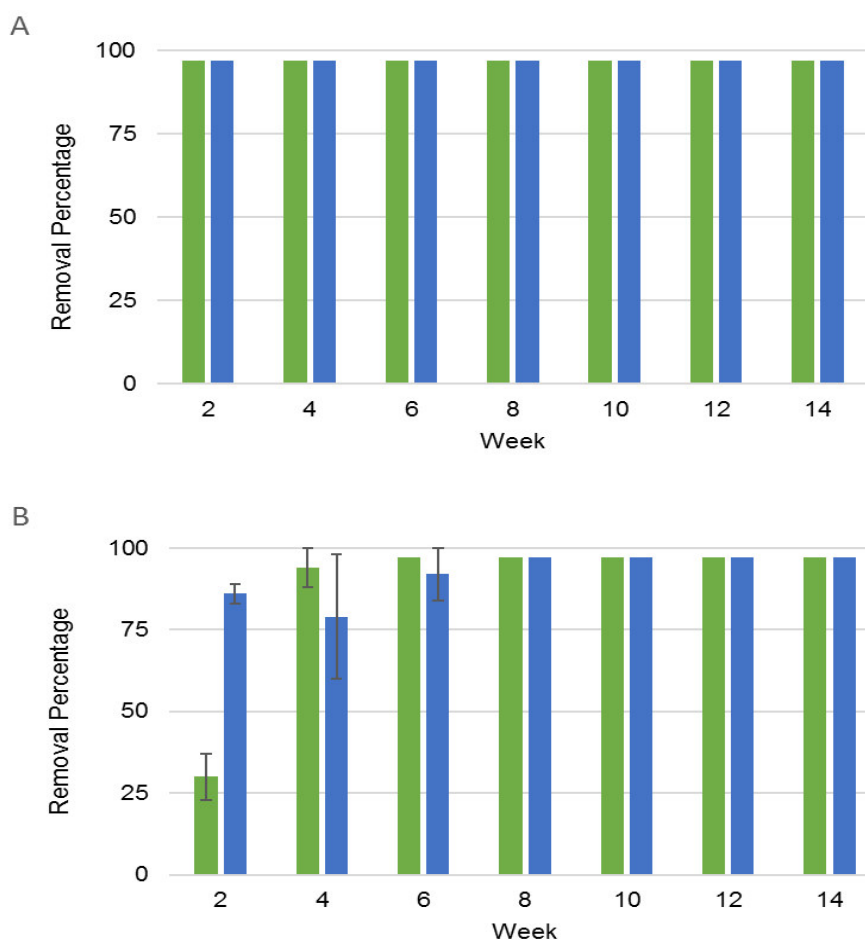


Figure 7 - Removal percentages (average and standard deviation, n=3) of Bzf from solution in microcosms with both inocula (Green – Activated Sludge, Blue – Estuarine Sediment), under both incubation conditions (A – Static, B – Agitation)

3.1.1.1.2. Abiotic Degradation and Adsorption Potential

The results obtained from the analysis of abiotic controls (only culture medium without inocula) indicated that Bzf is not degraded by abiotic processes, since it was

detected in the expected concentration in solution (1 mg/L). Furthermore, differences were not observed in the different incubation conditions.

Regarding Bzf adsorption potential, no significant tendency to be adsorbed to sterilized cells or sediment was observed, in either incubation conditions. In fact, only in some sporadic cases Bzf in solution was lower than doped concentrations (concentration range 0.7-1 mg/L, results not shown), however these values corresponded to a few replicas in the several analyses performed, and the tendency was not observed in the following sampling times.

3.1.1.2. Paroxetine

3.1.1.2.1. Enrichment culture

Removal percentage of Prx was, in general, not affected by the different incubation conditions (Fig. 8). Under static conditions (Fig. 8A) Prx was not detected (<0.03 mg/L) in solution during the time of the experiment either in microcosms of activated sludge or estuarine sediment. In agitation conditions (Fig. 8B), although in microcosms from estuarine sediment Prx was not detected in solution, it was not completely removed in microcosms inoculated with activated sludge after 2 weeks of the experiment (ca. 94% removal). But, in this late case, the maximum removal efficiency (Prx concentration <0.03 mg/L) was achieved in the following cycles.

3.1.1.2.2. Abiotic Degradation and Adsorption Potential

Prx demonstrated to be degraded by abiotic processes. After two weeks of experiment, Prx showed abiotic removal of 63% and 60% in static and agitation conditions, respectively. In the following sampling periods, these values decreased and remained stable (ca. 45%) during the remaining time of the experiment.

Analyzing the results obtained from adsorption controls it is possible to verify that Prx had potential to be adsorbed by particles. Since adsorption controls consisted in sterilized inoculum, biomass growth did not occur. Thus, Prx adsorption potential varied in function of the presence of particles, which decreased over time due to the medium renewal that were performed at the end of each period of two weeks (Fig. 9). In fact, two weeks after the beginning of the experiment, Prx was not detected in solution in microcosms with sterilized estuarine sediment, while in microcosms with sterilized activated sludge it was detected but in very low amounts (0.07 mg/L).

In the case of the adsorption controls with estuarine sediment, this tendency continued until week 6 after the beginning of the experiment, with Prx not being detected.

Only after 10 weeks it was possible to detect Prx in solution (removal of 54% and 69% in static and agitation conditions, respectively). At this time, solution was found to be very clear (OD~0.030, $\lambda=600$ nm), since sediments particles were largely removed from solution due to repeated medium renewal each two weeks.

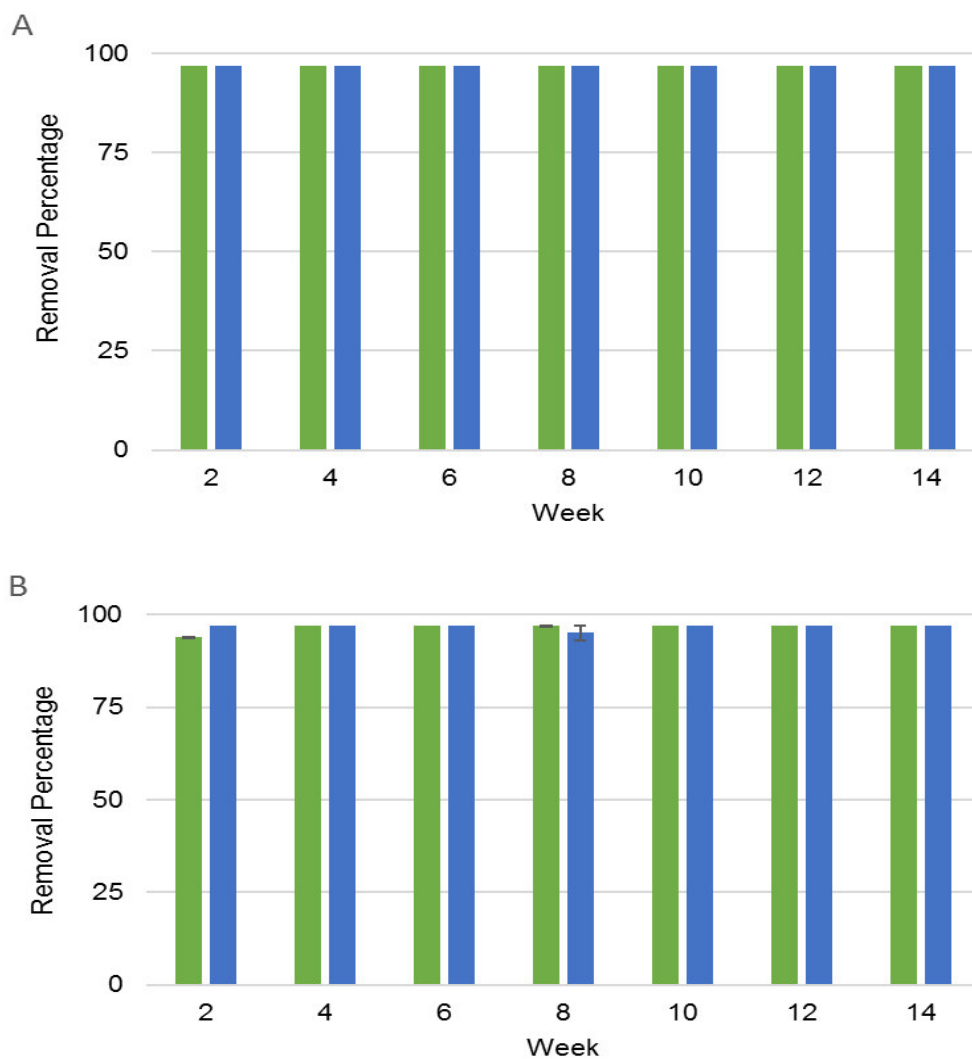


Figure 8 - Removal percentages (average and Standard Deviation, n=3) of Prx from solution in microcosms with both inocula (Green – Activated Sludge, Blue – Estuarine Sediment), under both incubation conditions (A – Static, B – Agitation)

Regarding adsorption controls of activated sludge, at week 6, a large part of the particles was already removed from solution, being possible to detected Prx in solution. Incubation conditions had no effect in the adsorption potential, with removal percentages of 75% and 65% in static and agitation conditions, respectively. Removal percentages were even lower after 10 weeks.

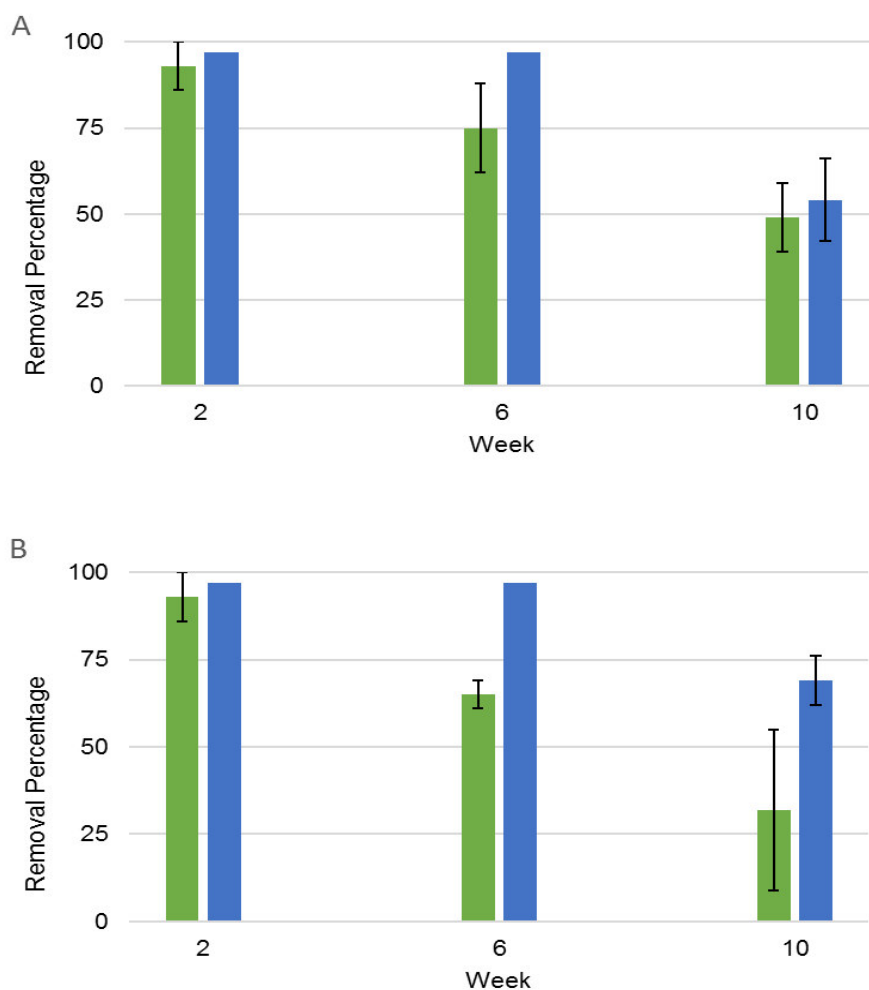


Figure 9 - Removal percentages (average and standard deviation, n=3) of Prx from solution in adsorption controls for both sterilized inocula (Green - Activated Sludge, Blue - Estuarine Sediment), under both incubation conditions (A - Static, B - Agitation)

3.1.2. Fluoride Anion Release

Since Prx is a fluorinated compound, the quantification of fluoride anion in solution can act as an indicator of the degradation of this pharmaceutical. However, the presence of organic particles and sediments interfered with the measurement of this parameter until 4 weeks after inoculation with activated sludge and after 8 weeks in microcosms inoculated with estuarine sediment (Table 5).

In general, microcosms with activated sludge presented high values of defluorination efficiency, at week 6. However, it was in static conditions that defluorination efficiency reached the higher values. In agitation conditions, it is possible to verify that defluorination efficiency increased with experimental time.

Analysis performed in abiotic controls indicated that defluorination did not occur by abiotic processes (fluoride anion not detected in solution), indicating that the release of fluoride anion only occurred through biologic processes.

Table 5 - Percentage of defluorination percentage (mean and standard deviation, n=3) in microcosms enriched with Paroxetine, over time

Condition Week	Activated Sludge		Estuarine Sediment	
	Static	Agitation	Static	Agitation
6	65±7	49±10	-	-
8	86±6	39±7	-	-
10	62±2	46±7	74±9	11±11
12	100±0	49±13	72±10	42±17
14	79±29	78±11	86±4	58±3

3.1.3. Biomass Growth

Biomass growth was estimated at the end of each feeding cycle, by spectrophotometry, at a wavelength of 600 nm, to understand how the community adapted to incubation conditions. The method measures the turbidity of the culture, which is an indicator of the biomass growth and how well distributed it is in the medium.

These results indicated that incubation conditions affected the biomass growth of activated sludge microorganisms (Fig. 10). Indeed, microbial cultures with and without pharmaceutical addition showed to be affected by agitation in the first weeks of the experiment (Fig. 10B), while in static conditions growth was favored in cultures in the same period (Fig. 10A). In static conditions a much higher biomass growth was observed in microbial culture enriched with Prx comparing with control. Over time, these cultures showed a certain tendency to stabilize.

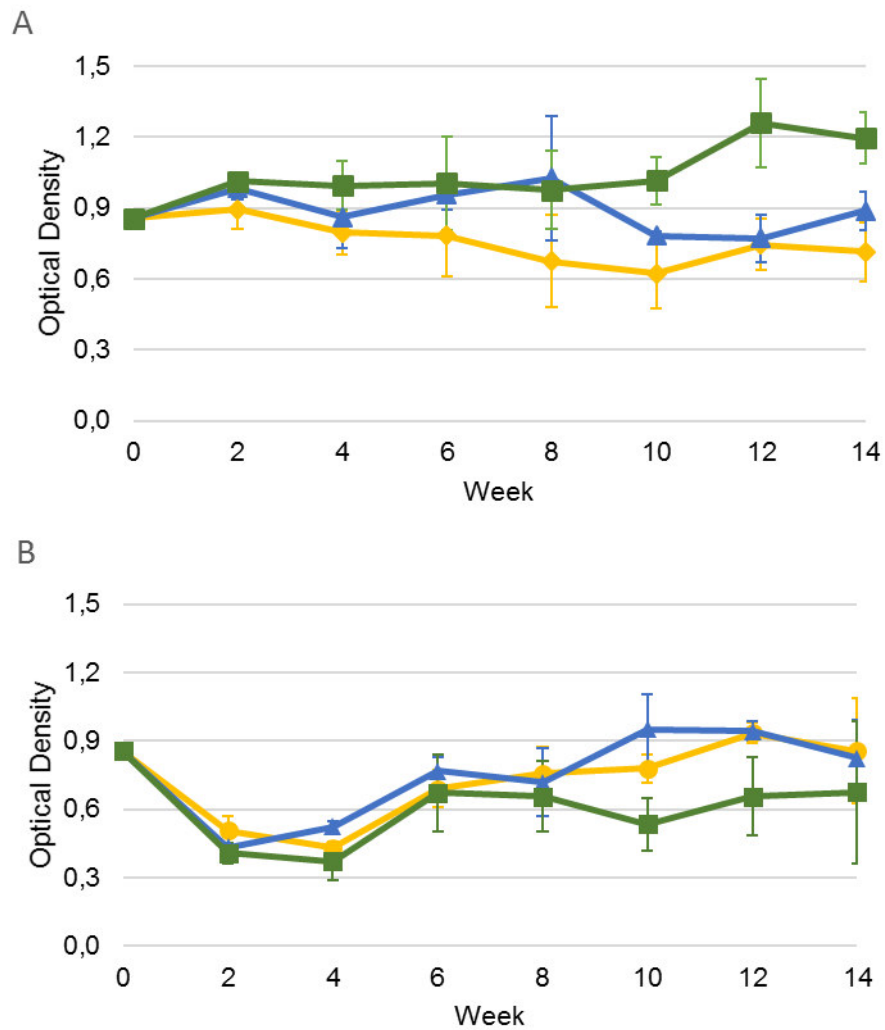


Figure 10 - Optical density (Abs at 600nm), measured over time, of cultures of activated sludge enriched with Bzf (Blue), Prx (Green) or without pharmaceutical addition (control) (Yellow) under both incubation conditions. A – Static, B – Agitation

For the cultures inoculated with estuarine sediment, biomass growth was only determined after 8 weeks of the experiment, since the presence of sediments interfered with the selected method. From this period on, it is possible to verify that, similarly to what occurred in microcosms from activated sludge, incubation conditions influenced biomass growth (Fig. 11). In fact, microcosms with the same treatment demonstrated a tendency to have higher biomass growth under static conditions than under agitation conditions. In static conditions, microcosms enriched with pharmaceuticals presented a clear tendency to have higher growth than the control in the same conditions (Fig. 11A). However, differences between treatments were not observed in agitation conditions (Fig. 11B). After 10 weeks of

experiment, flocculation occurred in microcosms under agitation conditions, with or without pharmaceutical enrichment. This can indicate the maladjustment of the community to the incubation conditions.

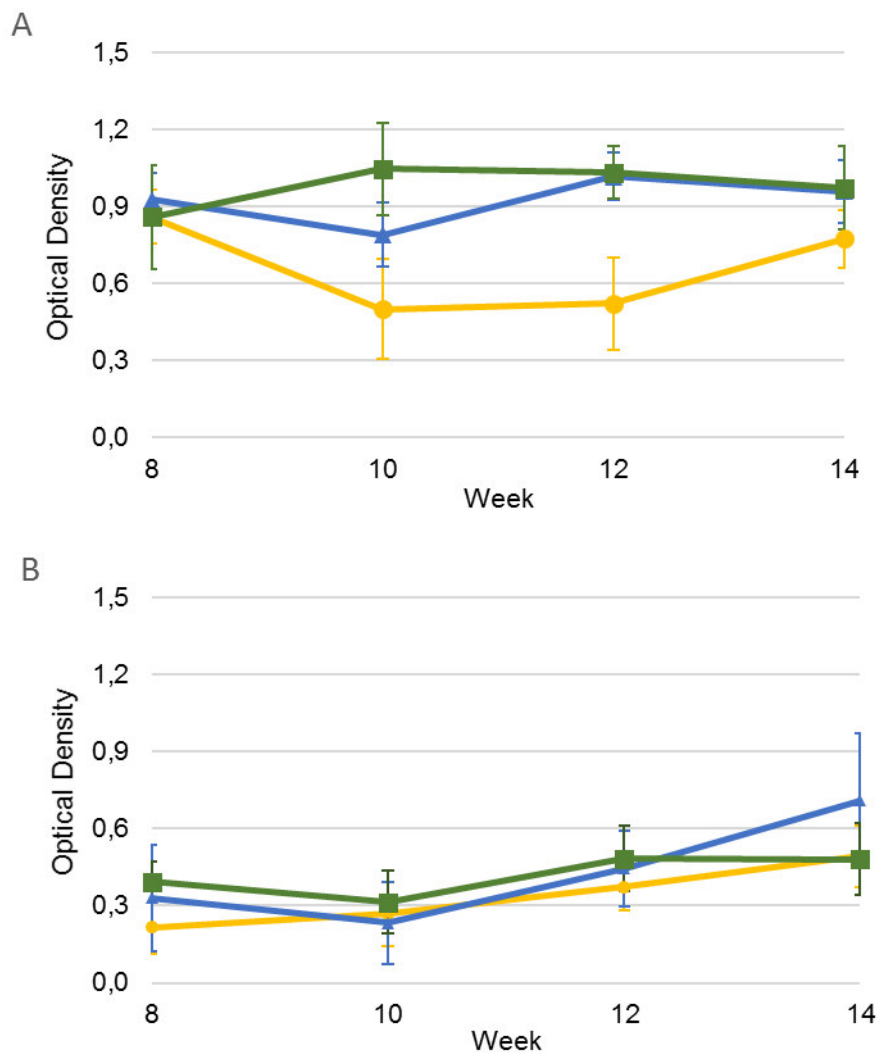


Figure 11 - Optical density (Abs at 600nm), measured over time, of cultures of estuarine sediment enriched with Bzf (Blue), Prx (Green) or without pharmaceutical addition (control) (Yellow) under both incubation conditions. A – Static, B – Agitation

3.1.4. Bacterial Richness and Diversity

The indexes of diversity and richness were calculated for each treatment, through ARISA profile in initial samples and samples collected after 2 and 14 weeks of incubation.

Richness results showed variations with treatment, incubation and time. However, it is possible to notice that initial samples of estuarine sediment have higher richness comparing with activated sludge. Furthermore, in Fig. 12 it is possible to observe that richness decreased in all samples of estuarine sediment, comparing to the initial value.

Microcosms enriched with activated sludge and Prx had to have different values of richness among incubation conditions ($p < 0.05$), being this tendency intensified with time.

In terms of diversity indexes, the present results showed significant differences ($p < 0.05$) between estuarine sediment in microcosms and initial sediment, with a loss in diversity (Fig. 13). Time also affected the diversity. Indeed, a decrease in diversity occurred at 14 weeks, however only in static conditions. Furthermore, the effect of Bzf was more effective than Prx comparing to control. However, this effect varied among conditions.

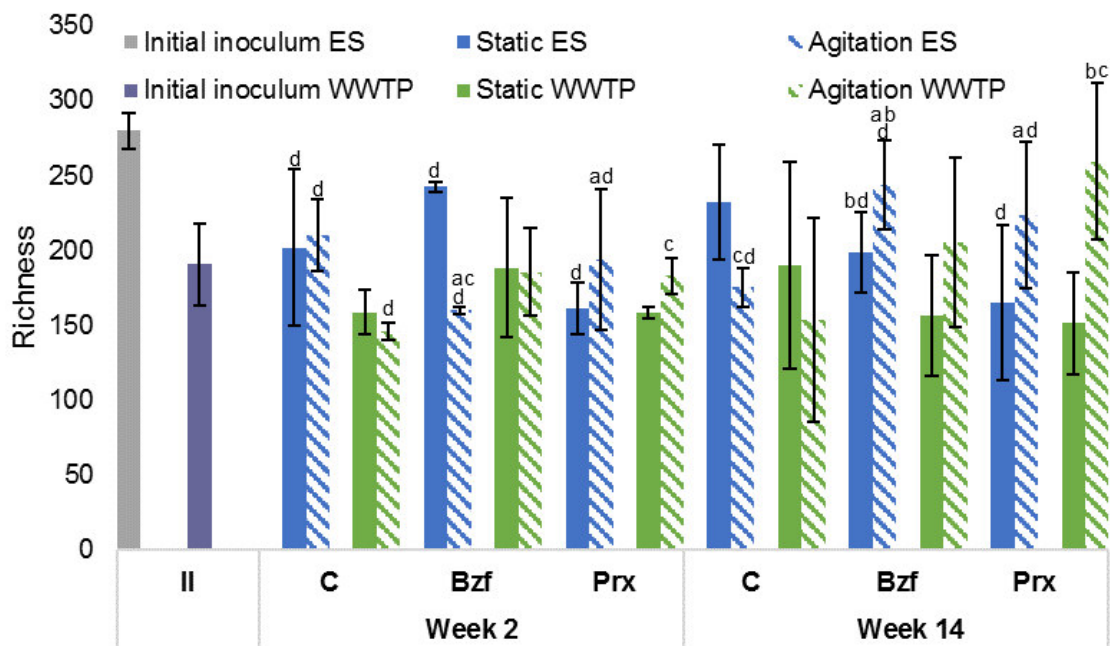


Figure 12 - Bacterial richness along experiment time. II – Initial inoculum; C - Control, Bzf – Bezafibrate treatment; Prx – Paroxetine treatment; WWTP – Inoculum from WWTP sludge; ES – Inoculum from estuarine sediment. a – significant differences comparing with respective control ($p < 0.05$); b – significant differences among same treatment, at different time ($p < 0.05$); c – significant differences among same treatment, with different incubation conditions ($p < 0.05$); d – significant differences between microcosm and initial samples ($p < 0.05$)

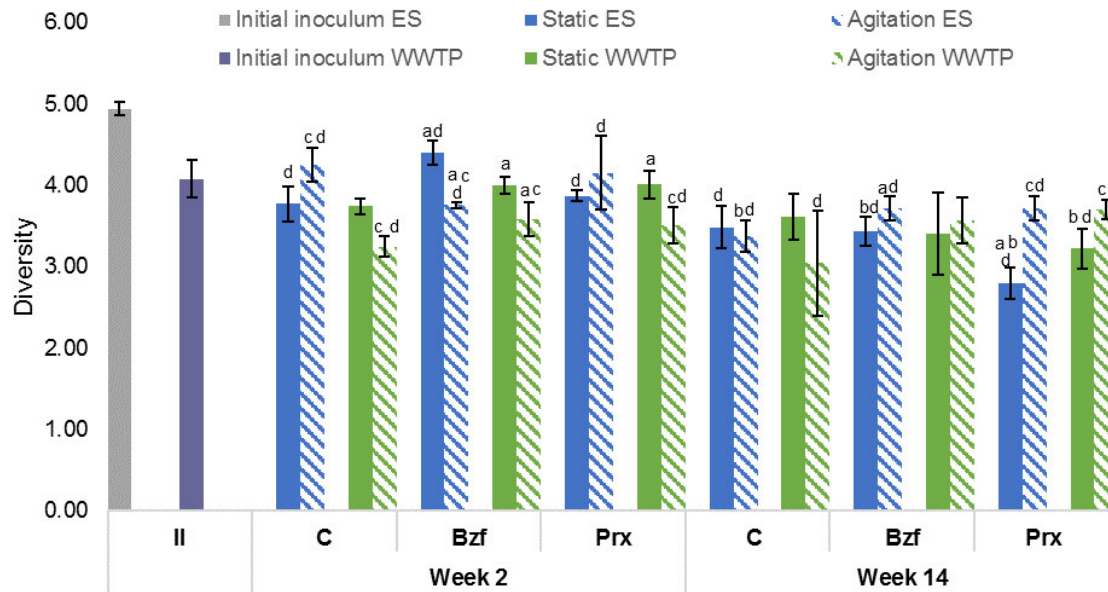


Figure 13 - Bacterial diversity along experiment time. II – Initial inoculum; C - Control, Bzf – Bezafibrate treatment; Prx – Paroxetine treatment; WWTP – Inoculum from WWTP sludge; ES – Inoculum from estuarine sediment. a – significant differences comparing with respective control ($p < 0.05$); b – significant differences among same treatment, at different time ($p < 0.05$); c – significant differences among same treatment, with different incubation conditions ($p < 0.05$); d – significant differences between microcosm and initial samples ($p < 0.05$)

3.1.5. Bacterial Community Structure

ARISA analysis was performed to characterize the bacterial community of the initial inocula and of the different treatments along the experiment. For each sample, ARISA fragment length (AFL) profiles were obtained. Different peaks correspond to different fragment lengths and therefore to different bacteria phylotypes. Differences in their genetic structure, or more specifically in the distribution of the different phylotypes among the different samples, are the most relevant feature.

Bacterial community evolution was evaluated based on similarity between samples with different pharmaceutical addition treatment, incubation conditions and time of experiment. Through ARISA results, two clusters were obtained for the bacterial community of each inoculum (Fig. 14), one for week 2 (Fig. 14A, Fig. 14C) and another for week 14 (Fig. 14B and Fig. 14D). Cluster analysis exhibit a good experimental replication as, for each treatment, replicates were clustered together, with one only exception. Samples are mainly grouped by incubation conditions (static or agitation), and, inside these groups,

higher similarity was observed among samples with pharmaceutical treatment comparing to control, except for sludge community, under static conditions at week 2.

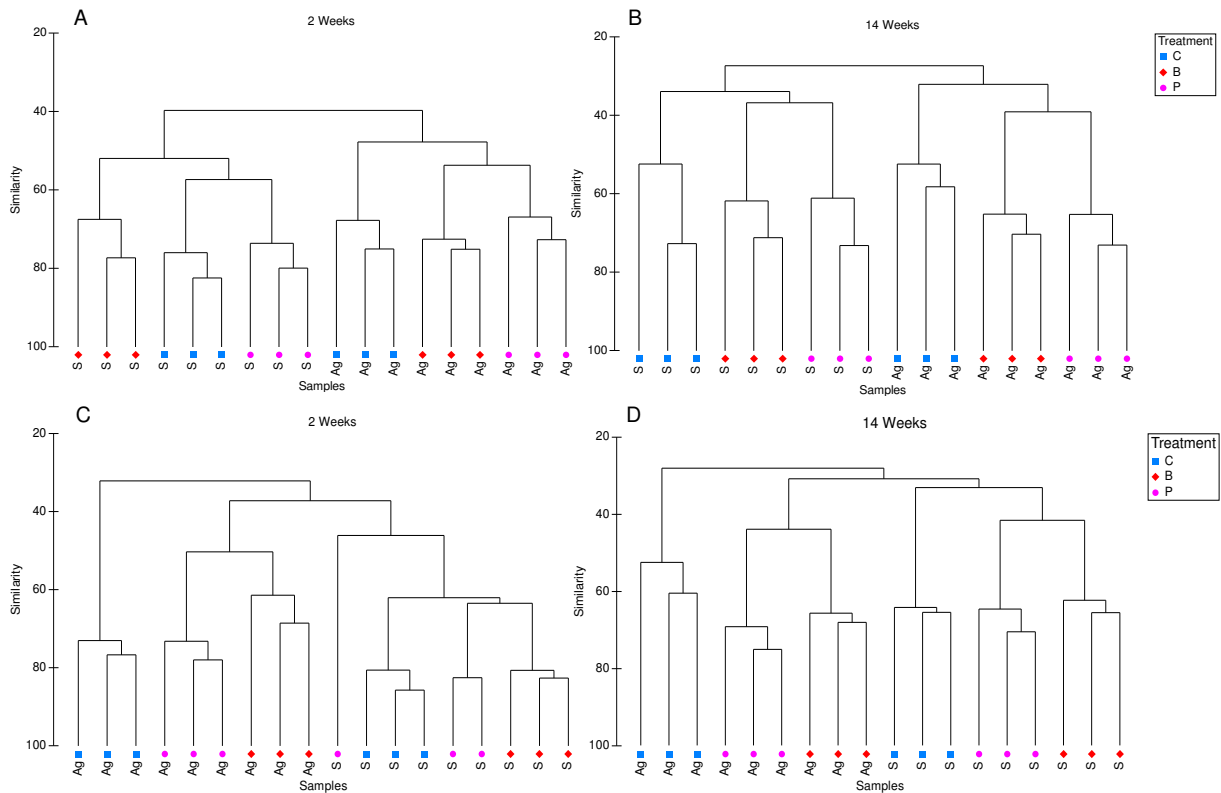


Figure 14 - Hierarchical clustering based on Bray-Curtis similarities from ARISA fingerprints of bacterial communities inoculated from activated sludge (A and B) and estuarine sediment (C and D) after 2 weeks (A and C) and 14 weeks (B and D) of experiment. S – Static; Ag – Agitation; C – Control; B – Bezafibrate treatment; P – Paroxetine treatment

Two MDS ordinations plots were assembled, for bacterial community of each inoculum (from estuarine sediment and from activated sludge), to visualize variation under different incubation conditions (static or agitation) (Fig. 15A; Fig. 16A) and variation along experiment time (2 and 14 weeks) (Fig. 15B; Fig. 16B).

Analyzing MDS ordinations, it is possible to observe that samples are grouped by time and that samples from the initial inocula are more similar to the samples from week 2 than to those of week 14. On the other hand, ordinations confirmed the division of samples according to the incubation conditions (static vs. agitation).

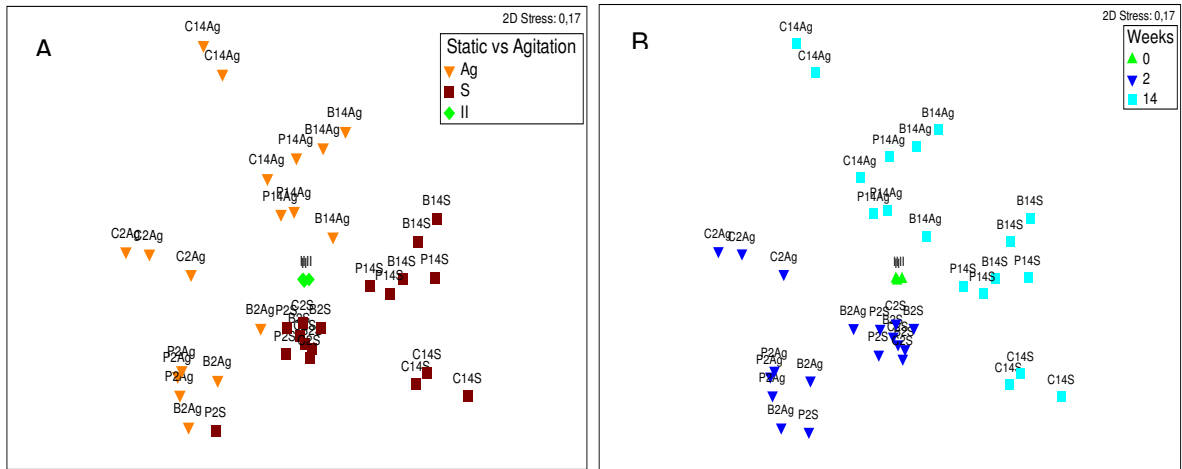


Figure 15 - Multidimensional scaling (MDS) ordination based on Bray-Curtis similarities from ARISA fingerprints of bacterial communities originated from estuarine sediments. A – Effect of incubation conditions; B – Effect of time. Ag – Agitation; S – Static; II – Initial Inoculum; C – Control; B – Bezafibrate treatment; P – Paroxetine treatment; 2 and 14 correspond to sample time in weeks, 0 is applicable to initial sediment

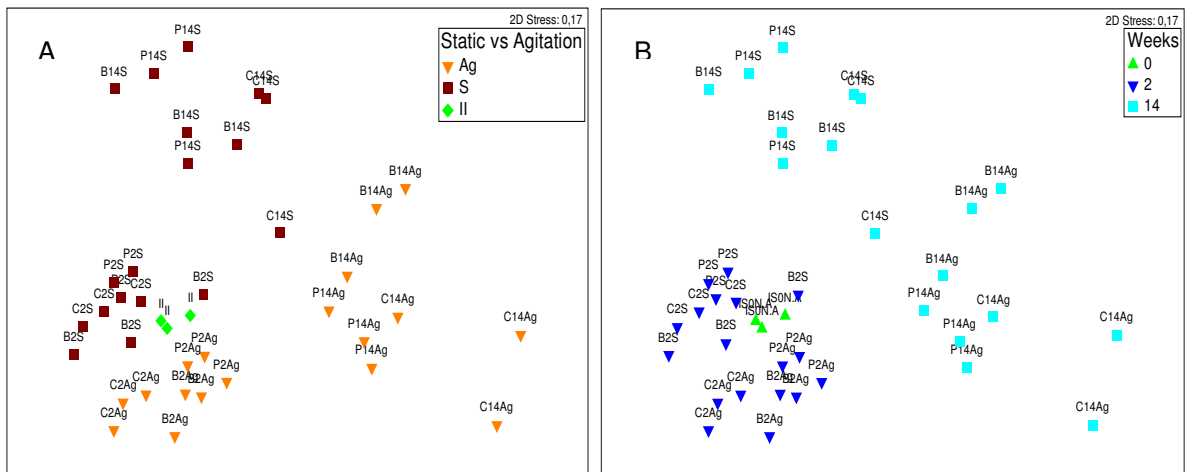


Figure 16 - Multidimensional scaling (MDS) ordination based on Bray-Curtis similarities from ARISA fingerprints of bacterial communities originated from activated sludge. A – Effect of incubation conditions; B – Effect of time. Ag – Agitation; S – Static; II- Initial Inoculum; C – Control; B – Bezafibrate treatment; P – Paroxetine treatment; 2 and 14 correspond to sample time in weeks, 0 is applicable to initial sludge

To understand the factors responsible for the shaping of the bacterial community structure, analysis of similarities (two-way crossed ANOSIM) was performed. Results showed a significant effect of both time and incubation conditions with significant differences among the different groups of samples (Table 6). For each experimental time (week 2 and week 14), the factor treatment showed to be the most important for the structure of the community, alongside with incubation conditions. Indeed, results indicate that whatever the origin of the inocula (WWTP or estuarine sediments), Bzf and Prx had a significant effect on bacterial community structure, either after 2 or 14 weeks of experiment.

Table 6 - Two-way crossed ANOSIM test for different time of exposure (week 2 and week 14), incubation conditions (static vs. agitation) and treatment (C – Control; Bzf – Bezafibrate; Prx – Paroxetine) effect, based on ARISA results from bacterial communities inoculated from activated sludge (AS) and estuarine sediment (ES)

		Statistic value (R)	Significance level (%)
TIME VS INCUBATION			
ES	Time	0.727	0.1
	Incubation	0.663	0.1
AS	Time	0.842	0.1
	Incubation	0.792	0.1
TREATMENT VS INCUBATION – 2 WEEKS			
GLOBAL TEST			
ES	INCUBATION	1	0.2
	TREATMENT	0.840	0.1
	PAIRWISE		
	BZF, C	1	1
	BZF, PRX	0.778	1
	PRX, C	0.778	1
GLOBAL TEST			
AS	INCUBATION	1	0.2
	TREATMENT	1	0.1
	PAIRWISE		
	BZF, C	1	1
	BZF, PRX	1	1
	PRX, C	1	1
TREATMENT VS INCUBATION – 14 WEEKS			
GLOBAL TEST			
ES	INCUBATION	1	0.3
	TREATMENT	1	0.1
	PAIRWISE		
	BZF, C	1	1
	BZF, PRX	1	1
	PRX, C	1	1
GLOBAL TEST			
AS	INCUBATION	1	0.3
	TREATMENT	0.992	0.1
	PAIRWISE		
	BZF, C	1	1
	BZF, PRX	1	1
	PRX, C	0.981	1

3.2. DISCUSSION

In the last years several authors published studies regarding pharmaceuticals removal in WWTPs, including Bzf and Prx. However, there is a lack of knowledge regarding removal processes. Indeed, pharmaceuticals removal from solution may be due to: i) complete degradation of the parental compound, ii) formation of intermediate products or iii) formation of (bio)transformation products (Musson *et al.*, 2010, Jelic *et al.*, 2011).

Several studies reported the capacity of microorganisms to remove pharmaceuticals (Yu *et al.*, 2006), pesticides (Barra Caracciolo *et al.*, 2013, Wang *et al.*, 2016), toxic metalloid (Dey *et al.*, 2016), hydrocarbons (Ribeiro *et al.*, 2013, Abed *et al.*, 2014, Sheu *et al.*, 2016), and other chemical compounds (Carvalho *et al.*, 2006, Zhu *et al.*, 2007) from the medium, using isolated bacterial strains or bacterial *consortia*, which are usually obtained from known contaminated sites.

Although there are a few reports of Prx occurrence in WWTP effluent and superficial water (Schultz and Furlong, 2008, Wu *et al.*, 2009), studies demonstrated that Prx can be efficiently removed from wastewater after treatment in WWTP (Schlüsener *et al.*, 2015). On the other hand, environmental occurrence of Bzf has been reported by several authors (Lindqvist *et al.*, 2005, Fent *et al.*, 2006, Luo *et al.*, 2014). Its removal rate in WWTP is not consensual among the different studies published, since removal rates are reported in a range from 10 to 75% (Jelic *et al.*, 2011, Luo *et al.*, 2014).

Sorption of a compound is usually predicted by its hydrophobicity and electrostatics interactions. Hydrophobicity, expressed as $\log K_{ow}$, is usually used to predict fate of micropollutants, while electrostatic interaction will translate the behavior of a certain chemical under different pH conditions (Tang *et al.*, 2014). Considering the high K_{ow} value of Bzf, it was expected that it would adsorb to sediments or cells, which did not occur in the present study. In fact, in adsorption controls Bzf was detected in very high concentrations in solution. In contrast with the present study, Tang *et al.* (2014) reported that Bzf adsorption was 16% and Jelic *et al.* (2011) reported an adsorption of 2%. But Calisto *et al.* (2015) highlighted the importance of pH, and how it influences the chemical structure of the compound. Adsorption capacity of Bzf can be affected by pH. Indeed, at neutral pH, studies showed that it did not adsorb due to electrostatic repulsive forces exerted by the negative electricity of the compound (Tang *et al.*, 2014, Semblante *et al.*, 2015).

Present results showed that Bzf was removed from solution in microcosms experiment, with microorganisms having an important role. In fact, Bzf was removed from

solution in high amounts, and considering its low adsorption, one can assume it was degraded by microorganisms. According to these results, Tang *et al.* (2014) reported the degradation of Bzf by activated sludge, and Collado *et al.* (2014) reported a high removal of this compound in secondary treatment of WWTP. There is a lack of information regarding Bzf degradation on estuarine environment, however, degradation by microorganisms from contaminated sites is well-established in laboratory conditions (Quintana *et al.*, 2005, Tang *et al.*, 2014). Abiotic controls were assembled to verify if degradation occurred through biotic or abiotic processes. Results indicated that Bzf was not significantly degraded by abiotic processes. Luo *et al.* (2014) reported that Bzf was very resistant to ozonation. Furthermore, Trovó *et al.* (2008) reported that Bzf was not degradable under solar irradiation, due to its incapability to absorb light above 300nm. This characteristic of Bzf can be related with its chemical structure, since chemical compounds with amide bonds ($RCONR_2$) are hardly degraded by UV light (Kim and Tanaka, 2009), indicating the resistance of this compound to abiotic degradation.

The present results indicated that Bzf was removed from solution either in microcosms with activated sludge or estuarine sediments. However, higher removal efficiencies were observed in static conditions, indicating that incubation conditions affected microorganisms' performance. Time also influenced the removal efficiency in agitation conditions, indicating that in these conditions microorganisms needed an adaptation phase.

In the present study, Prx was not detected in solution after 2 weeks of experiment and in any of the following cycles, in the different experimental conditions, either in the presence of estuarine microorganisms or activated sludge microorganisms. Nevertheless, according to our results, Prx was removed by adsorption and by abiotic degradation. Other authors (Kwon and Armbrust, 2008) reported that SSRIs have an adsorption capacity above 91%, while other studies have reported presence of Prx in biosolids and sediments (Radjenović *et al.*, 2009, Wu *et al.*, 2009, Chari and Halden, 2012). This behavior is in line with the predicted by the K_{ow} value. However, Prx high solubility in water can promote the formation of the molecule cationic form. Since adsorption is a natural process, adjusted by electrostatic interactions and dependent of the compound physicochemical properties, ionic binding and hydrophobic interactions with soil particles in solution can be promoted (Kwon and Armbrust, 2008, Brown *et al.*, 2015). Organic carbon has a straight relationship with adsorption process in distinct environmental scenarios, as activated sludge, soils and aquatic environments (Semblante *et al.*, 2015). According to K_D value, Prx has a great tendency to adsorb either to sludge or to natural sediments containing OC (Brown *et al.*, 2015). Actually, at environmental pH values (pH=7), positively-charged forms adsorb to OC

(negatively charged), through electrostatic interactions that are involved in adsorption process, being retained in sediments/sludge (Brown *et al.*, 2015, Semblante *et al.*, 2015). In abiotic controls, Prx was not detected in liquid phase until the total removal of sediments/cells, which can be explained by the potential sorption to particles. This characteristic of Prx can act as a limiting factor regarding its bioavailability in the water phase in the presence of solid material. On the other hand, sorption can also contribute to the biodegradation of compounds in the solid medium, since the interactions with bacterial surface can favor the uptake into cells or the interaction between the compound and the extracellular enzymes (Semblante *et al.*, 2015).

Our results indicated that Prx was removed in abiotic controls. Actually, pharmaceuticals can undergo abiotic transformations, although there are limitations associated with this process. Volatilization, hydrolysis and photolysis are the most common in the environment, however SSRIs compounds, as Prx, are not volatile, being more probably subjected to chemical degradation (Vasskog *et al.*, 2009, Semblante *et al.*, 2015). Kwon and Armbrust (2008) reported that Prx is not degraded by hydrolysis, however sunlight degrades it, being this process accelerated by the alkalization of the solution. Actually, it was reported that Prx is a photo-labile compound that absorbs light at wavelengths above 290 nm, being directly affected by photolysis (Kwon and Armbrust, 2008, Brown *et al.*, 2015). On the other hand, in environmental conditions Prx is not affected by direct photolysis due to the adsorption properties. The unavailability in solution allied with the natural organic matter in water, which undermines UV light penetration, will limit the action of photolysis (Kwon and Armbrust, 2008). Since microcosms were kept in the dark during experiment time, degradation of Prx by photolysis cannot be considered for the removal of the compound in abiotic controls.

Results indicated that Prx can be removed by adsorption, and be degraded by both biotic and abiotic processes. It was possible to verify that adsorption decreased over time, due to particles removal and its presence was very low after 10 weeks of experiment. At this time (10 weeks), Prx removal value in abiotic controls was similar to that observed in adsorption controls. Therefore, in inoculated microcosms the remaining Prx removal observed was probably due to biologic processes.

Vasskog *et al.* (2009) measured CO₂ produced and O₂ consumed, in an aerobic treatment with the purpose to estimate Prx removal from sewage, while (Bergersen *et al.*, 2012) measured the biogas produced by microorganisms and demonstrated that Prx can be biodegraded in anaerobic conditions. In both studies, Prx was highly removed, being the compound with the highest removal rate, but its biodegradation showed to be more efficient

in anaerobic conditions. This information is consistent with the present work, evidencing Prx degradation under different oxygen conditions. In fact, in both incubation conditions, either agitation, with an aerobic environment, and in static with a reduced amount of oxygen, Prx degradation was observed.

Removal of the halogen substituent from a compound is a fundamental reaction that occurs during its biodegradation (Carvalho *et al.*, 2006, Amorim *et al.*, 2013). Our results indicated that the presence of fluoride anion in solution was higher in static conditions either in cultures from activated sludge or estuarine sediment. However, time improved fluoride anion release in agitation conditions. These results indicate once more that the conditions of incubation and time affected the performance of the microorganisms.

Fluoride anion concentration results also corroborate the biotic degradation of Prx. In our study, the fluoride anion release suggests the presence of an enzyme with the capability to dehalogenate Prx. In fact, the absence of fluoride anion release in abiotic controls enhances this argument. However, a non-stoichiometric relation between fluoride anion release and Prx initial concentration was observed, which can be due to the formation of Prx transformation products, in which fluoride anion is incorporated, as reported in (Amorim *et al.*, 2013). These results indicate that Prx degradation was probably not complete, particularly in abiotic conditions.

The implementation of an external energy source (carbon) can enhance the efficiency of defluorination due to increasing growth of cells, and consequently decrease the time for removal of the contaminant occur (Amorim *et al.*, 2013, Amorim *et al.*, 2014, Carvalho *et al.*, 2016). Carvalho *et al.* (2016) used a single strain to study the biodegradation of moxifloxacin, obtaining high defluorination values (92%) due to the use of acetate as a bulk substrate, increasing the number of microorganism capable to react with the pharmaceutical. The same external energy source was used in the present study.

Acclimation of microorganisms to the incubation condition can act as a limiting factor to their degradation capacity. In our results, it is possible to verify the existence of different tendencies in biomass growth, mostly regarding the incubation conditions. Studies reported that the use of an additional source of carbon can enhance the microbial growth, instigating pharmaceutical removal (Amorim *et al.*, 2014, Carvalho *et al.*, 2016). Veach *et al.* (2012) reported that biomass growth was significantly affected by the presence of the pharmaceuticals. However, in the present study biomass growth was not significantly affected by pharmaceuticals presence. Indeed, in certain instances, biomass growth in controls was lower to the one verified in microcosms enriched with pharmaceutical,

indicating an extra energy source. So, these results indicated that microorganisms were resistant to both selected pharmaceutical compounds and that they were able to degrade them.

Microbial communities are ubiquitous organisms, responsible for the primary response to ecosystem perturbations. Thus, microorganisms, including bacteria, are vulnerable to exogenous physicochemical and biological stressors (Oladele, 1999, Juwarkar *et al.*, 2010). Microcosms are considered ecosystems models, in which is possible to set specific conditions to understand the effect of a pollutant in microbial communities (Barra Caracciolo *et al.*, 2013). In this study, microbial richness and diversity was measured, using ARISA, before and during the experiment. The response of bacterial community originated from activated sludge and from estuarine sediment was then evaluate regarding the presence of pharmaceuticals, time and incubation conditions.

Results from bacterial richness and diversity demonstrated that variations occurred, however they did not follow any pattern in the different parameters analyzed. At the end of week 2, it was possible to verify that the microbial communities present in microcosms with activated sludge and under Bzf treatment, had a diversity index superior to the one in control (without pharmaceutical addition), stabilizing over time. The decrease in microbial diversity in sediments from microcosms compared to initial sediment can be an indicator of the capability of the pharmaceutical to act as a selective force. The presence of significant differences among some samples in terms of diversity indicates that the community had the ability to adapt to the presence of these compounds.

Shifts on bacterial community structure were analyzed by ARISA, a tool useful to study the genetic structure of complex communities, through DNA fingerprinting. Time was one of the factors that affected communities, as it is possible to verify by the decrease in similarity of communities exposed to the same pharmaceutical compound and incubation conditions. Indeed, the presence of sediment at week 2 and its absence at week 14, can be relevant for these results, since microbial degraders are more commonly associated to surface of sediments and sediments lost can results in loss of some species (Wang *et al.*, 2016). Results showed that incubation conditions also had a relevant effect in microbial community with clear differences between conditions. In each period, samples from static incubations were grouped together among them, the same occurring for samples from agitation incubations, for microbial communities with different origins. These differences are in accordance with what was visualized during all the experiment, agitation cultures soon started to flocculate, while static cultures have kept a turbid solution during experiment time, indicating stress signs from agitations conditions. Agitation promoted the incorporation of

oxygen in the liquid media, while in static conditions oxygen saturation had lower values. Oxygen content is considered an abiotic factor, capable of influencing the metabolic activities of microorganisms (Tran *et al.*, 2013). Furthermore, oxygen can be used as an electron acceptor, stimulating microbial degradation of pollutants (Juwarkar *et al.*, 2010). Indeed, shifts in bacterial community occurred over time, promoting the emergence of a different bacterial community, able to degrade Bzf. In fact, Bzf degradation increased with time. Communities exposed to Prx also experienced shifts. In fact, although the compound removal from solution did not change over time, defluorination improvement suggests that a microbial community able to dehalogenate Prx with more efficiency emerged.

Analysis of similarity showed statistically significant effect of time. Presence of pharmaceutical compound and incubation conditions on microbial communities also showed to be statistically significant in each time period. Despite a few studies reporting the environmental presence of Bzf and Prx, the effect of these pharmaceuticals on microbial communities is not well known. However, since pharmaceutical compounds are designed to be biologically active, it is possible that they are capable of causing effects in living organisms (Boxall, 2004, Barra Caracciolo *et al.*, 2015). Thus, bacterial community can be affected by the presence of these compounds and their toxic effects. Fernandes *et al.* (2015), reported that estuarine communities can be affected by the presence of enrofloxacin, while Chen *et al.* (2014) reported, in their study, that variations on bacterial community present in sludge can occurred during pharmaceuticals removal. Sensitive species may be eliminated, while resistant species will have the ability to proliferate, since they are capable to produce degradative enzymes. Microbial consortium can also be constituted by resistant bacteria, capable of using secondary products, participating also in biodegradation processes (Oladele, 1999).

3.3. CONCLUSIONS

The results obtained in this experiment demonstrated the efficiency of autochthonous microorganisms derived from estuarine sediments and associated WWTP for the removal of the selected pharmaceuticals (Bzf and Prx). Despite Prx can also be removed by adsorption to particles and by abiotic processes, microorganism's presence is essential to the dehalogenation of the molecule.

The evaluation of community structure demonstrated that time, incubation conditions and pharmaceutical treatment influenced the bacterial community present in microcosms. This effect did not affect the capability of removing the pharmaceuticals, which, in some cases, increased with time.

CHAPTER 4

BIOREMEDIATION EFFICIENCY OF MICROORGANISMS ISOLATED FROM PHARMACEUTICAL ENRICHED *CONSORTIA*

4. BIOREMEDIATION EFFICIENCY OF MICROORGANISMS ISOLATED FROM PHARMACEUTICAL ENRICHED *CONSORTIA*

4.1. RESULTS

The obtained results reported at this section were obtained from the second experiment of this work. The aim of this experiment was to evaluate the efficiency of isolated strains, obtained from solution culture at the end of experiment one, in degrading the pharmaceuticals in study.

Results are reported for the 5 *consortia* of isolates : two *consortia* derived from Prx enrichment, under static conditions, in which one was obtained from estuarine sediment (P1) and the other from activated sludge from WWTP (P2) inocula, two *consortia* derived from Bzf enrichment, under static conditions, in which one was obtained from estuarine sediment (B1) and the other from activated sludge from WWTP (B2) inocula, and another *consortia* was derived from Bzf enrichment, under agitation conditions, obtained from activated sludge from WWTP inoculum (B3).

In this section, the results reported are in terms of number of different colonies per *consortia*, obtained in solid media, either in MM-pharmaceutical and PCA, pharmaceutical removal percentages in microcosms inoculated with the *consortia* of isolated colonies, and fluoride anion release in Prx treatments.

4.1.1. Isolated bacterial strains

Bacterial strains were isolated from the different *consortia* in solid-medium plates to assess the different phenotypes present. At first, 100 μL of each microbial culture solution (dilutions of 1×10^{-4}), obtained in the first experiment, were spread in a plate media. The inoculation of the different media allowed to estimate the different cultivable bacteria able to grow in a medium in which the pharmaceutical is present, as well as the number of colonies present in the whole consortium.

Present results indicate that the number of colonies with different phenotypes was higher in PCA media, comparing with the number of microorganisms obtained in MM-pharmaceutical media (Table 7). The number of colonies obtained in PCA medium was 7 for the two *consortia* originated from Prx enrichment culture under static conditions (P1 and P2), 6 for the two *consortia* originated from Bzf enrichment culture under static conditions (B1 and B2), and 9 for the *consortia* originated from Bzf enrichment culture under agitation conditions (B3). On the other hand, in the medium enriched with each of the pharmaceutical

compounds (MM-pharmaceutical) the number of colonies observed varied between 2 (P2), 3 (P1, B2, B3) and 4 (B1) phenotypes. So, the number of isolated bacterial strains present in each consortium able to grow in the presence of each pharmaceutical compound does not correspond to the total number of phenotypes present in the respective consortium. Furthermore, the origin of the consortium also influenced the number of microorganisms able to proliferate in a contaminated medium. The incubation conditions, and indirectly the amount of oxygen present in the medium, also influenced the number of different strains developed.

Table 7 - Bacterial strains isolated from the different consortia in PCA media (Blue) and MM-pharmaceutical media (Yellow). (x) indicates the presence and (-) the absence. P1 – Paroxetine, Estuarine Sediments, Static; P2 – Paroxetine, Activated Sludge, Static; B1 – Bezafibrate, Estuarine Sediments, Static; B2 – Bezafibrate, Activated Sludge, Static; B3 – Bezafibrate, Activated Sludge, Agitation

		Colony Identification Number											
		1	2	3	4	5	6	7	8	9	10	11	12
Consortium	P1	X	X	X	X	X	X	X	X	X	X	-	-
	P2	X	X	-	X	X	X	X	X	X	X	-	-
	B1	X	X	X	X	X	X	X	X	X	X	-	-
	B2	X	X	X	X	X	X	X	X	X	-	-	-
	B3	X	X	X	X	X	X	X	X	X	X	X	X

4.1.1. Pharmaceuticals Removal

Pharmaceuticals removal percentage was determined through the analysis of the concentration of each pharmaceutical compound in microcosm solution.

Three *consortia* potentially able to degrade Bzf, originated from the first part of this work, were reconstructed in new culture medium. So, the microbial communities were previously exposed to Bzf presence. Results (Fig. 17) showed that Bzf removal percentage (79%) in consortium derived from the estuarine sediment, B1, after 2 weeks, was significantly ($p < 0.05$) higher than the removal percentage observed for the *consortia* obtained from the activated sludge, B2 (31%) and B3 (57%). For B1, removal percentages were higher than 97% (Bzf not detected in solution) in following weeks, the same being noticed for B2. As for consortium B3, the only maintained under agitation conditions, despite the considerable Bzf removal percentage observed at the end of the 2nd week, Bzf removal decreased and by the end of the 6th week it was only of 23%.

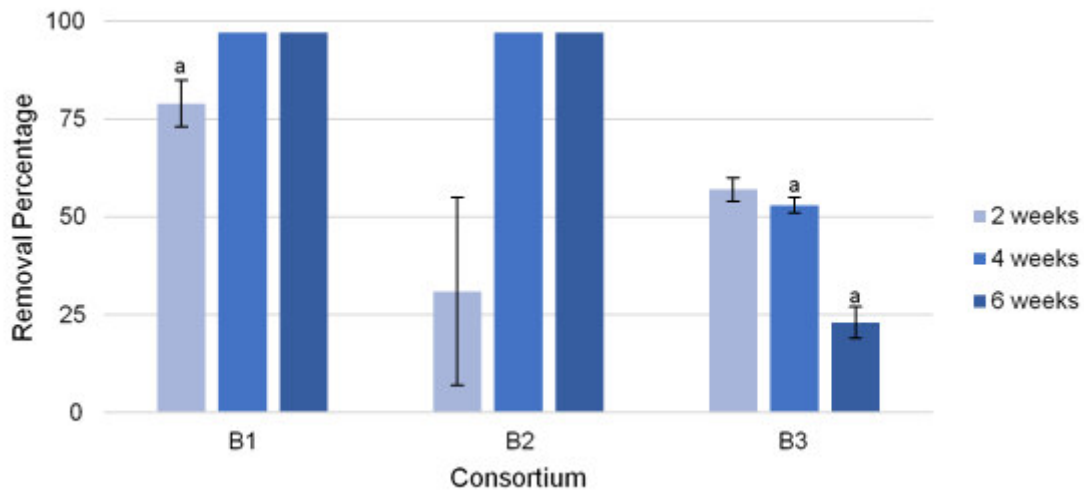


Figure 17 - Removal percentage of Bzf in the consortia of isolates, over time. B1 – Estuarine Sediments, Static; B2 – Activated Sludge, Static; B3 - Activated Sludge, Agitation. a – different from other consortia at same period ($p < 0.05$)

The reconstruction of two *consortia* potentially able to degrade Prx was performed using the microbial cultures of microcosms previously exposed to Prx in static conditions. Results (Fig. 18) indicate that high Prx removal percentage (93%) was obtained after 2 weeks of experiment for the consortium obtained from the estuarine sediment, P1, being the high removal percentages maintained over time. For the consortium derived from activated sludges, P2, Prx removal percentage was 59% after 2 weeks of experiment, increasing to 75% by the 6th week. In each sampling period (in weeks), differences between the two *consortia* were significant ($p < 0.05$).

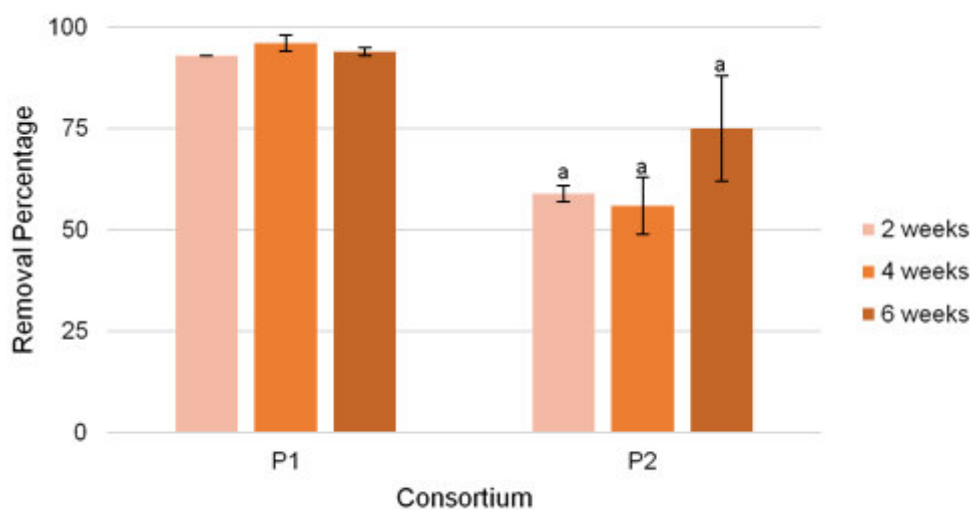


Figure 18 - Removal percentage of Prx in the consortia of isolates, over time. P1 – Estuarine Sediments, Static; P2 – Activated Sludge, Static. a - different between consortia at same period ($p < 0.05$)

4.1.1. Fluoride Anion Release

The results of defluorination obtained for medium inoculated with each of the two *consortia* produced with isolated strains previously exposed to Prx showed variations in the defluorination percentages over time (Fig. 19). The defluorination percentage increase over time until the 6th week. By this time defluorination percentage was significantly lower ($p < 0.05$) for the consortium originated from activated sludge (P2) than for the consortium originated from estuarine environment (P1).

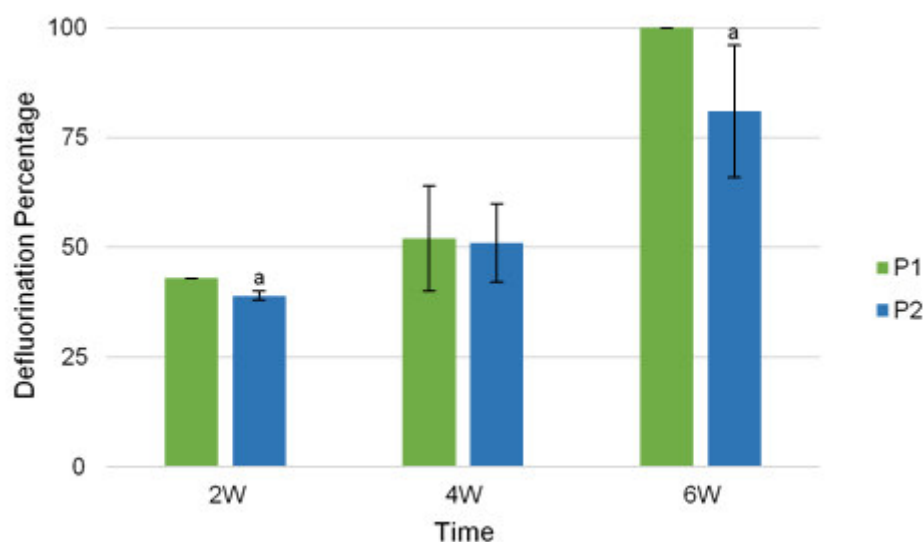


Figure 19 - Percentage of defluorination in the consortia of isolates, over time, in Prx microcosms. P1 – Estuarine Sediments, Static; P2 – Activated Sludge. a – Different between consortia, at same period ($p < 0.05$)

4.2. DISCUSSION

As mentioned, each microbial consortium exposed during several weeks to each pharmaceutical compound was cultivated in solid medium to differentiate bacterial strains present in each one of them. One should be aware that, nevertheless, several strains present in the initial *consortia* cannot be cultivable in solid medium (Hosokawa *et al.*, 2009). Bacterial strains were grown in both media with and without the respective pharmaceutical compound.

In this study, results indicated that the number of bacterial strains able to grow in a nutrients medium (PCA) was higher than the number of strains able to grow in a medium enriched with pharmaceuticals (MM-pharmaceutical). The presence of xenobiotics in the environment can affect microbial density (Oladele, 1999). Indeed, the xenobiotic presence at high concentrations can decrease the density of the sensitive species, while some

species can resist to its presence. In addition, these species can be able to degrade the xenobiotic. Resistant species have the capacity to produce biodegradative enzymes and use the excess of toxicant as nutrient source, being able to proliferate in the presence of the xenobiotic (Oladele, 1999).

The obtained bacterial strains were combined, creating a new microbial consortium with an expected potential to degrade the respective pharmaceutical compound, Bzf or Prx. Each one of these new *consortia* was re-suspended in new MM doped with the respective pharmaceutical compound and compound degradation/removal was evaluated, in co-metabolism with acetate.

Results indicated that Bzf removal varied along time and with the microbial consortium. For *consortia* B1 and B2 maintained under static conditions, the highest Bzf removal percentages (>97%) were achieved by the end of week 4. However, by the end of week 2, Bzf removal percentage was higher for consortium B1, originated from estuarine sediment, than for consortium B2, originated from activated sludge. Discrepancy in the performance between these two *consortia* can be related with *consortia* acclimatization. Inoculation was performed with similar amounts of biomass, however, the equilibrium in *consortia* can occur in a different way, creating a lag time (Zhu *et al.*, 2007, Yamamoto *et al.*, 2009). Furthermore, the passing from a solid to a liquid medium can also influence acclimatization time (Wang *et al.*, 2016).

As for consortium B3, originated from activated sludge under agitation, results indicate that this consortium was not able to reach a high Bzf removal percentage, even decreasing after 6 weeks. The decrease in efficiency removal of Bzf observed for *consortia* B3 can be due to the incapability of the consortium to completely remove Bzf from solution. In fact, the overload of the pharmaceutical over feeding cycles may have generated toxicity to the species able to degrade the compound. In stress conditions, as exposure to pharmaceuticals, the microbial community must develop detoxication mechanisms to be able to proliferate (Dey *et al.*, 2016). High concentration of toxicants can increase lag time, particularly if the number of microorganisms with degradation capability is low comparing to the amount of chemical compound in this medium (Wang *et al.*, 2016). So, this consortium is probably not the most suitable one to degrade Bzf, indicating that the agitation conditions, in which the consortium was maintained, were effectively not the most appropriated ones. In fact, as verified at the previous experiment (Chapter 3), different incubation conditions induced differences between the two communities from the same source, in microcosms enrichment. That way, differences in removal of Bzf from *consortia* originated under different agitation conditions is not surprising.

Regarding Prx removal, the two *consortia* had different origins (P1 from estuarine sediment and P2 from activated sludge), but were kept under the same static conditions.

For consortium P1 high removal percentages (> 90%) were observed already after 2 weeks of inoculation, being this removal percentage maintained over the following weeks. As for consortium P2, removal percentages only reached the maximum value after 6 weeks. However, this maximum was lower than the one observed for consortium P1. Release of fluoride anion results are in agreement with the results obtained for P2 *consortia*. This is, defluorination of Prx was proportional to removal rate at weeks 4 and 6, indicating that Prx was being effectively degraded into a non-fluorinated compound. For consortium P1, despite the immediately high Prx removal percentages, defluorination efficiency only reached the maximum value at 6th week, indicating that Prx degradation pathway over time differed among the *consortia*. One should be aware that as observed in the previous experiment, Prx can undergo abiotic degradation too. Nevertheless, fluoride anion concentration at week 6 was higher for consortium P1, originated from estuarine sediment indicating that this consortium was more effective in Prx degradation/removal. Bioremediation techniques require microbial *consortia* or microbial processes that make possible the decontamination of polluted sites (Juwarkar *et al.*, 2010). Regarding the compounds in study, the key reaction during microbial degradation is dehalogenation, without forming intermediary products containing the halogen (Janssen *et al.*, 2001). Dehalogenation consists in a substitution of the halogen component. In this way, the chemical compound can be easily degraded (Janssen *et al.*, 2001). The increase in defluorination efficiency can be a response of the *consortia* to the presence of contaminant, since enzymes synthesis (or cessation) is controlled at molecular level, through the activation of specific genes (Oladele, 1999).

Comparing results obtained at the present experiment with removal results obtained in the previous experiment (Chapter 3), it is possible to observe some differences in microcosms assembled with microorganisms from activated sludge. Indeed, *consortia* with microorganisms of activated sludge treated with Bzf that derived from agitation conditions (B3) had lower efficiency removals comparing with the observed in previous experiment, as well as for *consortia* derived from activated sludge treated with Prx (P2). Over time, removal efficiency did not achieve the values observed for the first experiment. However, in case of *consortia* exposed to Prx, defluorination efficiency achieved the maximum observed previously in the first experiment. Regarding *consortia* originated from the estuarine sediments, no differences were found in terms of pharmaceuticals removal with, in case of Prx, high defluorination rates, indicating the efficiency of this *consortia* to degrade Prx.

Results obtained for both pharmaceutical compounds highlight that *consortia* obtained from estuarine sediment had a quicker acclimatization in microcosm, comparing to *consortia* from activated sludge. Moreover, at least for Prx, the consortium with bacterial

strains obtained from estuarine sediment seemed to be more effective for the compound degradation.

WWTP were designed to remove organic matter from sewage generated in households and hospital effluents, being under specific operation and regulation (Halling-Sørensen *et al.*, 1998). Estuarine environments have a huge anthropogenic interest. The rapid urbanization and industries and shipping presence nearby, generated contamination through urban runoff and industrial effluents transport of chemicals (Sun *et al.*, 2012). Thus, strains present in each *consortia*, may be different not only because of site characteristics but also due to the different previous exposition to chemicals at the source.

4.3. CONCLUSIONS

Results obtained indicated that isolated strains derived from enrichment culture from the previous experiment were able to degrade the studied pharmaceuticals, however different values of efficiency were observed. *Consortia* derived from estuarine sediment required less time of acclimation to the experimental conditions, since at the end of the second week, high removal efficiencies (>97%) were already observed. However, isolated strains from activated sludge showed to remove both pharmaceuticals, more efficiently in static conditions. Defluorination of Prx increased over time in both *consortia* exposed to this pharmaceutical, however the maximum value of defluorination was achieved only in isolated strains from estuarine sediment. Future identification of the different strains isolated from the different *consortia* is important information to understand which type of bacteria are involved in degradation process of the pharmaceuticals. This way, new knowledge will be provided about this subject.

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The present work demonstrates the ability of autochthonous microorganisms from Douro estuary sediment and from activated sludge from a local WWTP to degrade Bzf or Prx in different experimental conditions (agitation vs. static). Although abiotic processes may lead to partial removal of Prx, dehalogenation only occurred in the presence of microorganisms, indicating the importance of their presence for the degradation of Prx. Regarding Bzf, removal was only due to the presence of microorganisms as abiotic degradation was not significant.

The initial microbial community present in estuarine sediment had higher bacterial richness and diversity, comparing with that from the activated sludge. Important changes occurred in the microbial community structure, influenced by time of experiment, incubation conditions and pharmaceuticals presence. Indeed, incubation conditions affected not only the bacterial community structure, but also the efficiency of pharmaceuticals removal that was initially lower under agitation than under static conditions. Still, despite the alterations in community structure observed along the study, high efficiency of pharmaceuticals removal was obtained at the end of the experiment.

Consortia of isolated strains derived from enrichment culture were able to degrade the studied pharmaceuticals, however different values of efficiency were observed. Higher efficiencies of pharmaceutical removal were observed in *consortia* originated from estuarine sediments. Regarding the *consortia* derived from activated sludge, pharmaceuticals removal was more efficiency under static than under agitation conditions. Defluorination of Prx increased over time in both *consortia* exposed to this pharmaceutical, however the maximum defluorination rate was achieve only in the consortium originated from estuarine sediments.

Thus, these results demonstrate that autochthones microorganism communities from estuarine environment and associated WWTP, have the potential to be used in bioremediation techniques for the removal of pharmaceuticals under specific conditions. Identification of isolated strains will provide new knowledge about bacteria potentially involved in Prx and Bzf removal and degradation processes.

REFERENCES

Abed, R. M. M., Al-Sabahi, J., Al-Maqrashi, F., Al-Habsi, A. and Al-Hinai, M., 2014. Characterization of hydrocarbon-degrading bacteria isolated from oil-contaminated sediments in the Sultanate of Oman and evaluation of bioaugmentation and biostimulation approaches in microcosm experiments. *International Biodeterioration & Biodegradation* 89: 58-66.

Allison, S. D. and Martiny, J. B. H., 2008. Resistance, resilience, and redundancy in microbial communities. *Proceedings of the National Academy of Sciences of the United States of America* 105(Suppl 1): 11512-11519.

Altieri, M. A., 1999. The ecological role of biodiversity in agroecosystems. *Agriculture, Ecosystems & Environment* 74(1-3): 19-31.

Amorim, C. L., Carvalho, M. F., Afonso, C. M. M. and Castro, P. M. L., 2013. Biodegradation of fluoroanilines by the wild strain *Labrys portucalensis*. *International Biodeterioration & Biodegradation* 80: 10-15.

Amorim, C. L., Ferreira, A. C. S., Carvalho, M. F., Afonso, C. M. M. and Castro, P. M. L., 2014. Mineralization of 4-fluorocinnamic acid by a *Rhodococcus* strain. *Applied Microbiology and Biotechnology* 98(4): 1893-1905.

Barra Caracciolo, A., Bottoni, P. and Grenni, P., 2013. Microcosm studies to evaluate microbial potential to degrade pollutants in soil and water ecosystems. *Microchemical Journal* 107: 126-130.

Barra Caracciolo, A., Topp, E. and Grenni, P., 2015. Pharmaceuticals in the environment: Biodegradation and effects on natural microbial communities. A review. *Journal of Pharmaceutical and Biomedical Analysis* 106: 25-36.

Bergersen, O., Hanssen, K. Ø. and Vasskog, T., 2012. Anaerobic treatment of sewage sludge containing selective serotonin reuptake inhibitors. *Bioresource Technology* 117: 325-332.

Blaise, C., Gagné, F., Eullaffroy, P. and Féraud, J. F., 2008. Ecotoxicity of selected pharmaceuticals of urban origin discharged to the Saint-Lawrence river (Québec, Canada): a review. *Brazilian Journal of Aquatic Science and Technology*; v. 10, n. 2 (2006).

Boxall, A. B. A., 2004. The environmental side effects of medication. *EMBO Reports* 5(12): 1110-1116.

Brown, A. K., Challis, J. K., Wong, C. S. and Hanson, M. L., 2015. Selective serotonin reuptake inhibitors and β -blocker transformation products may not pose a significant risk of toxicity to aquatic organisms in wastewater effluent-dominated receiving waters. *Integrated Environmental Assessment and Management* 11(4): 618-639.

Calisto, V., Ferreira, C. I. A., Oliveira, J. a. B. P., Otero, M. and Esteves, V. I., 2015. Adsorptive removal of pharmaceuticals from water by commercial and waste-based carbons. *Journal of Environmental Management* 152: 83-90.

Cardinale, M., Brusetti, L., Quatrini, P., Borin, S., Puglia, A. M., Rizzi, A., Zanardini, E., Sorlini, C., Corselli, C. and Daffonchio, D., 2004. Comparison of different primer sets for

use in automated ribosomal intergenic spacer analysis of complex bacterial communities. *Applied and Environmental Microbiology* 70(10): 6147-6156.

Carvalho, M. F., Ferreira Jorge, R., Pacheco, C. C., De Marco, P., Henriques, I. S., Correia, A. and Castro, P. M., 2006. Long-term performance and microbial dynamics of an up-flow fixed bed reactor established for the biodegradation of fluorobenzene. *Applied Microbiology and Biotechnology* 71(4): 555-562.

Carvalho, M. F., Maia, A. S., Tiritan, M. E. and Castro, P. M. L., 2016. Bacterial degradation of moxifloxacin in the presence of acetate as a bulk substrate. *Journal of Environmental Management* 168: 219-228.

Chari, B. P. and Halden, R. U., 2012. Validation of mega composite sampling and nationwide mass inventories for 26 previously unmonitored contaminants in archived biosolids from the U.S National Biosolids Repository. *Water Research* 46(15): 4814-4824.

Chen, Z., Wang, Y., Li, K. and Zhou, H., 2014. Effects of increasing organic loading rate on performance and microbial community shift of an up-flow anaerobic sludge blanket reactor treating diluted pharmaceutical wastewater. *Journal of Bioscience and Bioengineering* 118(3): 284-288.

Collado, N., Rodriguez-Mozaz, S., Gros, M., Rubirola, A., Barceló, D., Comas, J., Rodriguez-Roda, I. and Buttiglieri, G., 2014. Pharmaceuticals occurrence in a WWTP with significant industrial contribution and its input into the river system. *Environmental Pollution* 185: 202-212.

Cunningham, V. L., Constable, D. J. and Hannah, R. E., 2004. Environmental risk assessment of paroxetine. *Environmental Science & Technology* 38(12): 3351-3359.

Danovaro, R., Luna, G. M., Dell'anno, A. and Pietrangeli, B., 2006. Comparison of Two Fingerprinting Techniques, Terminal Restriction Fragment Length Polymorphism and Automated Ribosomal Intergenic Spacer Analysis, for Determination of Bacterial Diversity in Aquatic Environments. *Applied and Environmental Microbiology* 72(9): 5982-5989.

Dey, U., Chatterjee, S. and Mondal, N. K., 2016. Isolation and characterization of arsenic-resistant bacteria and possible application in bioremediation. *Biotechnology Reports* 10: 1-7.

Ettoumi, B., Guesmi, A., Brusetti, L., Borin, S., Najjari, A., Boudabous, A. and Cherif, A., 2013. Microdiversity of deep-sea Bacillales isolated from Tyrrhenian sea sediments as revealed by ARISA, 16S rRNA gene sequencing and BOX-PCR fingerprinting. *Microbes and Environments* 28(3): 361-369.

Farré, M. L., Pérez, S., Kantiani, L. and Barceló, D., 2008. Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment. *TrAC Trends in Analytical Chemistry* 27(11): 991-1007.

Fent, K., Weston, A. A. and Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology* 76(2): 122-159.

Fernandes, J. P., Almeida, C. M. R., Basto, M. C. P. and Mucha, A. P., 2015. Response of a salt marsh microbial community to antibiotic contamination. *Science of The Total Environment* 532: 301-308.

Fisher, M. M. and Triplett, E. W., 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Applied and Environmental Microbiology* 65(10): 4630-4636.

Gelsleichter, J. and Szabo, N. J., 2013. Uptake of human pharmaceuticals in bull sharks (*Carcharhinus leucas*) inhabiting a wastewater-impacted river. *Science of The Total Environment* 456–457: 196-201.

Gonçalves, C. M. O., Sousa, M. a. D. and Alpendurada, M. D. F. P. S. P. M., 2013. Analysis of acidic, basic and neutral pharmaceuticals in river waters: clean-up by 1°, 2° amino anion exchange and enrichment using an hydrophilic adsorbent. *International Journal of Environmental Analytical Chemistry* 93(1): 1-22.

Gravato, C., Guimaraes, L., Santos, J., Faria, M., Alves, A. and Guilhermino, L., 2010. Comparative study about the effects of pollution on glass and yellow eels (*Anguilla anguilla*) from the estuaries of Minho, Lima and Douro Rivers (NW Portugal). *Ecotoxicology and Environmental Safety* 73(4): 524-533.

Gwrc, 2009. Development of a common priority list of pharmaceuticals relevant for the water cycle. *Water Science and Technology* 59(1): 39-46.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P. F., Ingerslev, F., Holten Lützhøft, H. C. and Jørgensen, S. E., 1998. Occurrence, fate and effects of pharmaceutical substances in the environment- A review. *Chemosphere* 36(2): 357-393.

Hosokawa, R., Nagai, M., Morikawa, M. and Okuyama, H., 2009. Autochthonous bioaugmentation and its possible application to oil spills. *World Journal of Microbiology and Biotechnology* 25(9): 1519-1528.

Janssen, D. B., Oppentocht, J. E. and Poelarends, G. J., 2001. Microbial dehalogenation. *Current Opinion in Biotechnology* 12(3): 254-258.

Jelic, A., Gros, M., Ginebreda, A., Cespedes-Sánchez, R., Ventura, F., Petrovic, M. and Barcelo, D., 2011. Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment. *Water Research* 45(3): 1165-1176.

Juwarkar, A. A., Singh, S. K. and Mudhoo, A., 2010. A comprehensive overview of elements in bioremediation. *Reviews in Environmental Science and Bio/Technology* 9(3): 215-288.

Kim, I. and Tanaka, H., 2009. Photodegradation characteristics of PPCPs in water with UV treatment. *Environment International* 35(5): 793-802.

Kwon, J. W. and Armbrust, K. L., 2008. Aqueous solubility, n-octanol-water partition coefficient, and sorption of five selective serotonin reuptake inhibitors to sediments and soils. *Bulletin of Environmental Contamination and Toxicology* 81(2): 128-135.

Lacaze, E., Pédelucq, J., Fortier, M., Brousseau, P., Auffret, M., Budzinski, H. and Fournier, M., 2015. Genotoxic and immunotoxic potential effects of selected psychotropic drugs and antibiotics on blue mussel (*Mytilus edulis*) hemocytes. *Environmental Pollution* 202: 177-186.

Lara-Martín, P. A., González-Mazo, E., Petrovic, M., Barceló, D. and Brownawell, B. J., 2014. Occurrence, distribution and partitioning of nonionic surfactants and pharmaceuticals in the urbanized Long Island Sound Estuary (NY). *Marine Pollution Bulletin* 85(2): 710-719.

Lindqvist, N., Tuhkanen, T. and Kronberg, L., 2005. Occurrence of acidic pharmaceuticals in raw and treated sewages and in receiving waters. *Water Research* 39(11): 2219-2228.

Luo, Y., Guo, W., Ngo, H. H., Nghiem, L. D., Hai, F. I., Zhang, J., Liang, S. and Wang, X. C., 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Science of The Total Environment* 473–474: 619-641.

Madureira, T. V., Barreiro, J. C., Rocha, M. J., Rocha, E., Cass, Q. B. and Tiritan, M. E., 2010. Spatiotemporal distribution of pharmaceuticals in the Douro River estuary (Portugal). *Science of The Total Environment* 408(22): 5513-5520.

Mucha, A. P., Vasconcelos, M. T. and Bordalo, A. A., 2003. Macrobenthic community in the Douro estuary: relations with trace metals and natural sediment characteristics. *Environmental Pollution* 121(2): 169-180.

Mucha, A. P., Vasconcelos, M. T. S. D. and Bordalo, A. A., 2004. Vertical distribution of the macrobenthic community and its relationships to trace metals and natural sediment characteristics in the lower Douro estuary, Portugal. *Estuarine, Coastal and Shelf Science* 59(4): 663-673.

Musson, S. E., Campo, P., Tolaymat, T., Suidan, M. and Townsend, T. G., 2010. Assessment of the anaerobic degradation of six active pharmaceutical ingredients. *Science of The Total Environment* 408(9): 2068-2074.

Oecd (2013). *Health at a Glance 2013*, OECD Publishing.

Oladele, A. O. (1999). *Microbial Proteins as Biomarkers of Ecosystem Health. Integrated Assessment of Ecosystem Health*, CRC Press.

Pereira, A. M. P. T., Silva, L. J. G., Meisel, L. M., Lino, C. M. and Pena, A., 2015. Environmental impact of pharmaceuticals from Portuguese wastewaters: geographical and seasonal occurrence, removal and risk assessment. *Environmental Research* 136: 108-119.

Petrie, B., Mcadam, E. J., Scrimshaw, M. D., Lester, J. N. and Cartmell, E., 2013. Fate of drugs during wastewater treatment. *TrAC Trends in Analytical Chemistry* 49: 145-159.

Petrović, M., Gonzalez, S. and Barceló, D., 2003. Analysis and removal of emerging contaminants in wastewater and drinking water. *TrAC Trends in Analytical Chemistry* 22(10): 685-696.

Pubchem (National Center for Biotechnology Information. PubChem Compound Database (Bezafibrate); CID=39042, <https://pubchem.ncbi.nlm.nih.gov/compound/39042> (accessed Aug. 04, 2016).

Pubchem (National Center for Biotechnology Information. PubChem Compound Database (Paroxetine); CID=43815, <https://pubchem.ncbi.nlm.nih.gov/compound/43815> (accessed Aug. 04, 2016).

Quintana, J. B., Weiss, S. and Reemtsma, T., 2005. Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor. *Water Research* 39(12): 2654-2664.

- Radjenović, J., Petrović, M. and Barceló, D., 2009. Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. *Water Research* 43(3): 831-841.
- Ribeiro, C., Tiritan, M. E., Rocha, E. and Rocha, M. J., 2009. Seasonal and spatial distribution of several endocrine-disrupting compounds in the Douro River Estuary, Portugal. *Archives of Environmental Contamination and Toxicology* 56(1): 1-11.
- Ribeiro, H., Mucha, A. P., Marisa R. Almeida, C. and Bordalo, A. A., 2013. Bacterial community response to petroleum contamination and nutrient addition in sediments from a temperate salt marsh. *Science of The Total Environment* 458–460: 568-576.
- Richardson, M. L. and Bowron, J. M., 1985. The fate of pharmaceutical chemicals in the aquatic environment. *Journal of Pharmacy and Pharmacology* 37(1): 1-12.
- Rocha, M. J., Cruzeiro, C., Ferreira, C. and Rocha, E., 2012. Occurrence of endocrine disruptor compounds in the estuary of the Iberian Douro River and nearby Porto Coast (NW Portugal). *Toxicological & Environmental Chemistry* 94(2): 252-261.
- Schlüsener, M. P., Hardenbicker, P., Nilson, E., Schulz, M., Viergutz, C. and Ternes, T. A., 2015. Occurrence of venlafaxine, other antidepressants and selected metabolites in the Rhine catchment in the face of climate change. *Environmental Pollution* 196: 247-256.
- Schultz, M. M. and Furlong, E. T., 2008. Trace analysis of antidepressant pharmaceuticals and their select degradates in aquatic matrixes by LC/ESI/MS/MS. *Analytical Chemistry* 80(5): 1756-1762.
- Semblante, G. U., Hai, F. I., Huang, X., Ball, A. S., Price, W. E. and Nghiem, L. D., 2015. Trace organic contaminants in biosolids: Impact of conventional wastewater and sludge processing technologies and emerging alternatives. *Journal of Hazardous Materials* 300: 1-17.
- Sheu, Y. T., Lien, P. J., Chen, C. C., Chang, Y. M. and Kao, C. M., 2016. Bioremediation of 2,4,6-trinitrotoluene-contaminated groundwater using unique bacterial strains: microcosm and mechanism studies. *International Journal of Environmental Science and Technology* 13(5): 1357-1366.
- Silva, B. F. D., Jelic, A., López-Serna, R., Mozeto, A. A., Petrovic, M. and Barceló, D., 2011. Occurrence and distribution of pharmaceuticals in surface water, suspended solids and sediments of the Ebro river basin, Spain. *Chemosphere* 85(8): 1331-1339.
- Stenuit, B., Eyers, L., Schuler, L., Agathos, S. N. and George, I., 2008. Emerging high-throughput approaches to analyze bioremediation of sites contaminated with hazardous and/or recalcitrant wastes. *Biotechnology Advances* 26(6): 561-575.
- Sun, M. Y., Dafforn, K. A., Brown, M. V. and Johnston, E. L., 2012. Bacterial communities are sensitive indicators of contaminant stress. *Marine Pollution Bulletin* 64(5): 1029-1038.
- Tang, Y., Li, X. M., Xu, Z. C., Guo, Q. W., Hong, C. Y. and Bing, Y. X., 2014. Removal of naproxen and bezafibrate by activated sludge under aerobic conditions: kinetics and effect of substrates. *Biotechnology and Applied Biochemistry* 61(3): 333-341.
- Ternes, T. A., Bonerz, M., Herrmann, N., Teiser, B. and Andersen, H. R., 2007. Irrigation of treated wastewater in Braunschweig, Germany: An option to remove pharmaceuticals and musk fragrances. *Chemosphere* 66(5): 894-904.

- Tran, N. H., Urase, T., Ngo, H. H., Hu, J. and Ong, S. L., 2013. Insight into metabolic and cometabolic activities of autotrophic and heterotrophic microorganisms in the biodegradation of emerging trace organic contaminants. *Bioresource Technology* 146: 721-731.
- Trovó, A. G., Melo, S. a. S. and Nogueira, R. F. P., 2008. Photodegradation of the pharmaceuticals amoxicillin, bezafibrate and paracetamol by the photo-Fenton process—Application to sewage treatment plant effluent. *Journal of Photochemistry and Photobiology A: Chemistry* 198(2–3): 215-220.
- Van Dorst, J., Bissett, A., Palmer, A. S., Brown, M., Snape, I., Stark, J. S., Raymond, B., Mckinlay, J., Ji, M., Winsley, T. and Ferrari, B. C., 2014. Community fingerprinting in a sequencing world. *FEMS Microbiology Ecology* 89(2): 316-330.
- Vasskog, T., Bergersen, O., Anderssen, T., Jensen, E. and Eggen, T., 2009. Depletion of selective serotonin reuptake inhibitors during sewage sludge composting. *Waste Management* 29(11): 2808-2815.
- Veach, A., Bernot, M. J. and Mitchell, J. K., 2012. The influence of six pharmaceuticals on freshwater sediment microbial growth incubated at different temperatures and UV exposures. *Biodegradation* 23(4): 497-507.
- Verlicchi, P., Al Aukidy, M. and Zambello, E., 2012. Occurrence of pharmaceutical compounds in urban wastewater: Removal, mass load and environmental risk after a secondary treatment—A review. *Science of The Total Environment* 429: 123-155.
- Wang, S., Seiwert, B., Kästner, M., Miltner, A., Schäffer, A., Reemtsma, T., Yang, Q. and Nowak, K. M., 2016. (Bio)degradation of glyphosate in water-sediment microcosms – A stable isotope co-labeling approach. *Water Research* 99: 91-100.
- Waszak, I., Dabrowska, H. and Komar-Szymczak, K., 2014. Comparison of common persistent organic pollutants (POPs) in flounder (*Platichthys flesus*) from the Vistula (Poland) and Douro (Portugal) River estuaries. *Marine Pollution Bulletin* 81(1): 225-233.
- Who, 2012. Pharmaceuticals in drinking-water.
- Wu, C., Witter, J. D., Spongberg, A. L. and Czajkowski, K. P., 2009. Occurrence of selected pharmaceuticals in an agricultural landscape, western Lake Erie basin. *Water Research* 43(14): 3407-3416.
- Yamamoto, H., Nakamura, Y., Moriguchi, S., Nakamura, Y., Honda, Y., Tamura, I., Hirata, Y., Hayashi, A. and Sekizawa, J., 2009. Persistence and partitioning of eight selected pharmaceuticals in the aquatic environment: Laboratory photolysis, biodegradation, and sorption experiments. *Water Research* 43(2): 351-362.
- Yu, J. T., Bouwer, E. J. and Coelhan, M., 2006. Occurrence and biodegradability studies of selected pharmaceuticals and personal care products in sewage effluent. *Agricultural Water Management* 86(1–2): 72-80.
- Zhu, L., Ma, B., Zhang, L. and Zhang, L., 2007. The study of distribution and fate of nitrobenzene in a water/sediment microcosm. *Chemosphere* 69(10): 1579-1585.